THE IMPACT OF A MATERNAL CAFETERIA DIET ON OFFSPRING FOOD PREFERENCES AFTER BIRTH: THE ROLE OF THE MESOLIMBIC REWARD SYSTEM

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COMMONLY USED ABBREVIATIONS

6 A B C

6 PG	6-phosphogluconate
ACS	acyl CoA synthetase
ACOD	acyl CoA oxidase
ad libitum	to any desired extent
AMPH	amphetamine
AgRP	agouti-related protein
ANOVA	analysis of variance
BMI	body mass index
bp	basepairs
cAMP	cyclic adenosine monophosphate
CART	cocaine amphetamine related transcript
Cdk5	cyclin-dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoA	acyl-coenzyme A
CoV	coefficient of variance
CRF	corticotrophin releasing factor

D E F G

D1	dopamine 1 receptor
D2	dopamine 2 receptor
DARPP-32	dopamine- and cAMP-regulated phosphoprotein, Mr 32kDa
DAT	dopamine active transporter
DOPAC	3,4-dihydroxyphenylacetic acid
ELISA	enzyme linked immunosorbent assay
E	embryonic day
GABA	gamma-aminobutyric acid

H I J K L

JF	junk food
JF-W	junk food – withdrawal
L-DOPA	L-dihydroxyphenylalanine

M N O

MEFA	3-methy-N-ethyl-N(β-hydroxyethyl)-aniline
MAO	monoamine oxidase
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
NADH	reduced form of nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acid
NPY	neuropeptide Y
ob/ob	obese mouse

P Q R S

P	post-natal day
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
qRT-PCR	quantitative reverse transcription real time polymerase chain reaction
RNA	ribonucleic acid
SEM	standard error of the mean
SPSS	statistical package for social sciences
STAT3	signal transducer and activator of transcription 3

T U V W X Y Z

TH	tyrosine hydroxylase
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WHO	World Health Organisation

THESIS ABSTRACT

Maternal consumption of high-fat, high-sugar 'junk foods' during pregnancy and lactation is associated with increased risk of obesity in the offspring later in life. Recent studies have shown that these offspring are not only hyperphagic but also have an increased preference for junk foods. The mechanisms underlying the increased preference for junk foods are however still unclear.

To validate the rodent model of junk food exposure, we investigated the impact of chronic junk food feeding on physiological and behavioural parameters, as well as molecular changes within the mesolimbic reward system in adult rodents. These experiments indicated that chronic junk food intake increased fat mass in both male and female adult rats and the removal of the junk food diet was associated with significant weight loss and reduced food intake. Chronic junk food intake also resulted in changes in the expression of key genes within the mesolimbic reward system which were consistent with a desensitisation of the reward system. We also reported that these changes within the reward system were sex-specific. This study provided the basis for the central aims of the thesis which were to: 1) determine the effects of a maternal junk food diet on food preferences and expression of key components of the mesolimbic reward system in the juvenile and adult offspring, and 2) examine whether these changes in the reward pathway and the preference for fat could be reversed by providing offspring with a low-fat diet post-weaning.

Following the validation of the junk food diet rodent model, work from this thesis showed that peri-natal exposure to a junk food diet programmed an increased preference for fat in the offspring from weaning until adulthood. The drive for fat intake in the offspring of junk food fed dams (JF offspring) was associated with an upregulation of dopamine and opioid-related gene expression during the juvenile period followed by downregulation of these genes in adulthood, suggestive of desensitisation of the mesolimbic reward system in the JF offspring.

Studies in this thesis also showed that the excessive fat deposition and preference for fat was reversed when JF offspring were provided with a low-fat control diet for 3 weeks post-weaning. However, when access to junk food was reinstated at 6 weeks of age, JF offspring consumed more energy and carbohydrate, indicative of a hyperphagic response to

the junk food exposure. These offspring also exhibited changes in gene expression within the reward system, consistent with increased dopamine sensitivity which would be expected to render these JF offspring more susceptible to diet-induced obesity. When JF offspring were provided with a low-fat control diet from weaning until 3 months of age, they did not exhibit an increased preference for junk food when access was reinstated, indicating that a longer period of junk food abstinence post-weaning was able to reverse the increased appetite for junk foods. This intervention did not, however completely normalise the expression of key components of the reward system, particularly in the male JF offspring. Female JF offspring remained fatter than controls in adulthood even though there were no differences in food intake. Therefore, despite consuming a low-fat diet from weaning, some effects of a maternal junk food diet on the offspring persisted into adulthood.

Findings from this thesis have provided evidence and a mechanism through which maternal junk food intake during pregnancy and lactation can affect offspring food preferences later in life. The results demonstrated that nutritional insults during the development of the mesolimbic reward system can impair the ability to regulate palatable food intake, an effect which cannot be entirely reversed with a post-weaning low-fat diet.

DECLARATION

I declare that:

1. *This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university*
2. *To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.*

.....

Zhi Yi Ong

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PUBLICATIONS ARISING FROM THIS THESIS

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2. Muhlhausler BS, **Ong ZY**. The fetal origins of obesity: Early origins of food intake. *Endocrine, metabolic and immune disorders drug targets* 2011 Sep 1; 11(3):189-197

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4. Gugusheff JR, **Ong ZY**, Muhlhausler BS. A maternal "junk-food" diet reduces sensitivity to the opioid antagonist, naloxone in offspring postweaning. *FASEB Journal* 2013, Mar; 27(3):1275-1284

5. **Ong ZY**, Wanasuria AF, Lin MZP, Hiscock J, Muhlhausler BS. Chronic intake of a cafeteria diet and subsequent abstinence. Sex-specific effects on gene expression in the mesolimbic reward system. *Appetite* 2013, Feb 10 S0195-6663(13)00043-3. 10.1016/j.appet.2013.01.014

CHAPTER 1: LITERATURE REVIEW

1.1 The obesity epidemic

Obesity is defined in relation to body mass index (BMI) which is an estimate measure of body fat mass calculated by dividing body weight (kg) by height (m^2). The World Health Organisation (WHO) criteria states that a BMI of >25kg/m^2 is classified as overweight and a BMI >30kg/m^2 as obese. Recent data from WHO reported that approximately 500 million adults were classified as obese in the year 2008 (1). In the United States alone, there are currently more than 72 million obese adults and 32 million more are predicted to be obese by 2030 (2). The prevalence of obesity in children is also on the rise, with more than 40 million children under the age of 5 being classified as overweight in the year 2010 (1). With obesity having a major impact on population health and health care costs due to its associated co-morbidities such as type 2 diabetes and hypertension, it is essential to understand the mechanisms underlying the development of obesity and develop effective intervention strategies to overcome this epidemic. Whilst various approaches have been undertaken to counteract the rising prevalence of obesity, the problem remains, therefore highlighting the complexity of this multi-factorial issue.

Factors associated with the development of obesity range from genetics to behaviour to the environment. One of the major factors contributing to the increasing incidences of obesity is the excessive consumption of energy-dense 'junk foods' which is defined as nutrient poor, palatable foods that are high in fat, sugar and salt (3). Caloric intake has also been reported to have increased over the past decade in both industrialised and semi-industrialised countries (4).

Given the increasing incidence of obesity worldwide together with the ascent in caloric intake, it is not surprising that the number of women entering pregnancy overweight and/or obese is also on the rise. In the United States, data from the Pregnancy Risk Assessment Monitoring System (PRAMS) from 9 states indicated a 69.3% increase in pre-pregnancy obesity rates from 1992-1993 to 2002-2003 (5). More recently from the 2008 South Australian Pregnancy Outcome Database, it was reported that more than 50% of pregnant women were overweight and obese (6). Obesity during pregnancy can be harmful not only to the mother, but also to the infant, and these adverse effects include increased risks of pregnancy complications such as gestational diabetes (6), miscarriage (7) and stillbirth (8).

Importantly, it is now clear that the impact of maternal obesity on the offspring extends beyond the pre-natal and early post-natal period, and that children of obese mothers have a higher BMI and are at an increased risk of obesity and its co-morbidities later on in life compared to children of lean mothers.

1.1.1 Maternal obesity and offspring obesity

A wide range of epidemiological studies have supported a role of the intrauterine environment in programming the metabolic health of offspring throughout the life course. Studies conducted as early as 1954 reported positive correlations between maternal pre-pregnancy weight and gestational weight gain with offspring birth weight (9, 10). In the last decade, clinical studies have shown that obese mothers are more likely to give birth to large for gestational age (LGA) babies (11-14), who are at an increased risk of becoming obese before 12 years of age (15-17). In one of the largest cohort studies consisting of 10,683 adult British individuals whom detailed information on birth weight and current BMI were available, it was demonstrated that babies with the highest birth weights also had the highest adult BMI at 33 years of age (18). Importantly, the relationship between birth weight and adult BMI in this study was explained almost entirely by the presence of high pre-pregnancy weight and BMI of the mother of these individuals. These findings were further supported by a recent study in Jerusalem where maternal prepregnancy BMI was associated offspring adiposity and cardiometabolic risk factors at 32 years of age (19). Similar observations have also been reported in the cohorts from United Kingdom (20), northern Finland (21, 22) and Sweden (23), thus emphasising the fact that maternal obesity is a significant global issue which has the potential to adversely affect the health of future generations.

Although BMI and body weight have been widely used as a marker for obesity, these measures only provide a crude estimation for body fat mass and may not be applicable to all individuals. Individuals with the same BMI have been found to have very different percentage body fat mass when measured using dual x-ray absorptiometry (24). This indicates the limitation of BMI and highlights the need to determine body composition as a measure of adiposity, rather than using BMI alone. In the few studies which had information on the body composition of the subjects, body fat mass was reported to be higher at birth in neonates of overweight and obese mothers (25, 26) and is positively related to maternal body fat mass (27). This positive relationship between maternal obesity and offspring body fat mass also persists into childhood. Burdette and colleagues showed

that maternal pre-pregnancy obesity was related to higher body fat mass and BMI in the offspring at 5 years of age (28). Moreover, a recent study reported that a higher maternal pregnancy BMI can predict offspring adiposity in adulthood (29). These studies therefore suggest that heavier mothers give birth to heavier infants who will go on to have increased adiposity and higher BMI as adults, thus setting up an intergenerational cycle of obesity.

1.1.2 Maternal nutrient intake and offspring obesity

1.1.2.1 Humans

One possible mechanism linking maternal obesity and the development of obesity in the offspring is the exposure of the fetus to excessive supply of nutrients *in utero*. This was first demonstrated in infants of diabetic mothers who were exposed to high maternal glucose levels *in utero*. These infants had higher adiposity (30) and insulin levels (hyperinsulinaemia) at birth (31) and as children (32), followed by reduced glucose tolerance and an increased risk of developing diabetes during adulthood (33). Importantly, maternal obesity and weight gain during late pregnancy has also been directly related to high maternal energy intake (34, 35) and these mothers have an increased risk of giving birth to heavier babies (35). Therefore, evidence from these population studies points clearly towards a relationship between maternal overnutrition and maternal obesity/diabetes, as well as an increased susceptibility to obesity and its related metabolic disorders in the offspring later in life.

1.1.2.2 Sheep

The effects of maternal overnutrition on the offspring after birth have also been studied in large animal models. In the sheep, maternal overnutrition (~155% of recommended dietary intake) during pregnancy results in higher fetal glucose and insulin levels (36). Lambs of overnourished ewes consumed more food and had higher plasma glucose levels in the immediate postnatal period and a higher subcutaneous fat mass at 1 month of age when compared to lambs of control mothers during pregnancy (37). Importantly, the effects of maternal overnutrition in the sheep have also been shown to persist into adulthood. Long and colleagues demonstrated that offspring of mothers which were overnourished before and during pregnancy consumed more feed and had higher plasma leptin levels in adulthood (20 months of age) (38). These offspring of overfed ewes also exhibited impaired insulin sensitivity and glucose homeostasis (38). These findings support the human epidemiological studies in which exposure to maternal overnutrition *in utero* can increase adiposity, glucose and insulin levels in the infants and lead to the development of obesity in adult life.

1.1.2.3 Rodents

Similar to humans and sheep, it has been consistently reported in rodent models that exposure to maternal overnutrition during pregnancy and lactation can have profound effects on the offspring's health in later life. Chen and colleagues showed that offspring of

obese dams provided with a high-fat diet throughout pregnancy and lactation had increased body weight, greater adiposity and higher plasma concentrations of insulin and leptin at 20 days of age (39). Offspring of overnourished dams have also been shown to develop hyperphagia during the post-weaning period (40-42) and have higher fat mass as juveniles (40) and adults (42-44). Hence, these studies, together with those in humans and sheep, have provided compelling evidence that perturbations to the nutritional environment *in utero* and during lactation is associated with increased appetite and accumulation of body fat after birth, which increases the risk of developing obesity later in life.

1.1.3 Maternal nutrient intake and offspring food preferences

As indicated previously in both rodent and sheep models, offspring of overnourished and/or high-fat fed dams have been shown to exhibit an increased appetite after birth (37, 41). In addition, maternal diabetes induced by the administration of streptozotocin, even in the absence of maternal obesity, also results in offspring which were hyperphagic during post-natal life (45). Hyperphagia in offspring of overnourished and diabetic dams has been shown to be associated with the altered development of the hypothalamic appetite regulating neural circuitry (45-47), and changes in gene expression of the key orexigenic and anorexigenic neuronal peptides within the appetite regulating neural network of the offspring (37, 41, 48). These changes within the hypothalamic appetite regulatory network can lead to altered function of the appetite regulatory system and is therefore a potential mechanism by which maternal high fat and sugar levels during pregnancy and lactation can program the development of hyperphagia and the onset of obesity in the offspring later in life.

More recently, evidence has emerged demonstrating that not only food intake, but also the preference for certain foods, in particular those high in fat and sugar can be programmed early in life as a result of pre- and peri-natal exposure to an excess or deficient nutrient supply. This was first demonstrated by Bellinger and colleagues who showed that offspring of dams on a low-protein diet during pregnancy, when given a choice between high-fat, high-carbohydrate or high-protein rat pellets, displayed an increased preference for the high-fat but not high-carbohydrate or high-protein pellets at 12 weeks of age (49). This study suggested that the programming of obesity may be driven by alterations in the types of foods consumed, in addition to the overall food intake.

Since then, a number of rodent studies have also demonstrated an increased preference for fat in offspring exposed to maternal overnutrition during the peri-natal period. Exposure to a high-fat diet *in utero* was found to be associated with the preference for high-fat foods in male offspring at post-natal day 60 (P60) (50). In addition, a study conducted by Bayol and colleagues showed that offspring of dams fed on a cafeteria diet, which is a junk food diet well-established in rodent models, consisting of a range of common human 'junk foods' including muffins, jam doughnuts and biscuits, during pregnancy and lactation developed an exacerbated preference towards the highly palatable diet, in particular foods high in fat, carbohydrates and salt, after birth (40). Similarly, offspring of dams fed on a high-fat diet from the final week of gestation until the end of lactation exhibited an increased operant responding for high-fat foods but not high-carbohydrate diets (51). Interestingly, exposure to a high-fat diet during the first week after weaning also programmed an increased preference for high-fat foods during adult life in mice (52). Exposure to a high-carbohydrate diet during the peri-natal period, however, did not result in the preference for high-carbohydrate foods in adulthood, suggesting that the effects were fat specific. In contrast, Vucetic and colleagues found that offspring of high-fat fed dams had a higher preference not only for fat but also sucrose in adulthood (53), thus indicating that the programming of food preferences may not be limited to fat alone. Given that previous studies looked at the preference for total carbohydrate rather than sucrose, it is possible that a preference for sucrose, the simple form of carbohydrate, may be more likely to be programmed instead of carbohydrates which consist of both mono- and polysaccharides. Nevertheless, these studies collectively showed that food preferences can indeed be programmed during the perinatal period as a result of maternal nutrient intake during pregnancy and lactation, but whether this preference is specific for fat or sugar or both remains to be clearly demonstrated.

The programming of food preferences has also been demonstrated in human population studies. A study in the United Kingdom looking at 428 children of obese and lean parents showed that children of obese parents had a higher preference for high-fat foods and a lower preference for vegetables at 4-5 years of age (54). Whilst this study provided valuable information on the association between obese parents and offspring preference for high-fat foods in childhood, it is difficult, in population studies, to dissociate the pre-natal (*in utero* environment) and post-natal (milk composition and the family environment) contribution to the high-fat preference, and whether the pre-natal or post-natal environment plays a more significant role in determining the pre-disposition to offspring obesity.

Nevertheless, a more recent study conducted in a large birth cohort (The Alvon Longitudinal Study of Parents and Children, ALSPAC) showed that there was a strong association between maternal pre-natal fat, carbohydrate and protein intake with the child's intake of these same macronutrients. Paternal diet and maternal post-natal diet were however only weakly correlated with offspring food intake (55). This provides some evidence that maternal dietary intake during pregnancy may play an important role in the determination of food preferences in the offspring after birth.

The role of maternal pregnancy diet in predicting the child's food preferences has also been demonstrated in studies of maternal undernutrition. Children of undernourished mothers who during early gestation, went through the Dutch Famine 1944-1945, a period when food rations were limited to below 1000 calories per day, had a higher preference for fatty foods as adults which consequently contributed to the more atherogenic lipid profiles in these individuals (56). Interestingly, a cross-sectional, longitudinal, prospective cohort study conducted in Brazil showed that women who were born severely intrauterine growth restricted exhibited an increased preference for carbohydrates, but consumed less protein as adults (57). Fat intake was however not measured in this study. Evidence from these population studies highlights the importance of pre-natal dietary exposures on the determination of food choices in an individual later in life and also supports the hypothesis from animal studies that the preference for fat, and possibly sugar, can be programmed before birth.

In addition to an increased preference for particular macronutrients, it has also been shown that the preference for specific flavours can be programmed in human infants by exposure to that flavour *in utero* and during lactation. Mennella and colleagues conducted a study where pregnant mothers who were planning to breastfeed were randomly assigned to 1 of 3 groups: 1) carrot juice during pregnancy, water during lactation, 2) water during pregnancy, carrot juice during lactation, or 3) water during both pregnancy and lactation. The mothers were required to consume the carrot juice or water 4 times per week for 3 weeks during the last trimester of pregnancy and the first 2 months of lactation. After weaning, the children of these women were given infant cereal prepared with either carrot juice or water and the mothers were asked to rate the liking of the children towards each cereal. The positive and negative facial expressions of the children when given cereal prepared with water or carrot juice were rated by the mother. This study demonstrated that offspring exposed to the carrot flavour *in utero* and in the breast milk during lactation exhibited an increased liking

towards the carrot flavour after weaning when compared to those whose mother only had water (instead of carrot juice) during pregnancy and lactation (58). In another study, the taste preferences of children age 4 to 5 years old were tested on a range of foods and preference compared between infants fed on soy-based formula or milk-based formula consumed during infancy. Significant differences in food preferences were identified between those children fed on different formulas, with soy formula-fed infants showing a higher preference towards sour- and bitter- flavoured juices, compared to milk formula-fed infants (59). Findings from these studies therefore provide evidence that both the pre-natal and early infant periods are important for the programming of taste preferences in humans. Moreover, this has opened up another possible avenue for the programming of obesity in the offspring and highlights the importance of maternal diet not only during pregnancy but also during lactation in determining food preferences in the offspring.

1.1.4 Summary and speculations

The mechanisms underlying specific preferences for taste and specific macronutrients in different individuals and how this could be programmed by pre-natal and early post-natal exposure is still not well-understood. One of the postulated mechanisms influencing food preferences is through altered development of the mesolimbic reward system, which is an area in the brain highly implicated in studies of the addiction to drugs of abuse. The mesolimbic reward system is involved in the regulation of addiction and reward, and more importantly in the context of this thesis, the regulation of food preference/reward. The following sections of this Chapter will review on the current understanding of the role of the mesolimbic reward system in regulating food reward in adults, the development of this system throughout embryonic and early post-natal life, and existing evidence and suggestions that maternal nutrient intake during pregnancy and lactation can interfere with the development of the reward system, resulting in permanent alterations in reward signalling in adult life.

1.2 Neural regulation of food reward

Homeostatic regulation of food intake is controlled by the appetite regulatory neural network within the hypothalamus. In a fasted state, the orexigenic neuropeptides including neuropeptide Y (NPY) and agouti-related protein (AgRP) are activated to stimulate food intake; while anorexigenic neuropeptides such as pro-opiomelanocortin (POMC) and cocaine amphetamine related transcript (CART) are activated in response to increased

nutrient availability to reduce appetite (60). Despite the existence of this system, it is clear that many individuals tend to consume more than is required to meet their energy requirements. This is known as the non-homeostatic control of food intake, and is thought to be regulated by the mesolimbic reward system (60). This section will describe the molecular events within the mesolimbic reward system which occur in response to a rewarding stimulus and the subsequent development of addiction, with a focus on the impact of palatable diets on this system.

1.2.1 Mesolimbic reward system and addiction

Addiction is characterised by compulsive behaviours associated with the loss of control over the desire to obtain a rewarding substance, which can result in a negative emotional state when the substance is removed (61). The development of addiction is a complex process and differs according to the type of rewarding substance or specific drug of abuse. This process is regulated by the mesolimbic reward system in the brain, which is comprised of the ventral tegmental area (VTA) and the nucleus accumbens (NAc), with dopamine and opioids being the major neurotransmitters involved. Dopamine is produced in the VTA where the cell bodies of dopaminergic neurons reside. These neurons project to the NAc, which is subdivided to the shell and core regions, where dopamine is released. This area of the brain consists primarily of medium spiny neurons. Opioid neurons and receptors are also found in the VTA and NAc, and opioids at the VTA can activate dopamine neurons within the mesolimbic reward system (62). This section will provide an overview of the processes within the mesolimbic reward system.

The activation of the mesolimbic reward system begins with the stimulation of the dopaminergic neurons in the VTA, resulting in the phosphorylation of the rate limiting enzyme for dopamine production, tyrosine hydroxylase (TH), and thereby increasing dopamine production and release into the NAc. Dopamine then binds to its receptors on medium spiny neurons within the NAc. Concurrently, endogenous opioids are also released at the VTA and bind to their receptors on gamma-aminobutyric acid (GABA) interneurons to inhibit the release of the inhibitory neurotransmitter, GABA. GABA normally acts to inhibit dopaminergic neurons and therefore the inhibition of GABA release by opioids results in the disinhibition of the dopaminergic neurons and consequently leads to the increased release of dopamine at the NAc (62). Opioids also bind to their receptors within the NAc (63). Both the direct and indirect actions of opioids,

together with the activation of dopamine neurons can create a pleasurable feeling or 'high' associated with rewarding stimuli (Figure 1.1).

Within the dopaminergic neuron itself, there are a series of molecular events which regulates dopamine release in response to a rewarding stimulus. Activation of the neurons triggers the opening of Ca^{2+} channels and the influx of Ca^{2+} into the pre-synaptic terminal. This, together with cAMP, stimulates the phosphorylation of TH and this phosphorylated form of TH catalyses the rate limiting step in the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is subsequently catalysed to dopamine by the constitutively expressed enzyme L-aromatic amino acid decarboxylase (64). Dopamine is then packed into vesicles and released into the synapse. Once released, dopamine binds to its G protein coupled receptors, D1 and D2. D1 receptors interact with the $G\alpha_s$ family of G proteins to activate adenylyl cyclase (AC) which leads to cAMP production, whilst D2 receptors activate the $G_{\alpha i/o}$ family to inhibit AC and thus cAMP (65). Despite the opposing signalling pathways, activation of both D1 and D2 at the NAc is required for reward-motivated behaviour (66, 67). This may be due to the different populations of GABA-ergic medium spiny neurons within the NAc where D1 and D2 are located (65). Furthermore, D1 is exclusively located at the postsynaptic terminal but D2 is present at both the post-synaptic terminal and the pre-synaptic terminal. D2 receptors at the pre-synaptic terminal play a role in the negative feedback of dopamine signalling, as discussed below.

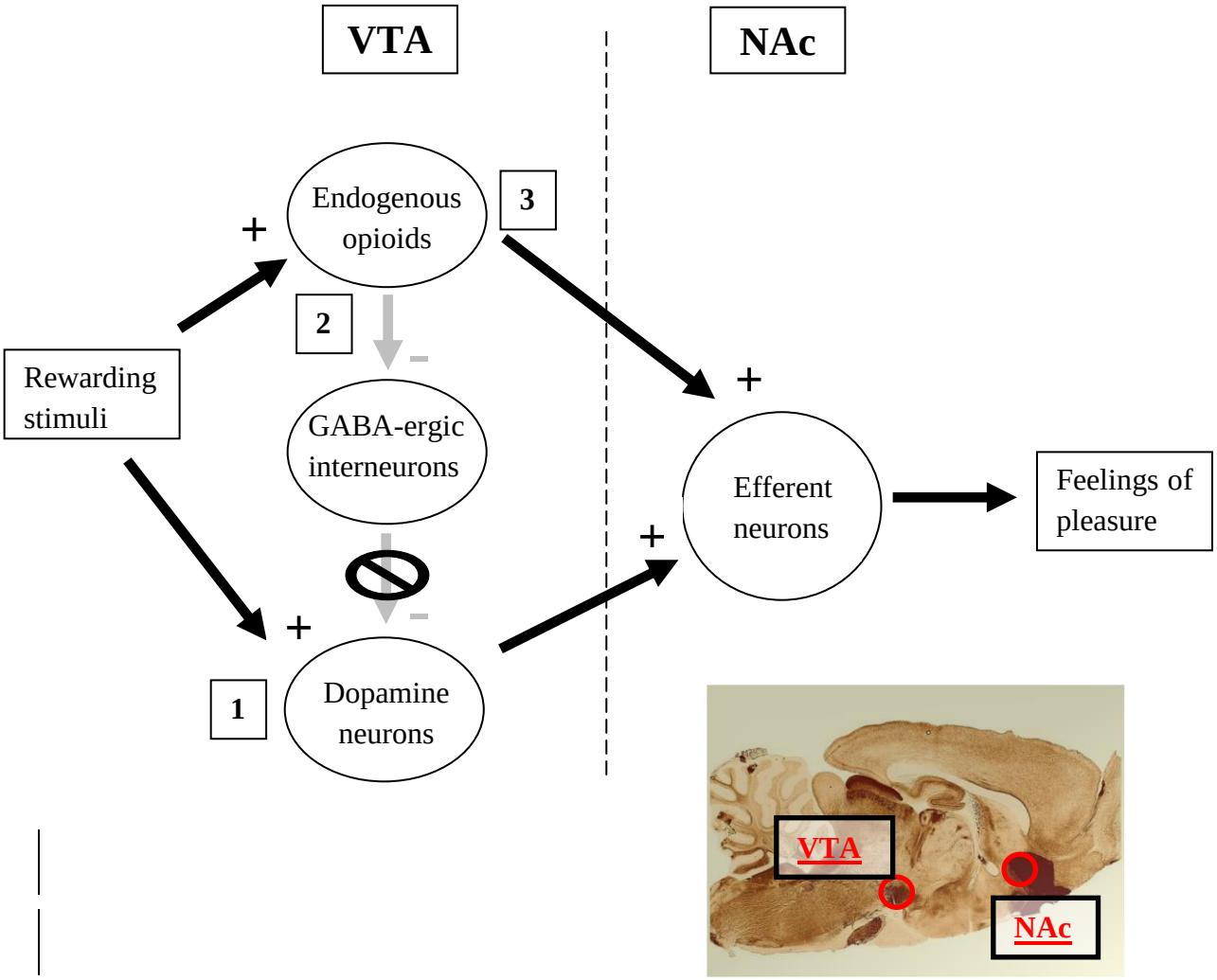
Upon binding to dopamine receptors, the remaining dopamine is taken up into the pre-synaptic terminal by the dopamine active transporter (DAT) where it gets broken down to 3,4-dihidroxyphenylacetic acid by monoamine oxidase (MAO-A and MAO-B). Dopamine that escapes being broken down is repackaged into vesicles by vesicular monoamine transporter (VMAT) to be 'recycled' and released into the synapse (64). As mentioned earlier, dopamine release is also regulated by D2 autoreceptors which are localised on the axons of dopaminergic neurons in the striatum and cell bodies and dendrites of dopaminergic neurons in the midbrain (68). These receptors detect the levels of dopamine in the synapse and change the impulse flow by opening potassium channels which hyperpolarises the cell. This closes Ca^{2+} channels and inhibits further dopamine synthesis and release (64) (Figure 1.2).

This tightly regulated system can however be disturbed by repeated activation of the reward pathways by the same rewarding stimulus. Chronic exposure to a specific drug of

abuse, for example, leads to molecular adaptations within the mesolimbic reward system resulting in desensitisation of this system, due to the downregulation of the dopamine and opioid receptors from repeated stimulation. As a result, the same stimulus is no longer capable of exerting the same magnitude of effect, and a stronger stimulus (or a greater quantity of drug) is required to elicit the same pleasurable sensation as experienced previously. This state, also known as tolerance, causes the affected individual to seek out higher quantities of the drug to achieve the desired pleasurable response.

It has also become clear that, in addition to drugs, a number of other stimuli including food, sex and gambling, also activate the mesolimbic reward pathway, and these are collectively known as ‘natural rewards’ (62). Addiction to natural rewards such as palatable foods is a more complicated process as it involves both peripheral and central signalling (63). The process by which these natural rewards stimulate the mesolimbic reward pathway is still unclear but recent studies have suggested that similar molecular mechanisms within the reward system are involved in response to natural rewards and drugs of abuse (63, 69), therefore implying that a common pathway underlies the addiction to these rewarding stimuli.

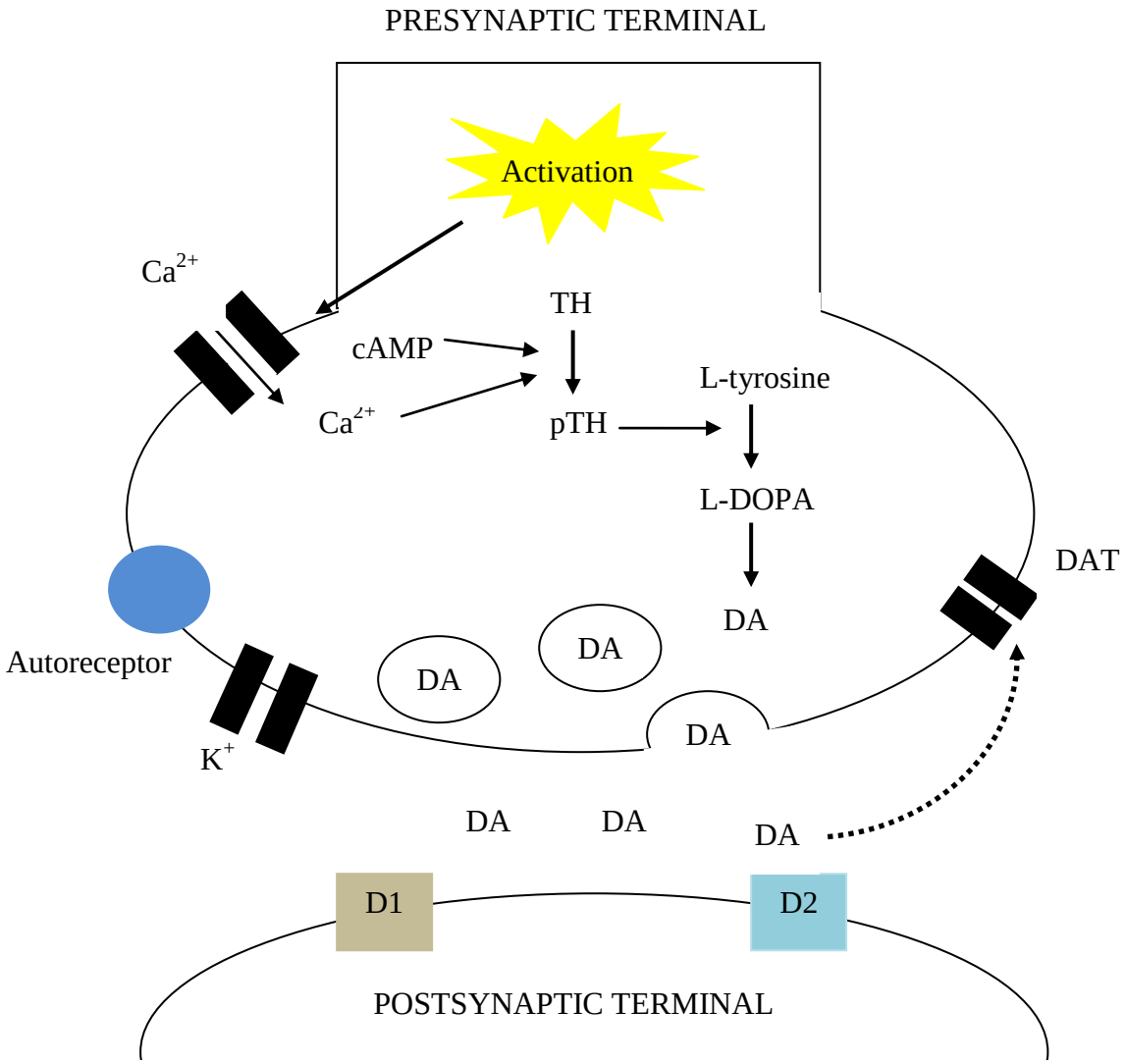
Figure 1.1



Simplified version of the activation of the mesolimbic reward system.

1) A rewarding stimulus such as drugs and palatable foods can stimulate the dopamine neurons at the VTA, resulting in the release of dopamine at the NAc. **2)** The rewarding stimulus can activate the release of endogenous opioids at the VTA, which inhibits GABA-ergic interneurons. GABA normally inhibits dopamine release. Therefore, this inhibition of GABA release disinhibits dopamine neurons resulting in increased dopamine release at the NAc. **3)** Opioids can also bind to their receptors located at the NAc. The activation of efferent target neurons at the NAc through **1), 2) and 3)** creates a pleasurable feeling associated with the rewarding stimuli. Black and grey arrows indicate neuronal activation and inhibition respectively. Neurons are represented in circles. Adapted from (62). Inset: Sagittal section of a rat brain. Red circles represent the location of VTA and NAc within a rat brain.

Figure 1.2



Molecular events of dopamine (DA) signalling within mesolimbic reward system

Activation of dopamine neurons opens Ca^{2+} channels, causing Ca^{2+} influx. Ca^{2+}, together with cAMP leads to phosphorylation of the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH). This catalyses L-tyrosine to L-dopa, which is then converted to dopamine. Dopamine is package in vesicles within the presynaptic terminal and is released into the synapse. Dopamine binds to both D1 and D2 receptors on the postsynaptic terminal. Excess dopamine is being taken up by the dopamine active transporter (DAT) to be recycled or degraded. The D2 pre-synaptic autoreceptors regulate dopamine release by opening K^{+} channels and closing Ca^{2+} channels which will hypopolarise the cell and reduce dopamine synthesis. Adapted from (64).

1.2.2 Dopamine and food intake

One of the links between addiction to drugs and palatable foods is the ability of dopamine to initiate the development of both these addictive behaviours. Dopamine administration to the NAc (one of the key areas of the mesolimbic reward system) has been shown to stimulate sugar intake (70) whilst NAc D1 antagonism reduces sucrose-seeking behaviour (71). Similar to drugs of abuse, palatable foods high in sugar and fat can stimulate the release of dopamine in the NAc (70, 72-75). In addition, palatable food intake has also been shown to alter receptor binding and gene expression of key components of the mesolimbic reward system, including dopamine receptors and transporters (76, 77). A rodent model of sugar addiction in which rodents were given intermittent access to 25% sucrose for 30 days showed an escalation in sucrose intake throughout the experimental period. By the end of the study, rats exposed to sucrose displayed increased D1 binding in the NAc, indicating heightened dopamine signalling (76). Exposure to highly palatable diets for 20 days in rats was also associated with increased expression of D2 dopamine receptors and reduced DAT activity in the NAc (77). These changes in both D2 receptors and DAT activity collectively led to an increase in extracellular dopamine and dopamine transmission in response to palatable food intake (77). The continuous intake of these foods can result in further alterations and adaptations within the mesolimbic reward system which have been suggested to ultimately contribute to the development of addiction.

As mentioned earlier, repeated activation of the dopaminergic neurons can result in desensitisation of the mesolimbic reward system, which is characterised by downregulation of dopamine receptors and reduced dopaminergic tone i.e. basal dopamine levels in the absence of a stimulus (78-81). The ability of palatable foods to stimulate the dopaminergic system has led to the hypothesis that chronic consumption of these foods can result in desensitisation of the reward pathway and the onset of palatable food addiction or dependence. Johnson and Kenny performed a seminal study which investigated the effects of chronic palatable food intake on the mesolimbic reward pathway, both in relation to gene expression and behavioural changes (82). They showed that rats chronically fed a palatable diet displayed compulsive eating behaviour and continued to consume the diet even when presented with an aversive stimulus (electrical foot shocks). Moreover, chronic palatable food intake was associated with the downregulation of D2 receptors in the striatum (82), which was also previously reported in drug addicts as well as in obese individuals (83). This was however in contrast to a previous study where D2 mRNA expression was upregulated in the NAc of rodents after 12 weeks of high-fat, high-sugar

diet (84). The different effects of palatable food intake on D2 mRNA expression in these 2 studies may be due to different timing of exposure and composition of the diet. Nevertheless, chronic palatable food intake was also associated with reduced D1 mRNA expression at the NAc (84) and VTA (85). In a separate study, D1 receptors were shown to be downregulated after a prolonged period of high-fat, high-sugar intake in obesity-prone rats compared to controls and obesity-resistant rats (86). Therefore, it is possible that chronic intake of palatable foods can lead to molecular changes within the central reward system consistent with desensitisation of the mesolimbic reward system.

In addition to altered expression of dopamine receptors, exposure to a palatable diet in rodents has also been associated with reduced TH expression (84, 85), an indication of reduced dopamine synthesis and lower basal dopamine levels within the mesolimbic reward system (87, 88). Obesity-prone rats have been shown to exhibit reduced basal dopamine levels (89), as a result of reduced dopamine synthesis and release (81). It would therefore appear that, an attenuated dopaminergic system and possibly a desensitised reward system, which is normally observed following prolonged consumption of palatable foods in normal rats, may be an inherent characteristic of obesity-prone rats. Moreover, diet-induced obese and obesity prone rats did not reduce their food intake despite the desensitised reward system but instead continued to consume similar quantities of the palatable diet. This suggests that the deficits in the dopaminergic reward system may be responsible for driving the palatable food intake in an attempt to compensate for the reduction in dopamine levels and restore brain dopamine back to normal levels (81).

Another characteristic of drug addiction is the onset of withdrawal symptoms in response to the removal of the rewarding stimulus, which is often associated with a negative emotional state. As with drugs of abuse, the removal of palatable foods after a period of prolonged exposure has also been shown to trigger signs of withdrawal. Le Magnen was one of the first to demonstrate that rats that had been chronically fed on a cafeteria diet developed morphine-like withdrawal symptoms when the diet was removed (90). However, it was not until a decade later that subsequent studies focussed on the addictive properties of individual macronutrients and their ability to precipitate withdrawal. Colantuoni and colleagues demonstrated that the removal of sucrose from rodents previously given intermittent access to a sucrose solution was associated with the onset of signs of withdrawal including teeth chattering and wet dog shakes (91), similar to those observed during opiate withdrawal. These withdrawal signs were, however, not observed in rats

which have previously been given *ad libitum* access to sucrose or a diet consisting of fat and sucrose (92) or fat alone (93), suggesting that the effects of withdrawal differ according to the specific macronutrients within the palatable diet and whether the diets were given intermittently or continuously.

The effect of abstinence from a high-fat diet appears to be associated to a greater extent with the increase in anxiety-related behaviours than classic opiate withdrawal signs. Teegarden and colleagues found that mice given free access to a high-fat diet displayed signs of anxiety including increased fecal boli production, increased locomotor activity and weight loss when the high-fat diet was replaced with standard rat chow (94). These mice also exhibited an increase in limbic corticotrophin releasing factor (CRF), which has been shown to be elevated in response to ethanol and drugs withdrawal due to increased anxiety (95, 96). Interestingly, these effects were not seen when mice lost access to a high-carbohydrate diet, suggesting that these effects were fat-specific (94). Increased anxiety, indicated by reduced time spent in the centre of an open field test, was also observed when rats given 4 weeks access to a palatable high-fat, high-sugar diet were withdrawn from the palatable diet (97). In contrast, Bocarsly and colleagues demonstrated that rats given intermittent or *ad libitum* access to a high-fat, high-sugar palatable diet did not display any signs of withdrawal, and when assessed using the elevated plus maze test, these rats also did not exhibit increased anxiety (93). Similarly, using the same test, Teegarden and colleagues did not observe any differences between control and high-fat fed rats in response to dietary withdrawal despite the increase in other anxiety signs as mentioned above (94). This therefore suggests that more robust measures are required to effectively test for anxiety in response to the removal of a palatable diet and that using one test for the determination of anxiety is unlikely to be sufficient. Nevertheless, after the removal of the palatable diet, the rats lost weight (93, 94) and consumed significantly less of the standard control chow when compared to rats which have never been exposed to the palatable diet (82) or to obesity resistant rats (97). Therefore, it is possible that the desire for the palatable diet, and the pleasurable sensation derived from consuming the diet, was able to override the normal physiological satiety signals in these rats (98).

In addition to the behavioural changes observed in response to palatable food withdrawal, rodents previously exposed to palatable diets have also been found to have lower dopamine levels within the mesolimbic reward system during the withdrawal phase compared to rodents which have only been exposed to a standard rat chow diet (91, 94), consistent with

the effects reported in alcohol withdrawal (99). Recently, Alsio and colleagues also showed that withdrawal from a high-fat, high-sugar diet led to changes in the expression of dopamine receptors within the NAc. In that study, D1 mRNA expression was significantly downregulated in obesity prone rats after they were withdrawn from a palatable diet (86). It is therefore possible that the disturbance in the dopaminergic system is associated with the onset of withdrawal symptoms.

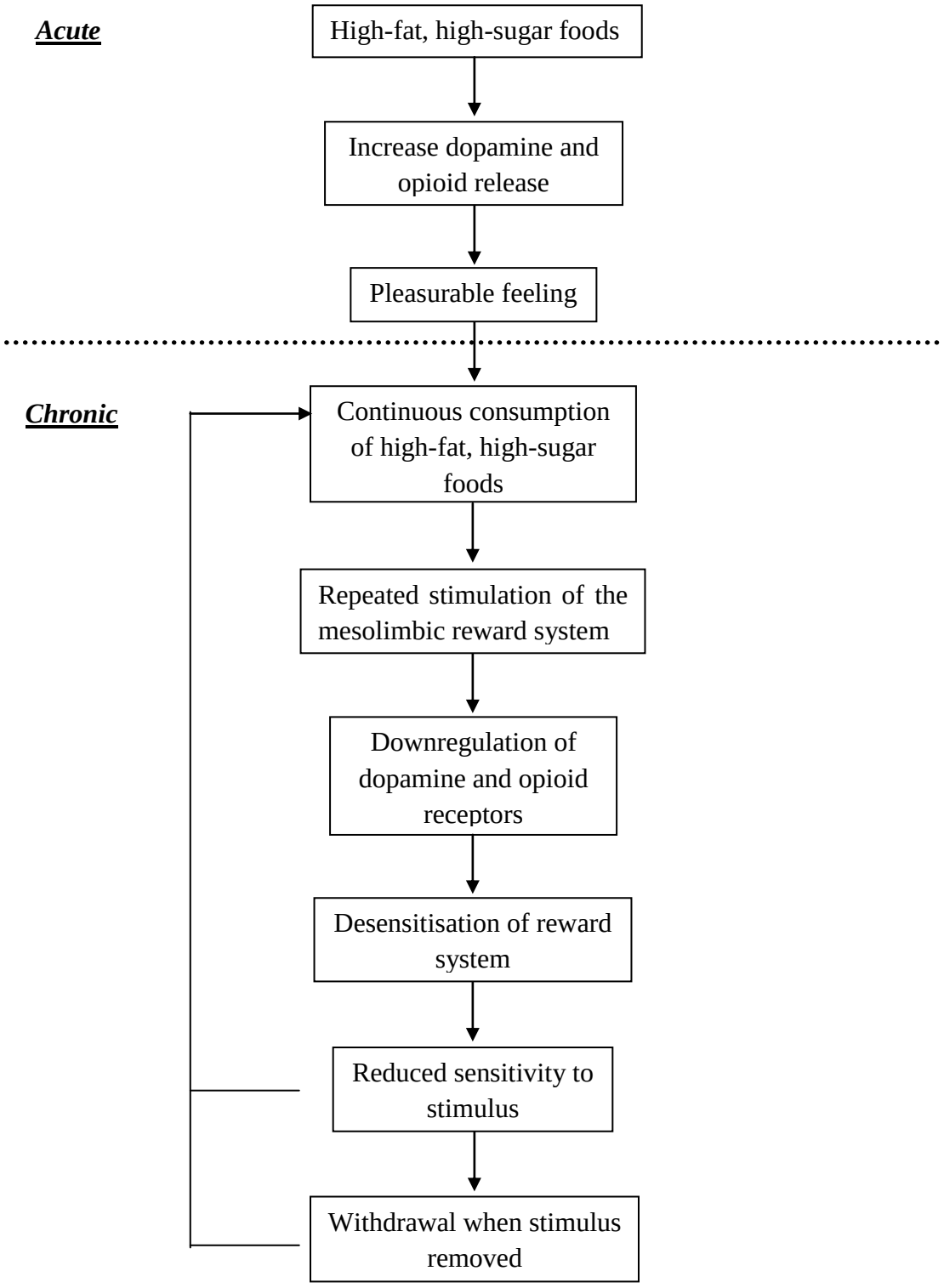
Whilst the specific signs of withdrawal appear to differ depending on the model of food addiction (high-fat diets, intermittent access to sucrose, cafeteria diet), it is clear that chronic high-fat, high-sugar foods intake impacts on dopamine signalling within the reward system which can potentially contribute to the development of 'food addiction', both at the molecular and behavioural levels (Figure 1.3).

1.2.3 Opioids and food intake

Another major class of neurotransmitter involved in the regulation of food reward within the mesolimbic reward system are the endogenous opioids including endorphin and enkephalin (100-102). Opioids can stimulate the intake of palatable foods by binding to the opioid receptors: μ, κ and δ; with the μ-opioid receptor subtype having the most significant role in the regulation of food intake and palatable food consumption (103). The μ-opioid agonist D-Ala2,Nme-Phe4,Glyol5-enkephalin (DAMGO) has been consistently shown to stimulate both normal food intake (102, 104, 105) and, more importantly, promote the consumption of palatable foods (106-109). Obesity prone rats, which are hyperphagic, have higher μ-opioid receptor expression in the hypothalamus and are more sensitive to DAMGO-induced high fat food intake (110). Conversely, but not surprisingly, the opioid antagonist, LY255582 (75) and μ-opioid antagonists, Naloxone (111, 112) and Naloxonazine (113), have been shown to inhibit palatable food intake. The effect of exposure to palatable foods on μ-opioid receptor expression and binding within the mesolimbic reward system has also been reported in numerous studies. Intermittent access to sucrose for 30 days resulted in a reduction in μ-opioid receptor binding in the NAc (76), an indication of reduced opioid signalling following repeated exposure to sucrose. Similarly, chronic high-fat feeding in rodents has also been associated with reduced μ-opioid receptors expression in both the NAc and VTA (114). Therefore, it is postulated that prolonged activation of the opioid signalling system could result in repeated receptor binding which eventually lead to the downregulation of opioid receptors, and presumably reduced sensitivity to subsequent opioid stimulation (Figure 1.3).

Interestingly, in naïve rats which have never been exposed to a palatable diet, when μ-opioid receptors were chronically blocked by prolonged administration of a μ-opioid receptor antagonist to the nucleus accumbens, the rats consumed less palatable foods, did not gain excessive body weight and were not fatter than their control counterparts (115). Similar observations were also reported in a μ-opioid receptor-deficient mouse model, which were shown to be resistant to diet-induced obesity (116, 117). This is in contrast to desensitisation of the reward system where chronic exposure to the palatable foods and repeated stimulation of the reward system is required to result in receptor downregulation. These obese-resistant rats are naïve and have no prior exposure to palatable foods. It is possible that when the reduced μ-opioid receptor is inherent or pharmacologically induced, the reduction in palatable food intake is due to reduced reward signalling. The limitation of the knock-out studies however is that μ-opioid receptor knock-out was not region-specific. It is therefore not possible to say with certainty whether the effects observed with the μ-opioid receptor deficient mouse model were exclusively due to the knock-down of μ-opioid receptors in the mesolimbic reward system or a combination of the effects of reduced μ-opioid receptors both peripherally as well as centrally. Nevertheless, these studies provide valuable insights and support for the role of μ-opioid receptor in the regulation of palatable food intake and reward, and the potential contribution of altered function of the reward system to the development of obesity.

Figure 1.3



A proposed mechanism of the development of palatable food addiction in response to acute and chronic high-fat, high-sugar intake

1.2.4 The relationship between dopamine and opioids in the mesolimbic reward system

As indicated above, opioids can activate the release of dopamine, at the VTA and the presence of dopamine and opioids within the mesolimbic reward system suggests that these neurotransmitters may interact synergistically to elicit rewarding effects. The increase in food intake initiated by DAMGO has been shown to be inhibited by simultaneous infusions of dopamine D1 receptor antagonist, SCH 23390 to the NAc and VTA, but not by infusions of dopamine D2 receptor antagonist, Raclopride (102, 105). Similarly, intra-VTA infusion of the μ-opioid antagonist, Naloxonazine, inhibited palatable food intake and morphine-induced dopamine release at the NAc (113), thus supporting the role of opioids in the VTA in activating dopamine neurons.

Whilst these data suggest that there may be an interaction between dopaminergic and opioid systems in regulating the rewarding properties of food, the precise relationship between these two neurotransmitters remains poorly defined. In fact, a study by Will and colleagues suggested that dopamine and opioids acted independently in the regulation of food intake, since neither D1 nor D2 receptor blockade within the NAc was able to block DAMGO-induced food intake (108), which is in complete contrast to the results presented in the previous paragraph. These differences may be attributed to different doses, sites and timing of drug administration and indicates that more research is required to fully elucidate the clearly intricate relationship between the dopamine and opioids in the regulation of palatable food intake.

1.2.5 Leptin, insulin and the mesolimbic reward system

Understanding the processes which are involved in the development of ‘addiction’ to palatable foods can be challenging due to the likely involvement of multiple peripheral and central signals involved. Two of the more common peripheral signals known to interact with the mesolimbic reward system are leptin and insulin.

Leptin is a hormone secreted by the adipose tissue. Circulating leptin is directly correlated with body fat mass and thus plays a role as a sensor of the body’s energy status (118, 119). Leptin’s role in the control of food intake at the hypothalamic level has been well-documented. Leptin inhibits appetite by binding to leptin receptors located on arcuate nucleus neurons within the hypothalamus. In addition to the hypothalamus, leptin receptors are also localised on VTA dopamine neurons and the binding of leptin to its receptors at the VTA modulates dopamine-induced food intake (120, 121) via the Jak-2 signalling

pathway (122). Leptin acts by decreasing the firing rate of dopamine neurons, which contributes to a reduction in food intake in rats after leptin infusions to the VTA (121). Infusion of leptin into the lateral ventricle of rats has also been shown to reduce extracellular dopamine levels and feeding-induced dopamine release at the NAc (123). In addition to reductions in food intake, leptin's actions at the VTA also appear to play an important role in regulating the intake of palatable foods. Using a two bottle preference test where rats were given a choice between water and 0.2%w/v sucrose solution, rats with leptin receptor knockdown at the VTA had an increased preference for sucrose solution. Furthermore, when the diet of these rats was switched from a standard rat chow to a high-fat chow, the leptin receptor knockdown rats became hyperphagic, whereas control rats showed no difference in the consumption of standard rat chow and high-fat chow (121). Taken together, these evidence demonstrated that leptin not only mediates food intake but also regulates the rewarding properties of food by inhibiting dopamine neurons, reducing dopamine release and thus effectively decreasing the rewarding potency of palatable foods.

It also appears however, that the ability of leptin to inhibit palatable food intake can be compromised under certain circumstances, for example leptin resistance induced by chronic overfeeding. In rats chronically fed on a high-fat diet, intraventricular administration of leptin failed to decrease sucrose self-administration, suggesting that chronic exposure to a palatable diet can induce leptin resistance at the level of the central reward pathway (124). Indeed, Matheny and colleagues demonstrated that chronic high-fat feeding resulted in reduced signal transducer and activator of transcription 3 (STAT3) phosphorylation in the VTA of rats, which is an indication of impaired leptin signalling and hence leptin resistance within the mesolimbic reward system (125). Whilst leptin appears to inhibit dopamine release in rodents, leptin deficient ob/ob mice exhibit reduced dopamine release upon electrical stimulation of dopaminergic cells (126), which is explained by lower availability of vesicular somatodendritic dopamine stores compared to their wild type counterparts (127). The reason for this is still unclear but it is possible that leptin deficiency resulted in an impaired development of the dopamine neurons within the reward system during early in life. These studies collectively highlight that the relationship between leptin and the mesolimbic reward system is likely to be altered by chronic palatable food intake.

Interestingly, in one study, rather than inhibiting dopamine neurons, acute leptin administration in control rats increased amphetamine (AMPH)-induced dopamine release

in the NAc via an increase in TH and DAT activity (128). This suggests that leptin is also capable of regulating dopaminergic activity at the level of the NAc by stimulating dopamine release. This finding seems to be in contrast to previous studies in which leptin inhibited dopamine neurons in the VTA and hence reduced dopamine release to the NAc (121). Whilst the specific mechanism of action for leptin within the mesolimbic reward system, particularly at the level of the NAc remains uncertain, it is clear that leptin plays a crucial role in modulating dopamine release by altering the dopamine system within the mesolimbic reward pathway in response to palatable diets, and is therefore important in the regulation of food reward.

Similar to leptin, insulin receptors have also been shown to co-localise with dopaminergic neurons in the VTA (120). Insulin is an important sensor of the energy status within the body and binds to its receptors within the hypothalamus to control appetite. Whilst the role of insulin in the regulation of food reward is not fully understood, the presence of insulin receptors on VTA dopamine neurons indicates a potential role of insulin in modulating dopamine activity within the mesolimbic reward pathway. Indeed, insulin has been shown to increase expression and activity of DAT, which in turn results in reduced dopamine activity (129). This reduction in dopamine activity may be one mechanism by which insulin administration decreases sucrose self-administration (124) and inhibits high-fat diet conditioned place preference (a test which associates a rewarding stimulus with an environment. Typically, the animal is placed into two environments, one associated with the rewarding stimulus and one without. Their 'preference' for the environment will be determined, in the absence of the stimulus.) (130). Hypoinsulinaemia, as a result of food restriction or drug administration (131, 132); or insulin resistance, due to chronic high-fat diet intake (133), are each associated with reduced striatal dopamine clearance and DAT function. Interestingly, the high-fat intake of insulin resistant diet-induced obese rats was reversed when insulin signalling was restored by viral injection of insulin receptor substrate 2 (IRS2) (133). These studies therefore provide evidence for the role of insulin in modulating mesolimbic dopamine activity and that insulin is one important peripheral signal linking palatable food intake with neuronal adaptations within the mesolimbic reward system in the regulation of food reward.

1.2.6 Summary

The effects of palatable food intake on the mesolimbic reward system and the reduced sensitivity of the reward system associated with prolonged consumption of these foods

may shed some light as to why many obese individuals experience difficulty in controlling their food intake, particularly high-fat, high-sugar foods, despite knowing their negative health consequences (134). Differences in the response to the rewarding properties of food and the possibility of leptin and insulin resistance in obese individuals may also explain why obese individuals are more likely to consume more high-fat foods when compared to lean individuals (135). The interaction between the mesolimbic reward system and palatable food intake is evident in adults. However, the question of whether palatable food intake during pregnancy and lactation can alter the development of the mesolimbic reward system and subsequent preference for palatable foods in the offspring later in life requires understanding of the development of the mesolimbic reward system.

1.3 Development of the mesolimbic reward system

The development of a number of systems in the brain has been well-characterised in rodents, in particular the appetite regulatory network. It is important to note that, whilst the majority of studies investigating the mechanisms underlying programming of food intake and food reward have used rodent models, rodents and humans have distinct brain developmental patterns. Based on brain morphology, the rat brain at embryonic days 11-21 is equivalent to that of the human brain at embryonic weeks 4-16 (136). Unlike humans, the development of the brain in the rodent extends into post-natal life and the first week of post-natal life in the rodent in relation to brain development, is approximately equivalent to the third trimester in humans (137). The development of the appetite regulatory network in the rodent begins as early as embryonic day 12 (E12) (138) and does not fully mature until at least post-natal day 18 (P18) (139); in humans however, the development of the appetite regulatory network is believed to occur during the third trimester (140). The ontogeny of the mesolimbic reward system in the rodent follows the similar pattern to that of the appetite regulatory network such that dopamine and opioid systems reaches maturity during the third week of post-natal life. This information is less clear in humans.

1.3.1 Dopamine

Mesolimbic dopamine neurons originate from the VTA and project to the NAc. In the rodent, the development of dopamine neurons in the midbrain (which includes the VTA) begins as early as E11 in the mouse (141) and E12 in the rat (142). These neurons migrate to the forebrain, towards the striatum (which includes the NAc) at around E15 to E16 (143). Dopamine neurons do not fully differentiate until P0, which means that prior to this, dopamine neurons projecting from the midbrain to the forebrain consist of neurons from

both the substantia nigra (SN) and VTA. By P12, the configuration of dopamine pathways is similar to that of an adult rodent (144).

In the striatum, where the projections of the dopamine neurons from the VTA terminate, neurogenesis of the efferent neurons begins at E12 and continues throughout embryonic life. Synaptic connections are detectable at birth and are mainly axodendritic (axon-dendrite). A small number of axosomatic (axon-soma) connections are formed during the first 2 post-natal weeks (145). During the second post-natal week, maturation of efferent medium spiny neurons occurs; the number of spines on the neuron increases drastically and peak during the second post-natal week, resulting in the formation of more synaptic connections in the NAc (145). Therefore, from the third week of post-natal life, there is a substantial increase in the number of axospinous (axon-spines) connections. The morphological changes of the medium spiny neurons in the striatum parallel the maturation of the dopamine system, indicating that the dopamine system is relatively mature during the second post-natal week (145). By the end of the third week of post-natal life, synaptic connections, the morphology of dopaminergic neurons and its efferent targets are similar to those in the adult rodent (145).

The presence of synapses at birth implies that dopamine receptors could also be formed prior to birth and may potentially be functional from birth. The mRNA expression for both the D1 and D2 receptors is detected as early as E14 in the striatum and expression levels continue to increase throughout the embryonic period (146, 147). D1, D2 mRNA expression subsequently declines at birth, increases again after P5 and peaks at P28 (148). Whilst the receptors are expressed pre-natally, D1 and D2 receptor binding does not appear to develop until the post-natal period, suggesting that that ability of rat fetuses to respond to dopamine *in utero* is limited (147).

The question remains as to whether or not these receptors are present in the VTA at similar stages of development as in the striatum, as D2 autoreceptors within the VTA also play an important role in the regulation of dopamine synthesis and release. The ontogeny of DAT expression and functionality, an important regulator of dopamine turnover within the mesolimbic reward system, during the pre-natal and/or post-natal period also remains to be investigated.

The majority of the studies on the ontogeny of the mesolimbic reward system have been conducted in rodents. The availability of human tissue for the investigation of the development of the reward system is very limited. Nevertheless, there is evidence that in humans, development of the dopaminergic system begins as early as 6 to 8 weeks of gestation (149) and by 12 weeks of gestation, TH protein and D1 mRNA levels are already detected in the fetus striatum (150, 151). D2 receptors and DAT are identified in the fetus striatum only at 21 and 32 weeks of gestation respectively (152). It is possible that in humans, the development of the dopaminergic reward system is not complete until late in gestation.

1.3.2 Opioids

As with dopamine, the development of the opioid system in a rodent also occurs before birth and continues into post-natal life. Endogenous opioids including endorphin and enkephalin are detected throughout the brain during early embryonic period and increase to levels similar to that of an adult during early post-natal life (153, 154). The endorphin and enkephalin systems are believed to follow distinct developmental trajectories. Immunoreactivity of endorphin is detected as early as E13 in the rat brain and increases steadily into post-natal life, whilst enkephalin only appears later in development (E16) at very low levels, and then increases rapidly through the remainder of gestation into post-natal life. The development of these opioid systems is also region specific. In the striatum, the increase in endorphin concentrations is marginal from E16 to P25, whereas enkephalin concentrations undergo a 50× increase across the same period. In the medulla and midbrain however, endorphin concentrations increase at a greater rate across gestation when compared to enkephalin (153). It therefore appears that the development of both the endorphin and enkephalin systems is independent of each other.

Functional opioid receptors are also detected during embryonic life with μ-opioid receptor binding present in the striatum as early as E14 and reaching adult levels at birth (155). The different opioid receptor subtypes, μ-, κ- and δ- develop at different stages of pre- and post-natal life. μ-opioid receptor density peaks at P2 in the NAc and subsequently decreases to adult levels. Conversely, κ- opioid receptors are expressed at low levels at P2 and decrease further across early post-natal life. δ-opioid receptors, on the other hand, are sparse at P2 but gradually increase in density to reach adult levels during the third post-natal week (154, 156).

In humans, the development of the opioid system is less well-defined. Available evidence indicates that the endogenous opioids, β-endorphin and encephalin were respectively detected in the striatum during early gestation (149) and at 12 weeks gestation (151). The presence of opioid receptors during development was tested using ligand binding studies and it was found that μ- and κ-opioid receptors were already available for binding at 20 weeks of gestation (157).

1.3.3 Summary

Ontogeny of the dopamine and opioid signalling systems have been described in several studies. However until now, our understanding of the development of these systems is still inadequate. In addition, the factors regulating the development of the mesolimbic reward system are not well-understood, including whether leptin plays a role in axonal outgrowth in development of the reward system as seen with the appetite regulating neural network (158). A better understanding of the development of dopamine and opioid systems will be of considerable value in helping to determine how perturbations during the critical developmental periods can alter dopamine function/structure of these systems in the offspring.

1.4 Mechanisms underlying programming of food preferences

It is known that the development of the dopamine and opioid systems in the rodent begins before birth and continues into the first few weeks of post-natal life. As a result, any perturbations such as stress or changes in maternal diet, during these periods have the potential to alter the development of this system. In the context of the current obesogenic society, one such perturbation would be the prolonged exposure to maternal high-fat, high-sugar diets during pregnancy and lactation. Given that palatable food intake is associated with the activation of the mesolimbic reward system in adults, it has been suggested that maternal palatable diets during pregnancy and lactation can alter the development of the reward system in the offspring, resulting in persistent changes within the reward circuitry which can then program the higher preference for foods high in fat and sugar in post-natal life.

1.4.1 Maternal nutrient intake and mesolimbic reward system in the offspring

The first study demonstrating the effect of altered maternal nutrient intake on the mesolimbic reward system of the offspring was conducted by Naef and colleagues in 2008 (159). In this study, offspring of dams maintained on a high-fat diet during the last week of

gestation and lactation had higher concentrations of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the NAc, and higher TH in the VTA at P90, compared to their control counterparts (159). These offspring of high-fat fed mothers displayed reduced sensitivity to amphetamine-induced locomotion. Amphetamine acts by stimulating dopamine signalling to increase locomotor activity. Reduced sensitivity to amphetamine therefore implies that these offspring have reduced dopamine sensitivity despite having elevated dopamine levels in the brain. In contrast, Shalev and colleagues found that offspring of dams fed a palatable liquid diet from before pregnancy until the end of lactation exhibited a higher amphetamine-induced locomotor activity response when weaned on a palatable diet, but not when weaned on a control diet (160). This higher amphetamine-induced locomotor activity in adulthood was also reported in another study where offspring were exposed to palatable diets either *in utero* or during suckling (161). These differences in response to amphetamine may be due to the different composition of maternal diet, since Naef and colleagues used a diet which was exclusively high in fat while the studies of Shalev et al and Bocarsly et al used a diet which was high in both fat and sugar. Despite the conflicting results, these studies support the role of maternal diet in altering the development of the mesolimbic reward system of the offspring; the magnitude and direction of the change however, remains to be further clarified.

In addition to functional changes, existing studies have also demonstrated changes in key genes within the mesolimbic reward system of offspring previously exposed to a maternal high-fat diet. Using the same model of maternal high-fat feeding, Naef and colleagues showed that adult offspring of high-fat fed dams had lower D2 receptor expression in the VTA compared to controls, which supports their previous hypothesis of lower dopamine sensitivity in offspring of high-fat fed dams. Importantly, these offspring of high-fat fed dams also exhibited an increased preference for high-fat foods and were willing to work harder to obtain the palatable foods than offspring of control dams, demonstrating for the first time that changes in key components of the reward system induced by maternal overfeeding was associated with an increased preference and motivation to obtain high-fat foods (51). Similarly, in mice, offspring of dams fed on a high-fat diet throughout pregnancy and lactation were shown to have an increased preference for fat and sucrose at 6 months of age. These offspring also had higher DAT mRNA expression in the VTA and NAc, and lower TH, D1, D2 and dopamine- and cAMP-regulated phosphoprotein (DARPP-32) in the NAc, indicating that the sensitivity of the dopamine system in these offspring was reduced. These offspring also exhibited higher expression of

preproenkephalin (PENK) and μ-opioid receptor in the NAc (53), suggesting an increase in opioid sensitivity during adulthood. The authors of this study postulated that this increase in opioid molecules may be mediating the changes in dopamine gene expression in the adult offspring of high-fat fed dams. An alternate possibility is that a maternal high-fat diet has differential impacts on the opioid and dopamine systems in the offspring during development early in life. This however, remains to be fully investigated.

As indicated previously, the preference for high-fat foods due to maternal diet was first demonstrated in a maternal low-protein diet model. Whether a low protein diet during pregnancy and lactation can affect the reward system of the offspring in a similar manner to that of maternal high-fat feeding is however, uncertain. Using a maternal low protein diet model, Vucetic and colleagues found that offspring born to protein restricted dams (8.5% protein versus 18% protein in a control diet) had higher TH and DAT expression in the NAc and VTA in adulthood (162), which indicates higher dopamine synthesis and turnover. These offspring also had a lower preference for sucrose (162). Fat preference was however, not measured in this study. At the same time, the response of offspring to cocaine was enhanced (162), which could be explained by the increase in DAT since DAT is a key target for cocaine within the mesolimbic reward system. In summary, this study demonstrated that a maternal low-protein diet can alter dopamine gene expression within the mesolimbic reward system. Although both the low protein model and the high-fat, high-sugar feeding model may share some similarities in relation to the programming of a similar phenotype, the underlying mechanisms are likely to be different between models.

1.4.2 Post-weaning programming of food preferences and the mesolimbic reward system

Whilst the studies above provided evidence that maternal nutrient intake during pregnancy and lactation can alter the development of neural systems regulating food intake and reward in the offspring, recent studies have suggested that the programming of food preferences can also be influenced by dietary manipulation beyond the lactation period. Teegarden and colleagues reported that exposure to a high-fat diet at 3 weeks of age could program an increased preference for high-fat foods during adulthood, and this was not observed for mice exposed to a high-carbohydrate diet at the same age. This preference for high-fat foods was associated with changes within the mesolimbic reward system characterised by cyclin dependent kinase 5 (Cdk5; responsible for phosphorylation of DARPP-32) and DARPP-32 upregulation in the NAc. DARPP-32 inhibits D1 receptor activity, therefore the increase in Cdk5 and DARPP-32 indicates reduced dopamine

signalling, and suggests that the increased preference for high-fat foods may represent a compensatory response to increased dopamine signalling transmission (52). The authors of this study argued that the impact of high-fat feeding in the post-weaning period on dopamine signalling was unlikely to be related to changes in the development of the reward system, as the development is thought to be completed by the end of the third week of post-natal life, and suggested that the changes observed may be due to epigenetic modifications. However, this was not demonstrated experimentally. Whilst epigenetics may be playing a role, it is also clear from other studies that neural systems retain a high degree of plasticity well into the post-natal period (at least 8 weeks of age), and are therefore prone to nutritional manipulations well beyond weaning. This was demonstrated by Pinto and colleagues where leptin administration to leptin-deficient ob/ob mice at 8 weeks of age resulted in changes in NPY and POMC synaptic density and profiles in these ob/ob mice such that they were similar to that of a wild type mouse (163). The ability of leptin to rewire the arcuate nucleus at 8 weeks of age suggests that the neural pathways in the brain still retain some degree of plasticity post-weaning. It is however unclear if this is the case for the mesolimbic reward system.

These studies indicate that the post-weaning diet as well as nutritional exposures earlier in development can affect food preferences in later life. The mechanisms which underlie the effects of the pre-natal and/or early post-weaning nutritional environment on the development of the mesolimbic reward system are however, yet to be clearly defined. However, certain hormones, including leptin and insulin have been shown to be possible factors involved in the development of neural systems.

1.4.3 Role of leptin in the development of neural systems

The role of leptin in the developing neonate is distinct from that of the adult, since leptin levels during early neonatal period do not correlate with food intake or body fat mass (164). Moreover, administration of leptin during the early post-natal period in the rodent also fails to alter food intake (165, 166). Neonatal rodents have a unique pattern of plasma leptin levels throughout the suckling period which is independent of milk intake, in which plasma leptin levels increase progressively during the first post-natal week, before peaking during the second post-natal week and then declining to adult levels by weaning (41, 164). The increase and subsequent peak in leptin levels during the first and second post-natal week is also known as leptin surge. The physiological role of the leptin surge and the factors which regulate it is however still unclear. The fact that leptin deficient ob/ob mice have been

shown to display neuronal structural abnormalities, impaired brain myelination (167, 168) and disrupted projections of the appetite regulating neurons within the hypothalamus (158), it is likely that leptin, and the leptin surge play an important role in neuronal development and function, including the development of the appetite regulatory circuits.

Bouret and colleagues carried out a seminal study looking at the development of the appetite regulatory network in ob/ob mice and the ability of leptin to restore the projections from the arcuate nucleus that are normally absent in the ob/ob mice (158). They found that leptin administration from P4 to P12, which mimics the leptin surge that occurs in wild type mice, restored the arcuate projections to the paraventricular nucleus in the ob/ob mice by stimulating axon elongation and outgrowth. However, leptin treatment during adulthood (P80) failed to result in any restoration of these pathways (158). These studies demonstrated clearly that the action of leptin in neuronal development is limited to the early post-natal period during which the hypothalamic pathways are actively developing, suggesting a possible role of the leptin surge.

Whilst the mechanisms underlying the leptin surge remain unclear, it has been shown that the timing, magnitude and duration of leptin surge can be influenced by maternal diet during pregnancy and lactation. In pre-natally undernourished offspring, the post-natal leptin surge occurs prematurely (169), and leptin levels attained during the surge are significantly reduced compared to their control counterparts (170). This impaired leptin surge has been associated with reduced POMC gene expression as well as altered POMC neuronal projections from the arcuate nucleus (170), which may contribute to impaired control of food intake and the development of metabolic disorders later in life.

This impaired leptin surge can be restored to levels similar to that in control rodents by exogenous administration of leptin to the prenatally undernourished rodents from P3 to P10. This treatment regimen prevents the development of obesity and insulin resistance in undernourished offspring (171). Interestingly, when neonates of control dams were administered leptin during a time which mimicked the premature leptin surge seen in offspring of undernourished dams, these offspring became glucose intolerant (172), gained more weight and had higher serum leptin levels than their control counterparts when both groups were put on a high-fat diet during post-natal period (169). This further confirms the association between premature leptin surge and the predisposition to obesity and highlights

the importance of leptin levels during the first two weeks of life in the programming of metabolic disorders later in adulthood.

Exposure to excess nutrients *in utero* and during the suckling period can also lead to altered leptin surge in the offspring. In rats, Kirk and colleagues demonstrated that offspring of high-fat, high-sugar fed dams exhibited a prolonged leptin surge throughout the suckling period and had higher leptin levels than the control offspring from P7 onwards (41). These offspring also had reduced projections from the arcuate nucleus to the paraventricular nucleus of AgRP neurons in the offspring of overfed dams at P30, similar to that observed in offspring of undernourished dams. This is unexpected given that leptin aids in axonal elongation and outgrowth, and that low leptin levels during the leptin surge and a premature leptin surge have been shown to impair projections from the arcuate nucleus to the paraventricular nucleus. One possibility, however, is that these offspring have developed central leptin resistance due to excess leptin exposure from maternal sources pre and peri-natally, therefore limiting the ability of leptin to stimulate neuronal projections within the appetite regulatory system. The finding that leptin administration at P30 failed to reduce food intake and body weight of the pre-natally overnourished offspring further supports the postulation that these offspring already have some degree of leptin insensitivity during the early post-natal period (41). This is supported by Vickers and colleagues in which leptin administration to male neonates of control rat dams from P3 to P13 resulted in increased fat mass and hyperinsulinaemia in these offspring during adulthood (173). In contrast, Pico and colleagues demonstrated that oral administration of leptin to offspring of rat dams fed on a control diet during lactation resulted in offspring with lower body weight, less fat deposition, reduced caloric consumption and improved insulin sensitivity throughout life (174, 175). These offspring were more leptin sensitive and were resistant to diet-induced obesity (174). This is somewhat surprising as it is in complete opposition to previous studies and especially since high leptin levels during lactation is postulated to result in leptin resistance and impaired neuronal organisation within the brain. The source of these different findings is not well-understood but may be due to the different timing and dose of leptin administered to the offspring. Furthermore, the high-fat, high-sugar content of the maternal diet together with the high leptin and insulin levels present in maternal milk in the maternal overnutrition model which is absent from the oral leptin administration studies (174, 175), may also be driving the early onset of obesity in the offspring of overnourished dams.

Leptin plays a crucial role during the early post-natal period in regulating the formations of neuronal projections from the arcuate nucleus to the paraventricular nucleus which are critical for the regulation of appetite (158). Exposure to either deficient (169, 170) or excess (41) leptin concentration during the development of these neuronal connections can contribute to the increase risk of obesity later in life. If the effects of leptin on the regulation of the development of the appetite regulatory neural network can be translated to the development of the mesolimbic reward system, it is possible that too much or too little leptin, together with high-fat and high-sugar levels in the maternal milk during the peri-natal period, may adversely impact on the development of the mesolimbic reward system. This could then result in irreversible changes within the reward system and an increased preference for high-fat foods throughout life.

1.4.4 Role of insulin in the development of neural systems

Insulin plays an important role in the differentiation and maturation of neurons within the central nervous system (176) and insulin receptors have been identified on neurons and astrocytes during early neuronal development (177). As a result, exposure of neuronal systems to inappropriately high or low insulin levels early in life has the potential to impact on neuronal development in the offspring. Indeed, there is evidence that the hypothalamic appetite regulatory system is adversely affected by exposure to altered insulin levels during the peri-natal period.

Small litter rearing (3 pups/litter) to induce early post-natal overnutrition in rodents has been associated with altered development of the hypothalamic appetite neural network (178). In these offspring, high levels of insulin within the hypothalamus have been reported at day 14 of life when compared to offspring of normal litters. These offspring have malformations of galanin neurons within the hypothalamus in adulthood, thus it is possible that elevated insulin levels prevented normal hypothalamic development in these offspring (178). Similar to the offspring from small-sized litters, offspring of diabetic dams which are hyperinsulinaemic have an altered organisation of the hypothalamic appetite neuronal network (45, 47). This impaired formation of the hypothalamic appetite system was also reported in offspring suckled by diabetic dams, therefore highlighting the importance of perturbations during the lactation period as well as before birth when the neural systems are still actively developing (179).

Given that both insulin and leptin are adiposity signals that regulate energy homeostasis and that insulin resistance is often accompanied by leptin resistance (180), it is not surprising that in diabetic dams and their offspring, circulating leptin levels are higher than that of their control counterparts (45). Therefore, the possibility of leptin playing a role in the malorganisation of the hypothalamic appetite network in the offspring of diabetic dams cannot be ruled out. Nevertheless, intrahypothalamic insulin administration at P8 has been shown to lead to alterations in the morphology of hypothalamic nuclei after 7 days of treatment (181). This provides direct evidence that insulin is one of the major hormones involved in the development of the hypothalamic neurons and that perhaps, both insulin and leptin may be acting in concert with each other during brain development.

1.4.5 Summary

Given that the timing of development of the mesolimbic reward system is somewhat similar to that of the appetite regulatory neural network within the hypothalamus, the potential exists that changes in leptin and insulin levels during development may also impact on the development of the mesolimbic reward system. The role of maternal diet and peripheral factors on the development of the mesolimbic reward system warrants further investigation.

1.5 Reversal of maternal programming of offspring obesity

Previous studies have indicated that exposure to an inappropriate nutrient supply before birth and early in life can result in permanent changes within the systems regulating appetite and energy homeostasis, therefore programming the development of obesity in adult life. A growing number of studies have suggested that there are critical windows of developmental plasticity which not only represent the time during which individuals are most sensitive to nutritional insults but also a potential window of opportunity during which these programmed effects may be reversed.

1.5.1 Critical windows for the programming of food intake and obesity – Evidence from human studies

In a well-known historical cohort of 300,000 19 year old men whose mothers were pregnant during the Dutch famine 1944 to 1945, exposure to the famine during the first half of gestation was associated with a higher incidence of obesity in adulthood compared to those exposed during the last trimester and first few months after birth (182). This observation indicated that there were critical windows of development during which

exposure to nutritional insults had a particularly potent effect on an individual's susceptibility to obesity later in life. The authors of this study suggested that the reduced nutrient supply during the first half of gestation may have affected the initial differentiation of the hypothalamic appetite regulating centres which subsequently contributed to the dysregulation of appetite control and a heightened obesity risk. Indeed, adults exposed pre-natally to the Dutch Famine during the first trimester have a higher preference for high-fat foods compared to those who were not exposed, or those exposed to the famine during mid and late gestation (56). In contrast, Stein and colleagues found no difference in energy intake between individuals exposed to the famine *in utero* during the early, mid or late gestation (183). However, in the same study, when compared to sibling controls, those exposed to the famine *in utero* (regardless of the timing of exposure) had higher energy intake and lower physical activity (183). Therefore, it appears that offspring exposed to famine are more likely to overconsume and have an increased risk of developing obesity as adults, and possibly a higher susceptibility in those exposed to the famine during early gestation.

In addition to the Dutch Famine, several studies have also reported on the impact of the Chinese Famine. The Chinese Famine (1959 to 1961) is the most severe famine in history with a conservative estimate of 30 million deaths during that period (184). Food rations were unequally distributed, with significantly less food provided to the rural areas, however the exact values of caloric intake were not reported (185). Li and colleagues compared adults who were exposed to the Chinese Famine (1959 - 1961) *in utero*, during early childhood (age 1 and 2), mid childhood (age 3 and 4) and late childhood (age 5 and 6) and showed that individuals who were exposed to the Chinese Famine either *in utero* and during the first 2 years of life each had an increased risk of developing metabolic syndrome and hypertension in adulthood. The risks associated with the development metabolic disorders later in life were further amplified with exposure to a western diet during adulthood (186, 187). The finding that undernutrition during the first 2 years of life may impact on the onset of metabolic syndrome contradicts that of the reports from the Dutch Famine cohort, which found lower obesity rates in adults exposed to the Dutch famine after birth. This may be due to the greater severity of the Chinese famine compared with the Dutch famine. Moreover, the Dutch famine cohort only included those who were exposed to the famine during the first few months of life, whereas in the Chinese cohort, the study was conducted on children from 1 to 6 years of age. Li and colleagues were the first to examine the consequences of post-natal exposure to the Chinese Famine and

suggested that there may be several critical windows for the programming of obesity, one of which is during early childhood. In addition, results from these studies also highlighted the importance of a mismatch between the pre-natal and post-natal diets in exacerbating the effects of pre-natal and early life undernutrition on the programming of later obesity in the offspring (188).

In modern society, there is an increased likelihood of an individual being exposed to pre-natal overnutrition rather than undernutrition. However, longitudinal studies on the effects of overnutrition *in utero* on the subsequent development of obesity are limited. Whether there are critical windows during which exposure to overnutrition in early life can program the onset of obesity and its associated metabolic disorders later in life is still unclear. Nevertheless, it was demonstrated that maternal macronutrient intake during pregnancy but not after pregnancy, was highly correlated with offspring macronutrient intake at 10 years of age (55). Therefore it is possible that in humans, maternal consumption of high-fat, high-sugar foods during pregnancy can program an increased preference towards these foods in the offspring later in life, thereby contributing to the development of obesity. Although the authors did not investigate the maternal macronutrient intake at different stages of pregnancy and lactation, it is possible that the potential window of programming may lie within the pregnancy period and not during post-natal life. In seeking to identify the critical periods of developmental programming, studies using rodent models, due to their short gestation and lactation periods, have provided indications that exposure to overnutrition during different windows of pre- and post-natal development may be associated with differential effects on offspring food intake and the predisposition to later obesity.

1.5.2 Critical windows for the programming of food intake and obesity – Evidence from rodent studies

The lactation period in a rodent has been deemed as one of the critical windows for programming, as it is during this period that neuronal circuits within the brain, including the appetite regulating centre and the mesolimbic reward system, are in the process of rapid development and maturation (145, 158). As a result, these systems retain a high degree of plasticity during the lactation period, and are therefore sensitive to nutritional alterations which occur during this period. In rodents, reducing the litter size to 3 or 4 pups after birth has been consistently demonstrated to be associated with persistent alterations in the structure and function of the hypothalamic appetite regulating system, which can increase food intake and body weight of the offspring throughout post-natal life (189, 190).

In response to a high-fat diet challenge, these offspring consumed more energy and gained more weight compared to control offspring (190), suggesting a heightened response towards high-fat foods which is not only due to alterations within the appetite regulating circuits mentioned above, but also possibly the rewiring of the mesolimbic reward system during the lactation period.

To investigate the critical windows of the programming of food preferences, Bayol and colleagues investigated the effects of junk food exposure on the offspring at 3 different periods, namely gestation, suckling and post-weaning. Not surprisingly, offspring exposed to a post-weaning junk food diet had higher energy intake than those weaned on a control diet, independent of whether or not they had been exposed to the junk food diet earlier in life. Importantly however, when compared to offspring suckled on a control diet, exposure to the junk food diet during suckling was associated with elevated intake of junk food after weaning,, when they were weaned on the same junk food diet. This highlights the importance of the lactation period for the programming of the response to nutritional excess and its contribution to the programming of increased energy intake and adult obesity. This study also showed that offspring exposed to a junk food diet *in utero* but suckled by a control dam had lower BMI and consumed less junk food when provided with free access to a junk food diet post-weaning (JCJ) compared to those that were suckled by junk food dams (40). The energy intake, BMI, body fat mass and post-weaning growth rates of these JCJ offspring were comparable to offspring born to and suckled by control dams but fed on a junk food diet post-weaning (40), indicating that introducing a control diet during lactation could potentially reverse at least some of the negative consequences of being exposed to a high fat diet earlier in development. The significance of the lactation period was also demonstrated in a cross-fostering study where offspring of obesity prone dams cross-fostered to obesity-resistant dams were shown to have improved insulin resistance when compared to those cross-fostered to obesity prone dams (191). Conversely, when offspring of obesity resistant dams were cross-fostered to obesity prone dams during lactation, they consumed more of a high-energy diet and became obese and insulin resistant during adulthood (191). These studies therefore arrived at the same conclusion that the lactation period in rodents represents a key critical window in the programming of appetite and obesity.

In addition to the lactation period, exposure to a high-fat diet exclusively before birth has also been shown to be associated with the programming of appetite and later obesity. Chen

and colleagues determined the effects of maternal obesity and litter size reduction on offspring metabolic health outcomes later in life (48). Offspring of dams fed a high-fat diet during pregnancy were heavier and consumed more energy compared to offspring of control dams, irrespective of the litter sizes in which they were reared during lactation and the post-weaning diet (48). In another study, offspring exposed to a high-fat diet (50% energy as fat) *in utero* were heavier, had higher caloric intake, serum triglycerides, non-esterified fatty acid (NEFA), leptin, insulin and body fat at P70 when compared to controls, regardless of whether the offspring were exposed to a control diet (25% energy as fat) or high-fat diet during suckling. Importantly, offspring exposed to the control diet *in utero* and cross-fostered to dams on the high-fat diet during the suckling period did not display characteristics of obesity (50). It is important to note that the control diet used in this study consisted of 25% energy as fat which is more than twice the level provided by the standard rat chow (~12% energy as fat). Although cross-fostering studies provide important information on the dissociation between the effects of insults during pregnancy and lactation, it has been recently shown in mice that the cross-fostering method itself may pose as a programming factor, where normal mice which have been cross-fostered develop cardiometabolic dysfunction during adulthood (192). Therefore, data obtained from cross-fostering studies should be interpreted with caution, at least in studies involving mice. Nonetheless, these studies suggested that diet during pregnancy may be playing a more significant role in the programming of food intake and obesity than diet during lactation, which is in contrast to prior studies (40, 189, 190). The differences between studies may be due to the use of different rodent models and dietary treatments. More work is therefore required to dissociate the *in utero* and post-natal effects of maternal palatable diets on offspring food preferences, in order to better define the optimal periods for intervention.

1.5.3 Interaction between post-weaning and maternal diet

Previous studies have mainly focussed on the impact of maternal diet on the programming of offspring obesity and have provided suggestions that the adverse offspring health effects due to an inappropriate nutrition early in life are largely irreversible. There is however evidence showing that intervention applied during the postnatal period, including leptin supplementation during the juvenile period, can rewire the appetite regulatory network in ob/ob mice, suggesting that the critical developmental windows may extend into the post-weaning period (163). Emerging studies have thus provided suggestions that the post-weaning diet may, in some cases, have the potential to modulate the effects of nutrition exposures experienced during the perinatal period and therefore play a part in determining

the subsequent risk of obesity in the offspring. Indeed, introducing a high-fat diet post-weaning has been shown in several studies to exacerbate the offspring obesity phenotype associated with maternal high-fat diet such as increased body weight and food intake, regardless of whether the offspring were exposed to a high-fat diet *in utero* (43), during lactation (193) or both (40, 48, 190).

The studies suggesting that a post-weaning high-fat diet can exacerbate the impact of perinatal overnutrition on offspring metabolic health outcomes have led to the postulation that a post-weaning low-fat diet could ameliorate the effects associated with maternal diet and obesity on the offspring and this has now been examined in a number of studies. In one such study, offspring exposed to a palatable diet throughout pregnancy and lactation were found to be lighter, have a lower BMI, consume less energy (40) and have lower fat mass (160, 194, 195) compared to offspring weaned on a palatable diet. The phenotype of these offspring weaned on a control diet was similar to offspring which had never been exposed to a palatable diet (40, 160, 194). Similarly, Velkoska and colleagues found that after being weaned on a control diet, offspring from a reduced sized litter group were no longer heavier and hyperleptinaemic when compared to those from the control litter groups. However, these effects were only observed during adulthood (196). These studies provided some indication that an appropriate post-weaning diet could potentially ameliorate at least some adverse effects of exposure to an inappropriate supply of nutrients in utero and/or early in life.

In one study, offspring of dams fed a cafeteria diet before pregnancy, throughout pregnancy and lactation were shown to be leptin resistant at P30, and this effect persisted until at least P90 despite being weaned onto a control diet (41). Several other studies have also shown that whilst the young offspring of obese dams appear lean and glucose tolerant (41, 190), adverse metabolic health outcomes in the offspring such as increased body fat mass, body weight and plasma leptin levels, emerged later in adulthood, even after long term exposure to a control diet post-weaning (41, 42, 190). This indicates that the obesity phenotype may not be apparent during early in life, however, may emerge with advancing age and that this effect may be exacerbated by exposure to metabolic insults (eg. a high-fat diet) during post-natal life. The inconsistencies between studies may be due to different rodent models used, parameters analysed, the presence of post-weaning nutritional insults and the period of which the parameters were investigated. Whether the effects of maternal overnutrition, including the increased preference for palatable foods, can be ameliorated

with a healthy post-weaning diet remains to be established. Nevertheless, various studies have been carried out to reverse the impact of maternal obesity on offspring health outcomes through dietary interventions applied before and during pregnancy.

1.5.4 Dietary interventions for reversing the programmed development of obesity

Given that most overweight or obese pregnant women are usually overweight or obese even before pregnancy, an increasing number of studies have attempted to determine if a dietary intervention prior to conception can reverse any adverse events in the offspring associated with maternal obesity.

Gallou-Kabani and colleagues conducted a study in a rodent model where obese dams were given a control diet during the peri-conceptual, gestation and lactation periods (197). They found that half of the female offspring of these dams were resistant to a high-fat diet such that their caloric intake was not different from that of controls and when compared to their fat-sensitive littermates, they were not hyperinsulinaemic and did not gain as much weight (197). Similarly, Zambrano and colleagues found that by giving obese dams a control diet one month prior to conception, offspring of these dams at P21 were leaner than offspring of obese dams fed on an obesogenic diet throughout pregnancy and lactation, and had the same amount of body fat, plasma, leptin, triglycerides and insulin levels as controls. This reversal observed at weaning was however no longer present during adulthood (198).

Results from these rodent studies have been further supported by a recent study in a large animal model. In this study, ewes that were overfed before pregnancy were caloric restricted during the peri-conception period and as a result the female lambs of these ewes were no longer fatter than control lambs (199). These studies from both large and small animal models, although limited, provided evidence to suggest that maternal dietary control, even well before pregnancy, is required for obese or overweight individuals who are planning to conceive to avoid the development of obesity in their offspring later in life.

Whilst human studies are still at the early stages and long term effects of interventions on obese mothers and their offspring remains to be determined, interventions including dietary advice and physical activity do appear to have been successful at reducing gestational weight gain in obese mothers (200-202). This improvement is likely to prevent pregnancy complications and the birth of macrosomic babies. These studies have highlighted the

importance of dietary interventions for obese pregnant women in improving their dietary habits and possibly health outcomes during pregnancy as well as of their children.

1.5.5 Dietary interventions for reversing the programmed preference for fat and sugar

In light of the recent finding that food preferences can be programmed before birth, there is increasing interest as to whether or not these changes in food preferences can be reversed. Sanchez and colleagues conducted a study on normal healthy rats which have an inherent preference for fat (175). They found that leptin administration during lactation reduced the preference for fat in these offspring when compared to offspring without leptin treatment (175). Similarly, Bayol and colleagues also found that offspring of dams fed on a cafeteria 'junk food' diet comprised of a range of energy dense, high-fat, high-sugar foods, when suckled by dams on a control diet exhibited lower preference for fat, sugar and salt compared to offspring exposed to the junk food diet both *in utero* and during the suckling period, despite being weaned on the same junk food diet (40). These studies further support the importance of the lactation period as a potential critical window for intervention to reverse the preference for high-fat foods later in life.

In other rodent studies, even after being weaned on a control diet until 6 months of age, offspring of high-fat fed dams retained an increased preference for fat and sugar compared to those of control dams (51, 53). Interestingly, in a maternal low protein model, when offspring were weaned on a control diet, the preference for fat in the offspring of low protein dams was observed at 12 weeks but not at 30 weeks of age (49). Thus, while there is some evidence that the preference for fat can be reversed by interventions applied after weaning, the evidence is limited and it is not possible to draw definitive conclusions as to whether this is truly the case. Therefore, future work is required to achieve a better understanding of the mechanisms involved and obtain robust evidence regarding the potential opportunities for interventions.

1.5.6 Summary

Breaking the intergenerational cycle of obesity has proven to be a challenging task. Whilst it is clear that major development of neural circuits in rodents occur during the suckling period and that interventions applied during that critical window of development can potentially reverse the onset of obesity, emerging studies have also indicated the importance of the maternal diet well before pregnancy. The post-weaning diet also plays a role in the subsequent development of obesity where a diet high in fat and sugar can

potentiate the adverse effects of maternal overnutrition. A nutritionally balanced diet which is low in fat may also improve the metabolic health outcomes in offspring predisposed to obesity. However, this reversal may not be complete and insults later in post-natal life including a high-fat diet challenge or ageing may 'unmask' the programmed effects resulting in the inability of various systems in the body to cope with the metabolic challenge. The mechanism behind this is still unclear but a recent hypothesis highlights the role of age-associated decline in androgen in males and estrogen in females and the subsequent development of obesity.

1.6 Sex differences

According to the World Health Organisation report in 2012, the prevalence of obesity differs between males and females, with females having higher obesity rates than males (1). Females are also more resistant to weight loss (203) while males are more likely to develop obesity-associated metabolic disorders, including cardiovascular disease and type 2 diabetes (204). Despite the significant differences between males and females in the susceptibility to obesity and the regulation of energy homeostasis, the majority of studies to date have used males rather than females to avoid hormonal fluctuations as part of the estrous cycle in females. This section will focus on the limited evidence of sex differences in the regulation of energy homeostasis and reward, in both adult humans and rodents. With relevance to this thesis, this section will also review recent research looking at sexual dimorphism in the adaptation and responses of the offspring towards an excess supply of nutrients *in utero* as well as early in life.

1.6.1 Sex differences in response to high-fat intake

One of the factors mediating the difference in susceptibility to obesity is the differential regulation of energy homeostasis between sexes (205). On average, females have lower resting energy expenditure compared to males (206) and this lower energy expenditure is more likely to continue to reduce with age (207), thus making females more prone to weight gain and obesity. Fat distribution also varies between males and females, with males tending to deposit fat viscerally while females tend to accumulate more fat in subcutaneous depots (205). This effect is thought to be due to the presence of estrogen as postmenopausal women in whom estrogen levels are substantially reduced tend to exhibit a more similar fat distribution to men (205). Accumulation of visceral fat is associated with the development of cardiovascular disease and diabetes, which helps explain why males

and post-menopausal women are more prone to metabolic diseases despite the fact that females tend to gain more weight and have higher overall adiposity (208).

This differential susceptibility to obesity between sexes in humans has been replicated in rodent studies. In response to a chronic intake of high-fat diet, female rats have been consistently shown to gain more weight than males (209-212). The higher weight gain in female rats is due largely to the fact that they accumulate greater amounts of fat in adipose depots than males. However, despite the higher fat accumulation in adipose depots, females have higher fatty acid oxidation capacity within the muscle which protects them against some of the metabolic consequences of obesity (209). Even though male rats do not gain as much fat as females after exposure to the high-fat diet, they have reduced fatty acid oxidation in the muscle, which tends to be associated with increased fat accumulation in this tissue and also more triglyceride deposits in the liver (209). This decreased muscle fatty acid utilisation and fat accumulation in the liver may explain why males are more prone to obesity-related metabolic diseases. Furthermore, males are more sensitive to the catabolic actions of insulin, while females are more sensitive to leptin (213). This is supported by a study by Shi and colleagues where leptin was shown to exert a more potent effect in reducing food intake in females compared to males (214). Female rats with leptin receptors ablated from POMC neurons showed a greater deposition of visceral and subcutaneous fat whereas in males, fat deposition was not affected by this same treatment (214). In addition, female rats have lower baseline plasma glucose levels and a higher glucose clearance rate compared to males (214). Similarly, elevated insulin levels observed in male rats in response to chronic high-fat intake was not found in females (209), suggesting a sex-dependent regulation of both fat deposition and glucose homeostasis in adult rodents.

1.6.2 Sex differences in the programming of obesity and food preferences

It is clear that sexual dimorphisms exist in adult energy homeostasis at baseline and in response to a high-fat diet. The question therefore arises as to whether the effects of exposure to an insult early in life also differs between males and females, and there is increasing evidence to suggest that this is indeed the case. Earlier studies on maternal undernutrition consistently reported that males were more prone to develop hypertension and impaired energy homeostasis later in life, when compared to females (215-217). Studies looking at sex differences in the offspring in maternal overnutrition models are however, limited. In one study, Bayol and colleagues showed that female offspring that

had been exposed to cafeteria diet *in utero* and during suckling gained more weight and deposited more fat than their male counterparts (40). In the same group of rats, serum glucose levels were found to be elevated without an increase in serum insulin levels in females, whilst the opposite was observed in males (119). Furthermore, both male and female offspring of dams fed a diet rich in lard from before pregnancy until the end of lactation developed endothelial dysfunction, but only the female offspring were hypertensive (218). These findings suggest a greater impact of exposure to the high saturated fat diet early in life on the regulation of body weight, glucose homeostasis and vascular function in female offspring compared to the male offspring.

In contrast to the studies demonstrating that female offspring are more prone to the adverse effects of maternal overnutrition, there are studies which have also suggested that male offspring of overnourished dams are more susceptible. Samuelsson and colleagues showed that in response to maternal overnutrition during pregnancy and lactation, male offspring developed impaired glucose homeostasis and there were no sex differences in the development of hypertension in the offspring in adulthood (42). In addition, female offspring of obese mice that were caloric restricted during the peri-conceptional period, pregnancy and lactation were also found to be resistant to diet-induced obesity, however this effect was not seen in males (197). Interestingly however, it was demonstrated in a maternal high-fat diet model that male offspring of high-fat fed dams gained less weight and exhibited metabolic parameters (ie plasma glucose, insulin, leptin) similar to that of controls at 10 weeks of age, even when they were weaned on a high-fat diet, indicative of a protective effect of a maternal high-fat diet on the male offspring throughout life (219). Nevertheless, both male and female offspring of obese dams were less leptin sensitive when compared to offspring of control dams which were weaned on a control diet post-weaning, which suggests that maternal overnutrition throughout pregnancy and lactation programs leptin resistance in the offspring, irrespective of offspring sex (219). These studies, despite the conflicting findings, provide evidence for sexual dimorphism in the adaptations towards maternal overnutrition/obesity and highlight the need to analyse the responses of male and female offspring separately in programming studies.

In the context of this thesis and the programming of food preferences in the offspring, it is worth noting that in addition to energy homeostasis regulation, food preference also has a sex-specific component, both in adults and in offspring exposed to an inappropriate nutrient supply early in life. In obese adults, males have a higher preference for high-

protein and high-fat foods whilst females prefer high-carbohydrate and high-fat foods (135). It is believed that the high-fat component of the diet is driving the obesity phenotype in these individuals despite the different types of preferred foods between males and females. In rodents, it was the females which had a higher preference for fat and consumed more fat than the male offspring when they were provided with free access to a palatable diet (220). Interestingly, this increased preference for fat in females was abolished if their mothers had been caloric restricted but the same effect was not observed in males (220). Similarly, in a maternal low-protein diet model, female offspring of low-protein fed dams were found to have a higher preference for fat compared to controls, whereas food preferences were unaffected in males (49). The findings in rodent models are somewhat in contrast with a study in obese humans, in which females had a higher liking for high-carbohydrate foods compared to males (135). Nevertheless, it is clear that males and females have distinct food preferences and that these differences are likely due to the differential regulation of reward and motivation between sexes within the mesolimbic reward system, the system responsible for the development of addiction.

1.6.3 Sex differences in susceptibility to addiction

Sex differences have been reported at all stages of addiction, from the initiation phase to withdrawal and subsequent relapse (221). Although the rate of drug abuse is higher in males than females, females are more prone to increase drug use and have a harder time suspending drug use than males, suggesting that they are less able to overcome addictive behaviours (222). Estrogen has been implicated as one of the factors mediating the differences in susceptibility to addiction between males and females since estrogen is able to interact with and modulate the function of the mesolimbic reward pathway. Estrogen increases dopamine release, and administration of estrogen in ovariectomised female rats enhances the motivation to obtain cocaine, suggesting that it potentiates addictive behaviours (223). The key components of the mesolimbic reward system are also differentially expressed at basal levels in males and females, and basal dopamine levels and expression of D1 dopamine receptors in the striatum are higher in males than females (221, 224). This results in a higher threshold for reward activation in males, compared to females, which increases the propensity of females to become addicted after initial exposure to a rewarding stimulus (224).

1.6.4 Summary

Interventions and treatments for obesity to date have rarely taken into account the sexual dimorphism in body weight regulation and energy homeostasis. In rodents, it has been shown that maternal caloric restriction during lactation resulted in an improved regulation of body weight and food intake in male offspring but not in females (220), consistent with the result of human studies in which females are more resistant to weight loss (203). Another study in humans showed that intra-nasal insulin administration reduced body weight, adiposity and waist circumference only in males, but not in females (225). Given that males and females respond differently to different treatments and interventions, it is therefore important to understand the sex-specific mechanisms underlying both peripheral and central regulation of energy homeostasis and reward in order to effectively prevent and reduce the incidence of obesity in both males and females.

1.7 Experimental hypotheses

The increase in caloric intake is one of the major factors contributing to the elevated rates of obesity worldwide. The consumption of palatable foods beyond energy requirements suggests a non-homeostatic control of food intake. Acute consumption of palatable foods can result in activation of the mesolimbic reward system, and continuous consumption of these foods can eventually lead to desensitisation of the reward system, in a manner similar to the effects of drugs of abuse. Recent studies have also demonstrated that long term intake of a high-fat diet or intermittent access to sucrose can lead to signs of withdrawal when the diet is removed and replaced with a standard low-fat diet. Whilst these studies have highlighted the possibility of 'junk food addiction', the behavioural parameters and changes within the reward system in response to chronic junk food intake and abstinence remains to be investigated. Furthermore, the susceptibility towards the development of 'junk food addiction' and whether the effects of prolonged junk food intake on the mesolimbic reward system differs between males and females are still unclear. **In Chapter 2, I hypothesised that long term consumption of junk foods in adult rodents would lead to changes in expression of key genes in the NAc associated with desensitisation of the mesolimbic reward system and that, removal of these palatable foods after long term intake would result in signs of withdrawal and gene expression changes within the mesolimbic reward system. Given that there are sex differences in the development of obesity and addiction, I further hypothesised that the molecular changes within the mesolimbic reward system associated with chronic junk food intake would differ between males and females.**

This literature review has also highlighted the evidence that the development of obesity has its origins early in life and is related to the nature of maternal diet during pregnancy and lactation. Recent studies have shown that offspring of dams fed a cafeteria diet during pregnancy and lactation exhibit a higher preference for high-fat/ high sugar foods, therefore indicating that the development of obesity in the offspring may be driven at least in part by the increased preference for palatable foods in the offspring. The precise mechanisms underlying the programming of food preferences remain unclear. **I therefore hypothesised in Chapter 3, that a maternal junk food diet during pregnancy and lactation would program an increased preference for fat in the offspring during the juvenile period and adulthood. I also hypothesised that the preference for fat in the junk food offspring would be associated with changes in key components within the mesolimbic reward system.**

Whilst it has become increasingly clear that maternal overnutrition during pregnancy and lactation can program an increase in food/fat intake in the offspring after birth associated with changes within the mesolimbic reward system, there is limited information on how early the changes occurred. **Thus in Chapter 4, I hypothesised that the changes in gene expression within the mesolimbic reward system which predisposes the offspring to increased fat preference are already present at weaning.** It is also unclear if the effects of maternal junk food diet on the offspring can be ameliorated. Various studies have suggested that introducing the offspring to a low-fat diet at weaning has the potential improve the offspring's metabolic health outcomes. However, the literature is conflicting and whether the effects of maternal overnutrition on offspring food preferences can be altered by nutritional interventions after birth is still unclear. Therefore, in Chapter 4 and 5, the offspring were weaned on a low-fat control diet to investigate whether short term (Chapter 4) and long term (Chapter 5) exposure to the low fat diet could ameliorate maternal overnutrition-programmed preference for fat and its associated metabolic parameters. In both these chapters, offspring were subjected to a food preferences study to determine their food and macronutrient preference. **In Chapter 4, I hypothesised that a short term exposure to the low-fat control diet post-weaning in the offspring of junk food fed-dams could reverse fat deposition in the short term but not after being exposed to the junk food diet during the food preferences study. I also hypothesised that the short term exposure to the low-fat diet could not prevent the increased fat preference or normalise the expression of key components of the reward system. In Chapter 5, I hypothesised that the long term post-weaning low-fat diet would succeed**

in normalising fat deposition and preventing the exacerbated preference for fat in the offspring of junk food fed dams. I further hypothesised that this intervention would also be associated with normalisation of the expression of key genes within the mesolimbic reward system to control levels.

CHAPTER 2: CHRONIC JUNK FOOD FEEDING AND SUBSEQUENT ABSTINENCE IMPACTS ON BEHAVIOUR AND GENE EXPRESSION WITHIN THE MESOLIMBIC REWARD SYSTEM IN MALE AND FEMALE ADULT RATS

2.1 Introduction

The incidence of obesity has reached epidemic proportions in industrialised and semi-industrialised nations across the globe. According to the World Health Organisation, over 300 million adults worldwide were classified as obese in 2005, and this figure had risen to 500 million by 2008 (1). In the context of this obesity epidemic, there is a growing emphasis on the need to understand the physiological mechanisms which may contribute to the dysregulation of food intake and energy expenditure, and thus promote weight gain and obesity.

The causes of obesity are multi-factorial. Whilst genetics clearly plays a role in determining obesity risk at an individual level, the marked increase in the incidence of obesity in populations around the world over the past 2 decades has occurred during a time when the gene pool has remained relatively stable, and this has led to suggestions that environmental, rather than genetic factors, are the major contributors (1). One of the most obvious environmental factors contributing to weight gain and fat deposition is the increased consumption of energy dense, high-fat, high-sugar 'junk foods'. The caloric intake of individuals in developed and developing countries has risen steadily over the past decade (4) and importantly, obese individuals are found to exhibit a stronger preference for junk foods than lean individuals (226). These studies have raised questions as to the ability of the appetite regulatory system, the system that controls food intake and energy homeostasis, to function optimally in an over-nourished state.

The phenomenon whereby animals and humans continue to consume high-fat, high-sugar foods despite their negative health consequences is analogous to behaviour displayed by drug addicts. Indeed, there have now been a number of studies which have shown that

increased consumption of high-fat and high-sugar foods is associated with acute increases in dopamine synthesis and release, as is observed for drugs of abuse, and has led to the concept of food as a natural reward (227). The similarities between the effects of junk food and drugs of abuse has led to the suggestion that the overconsumption of junk foods could precipitate junk food addiction, leading people to seek out junk foods despite knowing their negative health consequences (227, 228). However, studies which have examined the effects of chronic junk food feeding and subsequent junk food abstinence on the mesolimbic reward pathway have been conducted almost exclusively in males, and whether comparable effects occur in females remains unclear.

This study therefore investigated the effects of 8 weeks of junk food intake and subsequent abstinence in both male and female adult rats on their body weight, food intake, activity levels and the expression of key reward components, including TH, DAT, D1 and D2 receptors, and the μ-opioid receptor in the NAc, an area in the brain where dopamine neurons project to within the mesolimbic reward system. This study also aimed to develop a cafeteria junk food diet-induced obesity rodent model which could induce changes within the reward pathway in adult rodents and could thus be used in investigating the impact of peri-natal junk food feeding on the reward system in the subsequent Chapters of this thesis.

2.2 Materials and Methods

2.2.1 Animals and feeding

This study was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science. Twenty one male and twenty one female Albino Wistar rats (200 - 300g) were used in this experiment. All rats were individually housed under a 12 hour light/12 hour dark cycle at a room temperature of 25°C. Rats were fed *ad libitum* on a standard rat chow (Specialty Feeds, Glen Forrest, Western Australia, Australia) and were allowed to acclimatise to the animal housing facility for one week prior to the start of the feeding protocol.

Male and female rats were randomly assigned to control (Control, n=10, 5 males, 5 females) or junk food (JF, n=32, 16 males, 16 females) groups. Control rats were given free access to standard rat chow while JF rats were provided with a cafeteria diet containing peanut butter, hazelnut spread, chocolate biscuits, savoury snacks, sweetened multi-grain breakfast cereal, ham and chicken flavoured processed meat and a mixture of lard and standard rat chow. The standard rat chow diet contained 67.1% carbohydrate, 21% protein and 11.9% fat whilst the cafeteria junk food diet contained 44.5% carbohydrate, 8.2% protein and 43.4% fat. Detailed nutritional information of the control and cafeteria diets is shown in Table 2.1. The food and macronutrient intake of rats in the Control and JF groups was determined every 2 days throughout the duration of the experiment. In the JF group, the amount of each type of junk food remaining at the end of the 2 day period was individually weighed and subtracted from the original quantity provided. All rats had *ad libitum* access to water. Rats were weighed once a week throughout the experimental period.

Rats in the Control and JF groups were maintained on their respective diets for 8 weeks. An 8 week experimental feeding period was selected for this study in order to determine the effects of continuous exposure to these junk foods. At the end of the 8 week period, a random sample of 11 male and 6 female rats in the JF group were killed and tissues collected. The remaining 5 male and 10 female rats had their junk food removed and replaced with control chow for a 72 hour period (Junk Food-withdrawal (JF-W) group). This 72 hour withdrawal period was chosen based on previous studies (91, 229) to allow identification of early withdrawal signs as a result of junk food removal, and the associated

molecular adaptations within the mesolimbic reward system. Both Control and JF-W rats were then killed and tissues collected.

2.2.2 Determination of spontaneous activity

Spontaneous activity levels were assessed in all experimental animals. All rats were acclimatised to the room in which this experiment was performed and to the experimental procedure 1 day prior to the start of the experiments. Spontaneous activity of all rats was assessed between 09:00am and 12:00pm, i.e. between 2 and 5 hours from the beginning of the light phase. Activity was observed in a clear plastic observational chamber (20cm × 30cm × 50cm) and video-recorded for a 20 minute period. Food was not provided during the 20 min observation period. The time of day at which assessments were conducted was alternated for the Control and JF groups, to avoid the introduction of bias due to systematic differences in the timing of the testing between different treatment groups. Due to the fact that the female cohort was studied separately to the male cohort, the protocol used to determine the spontaneous activity was slightly different for males and females. In males, the 20 minute video recording was used to determine how long (in seconds) each experimental animal spent being active (walking or running, rearing or grooming) and the amount of time they were inactive (sitting still, sleeping). The number of episodes of rearing and grooming during the 20 minute period was also documented. For female rats, a 10cm × 10cm grid was drawn on the base of the plastic box, and the level of activity was determined by recording the number of times within the 20 minute observation period the rats crossed over these grid-lines. All measurements were conducted by a single observer.

Table 2.1 Nutritional information of individual junk foods and standard rat chow

Values for fat, carbohydrate and protein are expressed as a percentage of energy.

	Energy (kJ/g)	Fat (%)	Carbohydrates (%)	Protein (%)	Sodium (mg/g)
Lard/ chow mixture	20.9	19	51	17	3
Peanut butter	26.2	50	25	21	6
Hazelnut spread	23.3	36	54	4	1
Chocolate biscuits	20.0	15	71	10	3
Savoury snacks	23.1	35	51	7	10
Sweetened cereal	27.3	3	43	1	1
Processed meat	7.3	12	7	10	12
Standard chow	18.0	5	60	20	4

2.2.3 Assessing the effect of junk food removal

In order to assess whether removal of junk food was associated with the onset of classic signs of withdrawal, behaviour was observed every 24 hours for 72 hours following junk food removal in the JF-W rats and a corresponding group of Control rats which had never been exposed to junk food. Behavioural measurements include: spontaneous activity (line crossings/movements), rearing, grooming, scratching and classic signs of withdrawal (piloerection, teeth-chattering, paw tremors and wet dog shakes). Behaviour was also determined prior to removal of junk food in all JF-W and Control animals to provide a measure of these activities at baseline and all rats were acclimatised to the experimental procedure prior to the first assessment period. For the behavioural studies, rats were removed from their home cage and placed in the clear plastic observational cage (20cm × 30cm × 50cm) for 20 minutes. The number of episodes of rearing, grooming, scratching and classic signs of withdrawal (piloerection, teeth chattering, paw tremors and wet dog shakes) during the 20 minute observation period were recorded. Spontaneous activity was assessed as described above and body weight and food intake were recorded daily. All measurements were conducted by a single observer.

2.2.4 Post-mortem and tissue collection

Rats were not fasted prior to post-mortem. All rats were weighed and killed with an overdose of CO_2. Blood samples were collected by cardiac puncture into heparinsed tubes and spun at 3,500g for 15 minutes at 4°C. Plasma samples were stored at -20°C for subsequent analyses of glucose, insulin, NEFA and leptin concentrations. All internal and subcutaneous fat depots were dissected and weighed to provide an accurate measure of total body fat mass.

2.2.5 Determination of plasma glucose and NEFA concentrations

Plasma glucose concentrations were determined using the Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA) which utilises the hexokinase/glucose-6-phosphate dehydrogenase method. This method converts glucose to 6-phosphogluconate (6-PG), NADH and hydrogen ions. The level of NADH formed represents the amount of glucose in the sample and can be measured at the absorbance of 340nm. Plasma concentration of NEFA was measured using the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). The kit utilises an enzymatic method which produces acyl-coenzyme A (CoA) from fatty acids by the addition of acyl-CoA synthetase (ACS). The acyl-CoA is oxidized by added acyl-CoA oxidase (ACOD) with the generation of hydrogen peroxide

and in the presence of peroxidase (POD), this permits the oxidative condensation of 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm. Inter and intra-assay coefficients of variation (CoV) for both glucose and NEFA assays were both <5%.

2.2.6 Determination of plasma insulin and leptin concentrations

Insulin and leptin concentrations were measured by radioimmunoassay with Rat Insulin and Rat Leptin Kits (Linco Research, St. Charles, MO) respectively, according to manufacturer's instructions. Briefly, samples were incubated overnight with the antibody (guinea pig anti-rat insulin or anti-rat leptin antibody) and radioactive labelled (I^{125}) rat insulin or leptin, therefore creating competition between the antigen in the sample and the I^{125} antigen. Precipitating reagent consisting of the goat anti-guinea pig IgG serum was then added to form an antigen-antibody precipitate. A gamma counter was used to count radioactivity in all tubes. The higher the concentration of the antigen within the plasma sample, the less radioactive labelled antigen would be bound to the antibody, therefore, the lower the radioactive counts. Insulin and leptin concentrations in the plasma samples were calculated based on the standard curve obtained in each run. Two quality controls were included in each run. Inter- and intra-assay CoV were <10%.

2.2.7 NAc isolation

The whole brain was carefully removed and placed on a glass dish that had been cooled on dry ice. Isolation of the NAc was conducted based on the reference to a previously published study (230) and the Rat Brain Atlas (231). The NAc was dissected from a coronal slice (bregma -0.24mm to 1.68mm) which spanned from the optic chiasm to 2mm anterior to the optic chiasm. The cortex was then removed and the section was cut horizontally at the level of the rhinal fissure. The NAc was isolated by cutting 1mm above the rhinal fissure. The section was immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent determination of mRNA expression by qRT-PCR.

2.2.8 Determination of gene expression in the NAc

Total RNA from the NAc was isolated with Trizol reagent (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and purified using an RNeasy Mini Kit (Qiagen Pty Ltd, Doncaster, Australia). RNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm, and RNA integrity was confirmed using agarose gel electrophoresis. ~2μg RNA was then reverse transcribed to generate cDNA using

Superscript III reverse transcriptase (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and random hexamers.

Quantitative real time PCR was conducted using the SYBR Green system in an ABIPrism 7300 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primer sequences for TH, D1, D2, μ-opioid receptor and DAT are shown in Table 2.2. Rat β actin QuantiTect Primer Assay (Qiagen Pty Ltd, Doncaster, Australia) was used to determine β actin mRNA expression. All primers were sequenced prior to the experiment to ensure the authenticity of the DNA product and a qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Amplification efficiency of all primers was 0.997 - 0.999. A constant amount of cDNA (1μl) was used for each qRT-PCR measurement and three technical replicates were performed for each gene. Two quality controls were included on each plate in order to verify inter-plate consistency.

Each qRT-PCR reaction well (10μl total volume) contained 5μl of 2× SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA); 1μl of each primer giving a final concentration of 60nM to 900nM, 2μl of molecular grade H_2O and 1μl of a 50ng/μl dilution of the stock template. The cycling conditions consisted of 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The abundance of each mRNA transcript was measured and expression relative to that of β actin was calculated using Q-gene qRT-PCR analysis software (232).

Table 2.2 Primer sequences for TH, DAT, D1, D2 and μ-opioid receptor

		Sequence	Size (bp)	Accession Number	Working Concentration
TH	F	5’-GCC CCC ACC TGG AGT ATT TTG-3’	83	NM_012740	300nM
	R	5’-AGA CAC CCG ACG CAC AGA GC-3’			
DAT	F	5′-AGC TGG CTC TCG GAC AGT TC-3′	141	NM_012694	600nM
	R	5′-GTG CCC ATG CGA TGA TGA C-3′			
D1	F	5’-CGG GCT GCC AGC GGA GAG-3’	86	NM_012546	60nM
	R	5’-TGC CCA GGA GAG TGG ACA GG-3’			
D2	F	5’-AGA CGA TGA GCC GCA GAA AG-3’	96	NM_012547	900nM
	R	5’-GCA GCC AGC AGA TGA TGA AC-3’			
μ	F	5'-GTA GTG GGC CTC TTC GGA AAC-3'	75	NM_001038598	300nM
	R	5'-GTT GGT GGC AGT CTT CAT TTT G-3'			

2.2.9 Assessing the effect of chronic junk food feeding on subsequent food preferences

An additional 10 male and 10 female rats were given either the control (n=5 male, n=5 female) or the junk food (n=5 male, n=5 female) diet for a period of 8 weeks. At the end of the 8 week period, the rats were given free access to both the control and the junk food diet for 2 weeks and macronutrient intake recorded to assess the impact of prior chronic exposure to a junk food diet on food intake and preference during a subsequent exposure to the junk food diet. Macronutrient intake was analysed separately for week 1 and week 2 of the food preferences study.

2.2.10 Statistical analysis

Data are presented as mean ± SEM. The effect of 8 weeks of junk food feeding and withdrawal on food intake, body weight, body fat mass, plasma hormones and metabolites, and gene expression was determined by two-way ANOVA, with sex and treatment groups as factors. For food intake, the JF group included all rats exposed to the junk food diet, irrespective of whether they were subsequently exposed to junk food abstinence. Where a significant interaction between sex and junk food feeding was identified, data from male and female rats were analysed separately unpaired Student's t-test (food intake only) or by one-way ANOVA. The effect of junk food withdrawal on food intake, body weight and behaviour in male and female rats was determined by a multifactorial ANOVA with repeated measures, with sex and time post-withdrawal as factors. Where the ANOVA identified a significant effect, the Duncan's multiple range test was used post-hoc in order to determine significant differences between values. All statistical tests were carried out using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL) or Stata 10 (StataCorp LP). A probability <5% (P<0.05) was accepted as statistically significant.

2.3 Results

2.3.1 Macronutrient and energy intake of male and female rats

Daily energy and fat intakes were higher in rats on the palatable junk food diet compared to Controls ($P<0.001$) (Table 2.3). In males only, carbohydrate intake was higher in the JF group ($P<0.05$) (Table 2.3). Protein intake was lower in both male and female rats in the JF group when compared to Controls ($P<0.001$) (Table 2.3).

2.3.2 Effect of chronic junk food feeding on body weight and body fat

There was no difference in body weight between the Control and JF groups before commencement of the control or junk food diet in either male or female rats (Male Control: 278.7 ± 5.3g, Male JF: 268.4 ± 6.6g; Female Control, 253.4 ± 4.7g, Female JF: 258.6 ± 5.8g). In males, there was no significant increase in body weight in the JF and JF-W groups, however percentage body fat was higher in both the JF and JF-W males compared to Controls ($P<0.05$) (Table 2.4). In female rats, body weight and percentage body fat were significantly higher in the JF and JF-W groups compared to Controls at the end of the experimental period ($P<0.01$) (Table 2.4).

2.3.3 The effect of chronic junk food feeding on spontaneous activity in male and female rats

Spontaneous activity level in the male rats was recorded after 7 weeks on the junk food diet. Male rats in the JF group tended ($P=0.06$) to be less active compared to their Control counterparts, as indicated by the shorter time spent running, walking, rearing or grooming during the 20 minute observation period (Control: 854.8 ± 54.5s, JF: 594.0 ± 110.5s; n=5 for each group). There was no difference in the number of episodes of rearing and grooming between the Control and JF groups. In female rats, activity levels were measured in both the fourth and seventh week after the start of the junk food diet. In the fourth week after the start of junk food feeding, there was no difference in spontaneous activity level between the Control and JF groups (Control: 285 ± 36 line crosses, JF: 317 ± 23 line crosses; n=5 for each group). However, after 7 weeks on the junk food diet, spontaneous activity was significantly reduced in female JF rats (Control: 289 ± 18 line crosses, JF: 197 ± 29 line crosses; $P<0.05$).

Table 2.3 Daily energy, fat, protein and carbohydrate intake in the Control and JF groups in males and females

Data are expressed as mean ± SEM. *P<0.01, **P<0.001.

	Male		Female	
	Control n = 5	JF n = 11	Control n = 5	JF n = 11
Energy intake (kJ/day)	345 ± 10	557 ± 21**	367 ± 8	436 ± 14**
Fat intake (g/day)	0.91 ± 0.02	6.54 ± 0.29**	0.94 ± 0.02	5.90 ± 0.32**
Protein intake (g/day)	3.91 ± 0.11	2.57 ± 0.12**	3.99 ± 0.10	1.89 ± 0.03**
Carbohydrate intake (g/day)	12.0 ± 0.32	15.9 ± 0.77*	12.2 ± 0.28	12.2 ± 0.58

2.3.4 Effect of chronic junk food feeding and abstinence on plasma hormones and metabolites

Continuous junk food intake resulted in elevated plasma leptin concentrations in both male and female rats compared to those consuming the standard rat chow ($P<0.01$); however plasma leptin concentrations were no longer different from Controls after 72 hours of junk food abstinence (Table 2.4). In females, but not in males, the JF and JF-W groups also had higher plasma NEFA concentrations compared to Controls ($P<0.01$) (Table 2.4). Plasma glucose concentrations were also higher in the female JF group at the end of the 8 week period of junk food feeding ($P<0.01$), but were no longer different to Controls after 72 hours of junk food abstinence. There was no effect of junk food intake on plasma glucose or NEFA concentrations in males or on plasma insulin concentrations in either males or females (Table 2.4).

2.3.5 Effect of forced junk food abstinence on body weight, food intake and behaviour in male and female rats

The body weight of the JF-W rats decreased significantly over the 72 hour period of forced junk food abstinence, and both male and female rats lost an average of between 1.5% and 3.4% of their initial body weight during this period (Figure 2.1A, B). Both male and female rats also consumed significantly less chow during the period of forced junk food abstinence compared to Control rats which had never been exposed to junk food (Figure 2.1C, D).

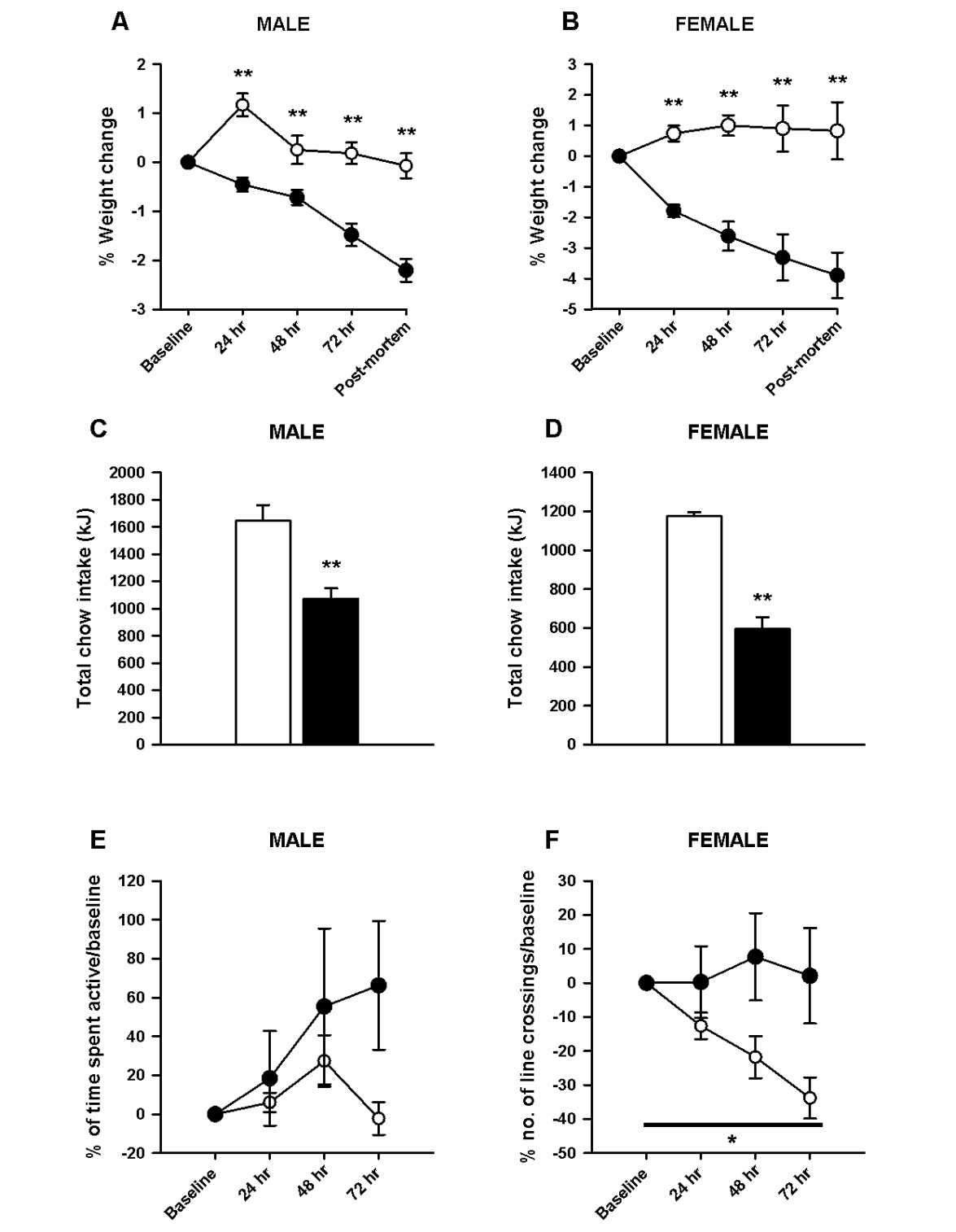
In male rats, spontaneous activity levels in the JF-W group tended ($P=0.06$) to increase during the 72 hour withdrawal period when compared to Control rats that had never been exposed to junk food (Figure 2.1E). The number of episodes of rearing and grooming were not different between Control and JF-W rats and did not change across the period of junk food abstinence (data not shown). In female rats, the spontaneous activity level of the JF-W group did not change across the withdrawal period whilst activity levels in the group which had never been exposed to junk food declined progressively relative to the levels observed at baseline at each successive assessment, consistent with a habituation to the assessment procedure ($P<0.05$) (Figure 2.1F). No rats in either feeding group or sex exhibited classic signs of withdrawal during the period of forced junk food abstinence.

Table 2.4 Body weight, percentage body fat, plasma leptin, NEFA, insulin and glucose levels of male and female Control, JF and JF-W rats at the end of experimental period.

Data expressed as mean ± SEM. Fat mass is presented as a percentage of total body weight. Different superscript letters indicate values within rows that are significantly different between Control, JF and JF-W groups for male and female rats. $P<0.01$ for female body weight, fat mass, leptin and glucose; $P<0.05$ for male fat mass and leptin.

	Male			Female		
	Control	JF	JF-W	Control	JF	JF-W
	n = 5	n = 11	n = 5	n = 5	n = 6	n = 10
Weight (g)	476.6 ± 4.4[a]	533.9 ± 22.6[a]	509.3 ± 32.3[a]	303.4 ± 5.4[a]	401.6 ± 22.7[b]	351.7 ± 11.3[c]
Fat mass (%)	9.2 ± 1.3[a]	15.3 ± 1.7[b]	18.0 ± 3.8[b]	9.4 ± 0.7[a]	20.7 ± 2.0[b]	14.0 ± 1.4[b]
Leptin (ng/ml)	18.7 ± 2.7[a]	47.6 ± 8.3[b]	31.7 ± 8.4[ab]	5.6 ± 1.0[a]	51.3 ± 8.0[b]	13.7 ± 2.3[a]
NEFA (meq/ml)	0.3 ± 0.05[a]	0.3 ± 0.04[a]	0.4 ± 0.05[a]	0.4 ± 0.04[a]	0.5 ± 0.04[b]	0.5 ± 0.03[b]
Insulin (µU/L)	3.0 ± 1.3[a]	2.8 ± 0.5[a]	3.9 ± 1.0[a]	1.6 ± 0.2[a]	2.0 ± 0.9[a]	1.6 ± 0.3[a]
Glucose (mmol/L)	13.1 ± 2.3[a]	12.4 ± 1.4[a]	13.6 ± 1.9[a]	11.2 ± 0.9[a]	17.3 ± 1.7[b]	9.8 ± 0.6[a]

Figure 2.1



The effects junk food removal on body weight (expressed as a percentage from weight prior to removal of junk food) (A, B), chow intake (C, D) and spontaneous activity levels (E, F) of Control (open circle/bar) and JF-W (closed circle/bar) groups in both male and female rats at 24, 48 and 72 hours post-junk food removal.

Values are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 5 in each group.

2.3.6 Effect of chronic junk food intake and abstinence on mRNA expression in NAc

2.3.6.1 TH and DAT

There was a significant sex by treatment interaction for TH mRNA expression in the NAc ($P<0.01$). In male rats, TH mRNA expression was lower in both JF and JF-W groups when compared to Controls ($P<0.001$) (Figure 2.2A). TH mRNA expression in the male rats was also negatively correlated with total energy intake (r=-0.74; $P<0.01$) and fat intake (r=-0.82; $P<0.01$) during the 8 week feeding period. There was no effect of chronic junk food feeding on TH mRNA expression in female rats (Figure 2.2B). DAT mRNA expression was not different between Control, JF and JF-W groups in either males or females (Figure 2.2C, D).

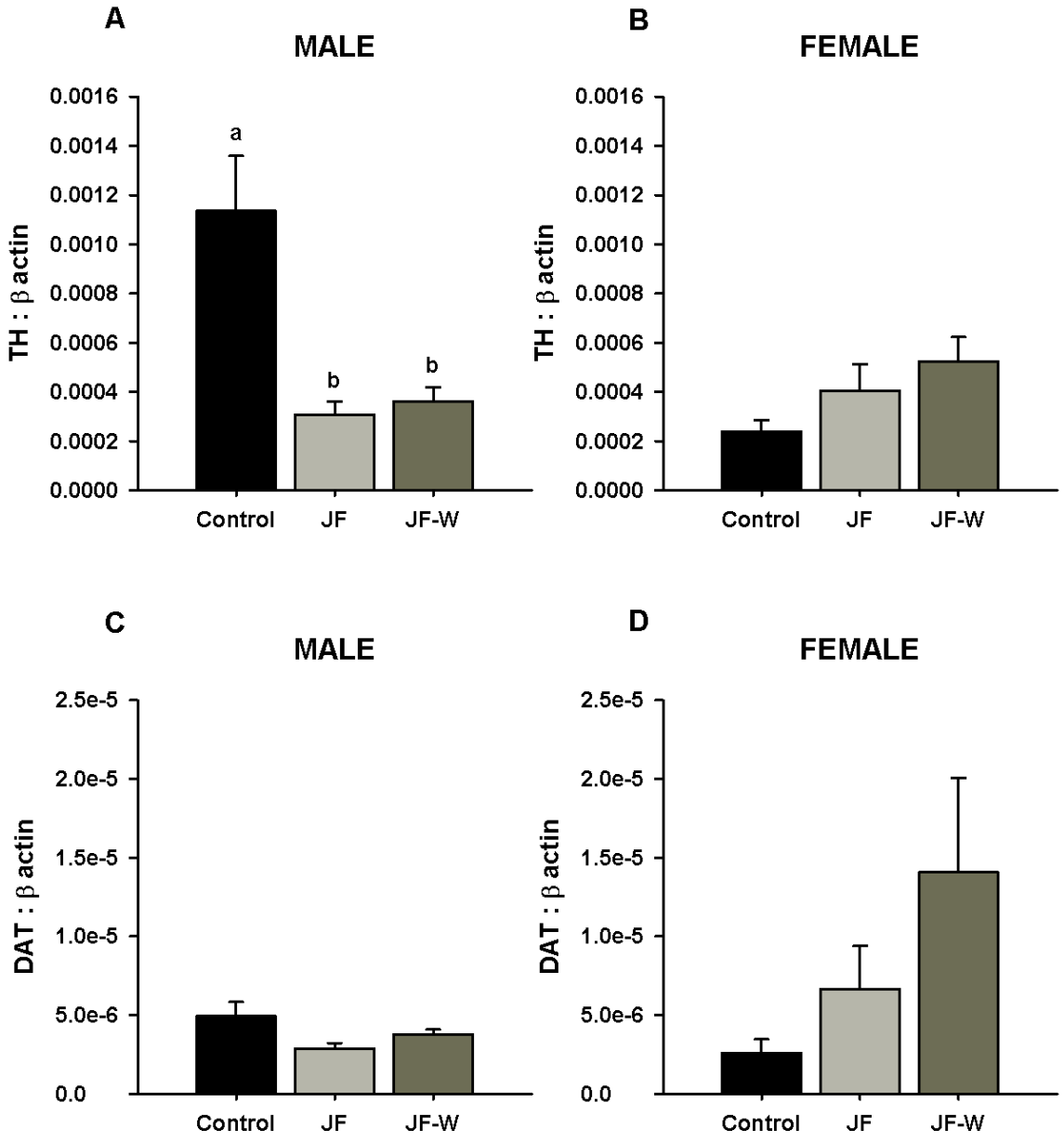
2.3.6.2 D1 and D2 receptors

There was a significant interaction between sex and treatment for D1 expression in the NAc ($P<0.01$). In male rats, D1 mRNA expression was higher in the JF, but not JF-W rats, compared to Controls ($P<0.05$) (Figure 2.3A). In female rats, however, D1 mRNA expression was lower in the JF and JF-W groups compared to the Controls ($P<0.01$) (Figure 2.3B). D1 mRNA expression in female rats was also negatively correlated with total energy intake (r=-0.71; $P<0.01$) and fat intake (r=-0.66; $P<0.01$) during the 8 week feeding period. There was no difference in D2 mRNA expression between Control, JF or JF-W groups in either males or females (Figure 2.3C, D).

2.3.6.3 μ-opioid receptor

The impact of chronic junk food feeding and subsequent junk food removal on the expression of the μ-opioid receptor was also different between male and female rats. In male rats, mRNA expression of μ-opioid receptor was lower in the JF and JF-W groups when compared to Controls ($P<0.01$) (Figure 2.4A). There was no difference in μ-opioid receptor mRNA expression between groups in female rats (Figure 2.4B).

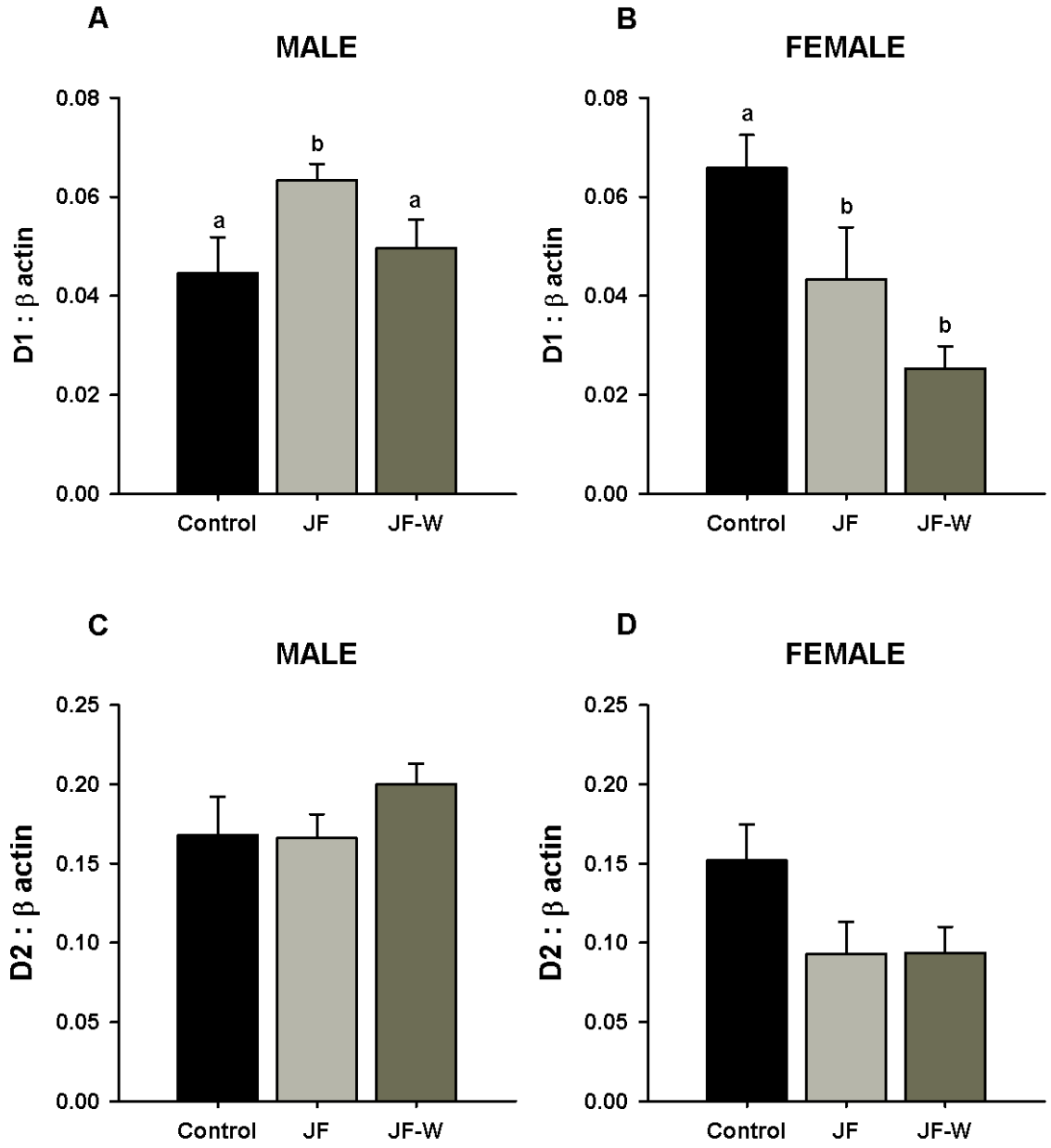
Figure 2.2



Normalised mRNA expression of TH (A, B) and DAT (C, D) in the NAc of Control (black bar), JF (light grey bar) and JF-W (dark grey bar) groups at the end of the experimental period.

Values are expressed as mean ± SEM. Different superscript letters indicate values which are significantly different (P<0.01). n = 5 - 11 in each group.

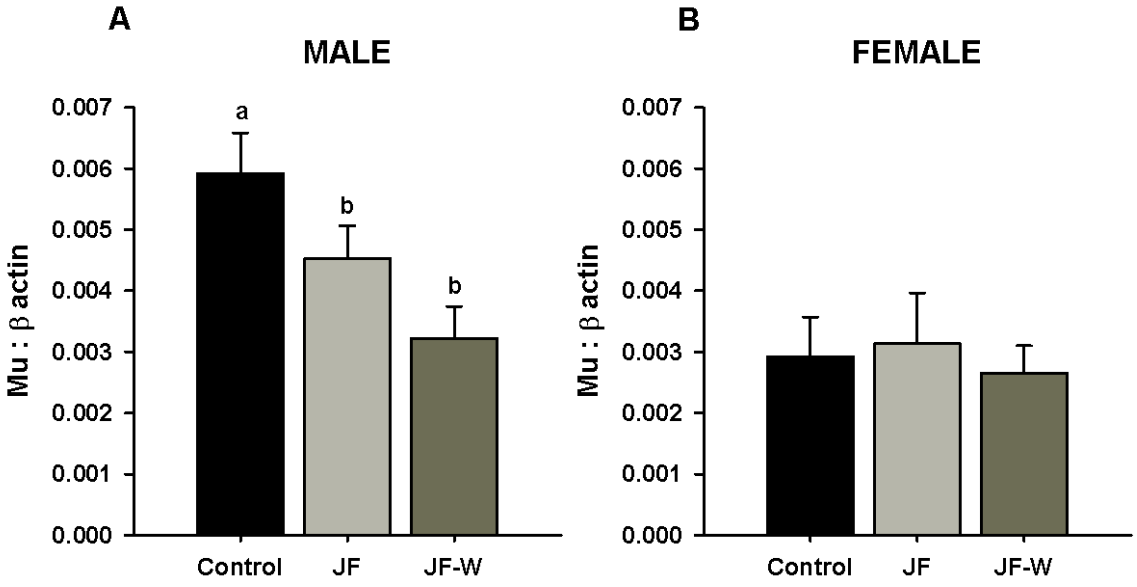
Figure 2.3



Normalised mRNA expression of D1 (A, B) and D2 (C, D) in the NAc of Control (black bar), JF (light grey bar) and JF-W (dark grey bar) groups at the end of the experimental period.

Values are expressed as mean ± SEM. Different superscript letters indicate values which are significantly different (P<0.05). n = 5 - 11 in each group.

Figure 2.4



Normalised mRNA expression of μ-opioid receptor in the NAc of Control (black bar), JF (light grey bar), and JF-W (dark grey bar) groups in both male (A) and female (B) rats.

Values are expressed as mean ± SEM. Different superscript letters indicate values which are significantly different ($P<0.05$). n = 5 - 11 in each group.

2.3.7 Sex differences in mRNA expression in Control rats

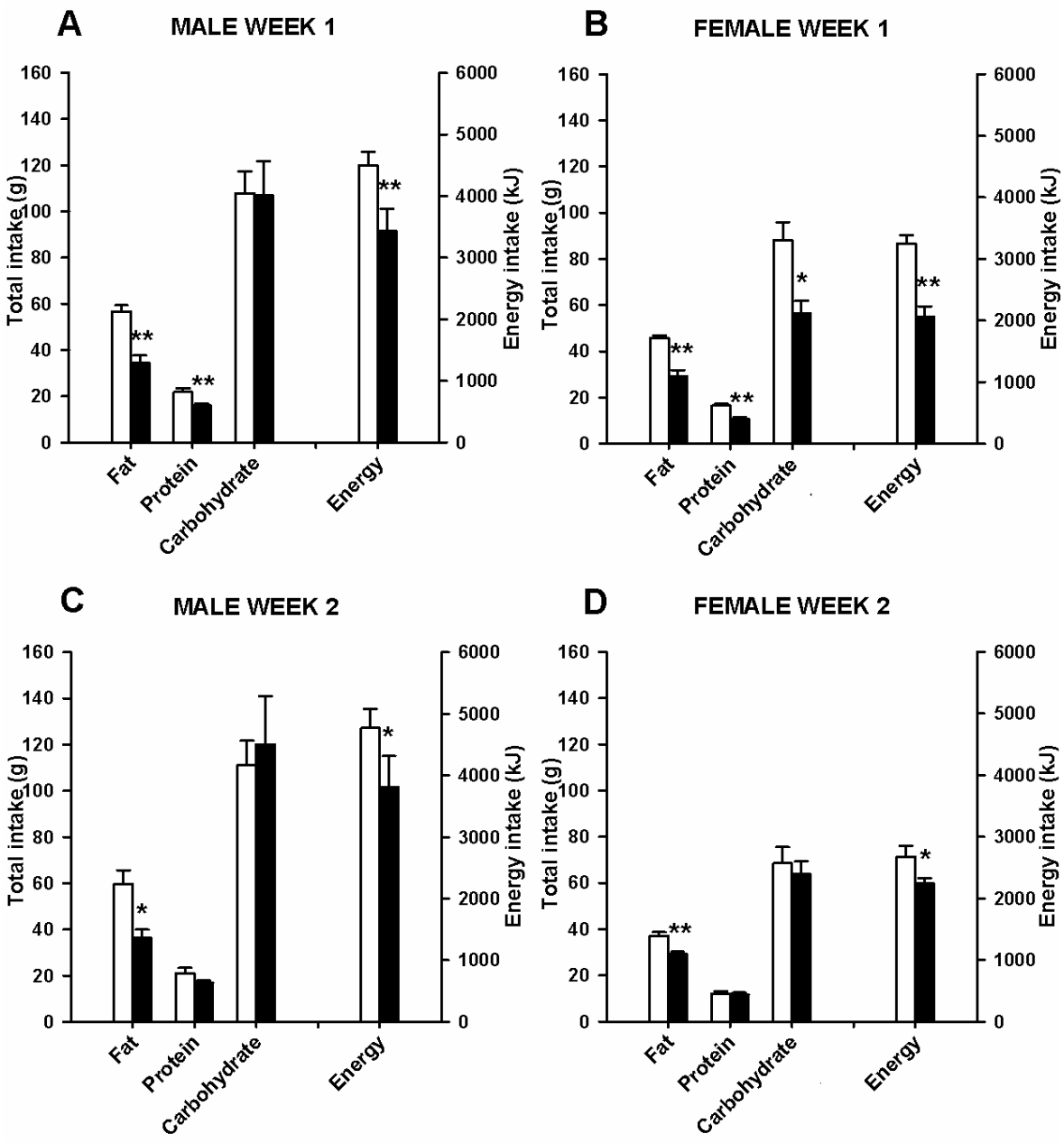
In addition to the different responses of the male and female rats to the highly palatable diet, there were also sex differences in the expression of TH and μ-opioid receptor mRNA in the NAc of Control rats not exposed to junk foods, with higher expression of both TH and μ-opioid receptor mRNA in males than females (TH: Male, $1.1\times10^{-3} \pm 2.2\times10^{-4}$, Female, $2.4\times10^{-4} \pm 4.5\times10^{-5}$; μ-opioid receptor: Male, $5.9\times10^{-3} \pm 6.7\times10^{-4}$, Female, $2.9\times10^{-3} \pm 6.5\times10^{-4}$; $P<0.05$).

2.3.8 Effect of chronic junk food intake on subsequent food preferences

During the first week of the food preference study, both male and female Control rats with no prior exposure to junk food consumed significantly more total energy than JF rats and derived a significantly higher proportion of their total energy intake from the junk food diet (Males: Control, 95 ± 1.6%, JF, 90 ± 1.7%; Females: Control, 99 ± 0.2%, JF, 97 ± 0.8%; $P<0.01$). Fat and protein intake in both males and females and carbohydrate intake in females were also higher in Control rats when compared to the JF rats ($P<0.05$) (Figure 5A, B).

During the second week of the food preferences study, total energy and fat intake remained higher in the Control rats compared to the JF rats in both males and females ($P<0.05$), however protein and carbohydrate intake was no longer different between the Control and JF groups (Figure 5C, D). In both males and females, the percentage of energy derived from the junk food diet remained higher in the Control rats compared to the JF rats in the second week of free access to the control and junk food diets (Males: Control, 98 ± 0.7%, JF, 91 ± 1.7%; Females: Control, 99 ± 0.1%, JF, 96 ± 1.7%; $P<0.01$).

Figure 2.5



Total fat, protein, carbohydrate and energy intake of both male and female Control (open bar) and JF (closed bar) rats during week 1 (A, B) and week 2 (C, D) of the food preference study.

Values are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 5 in each group.

2.4. Discussion

This study has demonstrated that chronic intake of a highly palatable high-fat, high-sugar junk food diet is associated not only with increased body fat mass, but also results in altered expression of key components of the mesolimbic reward system and significant reductions in food intake and body weight when access to the palatable diet is removed. Furthermore, this study has shown that the effects of the palatable diet and subsequent abstinence on the dopaminergic and opioid systems in the mesolimbic reward circuit are sexually dimorphic.

2.4.1 Continuous access to highly palatable diet, body weight, fat mass and plasma leptin levels

The finding that 8 weeks of continuous access to the palatable junk food diet resulted in increased fat deposition and elevated leptin concentrations in both males and females is in agreement with previous studies (233-235). Despite the increase in fat mass and leptin levels in male JF rats, there was no significant difference in body weight between the Control and JF male rats after 8 weeks on the palatable diet. This is different to the results of previous studies (234, 236). However, it is important to note that both Control and JF males in the current study were still actively growing during the feeding period. Moreover, the fact that the JF males were consuming more fat at the expense of high-protein foods (eg. lard and chow) may have also contributed to a lack of lean tissue accretion in the JF male rats during this period of rapid growth and a disproportionate increase in fat deposition.

2.4.2 Chronic intake of highly palatable diet and reduced locomotor activity

In addition to the increased energy intake, 8 weeks of chronic junk food intake was associated with reduced spontaneous locomotor activity in female rats, and a trend towards reduced activity levels in males. Reduced levels of physical activity are also reported in clinical studies of human obesity, and have generally been attributed to the increased basal energy requirements of a higher body mass (237, 238). In human studies, it is often difficult to determine whether reduced physical activity is a cause or consequence of a high body mass. However, it seems unlikely that the reduced activity was a cause of the increased weight gain in this study, at least in female rats, since it was only observed after 7 weeks on the junk food diet when body weight was already significantly elevated.

2.4.3 Impact of forced junk food abstinence following a period of prolonged intake

A 72 hour period of junk food abstinence after 8 weeks of junk food diet was associated with significant reduction in standard rat chow intake and body weight loss, consistent with previous findings (82, 94, 97). Importantly, this occurred despite a decrease in plasma leptin concentration compared to rats consuming the junk food diet, which would normally be expected to stimulate appetite. This suggests that the desire to obtain more palatable food may have dampened normal physiological hunger signals during this period (239).

In contrast to a previous study where removal of sucrose after an intermittent access schedule resulted in the development of classic signs of withdrawal similar to those seen in response to withdrawal (91), we did not observe any classic withdrawal signs following removal of the palatable diet. This may be due to differences in the composition of the diet and/or the feeding schedule (i.e. intermittent versus *ad libitum* access). It has previously been reported that rats given *ad libitum* access to sucrose for one month did not exhibit signs of withdrawal upon removal of access to sucrose (91), whilst intermittent access to sucrose, but not fat or a combination of sucrose and fat, has been associated with the onset of signs of opiate-withdrawal when access to the diets was removed (92, 93). In contrast, it was reported that removal of a high-fat diet after a period of *ad libitum* access did not elicit classic withdrawal signs, but instead resulted in symptoms of heightened anxiety (94). It therefore appears that separate components of the palatable diet, i.e. fat and sugar, have distinct effects on the reward system and elicit different behavioural responses following either intermittent or continuous exposure.

Nevertheless, when we measured the locomotor activity in these rats after removal of junk food, we found differences in activity levels between the Control and JF rats. In females, Control rats which had never been exposed to the palatable diet exhibited a progressive reduction in spontaneous activity over the testing period, consistent with habituation to the experimental procedure (240), as would be expected. However, this was not seen in female rats that had been exposed to the highly palatable diet. This finding suggests that chronic junk food intake may result in heightened anxiety in female rats, which has been linked to deficits in the capacity to habituate in previous studies (240, 241). In males, we observed a trend towards an increase in spontaneous activity levels 72 hours after the removal of junk food in the animals previously exposed to junk food. It is possible that these differences between males and females could be a result of the different methods of assessing activity levels, in particular the fact that male activity data included time spent rearing and

grooming in addition to movement around the cage. Even though we did not observe any differences in the total number of episodes of rearing and grooming, it is possible that the increased physical activity levels in male JF-W rats may reflect increased time spent rearing and grooming, which is an established sign of increased anxiety (242). What is important, however, is that the behavioural signs exhibited by both males and females during the period of junk food abstinence, i.e. loss of habituation and increased spontaneous activity are signs of heightened anxiety and similar responses have been reported in rats during withdrawal from barbiturates (243), ethanol (244) and cocaine (245). Whilst the behavioural data from the present study indicated the possibility of an increased anxiety in the JF-W rats, further studies using anxiety behavioural tests including the open field test and elevated plus maze test, as well as the measurement of central corticotrophin releasing factor (CRF) expression, which is elevated in response to stress and, alcohol and drug withdrawal (95, 96), are required to provide a more accurate assessment of whether the behavioural changes observed during the period of forced junk food abstinence are due to an increase in anxiety levels.

2.4.4 Highly palatable diets and gene expression in the NAc

2.4.4.1 TH and DAT

Dopamine is released both at the VTA and NAc and its production is catalysed by its rate limiting enzyme, TH, which is an indicator of dopamine levels within the mesolimbic reward system. In the present study, we found lower TH mRNA expression in the NAc of male, but not female rats, following an extended period of junk food intake. Whilst studies of addiction have typically focussed on TH mRNA within the VTA, where the dopaminergic cell bodies lie, recent findings have clearly shown that TH mRNA is also expressed at the terminals of dopamine neurons in the NAc (162, 246), albeit at lower levels. In the present study, the finding of a negative correlation between TH mRNA expression and average daily fat and energy intake in male rats suggests that TH expression in the NAc may be directly regulated by fat and energy intake. Therefore, the lower TH in the NAc may indicate a desensitisation of the reward system as a result of continuous consumption of the junk food diet, which is likely to promote further palatable food intake in an attempt to restore reward sensitivity.

A persistent reduction in TH levels in the NAc was observed during the period of forced junk food abstinence in the male JF-W rats. Previous studies have also reported lower dopamine levels within the NAc during withdrawal from sucrose and drugs of abuse (91,

99, 247). Interestingly, we found no evidence of altered TH mRNA expression in the NAc of female rats in either the JF or JF-W groups, suggesting that the effect of the palatable diet on dopamine synthesis in the NAc is likely to be sex-specific.

There was no effect of the junk food diet or a subsequent period of abstinence on the mRNA expression of DAT, the transporter responsible for clearing dopamine from the synapse, in the NAc of either male or female rats. This is different from the reduced DAT binding reported after acute exposure to a high-fat diet in mice (248). We cannot, however, exclude the possibility that DAT binding in the NAc may have been altered in rats exposed to the palatable diet in the absence of significant changes in DAT gene expression.

2.4.4.2 D1 and D2 receptors

We found different effects of chronic exposure to a palatable diet on D1 receptor mRNA expression in the NAc in males and females. Male rats exhibited a significant increase in D1 receptor expression after chronic exposure to the palatable diet, which may be a compensatory response to the reduction in dopamine synthesis (as indicated by lower TH mRNA expression) in male JF rats. The results of our study differ from previous studies, in which D1 receptor mRNA and protein concentrations in the NAc of male rats were reduced after chronic exposure to a high-fat diet (84, 85). These differences may be the result of the shorter period of exposure to the palatable diet in our study (8 weeks) compared to previous studies (12 weeks and 20 weeks). D1 receptor expression in males was, however, no longer different from Controls after 72 hours of junk food abstinence, suggesting a restoration of D1 mRNA expression following a relatively short period of withdrawal from the junk food diet. These results are in contrast to the study by Alsio and colleagues, in which D1 receptor expression was significantly reduced in obesity prone rats during a period of forced abstinence from a junk food diet, however these different results may be due to the longer withdrawal period (18 days) when compared to that in the present study (3 days) (86). It is therefore possible that an extension of the withdrawal period in the current study may lead to further changes in D1 expression within the NAc of male JF rats.

In females, however, the expression of the D1 receptor was significantly reduced at the end of the junk food feeding period, and remained significantly lower than Controls at the end of the 72 hour period of junk food abstinence. This study is the first to demonstrate changes in D1 receptor expression in females in response to high-fat, high-sugar feeding.

The functional desensitisation of dopamine D1-like receptors is also implicated in the development of tolerance in chronic cocaine users (249). The reduction in D1 receptor expression after a highly palatable diet implies that the dopamine transmission is reduced by high-fat and high-sugar feeding, and it is possible that this may play an important role in sustaining food-seeking behaviour. In addition and in contrast to males, it appears that the expression of D1 receptors in female rats is sensitive to increases in fat and energy intake, as shown by our finding of a negative correlation between D1 receptor mRNA expression and, fat and energy intake in the female rats.

We did not observe any effect of chronic junk food feeding or a subsequent period of junk food abstinence on the mRNA expression of D2 receptor in the NAc in either males or females. There are conflicting data from previous studies in relation to the effect of palatable diets on the expression of the D2 receptor in the NAc; one study reported an increase in expression following acute exposure to a high-fat diet (77), whilst expression was reduced after 4 and 5 weeks of high-fat, high-sugar food intake (82, 86) and unchanged following 20 weeks of exposure to high-fat diets (85). Therefore, it appears that there may be a time-dependent effect on D2 receptor expression in response to a palatable diet intake which requires further investigation.

2.4.4.3 μ-opioid receptor

The opioid system has been implicated in the regulation of hedonic feeding in studies where systemic injections of opioid agonists induce a robust increase in food intake, and preferentially stimulate the intake of palatable foods (104, 250). In the present study, we found that the expression of the μ-opioid receptor in the NAc was reduced in male rats, but not females, following 8 weeks of palatable food intake, and remained lower following the period of junk food abstinence. These results in the males support the finding by Vucetic and colleagues where 12 weeks of high-fat feeding reduced μ-opioid receptor at the NAc and VTA (114). However, a recent study found that in high-fat, high-sugar rats that have been pair-fed to consume the same amount of energy as controls, μ-opioid receptor expression in the NAc in these rats was not different from their control counterparts (86). This difference may be due to the shorter length of exposure to the diet as well as the limited access to the high-fat, high-sugar diet in that study. Nevertheless, previous studies have shown that ingestion of palatable diets stimulates the synthesis and release of endogenous opioids, including β-endorphin (251), such that extended periods of exposure to a highly palatable diet will be associated with exposure of the reward system to

chronically elevated opioid concentrations. This chronic exposure to endogenous opioids, in turn, has been shown in other studies to inhibit the synthesis of opioids and reduce μ-opioid receptor abundance (252-254), consistent with our findings from the male rats. The results of the present study therefore adds to a growing body of evidence that exposure to an extended period of palatable food intake results in reduced μ-opioid receptors and potentially a reduced sensitivity to opioid stimulation (255).

The fact that the mRNA expression of μ-opioid receptor remained reduced following a 72 hour period of junk food abstinence is unexpected, since the absence of junk food would presumably diminish endogenous opioid synthesis. Nevertheless, it is possible that the expression of this receptor can be increased after more extended periods of junk food abstinence as shown by a recent finding of higher μ-opioid receptor expression in the NAc 18 days after withdrawal from a high-fat, high-sugar diet (86). The reduction in μ-opioid receptor mRNA observed in the present study may also explain why we did not observe any opiate-like withdrawal signs as seen with sucrose withdrawal, after the removal of the junk food diet. The absence of any changes in μ-opioid receptor expression in female rats suggests a distinct molecular response to palatable food intake in females compared to males.

2.4.5 Sex differences in the mesolimbic reward system of Control rats

The findings of the present study identified sex-specific effects on the mesolimbic reward system in response to chronic junk food feeding. Sex-specific effects on the reward system have also been reported in studies of drugs of abuse (256, 257) and alcohol addiction (258), and may be related to the different basal dopaminergic tone between males and females (221). We reported that female rats which have never been exposed to the junk food diet expressed lower TH in the NAc compared to naive males. The lower dopaminergic tone in female rats is postulated to be associated with a low stimulatory threshold and therefore an increased susceptibility to addiction (221). Our finding of lower μ-opioid receptor mRNA expression in naive females is, however, in contrast with previous observations, where μ-opioid receptor binding and density has been found to be higher in females than in males in a number of brain regions (259-261). The reason for this is unclear but may be due to the different methods of μ-opioid receptor quantification between studies. Nevertheless, given that the reward system is sexually dimorphic both in the basal and stimulated state, the level of basal expression of key genes within the mesolimbic reward system could

potentially influence the system's response to rewarding stimuli, thus explaining the sex-specific effects on the mesolimbic reward system in response to junk food intake.

2.4.6 Effect of chronic junk food intake on subsequent food preferences

Despite the differential adaptation of the mesolimbic reward system to chronic junk food feeding between sexes, the gene expression data of both male and female rats suggest reduced sensitivity of the reward system after 8 weeks of junk food feeding. The effect of chronic junk food feeding on subsequent food choices in a paradigm where rats were given free access to both standard chow and a range of junk foods provided evidence that these changes in gene expression have functional consequences. We found that rats previously exposed to the junk food diet consumed less total energy and fat than naive rats when provided with free access to both control chow and a palatable diet throughout the 2 week test period. This is consistent with the molecular changes in the NAc of the junk food rats, which would be expected to be associated with reduced dopamine sensitivity in the mesolimbic reward system. Previous work has also demonstrated that rodents chronically fed on a palatable high-fat, high-sugar diet exhibited higher brain reward thresholds (82) and reduced reward sensitivity as indicated by reduced sucrose preference (114) and high-fat food intake (85). These studies therefore provide evidence that changes in gene expression within the reward system can have functional consequences on the sensitivity of the reward system. Given that the JF rats have had prior exposure to the junk food diet, a more reliable measure to determine reward sensitivity would be to provide a different rewarding substance (eg morphine) that is novel to both the Control and JF rats. Although the junk food diet provided may be a confounding factor in this experimental setup, it is important to note that the experimental period lasted for 2 weeks, which would have abolished the novelty aspect of the diet in the Control rats and that the effect observed (higher fat intake) is more likely due to the higher reward sensitivity in Control rats.

2.5 Conclusion

In summary, we have shown that continuous intake of a highly palatable high-fat, high-sugar diet results in significant changes in the expression of genes in the dopaminergic and opioid systems within the central reward circuitry. The reduction in the expression of the μ-opioid receptor in males was consistent with previous observations of the effects of opioid abuse and sucrose ingestion. Similarly, the molecular changes found in the dopaminergic system in males were characteristic of those described in previous studies of drugs of abuse. A different set of molecular responses within the dopaminergic and opioid systems were seen in females, suggesting the involvement of distinct neuronal pathways. To our knowledge, this is the first study to investigate sex differences in the reward pathway both at baseline and in response to chronic intake of a palatable diet, and the clear differences observed between the sexes highlight the need for further comparative studies. These data therefore add to the growing body of evidence that palatable foods have comparable effects on the mesolimbic reward pathway as characterised drugs of abuse, and that junk food addiction is a concept that is underpinned by sex-specific physiological mechanisms. We also validated the cafeteria junk food diet-induced obesity rodent model and provided evidence that junk food diet have effects on the mesolimbic reward system in adult rats. This diet was therefore employed in the remaining chapters of this thesis which investigated the changes within the mesolimbic reward system of the offspring in response to maternal junk food intake during pregnancy and lactation.

CHAPTER 3: MATERNAL JUNK FOOD FEEDING OF RAT DAMS ALTERS FOOD CHOICES AND DEVELOPMENT OF THE MESOLIMBIC REWARD PATHWAY IN THE OFFSPRING

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3.1 Introduction

Whilst the causes of obesity are multifactorial, the ready availability of palatable high-fat, nutrient-poor junk foods and reduced levels of physical activity have had a major impact on obesity rates over the past decade, and this has led to the coining of the term 'obesogenic environment' to describe modern society (262). It is also clear, however, that some individuals who are exposed to this environment become overweight and obese, while others do not, and that there are therefore additional factors which determine the propensity to obesity in any given individual.

In this context, a world-wide series of epidemiological and experimental studies have demonstrated that an individual's susceptibility to obesity has its origins early in life, and can be related to their nutritional experience during critical windows of fetal and early post-natal development (263). There is good clinical and experimental evidence that individuals who are exposed to an elevated nutrient supply *in utero* as a consequence of maternal diabetes, maternal overnutrition or maternal obesity, are at increased risk of obesity throughout the life course (31, 264). This is particularly relevant since up to 30% of human pregnancies in developed countries are now complicated by maternal obesity (265), and there is a need to define our understanding of the mechanisms through which peri-natal overnutrition results in an increased risk of obesity later in life.

One of the possible mechanisms underlying the development of obesity as a result of peri-natal overnutrition is the programming of increased food intake in post-natal life. Previous

studies have demonstrated that exposure to an increased nutrient supply *in utero* is associated with permanent alterations within the central neural network for appetite regulation in the offspring, resulting in hyperphagia after birth (37, 41, 266). Maternal consumption of a cafeteria style diet has also been demonstrated to increase the preference for palatable foods in the offspring (40, 53). Importantly, a recent clinical study reported that preference for fat, carbohydrate and protein in children could be predicted by maternal intake of these nutrients during pregnancy, providing evidence that this phenomenon also occurs in humans (55). These studies suggest that in addition to increased food intake, maternal overnutrition can predispose offspring to later obesity by shifting their food preferences towards high-fat, high-sugar foods. The mechanisms which underlie the programming of food preferences are, however, still unclear.

In adults, palatable, or high-fat, high-sugar foods activate the dopaminergic and opioid systems within the mesolimbic reward pathway, an area in the brain implicated in motivation and reward (60, 227, 267). Activation of the mesolimbic reward pathway creates a sense of pleasure and interferes with physiological satiety signals, which promotes the further consumption of palatable foods (98). Furthermore, prolonged exposure to excessive quantities of palatable foods in adult rodents is associated with behavioural and neurophysiological adaptations comparable to those seen in drug addicts, in particular desensitisation of the central reward pathway, which then drives continued overconsumption of these foods (62). Given the known effects of palatable foods on the adult brain, it has been suggested that peri-natal exposure to a maternal diet dominated by palatable foods can alter the development of the central reward circuitry, resulting in altered function of this system after birth and an increased propensity to overconsume palatable foods later in life.

In the present study, therefore, we have investigated the hypothesis that maternal junk food intake before and during pregnancy and throughout lactation is associated with an increased preference for high-fat and high-sugar junk foods in the offspring and that this is due to altered expression of key molecules (TH, D1, D2, DAT and μ opioid receptors) within the dopaminergic and opioid systems of the mesolimbic reward pathway.

3.2 Methods

3.2.1 Animals and feeding regime

This study was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science. Twelve female Albino Wistar rats (200 - 250g) were individually housed under a 12 hour light/12 hour dark cycle at a room temperature of 25°C. Rats were allowed to acclimatise for one week before commencement of the experimental procedure.

At the end of the acclimatisation period, rats were weight-matched and assigned to control (Control, n=6) or Junk Food (JF; n=6) groups. Control rats were given free access to standard rat chow (Specialty Feeds, Glen Forrest, Western Australia, Australia) while JF rats were fed on a cafeteria junk food diet as described in Chapter 2 (Table 2.1). Every 2 days, food was replenished and food intake determined by subtracting the amount of each food type remaining at the end of the 2 day period from the amount originally provided.

Vaginal smears were conducted daily from 2 weeks prior to mating to determine the stages of the estrous cycle. After 4 weeks on their respective diets, on the evening of diestrous/proestrous, two female rats were placed in a cage with a proven male for 24 hours. A total of 4 mating males were used and each male rat was mated with females from both Control and JF groups. Vaginal smears were performed the following morning to check for the presence of sperm to confirm successful mating and this was identified as gestation day 0. Female rats were then removed from the males and housed individually. After mating, female rats were maintained on their respective diets throughout pregnancy and lactation. All dams were weighed weekly.

Pups were born at day 21 - 22 gestation. Within 2 days of birth, litter sizes were adjusted to 8 pups, with 4 males and 4 females where possible. Pups were weighed every 2 days until weaning and once a week after weaning.

3.2.2 Determination of food preferences

After weaning, male and female pups were separated and pups of the same sex from the same litter were housed together in the same cage (3 to 4 rats per cage). All cages of pups were provided with both control rat chow and the cafeteria junk food diet and pups were allowed to select freely from both diets. Both control chow and cafeteria diets were provided *ad libitum*. The total intake of each food type in each post-natal week was

calculated and equally divided according to the number of pups per cage. The calculated food intake was normalised to the pup's body weight at the end of that respective week.

3.2.3 Post-mortem and tissue collection

The dam, one male and female pup from each litter were killed at weaning (P21) and a further male and female pup from each litter were killed at 6 weeks (P42) and 3 months of age (P90) for the determination of body fat mass and collection of tissues. All post-mortems were carried out in non-fasted rats during the light phase (0800am - 1200pm). All rats were weighed immediately prior to post-mortem and were then killed with an overdose of carbon dioxide. Blood samples were collected into heparinised tubes and centrifuged at 3,500g, 4˚C for 15 minutes. The clear layer of plasma was removed and stored at -20˚C for subsequent analyses of glucose, insulin, NEFA and leptin concentrations. Organs and individual fat depots including the retroperitoneal, omental, gonadal, interscapular and subcutaneous fat were dissected and weighed. The weight of the individual fat depots were used to determine total body fat mass. Brains from 6 week and 3 month old offspring were dissected to isolate the NAc and VTA. Each region was snap frozen separately in liquid nitrogen for subsequent molecular analyses.

3.2.4 Determination of hormone and metabolite concentrations

As described in Chapter 2, plasma concentrations of NEFA and glucose were determined using the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) and Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA) respectively. Assays and analyses were conducted using a Konelab 20 (Thermo Scientific, Vantaa, Finland). Insulin and leptin concentrations were measured by radioimmunoassay with Rat Insulin and Leptin Kits (Linco Research, St. Charles, MO). All assays were conducted according to manufacturer's instructions and intra- and inter-assay coefficients of variation were <10% for all assays.

3.2.5 Isolation of the NAc and VTA

The whole brain was carefully removed and placed on a glass dish that had been cooled on dry ice. The NAc was dissected as described in Chapter 2. Briefly, a coronal slice (bregma -0.24mm to 1.68mm) which spanned from the optic chiasm to 2mm anterior to the optic chiasm was obtained and cut horizontally at the level of the rhinal fissure. The NAc was isolated by cutting 1mm above the rhinal fissure.

The VTA was dissected from a coronal slice spanning from the mammillary nucleus to the medial interpeduncular nucleus (bregma -4.95mm to -6.84mm). The section was placed with the coronal surface facing upwards and the substantia nigra was removed by cutting at the medial lemniscus. The VTA was isolated by cutting 1mm above the mammillary nucleus. The samples were snap frozen in liquid nitrogen and stored at -80°C for subsequent determination of mRNA expression by qRT-PCR.

3.2.6 Determination of gene expression in the NAc and VTA

Total RNA from the NAc and VTA was isolated with Trizol reagent (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and purified using an RNeasy Mini Kit (Qiagen Pty Ltd Australia, Doncaster, Australia). RNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm, and RNA integrity was confirmed using agarose gel electrophoresis. ~2µg RNA was then reverse transcribed to generate cDNA using Superscript III reverse transcriptase (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and random hexamers.

Quantitative real time PCR was conducted with the SYBR green system using the Rotor-Gene 6000 real time rotary analyser (Corbett Life Science, Sydney, Australia). Primer sequences for TH, D1, D2, µ-opioid receptor and DAT are shown in Table 2. β actin QuantiTect Primer Assay was used to determine β actin mRNA expression (Qiagen Pty Ltd, Doncaster, Australia). All primers were sequenced prior to the experiment to ensure the authenticity of the DNA product and a qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. The primer concentrations were consistent for all genes, and the amplification efficiency of all primers was 0.997 - 0.999. A constant amount of cDNA (2µl) was used for each qRT-PCR measurement and at least three technical replicates were performed for each gene. Two quality controls were included on each plate in order to verify inter-plate consistency, and the inter-plate coefficient of variation was <5%.

Each qRT-PCR reaction (10µl total volume) contained 5µl iQ™ SYBR® Green supermix (Bio-Rad Laboratories, Hercules, CA), 1µl of each primer giving a final concentration of 60nM to 900nM, 1µl of molecular grade H_20 and 2µl of a 25ng/µl dilution of the stock template. The cycling conditions consisted of 50 cycles of 95°C for 1 second and 60°C for 20 seconds. At the end of each run, melt curves were obtained.

The abundance of each mRNA transcript was measured and expression relative to that of β actin was calculated using Q-gene qRT-PCR analysis software. This software provides a quantitative measure of the relative abundance of a specific transcript in different tissues by the comparative threshold cycle (C_t) method which takes into account any differences in the amplification efficiencies of the target and reference genes.

3.2.7 Statistical analyses

Data are presented as mean ± SEM. For all analyses, only one data point was included for each litter, and the dam was used as the unit of analysis in all statistical tests. Analyses for post-weaning pups including body composition, plasma hormone and metabolite concentrations and gene expression in the NAc and VTA were conducted by two-way ANOVA with maternal dietary treatment (Control versus JF) and sex as factors. Where significant interactions between sex and maternal diet were identified, data from males and females were analysed separately using a Student's un-paired t-test. Body weight and food intake of the offspring were analysed separately for males and females using Student's un-paired t-test due to the differences in weight gain between sexes.

The effect of junk food feeding on body weight gain over the experimental period in male and female offspring was analysed using repeated measures ANOVA. The repeated measures ANOVA was performed using Stata 10 (StataCorp LP, Texas, USA). All other analyses were performed using SPSS for Windows Version 18.0 (SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered statistically significant in all analyses.

3.3 Results

3.3.1 Dams

3.3.1.1 Body weight, body composition, plasma hormone and metabolite concentrations

There was no difference in body weights between Control and JF dams prior to mating (Control: 289.6 ± 4.7g; JF: 311.2 ± 10.8g), however, dams fed a junk food diet gained more weight as a percentage of their starting weight prior to mating (Control: 22.5 ± 0.81%; JF: 31.3 ± 3.63%; $P<0.05$). There was no difference in gestational weight gain between Control and JF dams. After delivery, both Control and JF dams lost weight. JF dams lost significantly more weight during the second week of lactation compared to Controls (Control: -44.9 ± 18.3g, JF: -104.7 ± 14.1g; $P<0.05$). At the end of lactation, the JF dams had higher omental and interscapular fat mass as a percentage of body weight ($P<0.05$) (Table 3.1), and also tended ($P=0.06$) to have a higher total percentage body fat compared to Control dams (Table 3.1).

Plasma concentrations of NEFA were higher in JF dams than Control dams at the end of lactation ($P<0.01$) (Table 3.1). There was no difference in plasma glucose, insulin and leptin levels between Control and JF dams (Table 3.1).

3.3.1.2 Nutritional intake of dams during pregnancy and lactation

During pregnancy, JF dams consumed more fat but less protein compared to Control dams ($P<0.01$) (Figure 3.1A). During lactation, fat intake remained higher in the JF dams, while carbohydrate and protein intake was lower when compared to Control dams ($P<0.01$) (Figure 3.1B). Total energy intake of dams during pregnancy and lactation was not different between Control and JF groups (Figure 3.1A, B).

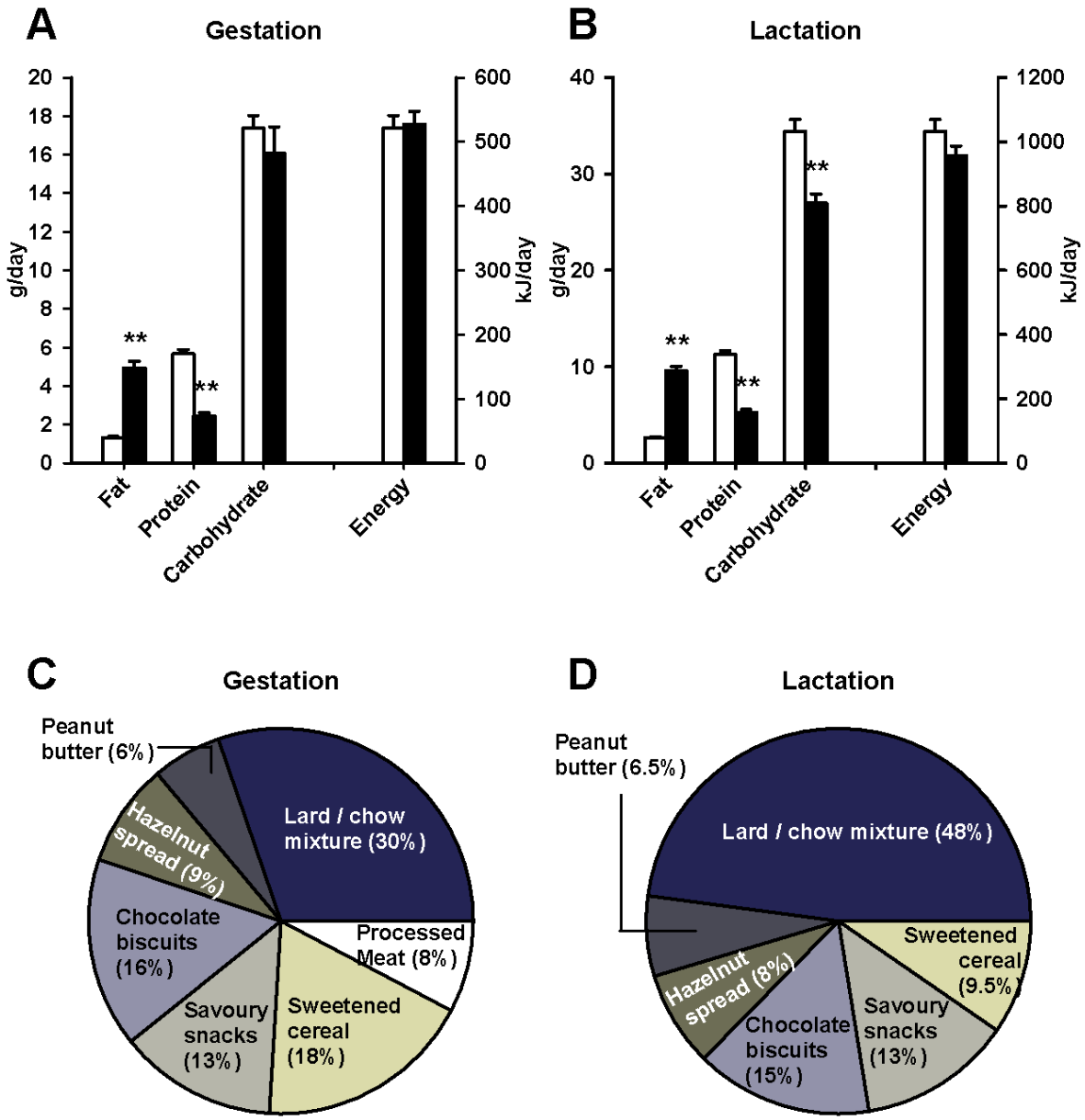
During pregnancy, the most preferred ‘junk foods’ consumed by the JF dams were the lard/chow mixture, sweetened breakfast cereal and chocolate biscuits (Figure 3.1C). Total energy intake and the quantity of each individual food consumed increased during lactation, with the lard/chow mixture, chocolate biscuits and extruded savoury snacks being consumed in the greatest quantities (Figure 3.1D).

Table 3.1 The mass of parametrial fat, retroperitoneal fat, omental fat, subcutaneous fat, interscapular fat and total body fat as a percentage of body weight (%), and plasma concentrations of NEFA, glucose, leptin and insulin in Control and JF dams at the end of lactation

Values are expressed as mean ± SEM. *P<0.05, **P<0.01, ^P=0.06. n = 6 in each group.

	Control	JF
Parametrial fat (%)	1.3 ± 0.4	2.4 ± 0.5
Retroperitoneal fat (%)	1.2 ± 0.2	2.0 ± 0.4
Omental fat (%)	0.8 ± 0.1	1.6 ± 0.3 *
Subcutaneous fat (%)	0.4 ± 0.1	0.7 ± 0.1
Interscapular fat (%)	0.4 ± 0.0	0.7 ± 0.1 *
Total fat (%)	4.0 ± 0.7	7.0 ± 1.5^
NEFA (meq/mL)	0.3 ± 0.1	0.6 ± 0.1 **
Glucose (mmol/L)	10.5 ± 1.2	9.9 ± 0.6
Leptin (µg/L)	3.4 ± 0.8	4.3 ± 1.0
Insulin (µU/mL)	4.6 ± 2.3	1.5 ± 0.4

Figure 3.1



Intake of fat, protein, carbohydrate and total energy of Control (open bars) and JF (closed bars) dams during gestation (A) and lactation (B). Percentage of total food intake of JF dams comprised of different junk food during gestation (C) and lactation (D).

Values in (A) and (B) are expressed as mean ± SEM. Values in (C) and (D) are expressed as a percentage of total foods consumed. $^{**}P<0.01$. n = 6 in each group.

3.3.2 Offspring

3.3.2.1 Birth outcomes

There was no significant difference in litter size (Control: 14 ± 1 pups/litter, JF: 12 ± 2 pups/litter) and the percentage of male (Control: 64.1 ± 5.3%, JF: 59.2 ± 3.1%) and female (Control: 35.9 ± 5.3%, JF: 40.8 ± 3.1%) offspring in each litter between the Control and JF groups. There were no pups born dead in either group. All offspring survived until weaning.

3.3.2.2 Effect of maternal junk food feeding on offspring body composition

Suckling period (P0 - P21)

Mean body weights of offspring at birth were not different between the Control and JF groups (Control: 7.0 ± 0.3g, JF: 6.6 ± 0.3g). However, male and female JF offspring were significantly lighter than their Control counterparts from 3 days after birth and remained lighter throughout the suckling period ($P<0.01$) (Figure 3.2A, B). The weights of both male and female JF offspring at weaning were significantly lower than their Control counterparts ($P<0.01$) (Figure 3.2A, B), however, JF offspring had a higher percentage of body fat ($P<0.01$) (Table 3.2).

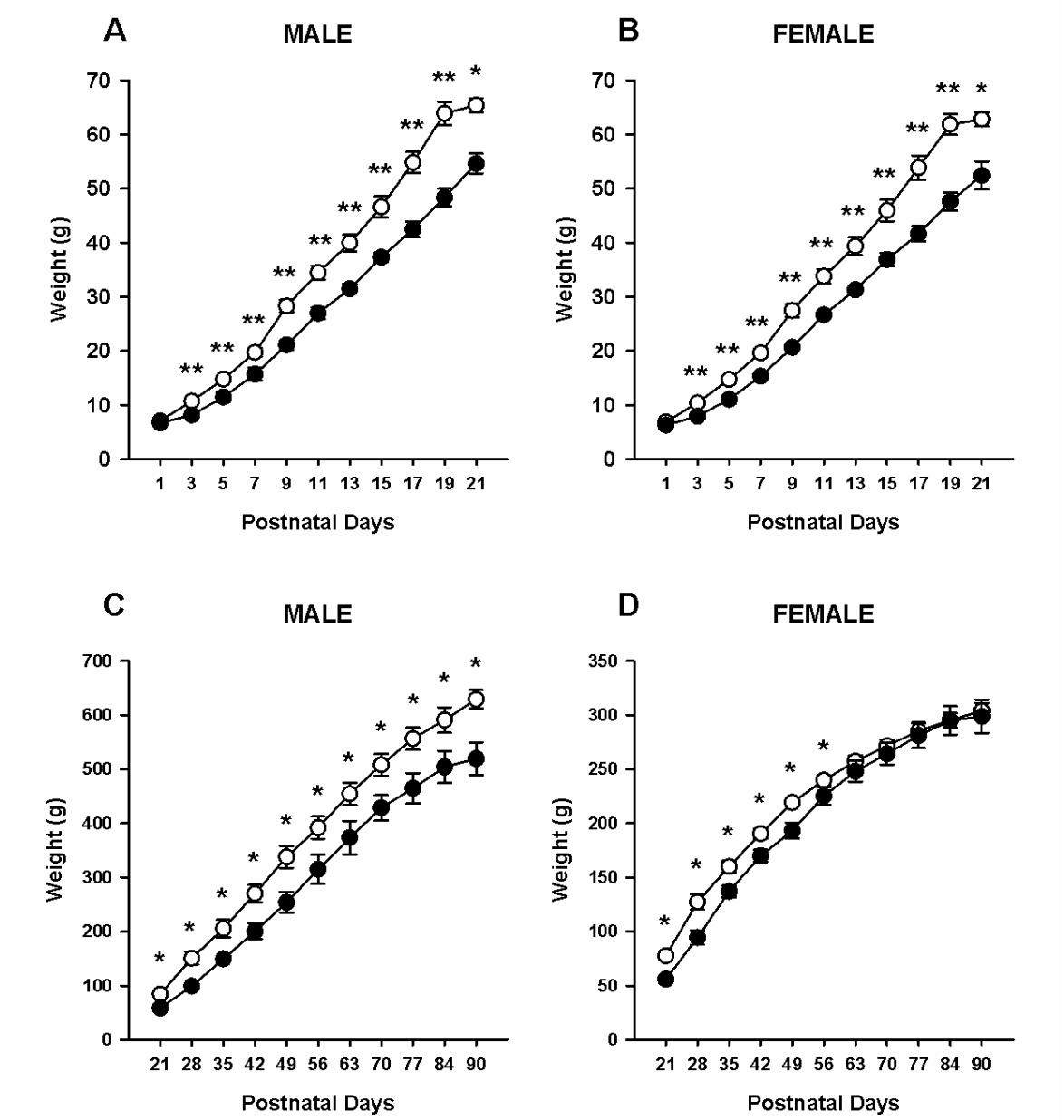
Juvenile period (P22 - P42)

The JF offspring remained lighter than their Control counterparts in the period after weaning and were still significantly lighter at 6 weeks of age (P42) in both males and females (Figure 3.2C, D). In females only, there was a significant group by time interaction for body weight in the period after weaning, and female offspring from JF dams grew at an accelerated rate compared to offspring from the Control dams during this period ($P<0.05$) (Figure 3.2D)**.** At 6 weeks of age, there was no significant difference in percentage body fat between Control and JF offspring (Table 3.2).

Adult period (P43 - P90)

Male JF offspring remained lighter than Control offspring throughout the adult period ($P<0.05$) (Figure 3.2C). In females, JF offspring were lighter than Control offspring from P42 – P56, but were not different from Control offspring from P56 onwards (Figure 3.2D). At 3 months of age (P90), there were no significant differences in percentage body fat between Control and JF offspring (Table 3.2).

Figure 3.2



Absolute body weights (g) of male and female Control (open circles) and JF (closed circles) offspring during the suckling period (P1 - P21) (A, B) and after weaning (C, D).

Values are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 4 - 6 in each group.

Table 3.2: Total body fat as a percentage of body weight in Control and JF offspring at 3 weeks, 6 weeks and 3 months of age

Values are expressed as mean ± SEM. ***P*<0.01. n = 4 - 6 in each group.

	Male		*Female*	
	Control	JF	Control	JF
% Fat (3 weeks)	5.8 ± 0.3%	8.3 ± 0.7%**	8.0 ± 0.6%	9.0 ± 0.4%**
% Fat (6 weeks)	9.1 ± 0.4%	10.7 ± 0.8%	9.1 ± 0.3%	8.1 ± 0.7%
% Fat (3 months)	19.0 ± 1.2%	17.1 ± 1.7%	15.4 ± 1.1%	15.5 ± 1.3%

Table 3.3: Plasma concentrations of NEFA, glucose, leptin and insulin in Control and JF offspring at 3 weeks, 6 weeks and 3 months of age

Values are expressed as mean ± SEM. ***P*<0.01. n = 9 - 12 in each group.

	3 weeks		*6 weeks*		*3 months*	
	Control	JF	Control	JF	Control	JF
NEFA (meq/mL)	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Glucose (mmol/L)	10.0 ± 0.5	9.7 ± 0.8	10.8 ± 0.6	12.6 ± 0.7	14.4 ± 1.1	16.3 ± 1.5
Leptin (µg/L)	8.3 ± 0.6	14.8 ± 1.5**	8.6 ± 0.7	11.5 ± 1.1	23.4 ± 3.2	34.5 ± 6.1
Insulin (µU/mL)	0.9 ± 0.1	0.5 ± 0.1**	1.8 ± 0.3	1.3 ± 0.2	3.8 ± 0.7	2.9 ± 0.5

3.3.2.3 Effect of maternal junk food feeding on offspring plasma hormone and metabolite concentrations

Plasma leptin concentrations at weaning were significantly higher in JF offspring when compared to Controls ($P<0.01$) (Table 3.3). Plasma leptin concentrations also tended ($P=0.07$) to be elevated in JF offspring at 6 weeks of age, but were not different by 3 months. Plasma insulin concentrations were significantly lower at weaning in JF offspring ($P<0.01$) but were not different from Controls at 6 weeks or 3 months of age (Table 3.3). There was no effect of maternal junk food feeding on plasma glucose or NEFA concentrations at any age (Table 3.3). No interactions between maternal diet and sex were observed.

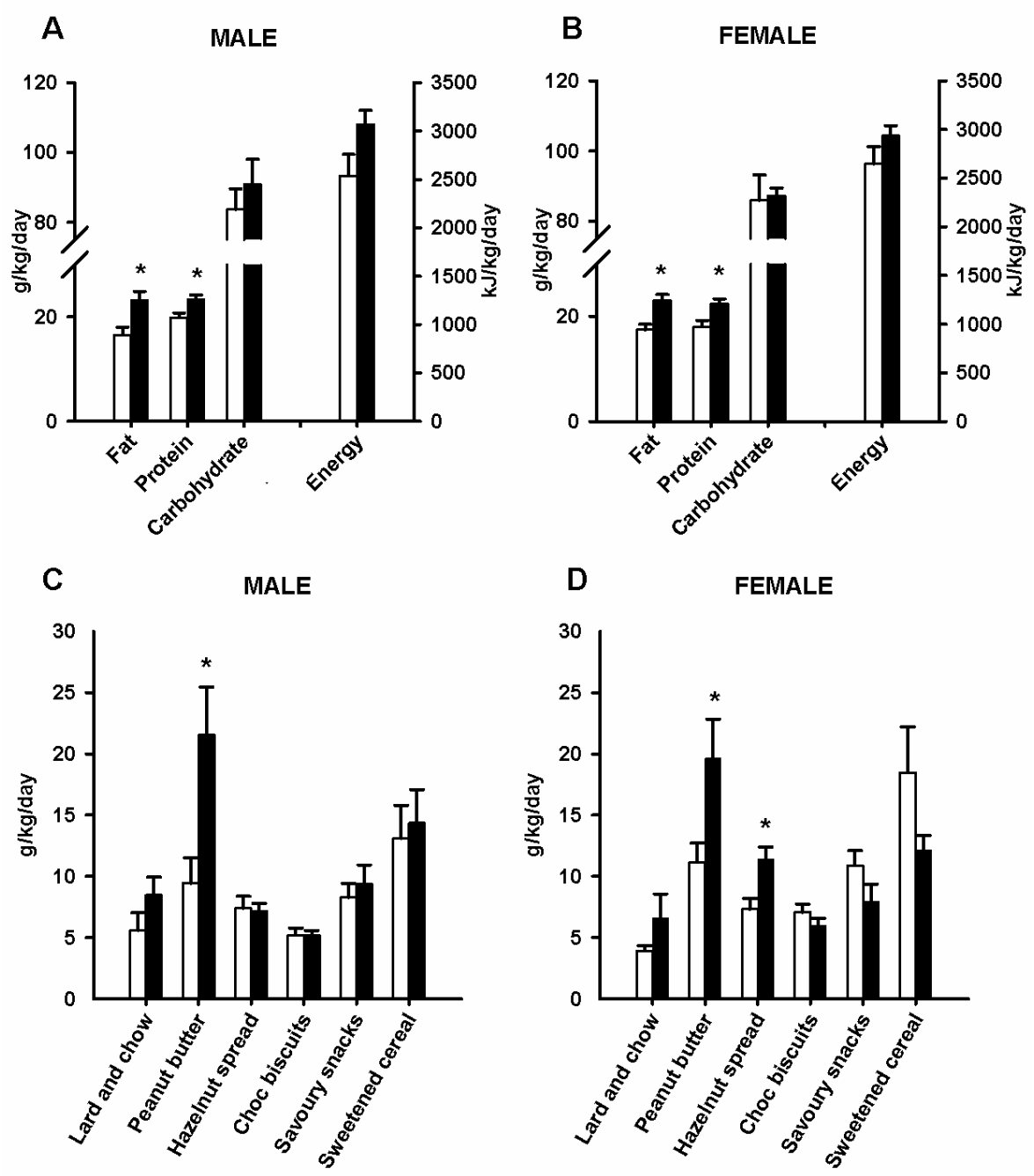
3.3.2.4 Effect of maternal junk food feeding on offspring food intake

Juvenile period (P22 - P42)

During the first 3 weeks after weaning, fat and protein intakes were higher in both male and female JF offspring ($P<0.05$) (Figure 3.3A, B). There was no difference between Control and JF groups in total energy intake and carbohydrate intake, although in males, total energy intake tended ($P=0.07$) to be higher in the JF group (Figure 3.3A, B).

When the intake of individual junk foods was analysed, peanut butter stood out as the most preferred food for both male and female JF offspring, and was consumed in significantly greater amounts when compared to Control offspring ($P<0.05$) (Figure 3.3C, D). In addition, female JF offspring also consumed more hazelnut spread during the juvenile period than female Control offspring ($P<0.05$) (Figure 3.3D). The amount of standard rat chow consumed was not different between groups (Male Control: 74.9 ± 3.34g/kg/day, Male JF: 80.7 ± 7.12g/kg/day; Female Control: 66.9 ± 4.46g/kg/day, Female JF: 76.9 ± 3.40g/kg/day).

Figure 3.3



Intake of fat, protein, carbohydrate and total energy in male (A) and female (B) Control (open bars) and JF (closed bars) offspring during the juvenile period and intake of individual junk foods in male (C) and female (D) Control (open bars) and JF (closed bars) offspring during the juvenile period (P21 - P42).

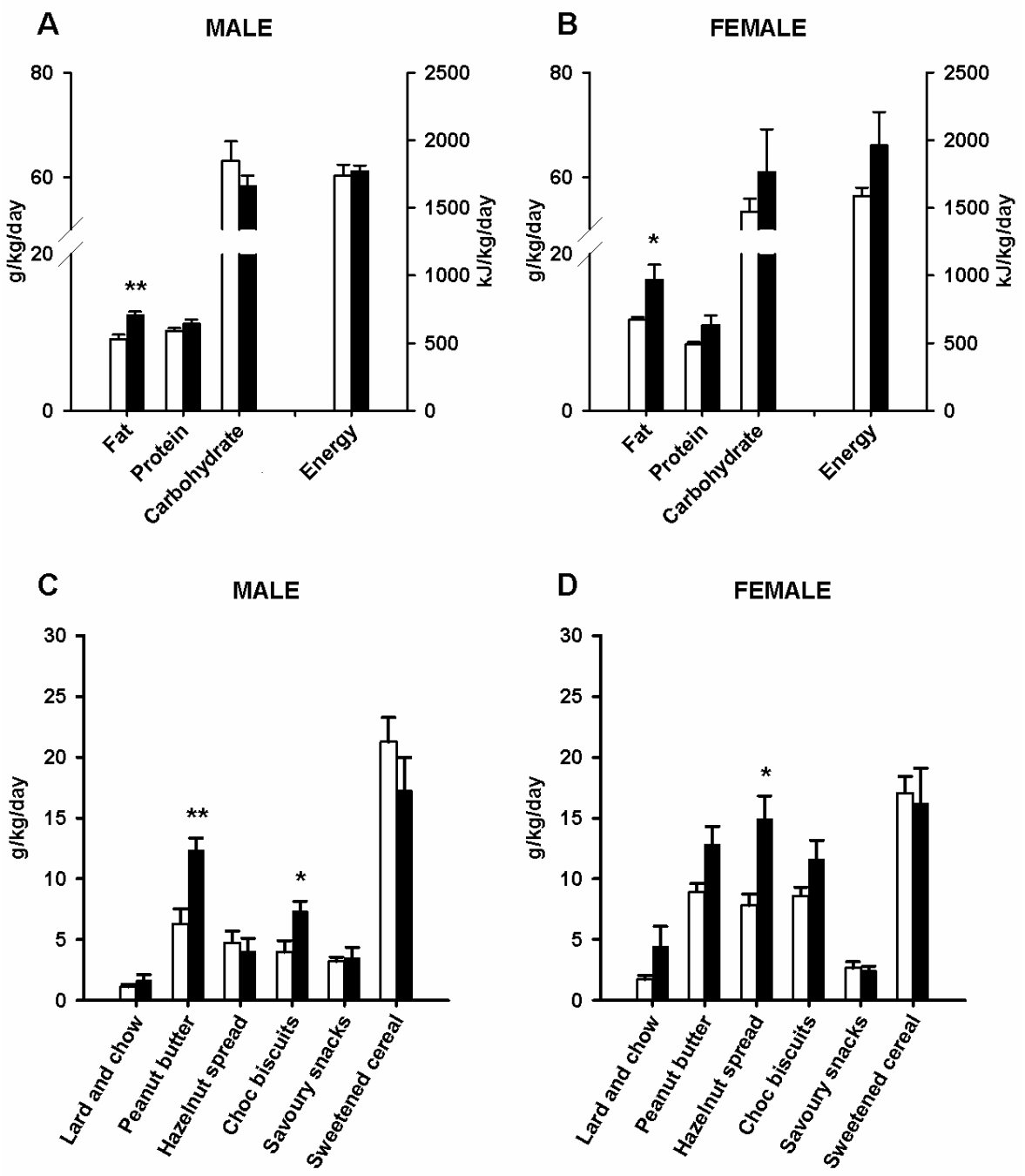
Values are expressed as mean ± SEM. **P*<0.05. n = 6 for each group.

Adult period (P43 - P90)

Similar patterns of food intake continued into early adulthood; the higher intake of fat was maintained in the JF offspring ($P<0.05$), but there was no longer any difference in protein intake between the treatment groups (Figure 3.4A, B). Total energy and carbohydrate intakes were also not different between Control and JF offspring (Figure 3.4A, B).

Peanut butter consumption remained higher in adult male offspring of JF dams compared to Control offspring ($P<0.01$) (Figure 3.4C). Male JF offspring also had a higher intake of chocolate biscuits during adulthood compared to Control offspring ($P<0.05$) (Figure 3.4C). In adult female offspring, the consumption of hazelnut spread was higher in the JF group compared to Controls ($P<0.05$) (Figure 3.4D). Peanut butter intake also tended ($P=0.07$) to be higher in adult female JF offspring compared to Controls (Figure 3.4D). There was no difference in the intake of standard rat chow between groups in either male or female offspring (Male Control: 39.0 ± 1.38g/kg/day, Male JF: 35.9 ± 2.13g/kg/day; Female Control: 23.3. ± 1.89g/kg/day, Female JF: 28.6 ± 3.20g/kg/day).

Figure 3.4



Intake of fat, protein, carbohydrate and total energy in male (A) and female (B) Control (open bars) and JF (closed bars) offspring and intake of individual junk foods in male (C) and female (D) Control (open bars) and JF (closed bars) offspring during adulthood (P43 - P90).

Values are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 6 in each group.

3.3.2.5 Effect of maternal junk food feeding on gene expression in the NAc and VTA of the offspring at 6 weeks and 3 months of age

There were no interactions between sex and maternal diet on μ-opioid receptor, DAT, TH, D1 and D2 mRNA expression in the NAc and VTA.

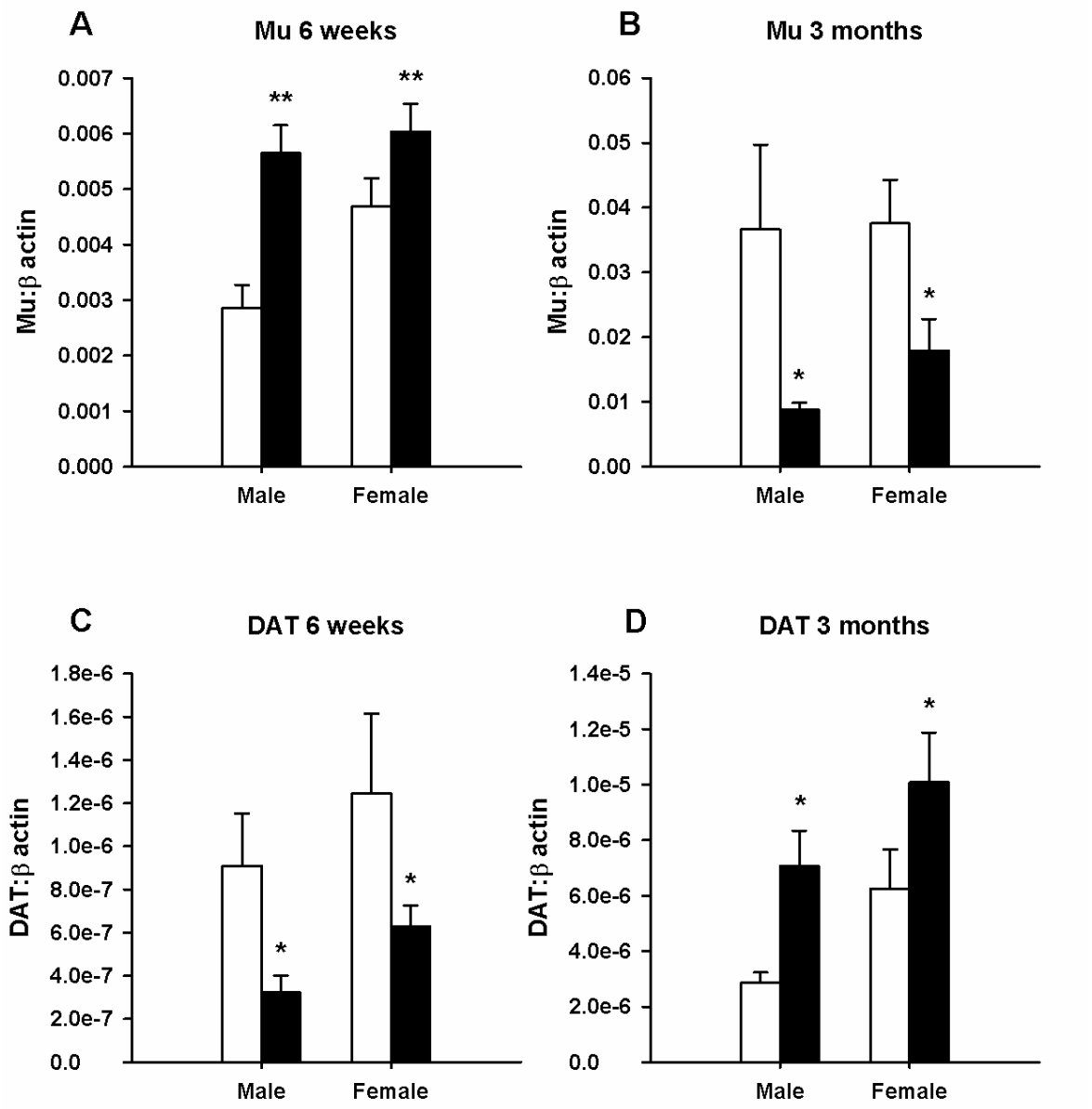
μ-opioid receptor mRNA expression in the NAc was higher in JF offspring at 6 weeks of age independent of sex ($P<0.01$) (Figure 3.5A). Similarly in the VTA, μ-opioid receptor mRNA expression tended to be higher in the JF offspring than the Controls (Control: 0.005 ± 0.001, JF: 0.010 ± 0.004; $P=0.07$). When data from all offspring were combined, μ-opioid receptor mRNA expression in the NAc was positively correlated with mean fat intake at 6 weeks of age (r= 0.524; $P<0.03$) and inversely correlated to the offspring's body weight at 6 weeks of age (r= -0.565; $P<0.02$).

This increase in μ-opioid receptor mRNA expression at 6 weeks of age did not persist, however, and at 3 months of age μ-opioid receptor mRNA expression in the NAc was significantly lower in the JF offspring compared to Controls ($P<0.05$) (Figure 3.5B). Interestingly, there was a 10-fold increase in μ-opioid receptor mRNA expression of the Control offspring from 6 weeks to 3 months of age, whereas in the JF offspring, the increase in μ-opioid receptor mRNA expression from 6 weeks to 3 months of age was only 3-fold.

DAT mRNA expression in the NAc was lower in the JF group compared to the Controls at 6 weeks of age ($P<0.05$) (Figure 3.5C). At 3 months of age however, DAT mRNA expression had increased in both groups and was significantly higher in the JF group compared to Controls ($P<0.05$) (Figure 3.5D). There was no difference in DAT mRNA expression in the VTA between the JF and Control groups (Table 3.4).

There was no effect of maternal junk food feeding on mRNA expression of TH or the D1 and D2 receptors in the NAc or VTA at 6 weeks or 3 months of age in either male or female offspring (Table 3.4).

Figure 3.5



mRNA expression of μ-opioid receptor (Mu) (A, B) and DAT (C, D) of Control (open bars) and JF (closed bars) offspring at 6 weeks and 3 months of age in both males and females.

Values are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 9 - 11 in each group.

Table 3.4: mRNA expression of TH, D1 and D2 (relative to β actin) in the NAc and VTA of Control and JF offspring at 6 weeks and 3 months of age

For ease of presentation, values for TH (NAc), DAT (VTA 6 weeks), D1 and D2 (NAc and VTA) are multiples of 1000. Values include both males and females and are expressed as mean ± SEM. n = 10 - 11 in each group.

	6 weeks		*3 months*	
	Control	JF	Control	JF
NAc				
TH	0.13 ± 0.02	0.18 ± 0.05	0.04 ± 0.00	0.04 ± 0.01
D1	38.5 ± 5.53	37.0 ± 4.08	16.9 ± 1.94	17.3 ± 1.62
D2	13.7 ± 1.64	14.4 ± 1.70	79.8 ± 16.10	56.7 ± 9.11
VTA				
TH	0.27 ± 0.07	0.15 ± 0.05	0.38 ± 0.03	0.35 ± 0.02
D1	1.3 ± 0.19	1.3± 0.38	0.11 ± 0.02	0.11 ± 0.02
D2	18.6 ± 5.15	16.5 ± 5.95	34.5 ± 4.87	36.9 ± 7.40
DAT	17.4 ± 5.04	6.8 ± 2.20	0.17 ± 0.05	0.13 ± 0.02

3.4 Discussion

This study has shown that offspring of dams consuming a junk food diet during pregnancy and lactation exhibit an increased preference for fat which persists from weaning until at least 3 months of age. Importantly, peri-natal exposure to a maternal junk food diet was associated with elevated expression of the μ-opioid receptor and reduced expression of the DAT at 6 weeks of age; changes consistent with heightened activation of opioid and dopaminergic activity within the central reward pathway, and these changes were reversed by the age of 3 months. These findings provide evidence that the development of the dopaminergic and opioid systems within the central reward circuitry is altered by exposure to a high-fat, high-sugar diet during the peri-natal period, and that this may be a potential mechanism through which peri-natal exposure to highly palatable diets increases the preference for high-fat foods in the offspring after birth.

3.4.1 Maternal junk food feeding and post-natal growth

We found that offspring of junk food fed dams were significantly lighter than Control offspring from 3 days after birth, and remained lighter throughout the suckling period. Previous studies of maternal high-fat feeding have provided conflicting results regarding effects on offspring body weight, with reports of increased (41, 53) and decreased (40, 219, 268) body weight at weaning. It is clear, however, that maternal obesity/high-fat diets do not always result in increased fetal and post-natal growth, and clinical studies have demonstrated that ~20% of obese mothers give birth to low birth weight infants (13, 269). The reduced post-natal growth in offspring of high-fat fed/obese mothers appears to be due to a lack of essential amino acids for growth and deficits in milk production (270, 271). The lack of amino acids for tissue accretion in early post-natal life is likely to have contributed, at least in part, to the reduced post-natal growth in the JF offspring in our study, since the JF dams consumed significantly less protein during pregnancy (7.2% vs 18.5%) and lactation (9.3% vs 18.5%) compared to Controls. The impact of reduction in maternal protein intake was similar to that in the rodent model of maternal low protein intake (9% vs 18% casein), which has consistently been shown to result in reduced body weight in the offspring at birth and in early post-natal life (49, 272).

Although milk production was not quantified in our study, there is evidence from clinical studies that obese mothers are at risk of breastfeeding difficulties as a result of delayed lactogenesis (273). It is therefore possible that a reduced milk supply, together with a

reduced availability of essential amino acids to support tissue growth, may have contributed to the lower body weight of JF offspring during the suckling period. Since there was no difference in the relative weights of major organs such as the liver and heart between the Control and JF offspring (data not shown) and body fat mass is positively correlated to bone mineral density and bone mineral content (44), the reduced body weight in the JF offspring was most likely due to deficits in lean tissue mass (274), rather than fat tissue deposition. This was supported by our finding that despite their lower body weight, the percentage body fat of the JF offspring was significantly higher than the Control offspring at the end of the suckling period. Fat deposition in the offspring has also been associated with the high fat content (271, 274, 275), high leptin (275) and insulin levels, but low essential fatty acids (191) levels in the milk of high-fat fed dams. Therefore, it is postulated that the milk composition of the JF dams may have contributed to the higher percentage of total body fat at weaning in the JF offspring. This observation is consistent with previous studies (40, 196) and provides evidence that peri-natal exposure to a high-fat diet is associated with an early increase in fat deposition.

The JF offspring in the present study remained smaller in the post-weaning period in both males and females, and did not catch up to their Control counterparts before 8 weeks (females) or 3 months (males) of age, despite higher fat and protein intakes. This is consistent with previous studies in both the maternal low protein (49) and maternal high-fat feeding (219) models, and suggests that deficits in lean tissue growth which are acquired before weaning are a critical determinant of lean body mass in adulthood. In contrast to the higher fat mass at weaning, there was no longer any difference in fat mass between the Control and JF offspring at 6 weeks and 3 months of age in either males or females, despite the increased fat intake in the JF group during this time. This is different from the results of several previous studies, which have reported that maternal high-fat feeding increases the susceptibility to diet-induced obesity in the offspring (41, 48). Nevertheless, our results are consistent with recent studies, which found that the effect of a high-fat diet after weaning on body weight and metabolic outcomes were not different between offspring born to Control or high-fat fed mothers (219) and that offspring born to dams consuming a high-fat diet were protected against diet-induced obesity (44). This has been postulated to be a consequence of the predicted adaptive response, whereby the fetus is capable of making adaptive changes in response to the *in utero* environment in anticipation of the environment in post-natal life (276, 277) (i.e. exposure to a high-fat environment during the peri-natal period would cause the fetus to become adapted to a

high-fat environment after birth). This suggests that *in utero* exposure to a junk food diet may result in physiological adaptations (eg. altered metabolic rate) in the fetus which 'prepare' the offspring for a high-fat, high-sugar environment after birth (219). Therefore, in our study, it is possible that the JF offspring are, to a certain extent, 'protected' from the effects of post-natal junk food diet and as a result, were not different from Control offspring in terms of metabolic outcomes. It is also possible, however, that the lack of a difference in fat mass between Control and JF offspring post-weaning was due to the fact that the total energy intake was not different between groups, and that total calories consumed is a more important determinant of weight gain and fat deposition. In addition, whilst the JF offspring consumed more fat, Control offspring consumed more carbohydrate as a percentage of total energy intake (data not shown), and it is becoming increasingly clear that high carbohydrate diets, especially those of high glycemic index, can promote weight gain and body fat mass to the same extent as high-fat diets (278).

3.4.2 Maternal junk food feeding alters food choices in the offspring

In the present study, we have confirmed the findings from previous studies in both humans and experimental animal models demonstrating that food preferences in the offspring post-weaning are influenced by maternal diet during pregnancy and lactation (40, 59, 279); and both male and female offspring of JF dams consumed more fat than Control offspring from weaning until at least 3 months of age. We also observed a preference for protein in the JF offspring, but only during the first 3 weeks after weaning. One possibility is that this was a compensatory response to the reduced protein intake by the JF dams during pregnancy and lactation. Previous studies have reported, however, that a maternal low protein diet has no effect on protein consumption in the offspring (49), and an alternate explanation may be that the increased protein intake was a secondary effect of the increased peanut butter intake, which was primarily driven by its high fat content (71%), although further studies are required in order to test this hypothesis. The fact that the 'junk foods' selected by the offspring were distinct from those which were selected by the dams suggests that it was the macronutrient content of the foods, rather than being exposed to their taste through the maternal milk, which was responsible for the increased preference for these foods in the offspring.

3.4.3 Maternal junk food feeding alters the opioid and dopaminergic signaling systems within the central reward pathway of the offspring

A key finding of the present study was that maternal junk food feeding resulted in altered expression of key components of the opioid and dopaminergic systems within the central reward pathway at both 6 weeks and 3 months of age. This is the first study to report longitudinal changes in the development of the reward pathway following peri-natal exposure to a high-fat, high-sugar diet, and adds to the growing body of evidence that peri-natal exposure to high-fat, high-sugar feeding is associated with altered development of the neural circuits that govern motivation and reward, and that this may explain the observed increases in fat intake in the offspring.

3.4.4 Increased mRNA expression of μ-opioid receptor and DAT in juvenile JF offspring

The altered profile of gene expression in the reward pathway at 6 weeks of age is consistent with heightened opioid and dopamine activity during this period; increased μ-opioid receptor mRNA expression in the NAc is associated with increased responsiveness to endogenous and exogenous opioids (53, 280), whilst the reduced expression of DAT would be expected to lead to reduced re-uptake of dopamine into the pre-synaptic neuron, thereby prolonging the transmission of dopamine to the post-synaptic terminal (281). The activation of opioid and dopaminergic systems in particular has been associated with an increased preference for palatable foods (101, 227), and it is therefore possible that the increase in the expression of μ-opioid receptor mRNA and reduced DAT mRNA in the NAc in early post-natal life may have driven the early preference for fat in the JF offspring. In support of this, μ-opioid receptor mRNA expression is increased in obesity prone rats prior to the onset of obesity in conjunction with an increased preference for high-fat foods, suggesting a potential causal relationship between increased μ-opioid receptor expression and increased fat intake (110). It is also important to note that in rats, the dopaminergic neurons in the central reward circuitry are present as early as E12 but do not fully develop until the second or third week of post-natal life (145, 282, 283), suggesting that nutritional perturbations during early post-natal life have the potential to influence the subsequent structure and function of this neuronal circuitry.

3.4.5 Reduced mRNA expression of μ-opioid receptor and DAT in adult JF offspring

The changes in gene expression seen at 6 weeks of age were completely reversed by the age of 3 months, when the expression of μ-opioid receptor mRNA was decreased and expression of DAT mRNA was increased relative to Controls. This pattern of gene

expression is consistent with reduced sensitivity to opioids and increased re-uptake of dopamine into the pre-synaptic neuron, and therefore collectively indicates reduced opioid and dopamine activity. These results are consistent with previous studies which have reported reduced sensitivity to amphetamine-induced locomotor activity and increased dopamine content within the NAc in the adult offspring of high-fat fed mice, which provided evidence for the desensitisation of dopamine receptors (159). Similarly, Vucetic and colleagues reported that offspring of dams fed on a high-fat diet had reduced expression of D1, D2 receptors and increased DAT expression at 6 months of age, indicating reduced activity of the dopaminergic system. This was further supported by reduced sucrose intake in the offspring of high-fat-fed dams, suggesting the dopamine system is now less sensitive to the rewarding value of palatable foods. Early exposure (3 weeks of age) to a high-fat diet in mice also resulted in a hypodopaminergic state in adulthood (52).

It is important not to disregard that palatable food intake can also alter dopaminergic and opioid systems within the central reward pathway (77, 87, 284). Therefore, this suggests that Control offspring in the present study may have altered dopaminergic and opioid systems as a result of chronic high-fat, high-sugar consumption from weaning until adulthood. However, the fact that we observed differences between the JF and Control offspring would appear to indicate that the impact of high-fat, high-sugar feeding on the central reward pathway may be further exaggerated in the JF offspring compared to the Controls and that, this may be a result of the altered reward systems during the peri-natal period in the JF offspring. Studies are therefore required to further investigate the development of the central reward pathway and how it is affected by maternal high-fat, high-sugar feeding.

Collectively, we and others have provided evidence that exposure to a high-fat and/or high-sugar diet before birth may increase the preference towards high-fat foods in the offspring after birth and this preference is associated with changes within the central reward pathway (82, 88, 94). Since the reward pathway has implications in the development of addiction, it is therefore possible that peri-natal changes within the reward pathway can potentially lead to ‘fat addiction’ and the inability to control food intake, as reported in obese individuals (134). In contrast to the altered expression of DAT and μ-opioid receptor in the JF offspring, there was no effect of maternal junk food feeding on the expression of the other

key components of the dopaminergic system at 6 weeks or 3 months of age, suggesting that these are less sensitive to programming by peri-natal exposure to high fat, high sugar diets.

3.4.6 Role of leptin and insulin in the development of the central reward circuitry?

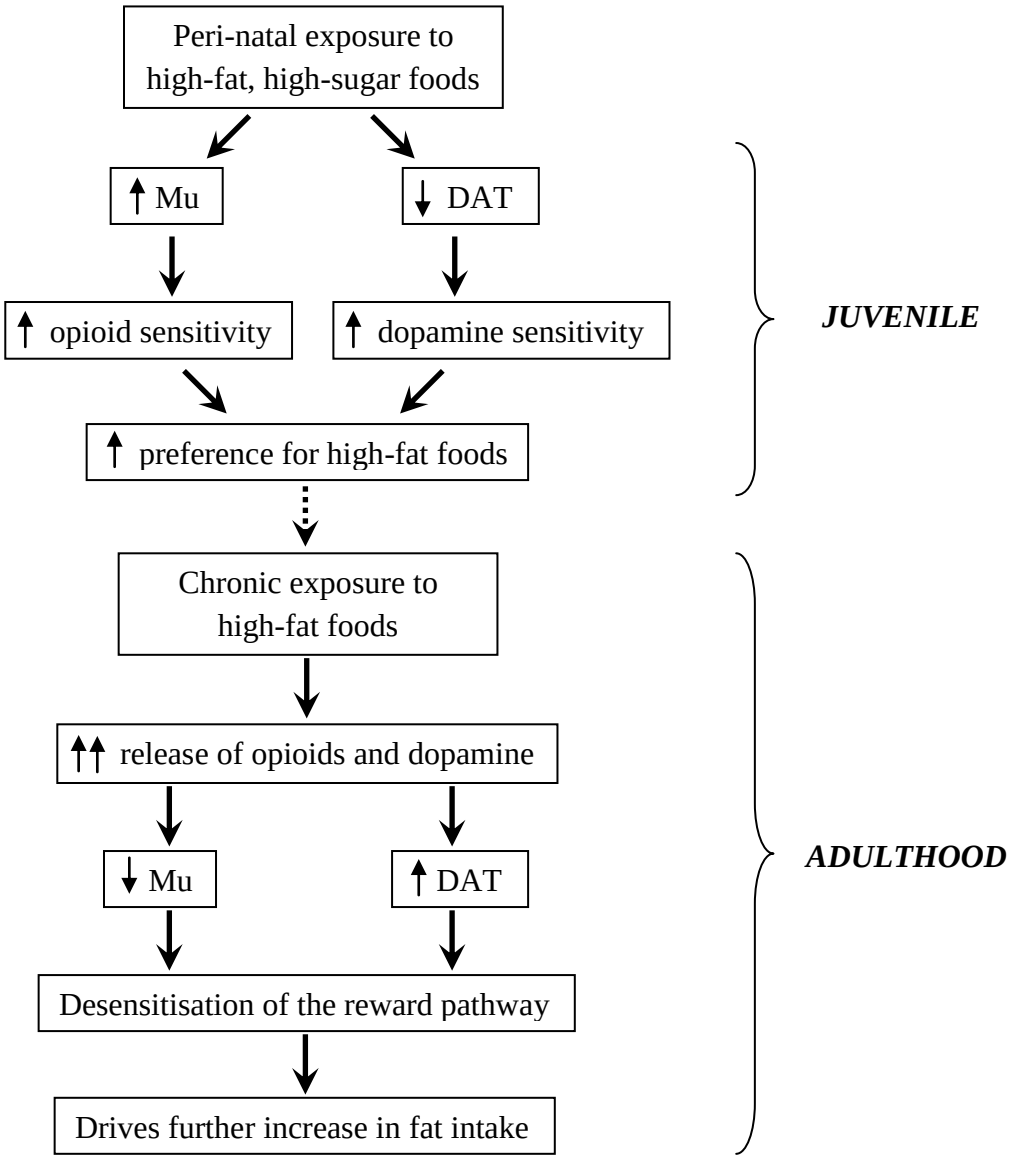
As noted earlier, development of dopaminergic neurons is not fully established until the second and third week of post-natal life and therefore the development of this pathway is sensitive to the nutritional environment during the peri-natal period. Leptin has been shown to profoundly influence the development of the central neural circuit which regulates appetite (41, 139, 285). Whilst there is no direct evidence that leptin influences the development of the central reward circuitry, studies in adult rats have shown that leptin receptors co-localise with dopamine neurons in the VTA and that leptin acts to inhibit dopamine activity (121, 126). In the present study, the offspring of JF dams had significantly higher concentrations of leptin than the Control offspring at weaning, consistent with previous findings where elevated leptin levels were prolonged during the sucking period in offspring of high-fat fed dams (41). It is therefore possible that the increased leptin concentrations in the JF offspring may lead to altered development of the central reward circuitry. However, further studies are required to confirm this.

In addition to increased leptin levels, JF offspring were also hypoinsulinaemic. The reduced insulin levels may be due to low maternal carbohydrate intake during pregnancy and lactation, and/or a consequence of elevated leptin levels in the offspring, which can act to suppress insulin secretion from the pancreas through the adipoinsular axis (286). Insulin during early developmental periods is important in neuronal maturation (176). Similar to leptin, hyperinsulinaemia during the perinatal period has been shown to be associated with impaired organisation of the hypothalamic appetite regulator network (47, 287), but no evidence on the development of the central reward circuitry thus far. Insulin receptors are also present on dopaminergic neurons within the VTA (120) and insulin increases DAT activity (129) to regulate food reward (124, 130). Hence, based on the available evidence, it is speculated that alterations in insulin and leptin levels in the offspring early in life can influence the development of mesolimbic reward pathways and potentially affect their food preferences later on in life.

3.5 Conclusion

The results of this study add to the growing body of evidence that exposure to a high-fat, high-sugar junk food diet during the peri-natal period results in persistent alterations in the control of food intake. Specifically, exposure to a junk food diet *in utero* and during the suckling period was associated with an increased intake of fat in post-natal life when offspring were given free access to a range of foods of varying macronutrient composition. Importantly, we have reported that offspring exposed to maternal junk food feeding exhibit altered expression of key components of the central reward pathway at 6 weeks of age which are indicative of heightened opioid and dopaminergic activity, providing a potential mechanism for the increased fat intake in early post-natal life. The offspring of JF dams also exhibited a transition from increased to suppressed opioid and dopaminergic activity between 6 weeks and 3 months of age, which may indicate an increased susceptibility to neuronal changes within the central reward pathway and subsequent development of junk food dependence associated with excessive intake of palatable foods (Figure 3.6). Thus, alterations in the function of the pathway regulating motivation and reward represents one potential mechanism whereby exposure to high-fat, high-sugar diets in early life may lead to the subsequent development of childhood and adult obesity.

Figure 3.6


Peri-natal exposure to high-fat, high-sugar foods
↑ Mu
↓ DAT
↑ opioid sensitivity
↑ dopamine sensitivity
↑ preference for high-fat foods
JUVENILE
Chronic exposure to high-fat foods
↑↑ release of opioids and dopamine
↓ Mu
↑ DAT
Desensitisation of the reward pathway
Drives further increase in fat intake
ADULTHOOD

Summary of proposed mechanism for the establishment of a high-fat food preference and intake in offspring of junk food fed dams during the juvenile and adult period.

Peri-natal exposure to high-fat, high-sugar foods results in increased μ-opioid receptor (Mu) and decreased DAT expression in the NAc of the juvenile offspring, which is associated with heightened opioid and dopamine activity within the central reward pathway. Increased opioid and dopaminergic activity promotes an increased drive to consume high-fat foods during the juvenile period. The elevated activity within the central reward pathway, together with the high intake of high-fat foods leads to further increase in opioid and dopamine release. Between 6 weeks and 3 months of age, the persistent exposure to elevated dopamine and opioid concentrations results in a compensatory downregulation of μ-opioid receptor and upregulation of DAT in the JF offspring (i.e. desensitisation of the reward pathway). This neuronal desensitisation, which is a characteristic of drug and alcohol abuse, drives the animal to continue to seek out fat during adulthood in an attempt to maintain the same degree of activation of the reward circuitry. The dashed arrow represents the transition from juvenile period to adulthood.

CHAPTER 4: A POST-WEANING LOW-FAT CONTROL DIET REVERSES THE PREFERENCE FOR FAT BUT NOT OVERALL APPETITE FOR JUNK FOOD IN JUVENILE OFFSPRING OF JUNK FOOD FED DAMS

4.1 Introduction

Both human and animal studies have provided compelling evidence that maternal overnutrition during pregnancy and lactation is associated with an increased risk of obesity and its related metabolic disorders in the offspring later in life (30, 33, 37, 42). These studies have shown that exposure to an increased nutrient supply *in utero* or during the early post-natal period can also lead to alterations within the hypothalamic appetite regulating network of the offspring, resulting in persistent hyperphagia during post-natal life (37, 41, 48), thereby predisposing the offspring to later obesity.

More recently, data has emerged which suggests that exposure to an increased nutrient supply in early life not only results in altered appetite regulation but also programs a shift in macronutrient preferences in the offspring, resulting in an increased preference for fat and sugar in post-natal life (40, 52, 53). This early programming of food preferences may therefore be one important mechanism through which pre-natal exposure to an increased nutrient supply programs an increased propensity to obesity later in life. These findings are consistent with results presented in Chapter 3 of the thesis, in which exposure to a maternal high-fat, high-sugar diet *in utero* and during the suckling period was associated with an increased preference for fat from weaning until at least 3 months of age.

Whilst the mechanisms underlying the early life origins of food preferences are not well understood, there is emerging evidence to suggest that exposure to an excessive supply of palatable foods high in fat and sugar can alter the development of the mesolimbic reward system, resulting in structural and functional changes in the way this system responds to natural rewards, including palatable foods, later in life. Previous studies have reported altered expression of key components of the dopamine and opioid systems within the NAc and VTA, including a lower D2 receptor expression and higher μ-opioid receptor expression in adult offspring of dams fed a high-fat diet during pregnancy and lactation (51,

53, 159). In Chapter 3 of this thesis, it was demonstrated for the first time that altered expression of the μ-opioid receptor and dopamine active transporter (DAT) was already present in offspring of dams fed a palatable diet as early as 3 weeks after weaning, and that these changes occurred in conjunction with an increased preference for fat in these offspring during this period. These data indicate that changes in the development of the mesolimbic reward system as a result of nutritional insults during the peri-natal period are likely to occur very early in life, and that there may be a causal link between altered development of mesolimbic pathways and the increased preference for fat in the offspring later on in life.

It is still unclear, however, how early in development do the effects of peri-natal exposure to a palatable diet on gene expression within the mesolimbic reward system emerge and whether the increased preference for fat in the offspring can be ameliorated by nutritional interventions which are applied after weaning. Therefore, the aims of this Chapter were to: 1) determine whether altered expression of key components of the mesolimbic reward system in offspring of junk food fed dams were already present at weaning and 2) investigate whether the impact of peri-natal junk food exposure on food preferences and expression of key components of the mesolimbic reward system during the juvenile period liocould be modulated by providing the offspring with a low-fat control diet post-weaning.

4.2 Methods

4.2.1 Animals and feeding regime

This study was approved by the Institute of Medical and Veterinary Science and University of Adelaide Animal Ethics Committee. Twenty three female Albino Wistar rats (~200g) were brought into the animal facility and housed individually in a 12hr light/12hr dark cycle environment at a constant temperature of 25ºC. Rats were allowed to acclimatise to the animal housing facility for 1 week prior to the commencement of the experimental procedure.

Following acclimatisation, rats were weight-matched and randomly assigned to one of two groups: 1) Control (n=11); or 2) Junk Food (JF, n=12). Rats in the Control group were given standard rat chow (Specialty Feeds, Glen Forrest, Western Australia, Australia) while rats in the JF group received a range of high-fat, high-sugar 'junk foods' including peanut butter, hazelnut spread, chocolate biscuits, savoury snacks, sweetened multi-grain breakfast cereal, and a mixture of lard and standard rat chow. Detailed nutritional composition of the junk food diet and standard rat chow has been provided in Table 2.1. Every 2 days, the remaining food was weighed and fresh food provided. Food intake and body weights were determined on a weekly basis. Rats were given free access to their respective diets 4 weeks before pregnancy, throughout pregnancy and lactation.

4.2.2 Mating

The mating protocol was as described in Chapter 3.

4.2.3 Offspring

Pups were born on day 21 - 22 of gestation. One day after birth (P1), pups were culled to 8 pups per litter, with 4 males and 4 females where possible. Pups were weighed every two days during the suckling period and weaned on P21. Pups of Control and JF dams are referred to as Control offspring and JF offspring respectively.

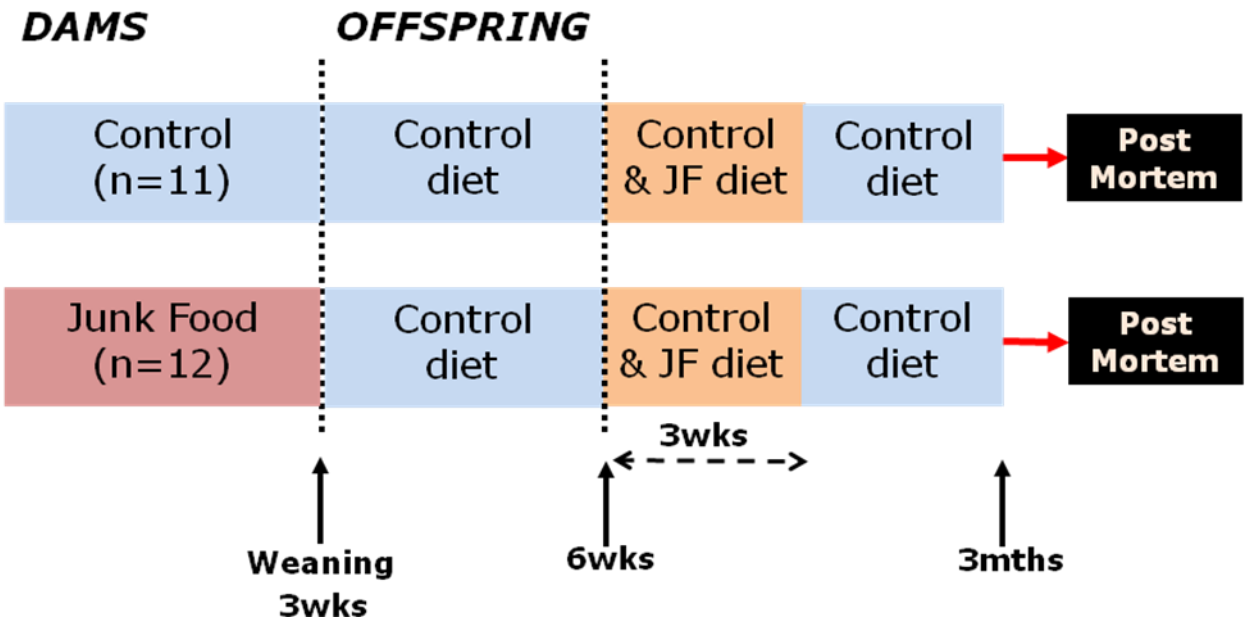
At weaning, offspring were group-housed according to litter and sex. All offspring were weaned onto a standard rat chow and maintained on this diet from 3 to 6 weeks of age. The standard rat chow was low in fat (4% of energy) and is referred to as the low-fat control diet.

One male and one female offspring from each litter were assigned to a food preferences study at 6 weeks of age. The offspring assigned to the food preferences study were given free access to both the control chow and the same range of junk foods provided to the JF dams for a period of 3 weeks. Food intake was determined every 2 days, averaged based on the number of offspring per cage and expressed relative to the body weight of the offspring. The amount of control chow and of each different type of junk food consumed was recorded and macronutrient preferences were calculated based on the nutritional composition of each food type. Body weight was determined weekly throughout the experimental period. After the completion of the food preference experiment, the offspring were returned to the low-fat control diet and chow intake monitored. At 3 months of age, these offspring were euthanised for the determination of body composition and collection of tissues for gene expression analyses. This time point was chosen to provide a brief 'wash-out' period after the 3 week junk food exposure during the juvenile period to avoid the direct effects of the junk food exposure/challenge on the parameters examined. A summary of the experimental protocol is shown in Figure 4.1.

4.2.4 Post-mortem

All rats were not fasted prior to post-mortem. One male and one female offspring from each litter were humanely killed at weaning (3 weeks of age), 6 weeks and 3 months of age with an overdose of CO_2. Immediately after euthanasia, blood samples were collected into heparinised tubes via cardiac puncture and centrifuged at 3,500g at 4ºC for 15 minutes. The top clear layer of plasma was collected and stored at -20ºC for subsequent analyses of plasma glucose, NEFA, insulin and leptin concentrations. Body weight, length (nose to the base of tail) and abdominal circumference were determined. All internal organs were weighed and all visible fat depots, including omental fat, retroperitoneal fat, gonadal fat, subcutaneous fat and interscapular fat, were dissected to determine the mass of individual fat depots and total fat mass. The weight of all internal organs and fat were expressed relative to body weight. The brain was removed and, NAc and VTA isolated as previously described in Chapter 3. All tissues were snap frozen in liquid nitrogen and stored at -80º for subsequent gene expression analyses.

Figure 4.1



Summary of experimental protocol

4.2.5 Determination of plasma glucose and NEFA concentrations

Plasma glucose and NEFA concentrations were determined using the Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA) and the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) respectively as previously described in Chapter 2. Assays and analyses were conducted using Konelab 20 (Thermo Scientific, Vantaa, Finland). Two quality control samples were included in each run and the inter- and intra-assay CoV were <5%.

4.2.6 Determination of plasma insulin and leptin concentrations

Plasma insulin concentrations were measured by rat insulin enzyme immunoassay (EIA) (ALPCO Diagnostics, Salem, NH) and leptin concentrations by rat leptin enzyme-linked immunosorbent assay (ELISA) (Crystal Chem Inc, IL). Both assays utilised the sandwich type immunoassay method. Briefly, the plasma sample and anti-insulin or anti-leptin antibody conjugated with the horseradish peroxidase enzyme was added to a microplate previously coated with mouse anti-insulin antibody and rabbit anti-leptin antibody for insulin and leptin assays respectively. The sample was then incubated to allow sufficient time for the binding of the antigen to the antibody. The excess unbound antibody was washed and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate added, which reacted with the horseradish peroxidase enzyme to give a blue colour. The stop solution (1N sulphuric acid) was added to stop the reaction. The microplate was then placed into the ELx808™ Absorbance Microplate Reader (BioTek Instruments Inc., VT) and the absorbance measured at 450nm with a reference wavelength of 620nm. The absorbance values at 450nm was normalised to the reference wavelength 620nm and subtracted from the absorbance of a blank sample to correct for non-specific background binding. The concentration of insulin or leptin in each plasma sample was determined from the standard curve using a 5-parameter logistic fit. Inter- and intra-assay CoV were <10%. All assays were conducted according to manufacturer's instructions with the exception of the modifications below.

4.2.6.1 Validation of insulin and leptin ELISAs

Prior to the actual experiment, linearity and spike-and-recovery tests were conducted to validate the assay for determining rat plasma insulin and leptin concentrations. The linearity test was conducted using serial dilutions of a plasma sample of known insulin/leptin concentrations. The concentrations of plasma hormones were determined and a linear curve plotted, with an R^2 of 0.995 - 0.999, therefore indicating that there was a

strong linear relationship between the concentration of leptin/insulin and the volume of plasma measured in the assay. The spike-and-recovery test was conducted to determine if there was a difference between the sample diluent and the biological sample matrix (ie plasma) which could affect the ability of the assay to accurately detect the antigens (insulin and leptin). For this test, a known amount of a selected insulin/leptin standard was added to a plasma sample of known insulin/leptin concentration. The observed concentrations of plasma hormones were compared against the expected concentrations and the percentage of recovery calculated. The percentage of recovery was 90 - 100% for the insulin assay and 85 - 95% for leptin assay, both of which were within the recommended range for the respective assays.

4.2.6.2 Variations from standard protocol

Plasma samples with leptin concentrations which were above the highest standard provided in the kit were diluted with the sample diluent such that the concentration fell within the range of the standard curve (0.2 - 12.8ng/mL). The actual concentration of leptin in the sample was then determined by multiplying the concentration of the diluted samples by the dilution factor applied.

4.2.7 RNA extraction and reverse transcription

Total RNA from NAc and VTA was isolated with Trizol reagent (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and purified using an RNeasy Mini Kit (Qiagen Pty Ltd, Doncaster, Australia). RNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm using NanoDrop 2000 Spectrophotometer (Thermo Scientific, DE, USA), and RNA integrity was confirmed using agarose gel electrophoresis. ~1μg RNA was then reverse transcribed to generate cDNA using Superscript III reverse transcriptase (Invitrogen Australia Pty Ltd, Mount Waverley, Australia) and random hexamers.

4.2.8 Determination of gene expression in the NAc and VTA

Quantitative real time PCR was conducted with the SYBR green system using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). Primer sequences for TH, D1, D2, μ-opioid receptor and DAT are shown in Table 2.2. β actin QuantiTect Primer Assay was used to determine β actin mRNA expression (Qiagen Pty Ltd, Doncaster, Australia). All primers were sequenced prior to the experiment to ensure the authenticity of the DNA product and a qRT-PCR melt curve analysis was

performed to demonstrate amplicon homogeneity. The primer concentrations were consistent for all genes, and the amplification efficiency of all primers was 0.997 - 0.999. A constant amount of cDNA (1µl) was used for each qRT-PCR measurement and at least three technical replicates were performed for each gene. Two quality controls were included on each plate in order to verify inter-plate consistency, and the inter-plate CoV were <5%.

Each qRT-PCR reaction (6µl total volume) contained 3µl iQ™ SYBR® Green supermix (Bio-Rad Laboratories, Hercules, CA), 0.6µl of each primer giving a final concentration of 60nM to 900nM, 0.8µl of molecular grade H_20 and 1µl of a 6ng/µl dilution of the stock template. The cycling conditions consisted of 40 cycles of 95°C for 1 second and 60°C for 20 seconds. At the end of each run, melt curves were obtained.

The abundance of each mRNA transcript was measured and expression relative to that of β actin was calculated using Q-gene qRT-PCR analysis software. This software provides a quantitative measure of the relative abundance of a specific transcript in different tissues by the comparative threshold cycle (C_t) method which takes into account any differences in the amplification efficiencies of the target and reference genes.

4.2.9 Statistical analyses

Data are presented as mean ± SEM. The dam was used as the unit of analysis. Maternal and offspring body weight were analysed using repeated measures ANOVA. Maternal mean daily food intake was analysed by dividing the total food intake during pregnancy and lactation by 21 days and compared between groups using unpaired Student's t-test. Plasma hormone and metabolite concentrations and gene expression of key components of the reward system in the offspring at 3 weeks, 6 weeks and 3 months of age were analysed using two-way ANOVA with sex and treatment groups as factors. When a significant interaction between sex and treatment was identified, the data was analysed separately for males and females using an unpaired Student's t-test.

Repeated measures ANOVA was performed using Stata 10 (StataCorp LP, Texas). All other analyses were performed using SPSS for Windows Version 18.0 (SPSS Inc., Chicago, IL). A probability of $P<0.05$ was considered statistically significant in all analyses.

4.3 Results

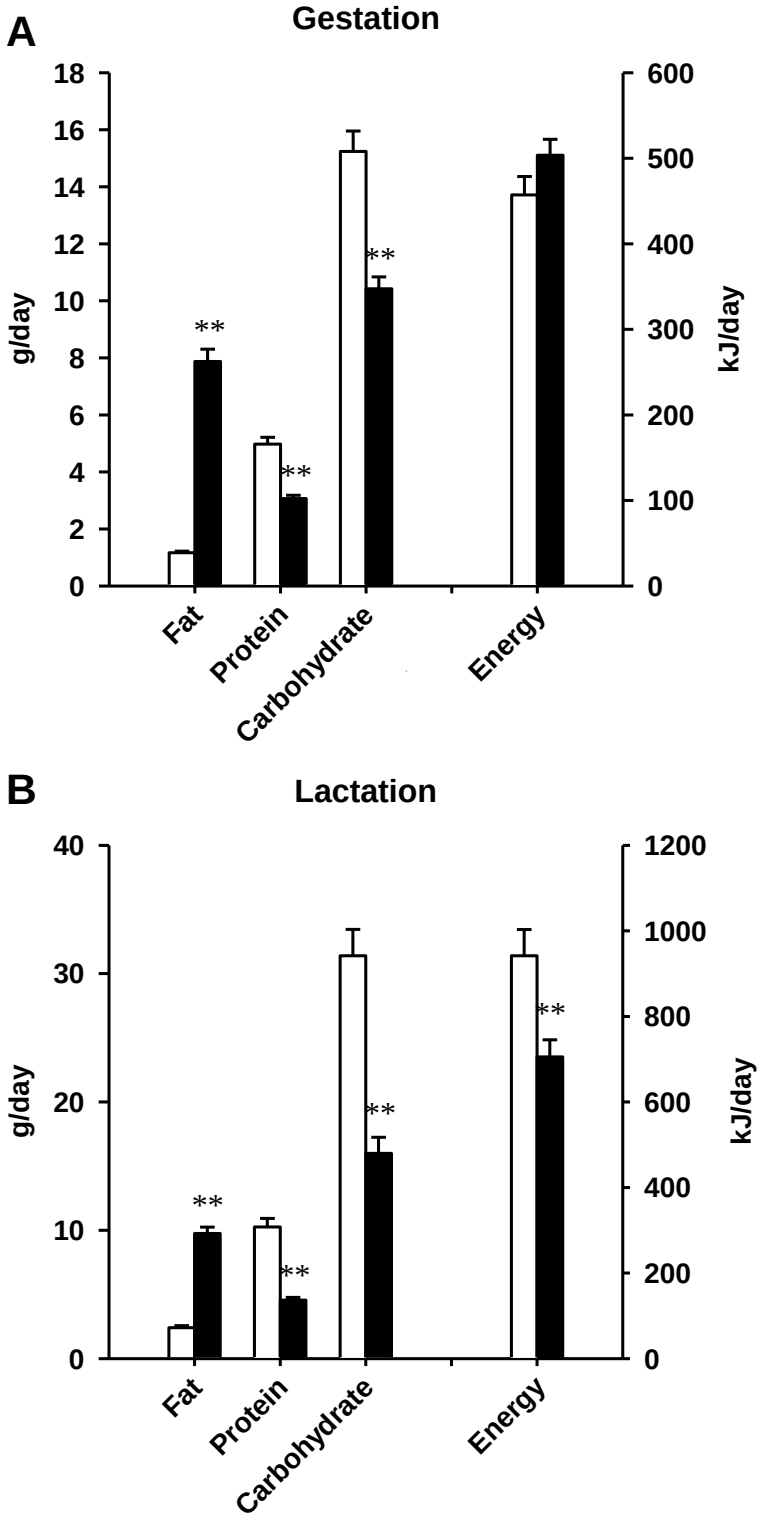
4.3.1 Dams body weight

Dams fed on the junk food diet were significantly heavier than Control dams after one week of junk food feeding (Control: 253.1 ± 3.7g, JF: 276.7 ± 4.8g; $P<0.01$) and remained heavier than Control dams during pregnancy (Control: 368.25 ± 7.4g, JF: 481.8 ± 12.4g; $P<0.01$). After delivery, JF dams lost more weight than Control dams during the second week of lactation (Control: -52.9 ± 11.4g, JF: -95.5 ± 13.4g; $P<0.05$) but remained heavier than the Controls at the end of lactation (Control: 336.0 ± 7.5g, JF: 410.6 ± 22.4g; $P<0.05$).

4.3.2 Dams food intake

Total energy intake was not different between the Control and JF dams during pregnancy. During lactation, however, energy intake was lower in the JF dams when compared to Controls (Figure 4.2). Fat intake of JF dams was higher, whilst protein intake was lower when compared to Controls throughout the experimental period ($P<0.01$) (Figure 4.2). Carbohydrate intake was also lower in the JF dams when compared to Controls during pregnancy and lactation ($P<0.01$) (Figure 4.2).

Figure 4.2



Intake of fat, protein, carbohydrate and total energy of Control (open bars) and JF (closed bars) dams per day during gestation (A) and lactation (B).

Values are expressed as mean ± SEM. ***P*<0.01 by unpaired Student's t-test. n = 11 - 12 in each group.

4.3.3 At weaning (3 weeks of age)

4.3.3.1 Offspring body weight, body composition and plasma hormones and metabolites

At weaning, both male and female JF offspring remained lighter ($P<0.01$), shorter ($P<0.01$), and had a smaller abdominal circumference ($P<0.01$) when compared to Controls (Table 4.1). Both male and female JF offspring had higher gonadal fat, omental fat, subcutaneous fat and total fat mass as a percentage of body weight when compared to Controls ($P<0.01$). In males only, retroperitoneal fat mass as a percentage of body weight was higher in the JF offspring compared to Controls ($P<0.01$). Relative interscapular fat mass also tended ($P=0.06$) to be higher in the JF offspring than Controls in both males and females (Table 4.1).

Plasma leptin concentrations were higher in the JF offspring compared to Control offspring in both males and females at 3 weeks of age ($P<0.05$). There was no difference in plasma glucose, NEFA and insulin concentrations between groups in either males or females (Table 4.1).

4.3.3.2 Gene expression of TH, D1, D2, DAT and μ-opioid receptors in the NAc and VTA of the offspring

At 3 weeks of age, DAT mRNA expression in the NAc was lower in the JF offspring when compared to Controls in both males and females ($P<0.05$). There were sex by treatment interactions for the mRNA expression of μ-opioid receptor in the NAc. In males, mRNA expression of the μ-opioid receptor in the NAc was higher ($P<0.01$) in the JF offspring when compared to Controls. There were however, no differences between groups in the expression of μ-opioid receptor mRNA in the NAc of female offspring (Figure 4.3).

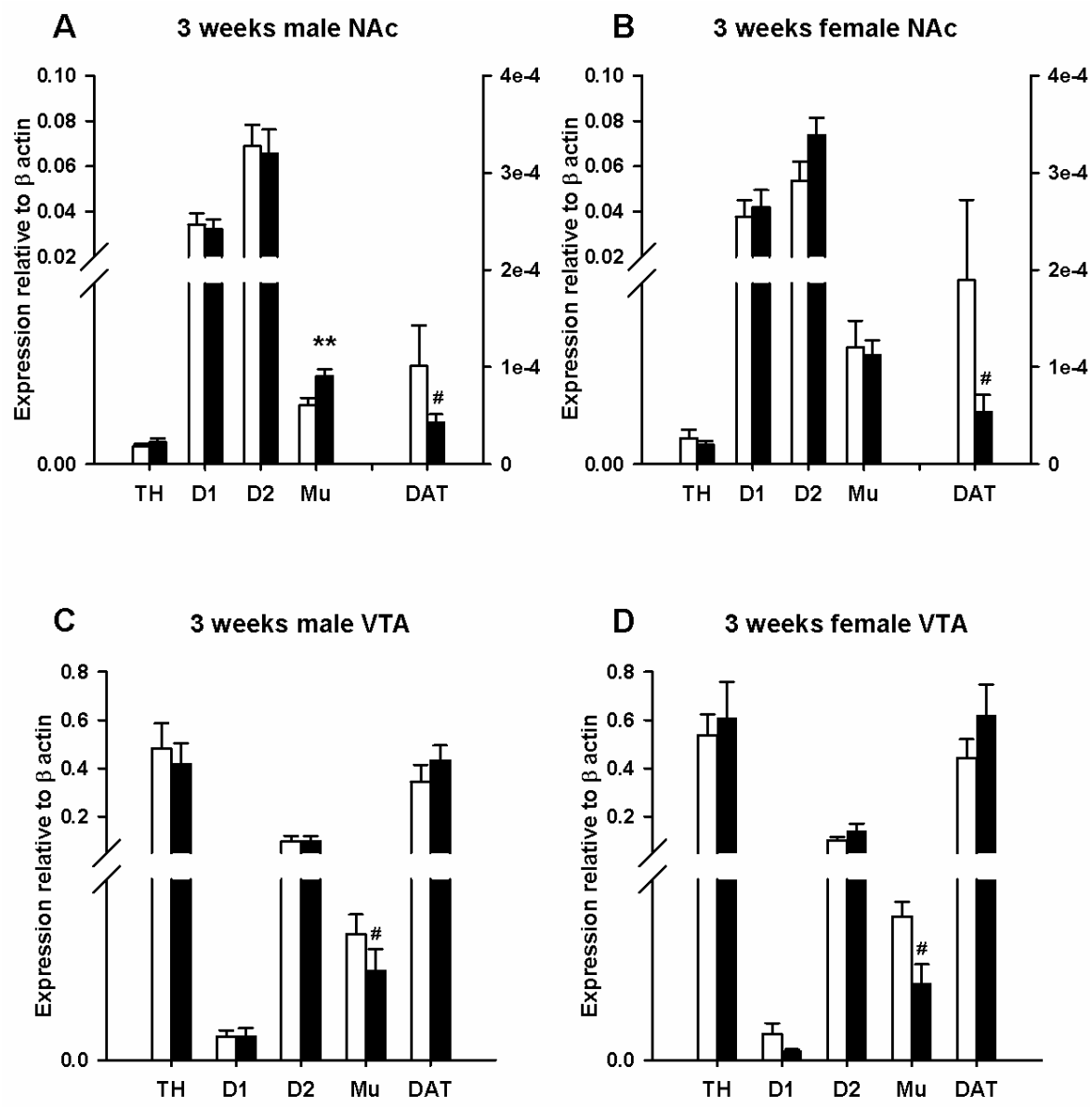
In the VTA, μ-opioid receptor mRNA expression was lower in the JF offspring when compared to Controls, independent of sex ($P<0.05$). There was no effect of maternal junk food feeding on the expression of TH, D1 or D2 in the NAc or VTA or the mRNA expression of DAT in the VTA in either male or female offspring (Figure 4.3).

Table 4.1 Metabolic parameters in Control and JF offspring at 3 weeks

Values are expressed as mean ± SEM. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. - = no difference, M = Maternal diet effect, S = Sex effect, M × S = Maternal diet and sex interaction. Student's T-Test, **P*<0.05 between male Control and JF offspring. n = 6 - 10 in each group.

	3 weeks of age				
	Male		Female		
	Control	JF	Control	JF	ANOVA
Body weight (g)	58.3 ± 1.3	49.1 ± 2.6	55.4 ± 1.1	47.6 ± 2.2	M
Length (cm)	14.8 ± 0.2	13.5 ± 0.2	14.9 ± 0.2	13.5 ± 0.2	M
Abdominal circumference (cm)	10.8 ± 0.2	10.1 ± 0.3	10.9 ± 0.2	10.0 ± 0.2	M
% fat mass					
Gonadal fat	0.7 ± 0.20	1.4 ± 0.43	0.8 ± 0.23	1.7 ± 0.40	M
Retroperitoneal fat	0.4 ± 0.04	0.8 ± 0.06*	0.4 ± 0.04	0.5 ± 0.05	M × S
Omental fat	0.6 ± 0.04	0.7 ± 0.03	0.6 ± 0.03	0.7 ± 0.03	M
Subcutaneous fat	3.3 ± 0.25	6.9 ± 0.55	3.5 ± 0.34	6.4 ± 0.58	M
Interscapular fat	0.7 ± 0.05	0.8 ± 0.05	0.8 ± 0.06	0.9 ± 0.04	-
Total fat	5.7 ± 0.28	10.7 ± 0.45	6.0 ± 0.36	10.1 ± 0.41	M
Plasma hormone & metabolites					
Glucose (mmol/L)	11.4 ± 0.57	11.8 ± 0.59	11.3 ± 0.52	13.1 ± 0.72	-
NEFA (meq/mL)	0.4 ± 0.03	0.4 ± 0.03	0.4 ± 0.03	0.6 ± 0.09	S
Insulin (ng/mL)	0.3 ± 0.07	0.4 ± 0.09	0.2 ± 0.03	0.6 ± 0.13	-
Leptin (ng/mL)	5.4 ± 0.59	15.7 ± 3.13	5.7 ± 0.63	14.4 ± 1.84	M

Figure 4.3



mRNA expression of TH, D1, D2, μ-opioid receptor (Mu) and DAT in NAc (A,B) and VTA (C,D) of Control (open bars) and JF (closed bars) offspring at 3 weeks of age in both males and females. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. ANOVA, $^{\#}P<0.05$ between Control and JF offspring; Student's t-test, $^{**}P<0.01$ between Control and JF offspring. Values are expressed as mean ± SEM. n = 7 - 11 in each group.

4.3.4 Six weeks of age

4.3.4.1 Offspring body weight, body composition and plasma hormones and metabolites

At 6 weeks of age, when both Control and JF offspring had been fed a standard rat chow since weaning, both male and female JF offspring were lighter ($P<0.05$), shorter ($P<0.05$) and had smaller abdominal circumference ($P<0.05$) compared to Controls. There was however, no difference in either body fat mass or the fat mass of any of the individual fat depots as a percentage of body weight between the Control and JF offspring in either males or females at this time (Table 4.2).

Female JF offspring had lower glucose levels than Controls at 6 weeks of age ($P<0.01$) (Table 4.2). There was however, no difference in glucose concentrations between Control and JF groups in male offspring. Plasma insulin concentrations tended ($P=0.06$) to be lower in the JF offspring compared to Controls in both males and females. There were no differences in plasma NEFA and leptin concentrations between Control and JF offspring in either males or females at 6 weeks of age (Table 4.2).

4.3.4.2 Gene expression of TH, D1, D2, DAT and μ-opioid receptors in the NAc and VTA of the offspring

D1 mRNA expression in the NAc at 6 weeks of age was lower in both male and female offspring of JF dams compared to Controls ($P<0.01$). DAT mRNA expression in the NAc tended ($P=0.06$) to be higher in the JF offspring compared to Controls in both males and females (Figure 4.4).

In the VTA however, D1 mRNA expression was higher in the JF offspring compared to Controls in both males and females ($P<0.05$). There were no differences in the expression of TH, D2 or the μ-opioid receptor in either the NAc or VTA between Control and JF offspring in either males or females (Figure 4.4).

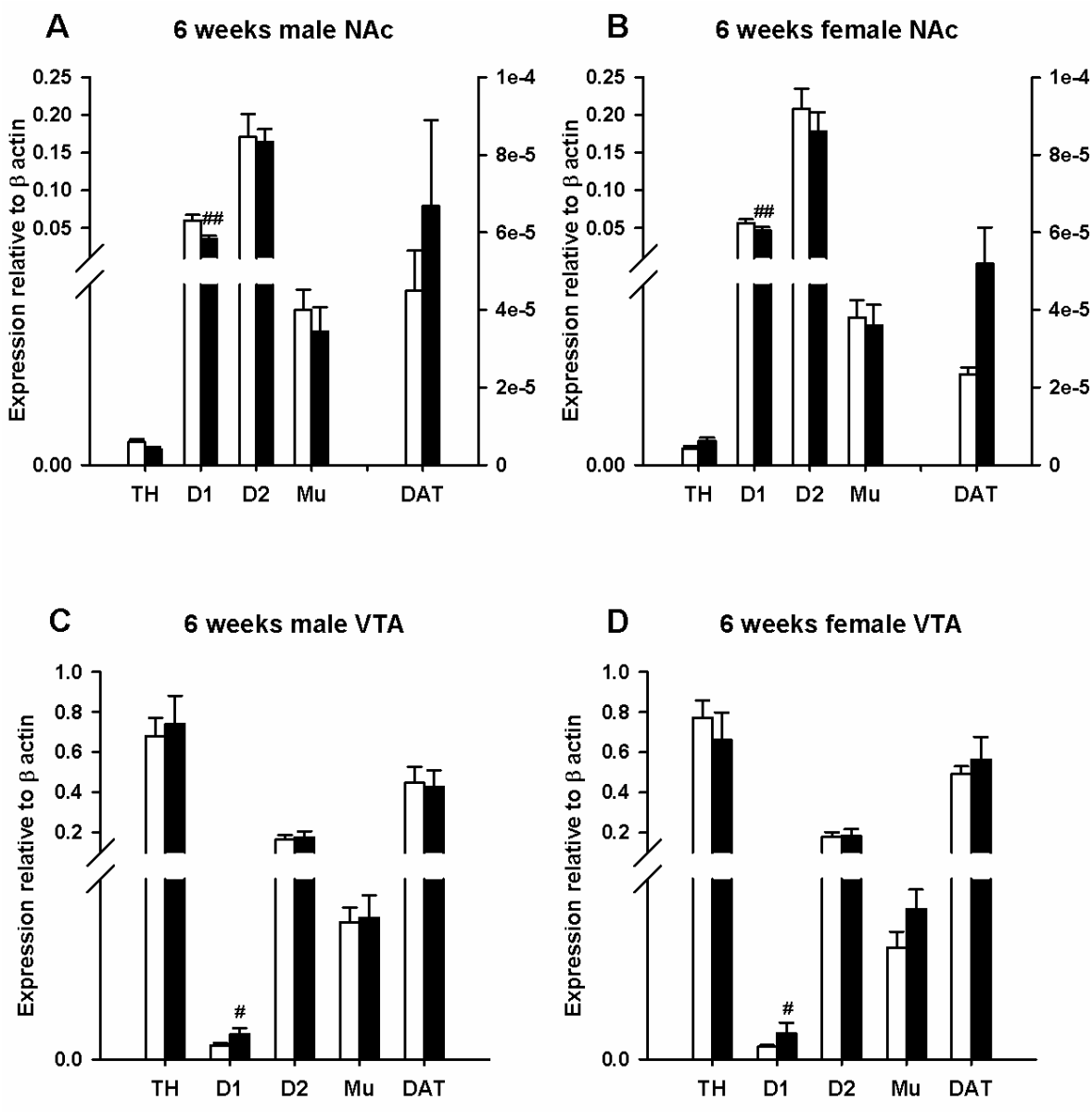
D1 expression at the VTA was positively correlated with carbohydrate intake during the food preferences study when the data from both male and female offspring were combined ($r = 0.52$; $P<0.01$).

Table 4.2 Metabolic parameters in Control and JF offspring at 6 weeks of age

Values are expressed as mean ± SEM. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. - = no difference, M = Maternal diet effect, S = Sex effect, M × S = Maternal diet and sex interaction. Student's t-test, ***P*<0.01 between female Control and JF offspring. n = 7 - 10 in each group.

	6 weeks of age				
	Male		Female		
	Control	JF	Control	JF	ANOVA
Body weight (g)	230.9 ± 5.5	187.6 ± 9.8	192.0 ± 6.3	160.3 ± 9.2	M, S
Length (cm)	23.1 ± 0.4	21.4 ± 0.5	21.6 ± 0.2	20.7 ± 0.4	M, S
Abdominal circumference (cm)	17.4 ± 0.3	15.8 ± 0.5	16.1 ± 0.4	15.0 ± 0.5	M, S
% fat mass					
Gonadal fat	0.9 ± 0.15	1.0 ± 0.27	1.0 ± 0.07	0.9 ± 0.09	-
Retroperitoneal fat	0.7 ± 0.05	0.7 ± 0.07	0.8 ± 0.05	0.7 ± 0.07	-
Omental fat	0.6 ± 0.03	0.7 ± 0.07	0.8 ± 0.04	0.7 ± 0.09	S
Subcutaneous fat	3.5 ± 0.32	3.9 ± 0.65	3.8 ± 0.44	3.3 ± 0.48	-
Interscapular fat	0.3 ± 0.03	0.4 ± 0.04	0.4 ± 0.05	0.3 ± 0.03	S, M × S
Total fat	6.0 ± 0.41	6.6 ± 0.64	6.8 ± 0.53	5.9 ± 0.65	-
Plasma hormone & metabolites					
Glucose (mmol/L)	13.4 ± 0.55	15.2 ± 1.00	18.2 ± 1.08	13.1 ± 0.59**	M × S
NEFA (meq/mL)	0.5 ± 0.05	0.6 ± 0.07	0.6 ± 0.07	0.6 ± 0.06	-
Insulin (ng/mL)	1.1 ± 0.29	0.5 ± 0.13	1.5 ± 0.38	1.0 ± 0.22	-
Leptin (ng/mL)	5.6 ± 0.89	4.7 ± 0.71	4.3 ± 0.66	4.2 ± 0.71	-

Figure 4.4



mRNA expression of TH, D1, D2, μ-opioid receptor (Mu) and DAT in NAc (A) and VTA (B) of Control (open bars) and JF (closed bars) offspring at 6 weeks of age in both males and females. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. ANOVA, $^{\#}P<0.05$, $^{\#\#}P<0.01$ between Control and JF offspring. Values are expressed as mean ± SEM. n= 7 - 10 in each group.

4.3.5 Food preferences study at 6 weeks of age

4.3.5.1 Body weight

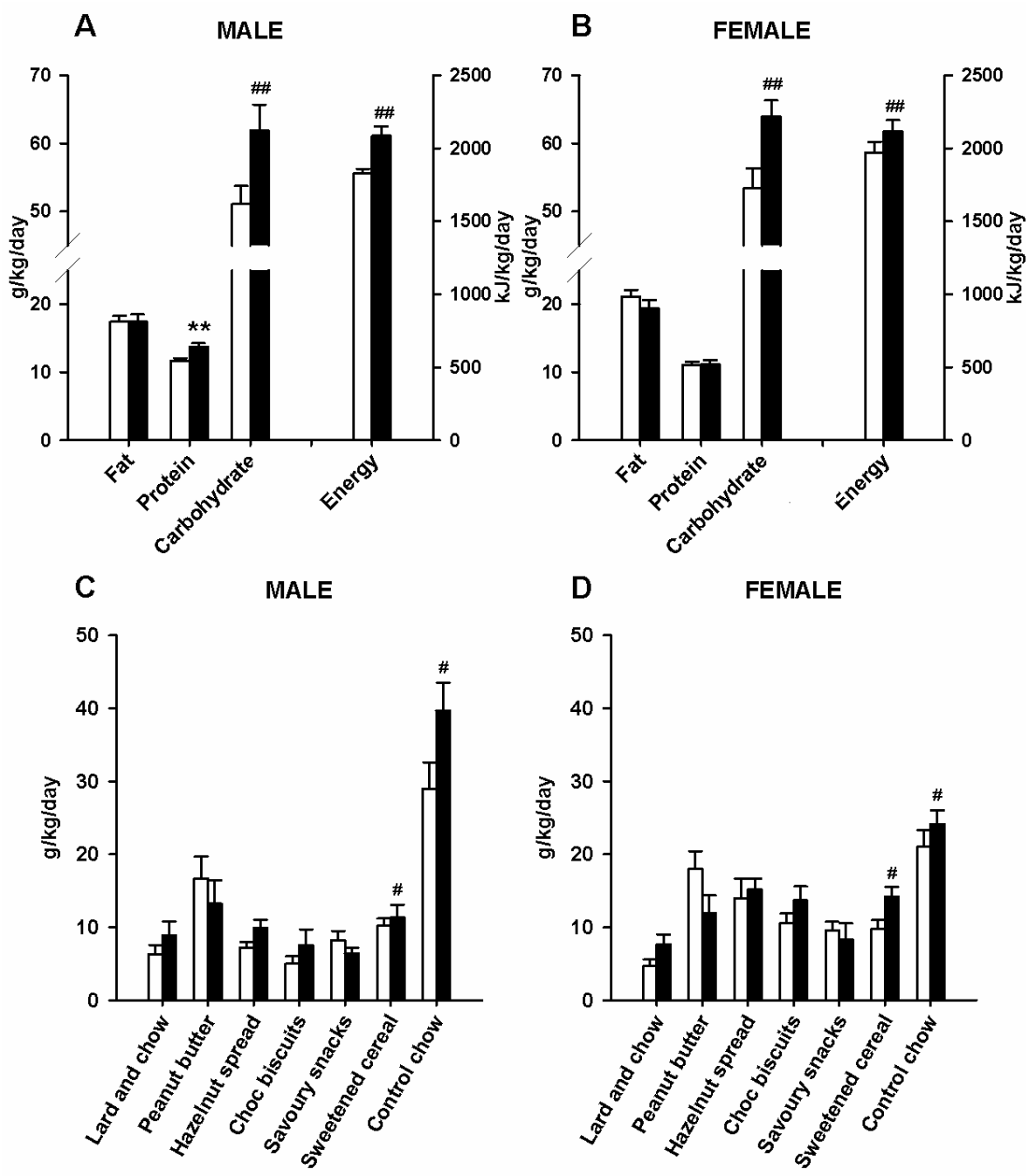
At the start of the food preference study, both male and female JF offspring were lighter than Controls (Male Control: 244.2 ± 4.0g, Male JF: 207.5 ± 7.2g; Female Control: 193.7 ± 6.0g, Female JF: 165.1 ± 4.5g, $P<0.01$). At the end of the food preferences study, both male and female JF offspring gained more weight as a percentage of their body weight prior to the food preferences study when compared to their Control counterparts (Male Control: 82.6 ± 2.7%, Male JF: 104.7 ± 2.6%; Female Control: 61.4 ± 5.5%, Female JF: 79.6 ± 5.8%; $P<0.05$). There were no longer any differences in body weight between groups at the end of the food preferences study in either males or females (Male Control: 442.1 ± 7.5g, Male JF: 428.1 ± 12.7g; Female Control: 304.1 ± 11.0g, Female JF: 303.2 ± 12.5g).

4.3.5.2 Food and macronutrient preferences

JF offspring consumed more total energy and carbohydrates compared to the Control offspring during the 3 week food preferences study in both males and females ($P<0.05$). In males, JF offspring also consumed more protein than Controls ($P<0.01$). There was no difference in fat intake between JF and Control groups in either male or female offspring (Figure 4.5A, B).

When the intake of individual junk foods was analysed, both male and female JF offspring were found to consume higher amounts of sugary cereal and standard rat chow during the 3 week food preference study compared to Control offspring ($P<0.05$). The JF offspring also tended ($P=0.06$) to consume more lard and chow mix compared to Controls (Figure 4.5C, D). There were also sex differences in the preference for individual foods which were independent of treatment group, with female offspring consuming more hazelnut spread and chocolate biscuits than males, and male offspring having a higher chow intake compared to females ($P<0.01$).

Figure 4.5



Intake of fat, protein, carbohydrate and total energy in male (A) and female (B) Control (open bars) and JF (closed bars) offspring and intake of individual junk foods and standard rat chow in male (C) and female (D) Control and JF offspring during the food preferences study. Data was analysed by two-way ANOVA. When a maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. ANOVA, $^{\#}P<0.05$, $^{\#\#}P<0.01$ between Control and JF offspring; Student's t-test, $^{**}P<0.01$ between Control and JF offspring. Values expressed as mean ± SEM. n = 10 - 11 in each group.

4.3.6 Post-food preferences study: chow intake at 9 weeks of age

When all offspring were returned to control chow, immediately following the food preferences study, both Control and JF offspring consumed less chow compared to their intake before the food preferences study. In males, there was a 64% reduction in chow intake per kg body weight in the Controls and 58% reduction in the JF offspring. In the females, the Control and JF offspring had a 54% and 57% reduction in chow intake per kg body weight respectively. These reductions were not significantly different between groups.

4.3.7 Three months of age

4.3.7.1 Body weight, body composition and plasma hormone and metabolites at post-mortem

At 3 months of age, there was no difference in body weight between Control and JF offspring in either males or females (Male Control: 508.5 ± 6.6g, Male JF: 513.6 ± 13.1g; Female Control: 311.7 ± 11.0g, Female JF: 329.0 ± 12.8g). Body length and abdominal circumference were also not different between Control and JF offspring. Gonadal, retroperitoneal, omental, subcutaneous, interscapular fat mass and total percentage body fat mass were all significantly higher in the JF offspring than Controls in both males and females ($P<0.05$) (Table 4.3).

Plasma leptin concentrations were higher in the JF offspring when compared to Controls in both males and females ($P<0.01$). There were no differences in plasma glucose, NEFA and insulin levels between the Control and JF groups in either males or females (Table 4.3).

Table 4.3 Metabolic parameters in Control and JF offspring at 3 months of age

Values are expressed as mean ± SEM. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. - = no difference, M = Maternal diet effect, S = Sex effect, M × S = Maternal diet and sex interaction. n = 9 - 11 in each group.

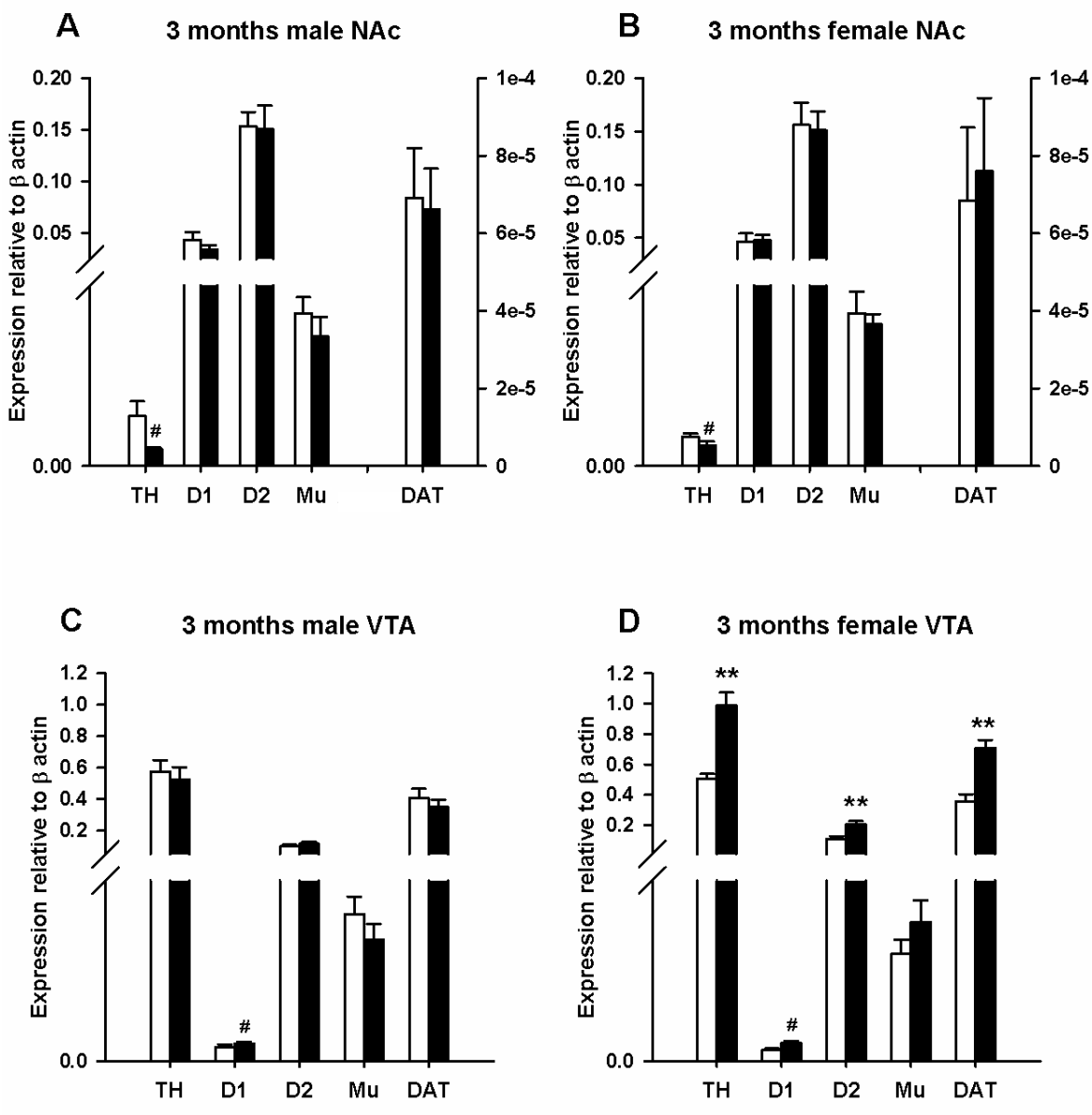
	3 months of age				
	Male		Female		
	Control	JF	Control	JF	ANOVA
Body weight (g)	508.5 ± 6.56	513.6 ± 13.11	311.7 ± 11.01	332.9 ± 13.48	S
Length (cm)	26.4 ± 2.64	29.0 ± 0.35	25.1 ± 0.25	25.2 ± 0.21	S
Abdominal circumference (cm)	20.4 ± 2.05	23.0 ± 0.44	18.8 ± 0.50	19.6 ± 0.51	S
% fat mass					
Gonadal fat	2.0 ± 0.16	2.4 ± 0.12	2.7 ± 0.23	3.7 ± 0.25	M, S
Retroperitoneal fat	2.2 ± 0.09	2.7 ± 0.22	2.0 ± 0.18	2.4 ± 0.29	M
Omental fat	1.4 ± 0.08	1.7 ± 0.08	1.3 ± 0.17	2.0 ± 0.20	M
Subcutaneous fat	5.4 ± 0.27	6.2 ± 0.52	4.7 ± 0.43	6.6 ± 0.46	M
Interscapular fat	0.3 ± 0.03	0.3 ± 0.02	0.3 ± 0.04	0.4 ± 0.05	S
Total fat	11.3 ± 0.55	13.3 ± 0.80	10.8 ± 1.03	15.1 ± 1.18	M
Plasma hormone & metabolites					
Glucose (mmol/L)	14.6 ± 0.55	15.1 ± 0.46	13.1 ± 0.45	13.8 ± 0.93	S
NEFA (meq/mL)	0.5 ± 0.04	0.4 ± 0.05	0.3 ± 0.03	0.4 ± 0.04	-
Insulin (ng/mL)	2.5 ± 0.28	3.4 ± 0.86	2.0 ± 0.29	2.4 ± 0.57	-
Leptin (ng/mL)	11.3 ± 0.86	13.9 ± 1.33	5.1 ± 1.02	10.0 ± 1.55	M, S

4.3.7.2 Gene expression of TH, D1, D2, DAT and μ-opioid receptors in the NAc and VTA of the offspring at 3 months of age

TH mRNA expression in the NAc was lower in the JF offspring compared to Controls in both males and females ($P<0.05$). D1, D2, μ-opioid receptor and DAT mRNA expression in the NAc were not different between Control and JF groups in either males or females at 3 months of age (Figure 4.6).

In the VTA, there were sex by treatment interactions for TH, D2 and DAT mRNA expression. In females, expression of TH, D2 and DAT mRNA were all higher in the JF offspring when compared to Controls ($P<0.05$) whilst there was no difference in the expression of these genes between groups in males. Both male and female JF offspring had higher D1 mRNA expression in the VTA when compared to Controls ($P<0.05$). There was no difference in μ-opioid receptor expression in the VTA between groups in either males or females (Figure 4.6).

Figure 4.6



mRNA expression of TH, D1, D2, μ-opioid receptor (Mu) and DAT in NAc (A) and VTA (B) of Control (open bars) and JF (closed bars) offspring at 6 weeks of age in both males and females. Data was analysed by two-way ANOVA. When maternal diet × sex interaction was identified, data was analysed by unpaired Student's t-test. ANOVA, $^{\#}P<0.05$, $^{\#\#}P<0.01$ between Control and JF offspring; Student's t-test, $^{**}P<0.01$ between Control and JF offspring. Values expressed as mean ± SEM. n = 8 - 11 in each group.

4.4 Discussion

This study demonstrated for the first time that some effects of maternal junk food feeding on the mesolimbic reward system of the offspring are already present at weaning. This study also showed that weaning offspring on a low-fat control diet reversed the effects of peri-natal junk food exposure on fat intake, body fat deposition and plasma leptin concentrations at 6 weeks of age. However, after being exposed to a palatable diet from 6 to 9 weeks of age, JF offspring became fatter and exhibited changes in the reward system that were consistent with sensitisation towards the palatable diet in comparison to Controls. This study therefore provided evidence that peri-natal exposure to a junk food diet is associated with altered development of the reward system and an increased propensity to overconsume high-fat, high-sugar foods, which cannot be corrected by providing a low-fat control diet post-weaning.

4.4.1 Impact of maternal junk food feeding at weaning

At weaning, we found that offspring of JF dams were phenotypically similar to those reported in Chapter 3, where the JF offspring were fatter, lighter and shorter compared to Controls. μ-opioid receptor expression in the NAc was also higher in male JF offspring and DAT in the NAc was lower at weaning in both male and female JF offspring when compared to their control counterparts. This higher expression of μ-opioid receptor and lower DAT in the NAc was also reported in 6 weeks old JF offspring in Chapter 3 of this thesis, when all offspring were given free access to both the junk food and control diet after weaning. This therefore suggests that these changes, indicative of increased opioid and dopamine activity within the mesolimbic reward system were already present at weaning and this increase in reward sensitivity may be responsible for driving the initial high fat intake in the JF offspring observed in Chapter 3.

In contrast to the NAc, both male and female JF offspring had lower μ-opioid receptor expression in the VTA when compared to the Controls. Given that opioids disinhibit dopamine neurons within the VTA, the lower μ-opioid receptor expression would indicate a lower dopaminergic tone within the mesolimbic reward system and reduced reward sensitivity. Reduced accumbens dopaminergic tone has been reported in obesity prone rats and this is thought to contribute to hyperphagia as a compensatory response to the reduced sensitivity to food reward in these rats (81). Therefore, it is possible that in the present study, the JF offspring exhibit reduced dopaminergic tone and the possible reduction in

reward sensitivity may also be playing a role in driving the increased preference for fat in the JF offspring when they were weaned on both the standard rat chow and junk food diet.

Given that both male and female JF offspring exhibited an increased preference for fat since weaning (as reported in Chapter 3), it is intriguing that the higher μ-opioid receptor expression in the NAc at 3 weeks of age was only observed in males, but not females. This sex difference in μ-opioid receptor expression has also been reported in adult rats following pre-natal morphine exposure, with morphine-exposed males, but not females, exhibiting an increase in NAc μ-opioid receptor density when compared to their control counterparts (260). This suggests that pre-natal insults have sex-dependent effects on μ-opioid receptor expression in the NAc. The functional implications of these differences in μ-opioid receptor expression within the NAc have yet to be determined but could potentially contribute to the differential susceptibility to addiction between males and females.

In addition to the sex-specific effect on μ-opioid receptor expression, the expression of these receptors was also region-specific, with μ-opioid receptor expression being increased in the NAc but reduced in the VTA in response to maternal junk food feeding. This region-specificity has also been reported in offspring of pre-natal morphine exposure (260) and may be due to the different ontogeny of the development of the opioid systems between the NAc and VTA (153). The expression of μ-opioid receptors in the striatum is first detected during the second half of pregnancy (155) and only reaches adult levels at the end of lactation (154, 156). In contrast, in the VTA, μ-opioid receptors are only detected at low levels at P4 (288).

Despite the sex-dependent and region-specific changes in μ-opioid receptor expression within the mesolimbic reward system, it is clear that maternal diet during pregnancy and lactation can directly impact on the development of the mesolimbic reward system of the offspring. These changes in the reward system observed at weaning are likely to impact on the subsequent response to being exposed to palatable foods later in life.

4.4.2 Impact of maternal junk food feeding at 6 weeks of age (before food preferences study)

In the present study, all offspring were weaned onto a low-fat control diet and maintained on this diet until 6 weeks of age. It was found that introducing this low-fat control diet to

peri-natally junk food exposed offspring normalised fat mass and plasma leptin concentrations of the JF offspring to levels similar to Control offspring after 3 weeks, suggesting a reversal of increased fat deposition. Body weight, however, was still lower in the JF offspring. This is in line with previous findings in models of maternal low-protein feeding, in which early deficits in protein supply and lean tissue mass could not be reversed by adequate nutrition after birth (49, 289, 290). This also supports the suggestion that reduced body weight in JF offspring is a consequence of the lower maternal protein intake during pregnancy and lactation.

Gene expression of key reward components was also determined at 6 weeks of age to identify changes within the reward system prior to re-introduction of the junk food diet for determination of food preferences. The changes in gene expression at 6 weeks of age were distinct to those at 3 weeks of age, which suggests that the period of low-fat feeding could modulate the expression of key genes within the reward system post-weaning. The offspring of junk food dams when weaned onto standard rat chow exhibited lower D1 mRNA expression in the NAc at 6 weeks of age, suggestive of a reduction in dopamine signalling/sensitivity within this region of the mesolimbic reward system. One possibility is that this was due to the removal of the junk food diet in the 3 weeks after weaning, since D1 receptor density in the NAc has been shown to decrease in response to cocaine withdrawal in rodents and non-human primates (291-293) and withdrawal of a palatable diet in adult rodents (86). Furthermore, this reduction in D1 receptor expression in the NAc has been associated with higher intakes of palatable foods during subsequent exposure to a palatable diet in adult rodents (86). It is therefore possible that the lower expression of D1 mRNA in the NAc in offspring of junk food dams at 6 weeks of age may contribute to the higher energy intake in the JF offspring upon subsequent exposure to the palatable diet.

In contrast to D1 expression in the NAc, D1 expression in the VTA was higher in the JF group when compared to the Controls in both male and female offspring. Whilst D1 in the NAc is responsible for overall dopamine transmission, D1 in the VTA has more of an auto-regulatory role and acts primarily to stimulate dopamine release within the VTA itself. D1 are located on GABA-ergic and glutamatergic afferents in the VTA and therefore causes the release of both GABA and glutamate to inhibit and stimulate dopamine neurons respectively (294-297). Interestingly, D1 receptor activation has a more potent effect on glutamate compared to GABA release, with the dose of D1 agonist required for glutamate release is 30× lower than that required for GABA release (298). This suggests dopamine

signalling via D1 in the VTA is involved in the positive feedback of dopamine transmission within this region. This is supported by studies of drug addiction where blockade of D1 receptors in the VTA reduced cocaine reward (299) and inhibited amphetamine-induced locomotor sensitisation in rodents (300). Therefore, the increased expression of D1 in the VTA would be likely to increase dopamine transmission in these juvenile JF offspring, which may also be responsible for an increased appetite for palatable foods.

4.4.3 Impact of short term exposure to a palatable diet during the juvenile period

Indeed, as postulated from the gene expression results prior to the food preferences study, both male and female JF offspring exhibited an increased appetite when provided with free access to both the junk food and control diet. The JF offspring had increased intake of carbohydrate, protein and total energy per kg body weight compared to their Control counterparts during the food preferences study. Interestingly, we found that carbohydrate intake of the JF offspring was positively correlated with D1 expression in the VTA at 6 weeks of age, which indicates that the higher VTA D1 may be driving the higher carbohydrate intake in the JF offspring. The role of D1 in promoting carbohydrate intake has been observed in previous studies where D1 antagonism has been shown to reduce sucrose seeking behaviour (71) and block carbohydrate preference (301). This suggests that, in addition to fat, the preference for carbohydrate may also be programmed before birth.

The relationship between increased perinatal exposure to carbohydrate and the preference for carbohydrate after birth remains unclear. Our finding of the high-carbohydrate preference in offspring of junk food dams is in contrast to a previous study where exposure to a high-fat or high-carbohydrate diet at 4 weeks of age did not result in an increased preference towards carbohydrate during adulthood (52). However, Vucetic and colleagues showed that offspring of high-fat fed dams have an increased liking towards both sucrose and fat (53). It is possible that the period early in life during which the high-fat or high-carbohydrate diet is provided may influence the effect of nutrient exposure on offspring's preference towards high-carbohydrate foods in adulthood. Furthermore, the preference for specific forms of carbohydrate, for example sucrose, which has been shown to display addictive properties in adults (91, 302), may be more likely to be programmed rather than total carbohydrate intake per se. In addition to carbohydrate, the male JF offspring also consumed more protein during the food preferences study. We propose that the higher

protein intake in the male JF offspring is unlikely to be a programmed effect of the junk food diet, but rather a response to compensate for the lower protein intake in JF dams, as discussed in Chapter 3.

Importantly, the fact that fat intake was not different between groups in this study indicates that introducing a low-fat control diet post-weaning can abolish the increased preference for fat in the juvenile JF offspring that was reported in Chapter 3. This reversal of increased fat preference is in contrast to previous studies of maternal high-fat feeding where the high-fat preference persisted in adult offspring that had been exposed to a palatable diet in early life, even when they were maintained on a low-fat control diet from weaning (51, 53). In these studies however, food preferences were studied in adult offspring following a much longer period of exposure to the low-fat chow diet rather than on juvenile offspring, as in the present study. Therefore, it appears that the ability of a post-weaning low-fat control diet to reverse fat preference of JF offspring may be a short term effect and may not be maintained into adulthood.

Given that these JF offspring consumed more energy during the food preferences study, it was not surprising that the JF offspring gained more weight during that 3 week period. Interestingly however, despite the normalisation of body fat mass and plasma leptin levels at 6 weeks of age, body fat mass and plasma leptin levels of the JF offspring were higher at 3 months of age, even after they were returned to a control low-fat diet for 3 weeks. This suggests that the offspring of junk food fed mothers, despite being leaner at 6 weeks of age, were more susceptible to diet-induced obesity when provided with free access to the junk food diet during the juvenile period compared to Controls.

Following the food preferences study, gene expression within the mesolimbic reward system was analysed at 3 months of age to determine the effects of a post-weaning junk food exposure on the mesolimbic reward system of the JF offspring. D1 receptor expression in the VTA remained higher in the offspring of junk food dams compared to Controls at 3 months of age. Female JF offspring also exhibited higher TH, D2 and DAT expression in the VTA which suggests an upregulation of dopamine synthesis and dopamine activity within the VTA. Previous studies of drug addiction have shown an association between addictive stimuli and the upregulation of dopamine synthesis during acute exposure, however this effect does not persist when the stimulus is removed (303, 304). Thus, in the present study, it is possible that peri-natal exposure to the palatable diet

altered the development of the mesolimbic reward system and consequently its ability to respond to subsequent junk food exposure, such that elevated expression of dopamine-related genes persists even when the junk food stimulus is withdrawn. Increases in dopamine levels within the mesolimbic reward system have been associated with increased intake of palatable diets in adult rodents (74, 75, 123), therefore this heightened dopamine activity in JF offspring may act to promote intake of palatable foods in the offspring, although this was not directly tested in the present study. The effects on gene expression within the mesolimbic reward system were more pronounced in the female JF offspring than in males, raising the possibility that female offspring have a heightened susceptibility to overconsume upon subsequent exposure to a palatable diet compared to males.

In contrast to the upregulation of dopamine-related genes in the VTA, TH mRNA expression in the NAc was lower in the JF offspring compared to Controls. Given that the gene expression was measured 3 weeks after the juvenile junk food exposure and that the effects of withdrawal on gene expression within the mesolimbic reward system occurs almost entirely at the NAc (305), it is speculated that this reduction in TH may be a response to the absence of the palatable diet to stimulate dopamine production in the 3 weeks preceding tissue collection.

4.5 Conclusion

In summary, results from the present study indicate that short term exposure to a low-fat control diet post-weaning can reverse fat deposition, hyperleptinaemia and the preference for fat in offspring of dams fed a junk food diet during pregnancy and lactation. This is significant as children with higher preference for fat tend to consume more high-fat foods and have greater adiposity (306, 307). These effects of the low-fat control diet on the JF offspring however did not persist after subsequent exposure to the junk food diet during the juvenile period and this secondary exposure to the junk food diet resulted in further adaptations to dopamine system within the mesolimbic reward pathway and increased adiposity in the JF offspring at the completion of the experiment. Therefore, the present study has shown that exposure to a junk food diet early in life results in programmed adaptations within the mesolimbic reward system as early as weaning, and that this is associated with increased susceptibility to diet-induced obesity which cannot be reversed by a short term low-fat control diet post-weaning.

CHAPTER 5: LONG TERM LOW-FAT CONTROL DIET POST-WEANING REDUCES JUNK FOOD INTAKE BUT NOT SUSCEPTIBILITY TO DIET-INDUCED OBESITY IN ADULT OFFSPRING OF JUNK FOOD FED DAMS

5.1 Introduction

It has become increasingly clear that the preference for specific foods and macronutrients can be programmed through excessive exposure to these foods before birth and in early life. The increased preference for the macronutrients, fat and sugar is likely to be one mechanism by which maternal overnutrition can program the onset of obesity in the offspring (40, 53, 55).

Whilst the early nutritional environment appears to be important for setting up a propensity to weight gain and obesity, there is emerging evidence demonstrating that the effects of early nutritional experience on the risk of obesity in the offspring can be modulated by dietary exposures after weaning (40, 119, 191, 193, 196). Providing rodent offspring with a high-fat diet post-weaning exacerbates the effects of a maternal high-fat diet on body weight gain and fat deposition, and is associated with poorer metabolic outcomes in adulthood compared to peri-natally overnourished offspring weaned onto a standard low-fat control diet (40, 41, 119, 160, 194).

The evidence that a post-weaning high-fat diet can exacerbate the effects of peri-natal overnutrition has led to the suggestion that the adverse metabolic effects of a maternal high-fat diet on the offspring could potentially be ameliorated or reversed by providing these offspring with a low-fat control diet post-weaning (40, 160, 194). In support of this, Velkoska and colleagues showed that offspring exposed to overnutrition during the suckling period were protected from increased body weight and fat deposition during adulthood when provided with a standard low-fat chow diet after weaning (196). In addition, offspring of high-fat, high-sugar fed dams weaned to a low-fat control diet had similar body weights and body fat mass as control offspring in adulthood and remained glucose and leptin-sensitive as adults (160). Nevertheless, there are also conflicting results suggesting that a maternal diet during pregnancy and lactation programs an increased body

weight and fat mass in the offspring independently of the post-weaning diet (41, 44). Therefore, it remains unclear whether the programming effects of peri-natal overfeeding on body weight, fat mass and in particular, food preferences can be reversed by nutritional interventions applied after birth.

In Chapter 4, it was reported that providing offspring of JF dams with a low-fat chow diet for 3 weeks after weaning reversed the increase in fat mass and leptin levels at 6 weeks of age. It was also found, however, that the increased adiposity and hyperleptinaemia in these offspring re-emerged when they were re-exposed to the junk food diet for a short period during the juvenile period. Moreover, the JF offspring consumed more energy and carbohydrate during the junk food exposure, and exhibited changes in gene expression of key components within the mesolimbic reward system. Together, these data suggested that the period of plasticity for the programming of body composition, food preferences and the mesolimbic reward system extends beyond the suckling period and into the juvenile period. It therefore appears that those offspring exposed to palatable diets during the peri-natal period may require more extended periods of abstinence from a palatable diet after weaning in order to override the programming effects of a maternal high-fat, high-sugar diet.

This study aimed to determine if maintaining the offspring on a low-fat control diet for a longer period of time (weaning to 3 months of age) could ameliorate the increased preference for fat and junk food intake, excessive accumulation of body fat and altered gene expression within the mesolimbic reward system in offspring of dams maintained on a junk food diet during pregnancy and lactation.

5.2 Methods

5.2.1 Animals and feeding regime

The same procedures were followed as described in Chapter 4. Briefly, 23 female Albino Wistar rats were brought into the animal facility and allowed to acclimatise for a week. The female rats were then assigned to one of two groups: 1) Control group (n=11), which were provided with a standard rat chow diet (Specialty Feeds, Glen Forrest, Western Australia, Australia) or 2) Junk Food group (JF, n=12), which were provided with a range of palatable junk foods as previously described in Chapter 2 (Table 2.1). Food intake was measured every 2 days and body weight determined every week.

5.2.2 Mating

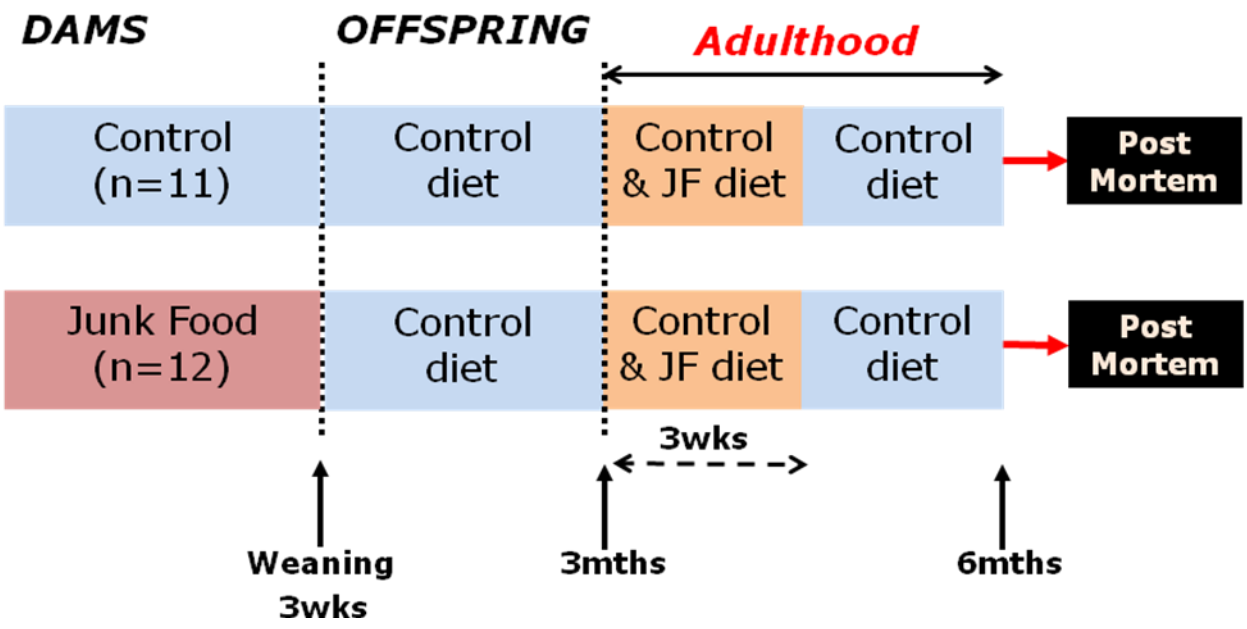
The same procedures were followed as described in Chapter 3.

5.2.3 Offspring

Pups were born on day 21 - 22 of gestation. At P1, litters were culled to 8 pups, with 4 males, 4 females where possible. Pups were weighed every 2 days from birth until weaning (P21). Pups of Control and JF dams are referred to as Control offspring and JF offspring respectively.

At weaning, offspring were group-housed according to litter and sex. All offspring were weaned on a standard rat chow and were maintained on this diet until 3 months of age. At 3 months of age, all offspring were assigned to a food preferences study for 3 weeks, during which they were given free access to both the standard rat chow and the same range of junk foods as provided to the dams. The amount of control chow and each different type of junk food remaining was recorded every 2 days and macronutrient preferences were calculated based on the nutritional composition of each type of food consumed. Food intake was averaged based on the number of offspring per cage and relative to the body weight of the offspring. Food intake was determined every 2 days and body weights determined every week. After 3 weeks, the offspring were returned to the control diet until 6 months of age. A summary of the experimental protocol is shown in Figure 5.1.

Figure 5.1



Summary of experimental protocol

5.2.4 Post-mortem and tissue collection

Offspring from the food preferences study were humanely killed at 6 months of age with an overdose of CO_2. These rats were not fasted. The same procedures for euthanasia and collection and storage of tissues were used as previously described in Chapter 4.

5.2.5 Determination of plasma glucose, NEFA, insulin and leptin concentrations

Assays for plasma glucose, NEFA, insulin and leptin concentrations were carried out as described in Chapter 4. Briefly, plasma glucose and NEFA concentrations were determined using the Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) and the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) respectively with Konelab 20 (Thermo Scientific, Vantaa, Finland). Two quality control samples were included in each run. Inter- and intra-assay CoV were <5%.

Plasma insulin concentrations were measured by rat insulin EIA (ALPCO Diagnostics, Salem, NH, USA) and leptin concentrations by rat leptin ELISA (Crystal Chem Inc, IL, USA) according to manufacturer's instructions. Inter- and intra-assay CoV for leptin and insulin assay were both <10%.

5.2.6 RNA extraction and reverse transcription

Procedures for RNA extraction and reverse transcription were carried out as described in Chapter 4.

5.2.7 Determination of gene expression in the NAc and VTA

Procedures for real time PCR were carried out as described in Chapter 4.

5.2.8 Statistical Analyses

Data are presented as mean ± SEM. The dam was used as the unit of analysis. Offspring body weight and food intake were analysed using repeated measures ANOVA. Plasma hormone and metabolite concentrations and gene expression of key components of the reward system in the offspring at 6 months of age were analysed using two-way ANOVA with sex and treatment groups as factors. When a significant interaction between sex and treatment was identified, data was analysed separately for males and females using an unpaired Student's t-test.

Repeated measures ANOVA was performed using Stata 10 (StataCorp LP, Texas, USA). All other analyses were performed using SPSS for Windows Version 18.0 (SPSS Inc., Chicago, IL, USA). A probability of $P<0.05$ was considered statistically significant in all analyses.

5.3 Results

5.3.1 Offspring: 3 weeks to 13 weeks of age

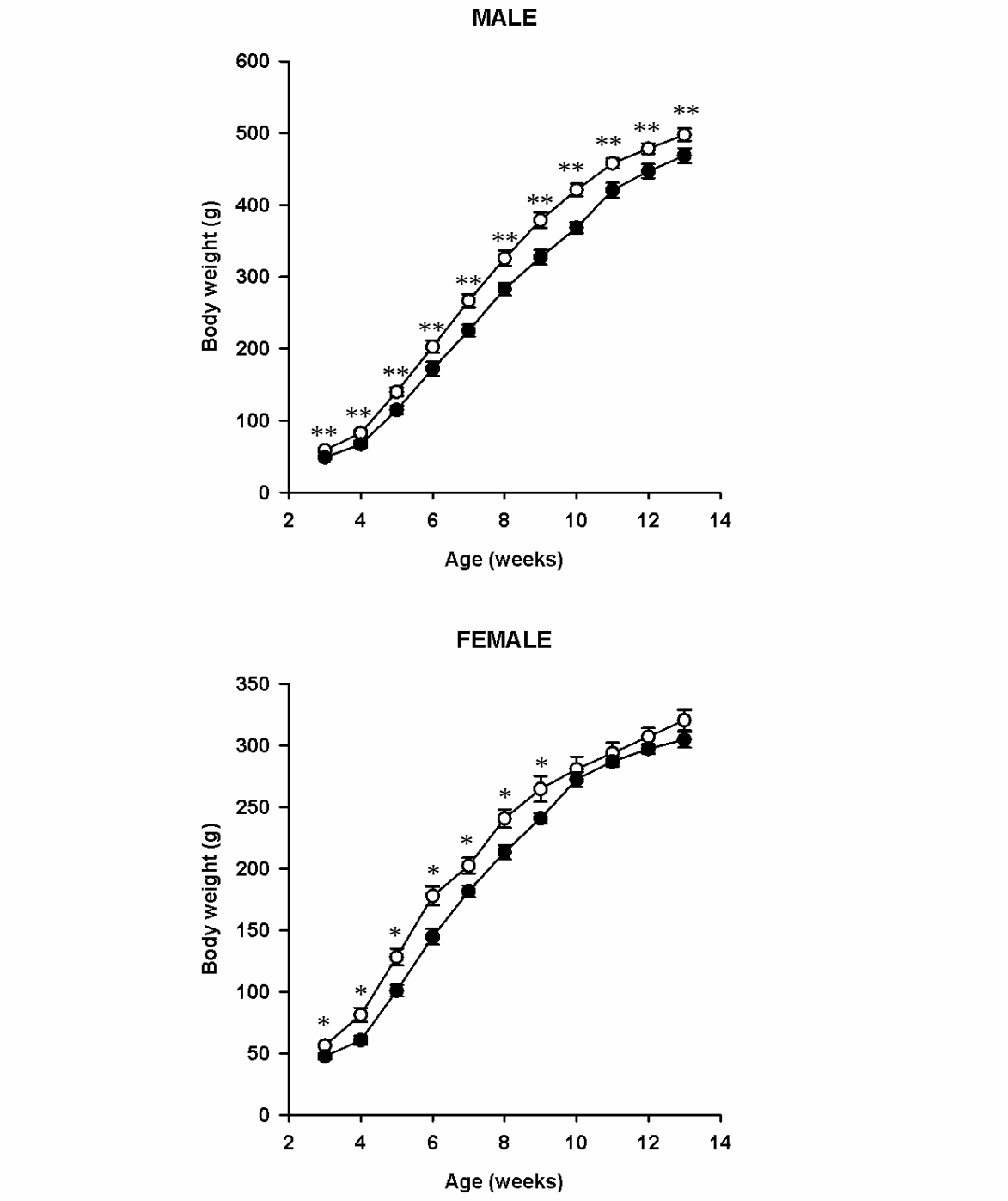
5.3.1.1 Body weight

At weaning, both male and female JF offspring were lighter than Controls ($P<0.01$). In males, body weight of JF offspring remained lower than Controls until 13 weeks of age ($P<0.01$). In females, however, body weight of JF offspring was lower than Controls until 6 weeks post-weaning ($P<0.05$), but was no longer different from Controls from 7 weeks post-weaning onwards (Figure 5.2).

5.3.1.2 Food intake

In males, chow intake was not different between JF and Control offspring during the first 6 weeks post-weaning. However, from 7 weeks post-weaning onwards, male JF offspring consumed more chow per kg body weight when compared to their Control counterparts ($P<0.01$) (Figure 5.3). In females, offspring of JF dams consumed more chow per kg body weight from weeks 2 to 4 post-weaning, when compared to Controls ($P<0.05$). There were no differences in chow intake between groups at any other time point (Figure 5.3).

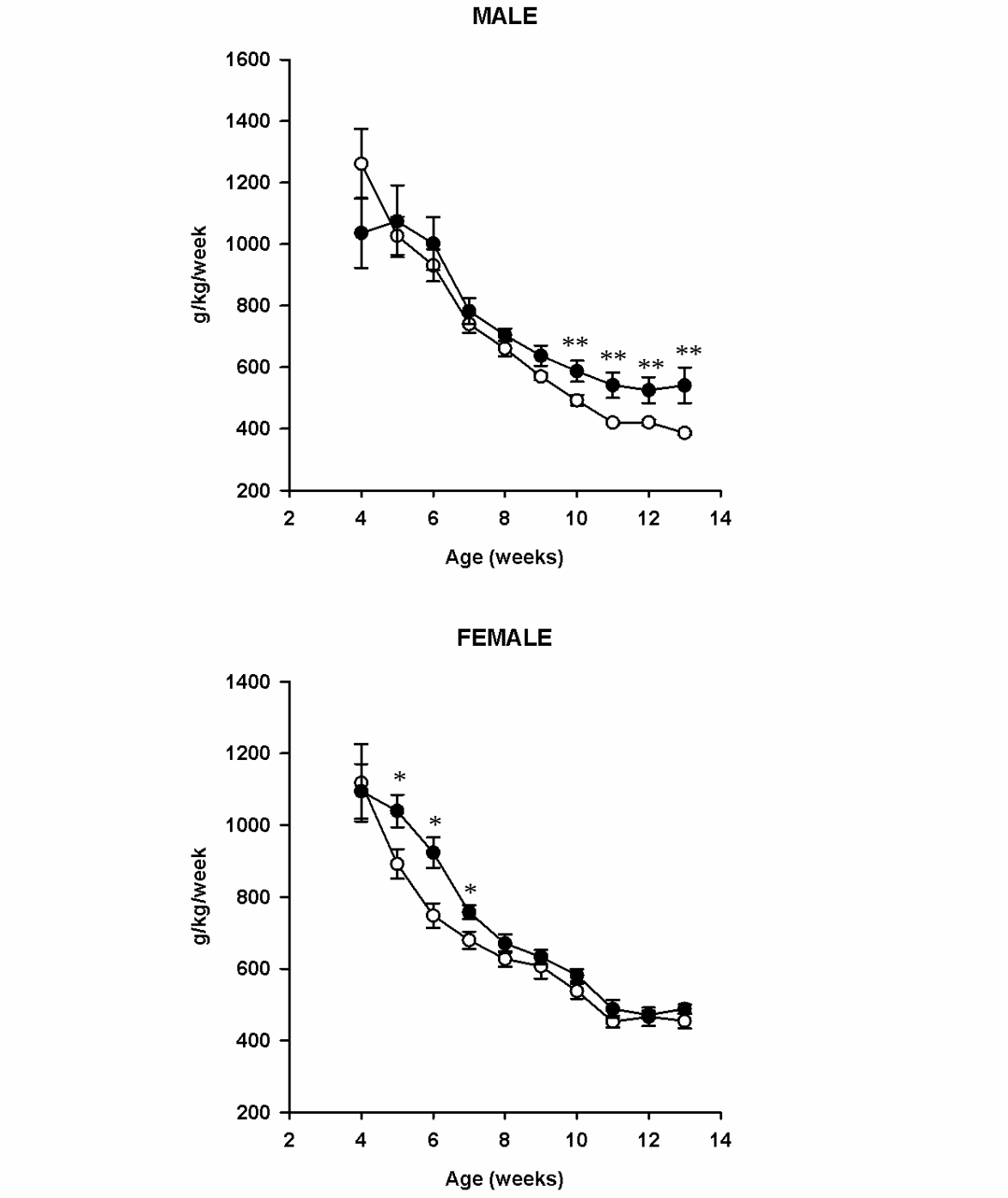
Figure 5.2



Absolute body weights (g) of male and female Control (open circles) and JF (closed circles) offspring from 3 - 13 weeks of age.

Values are expressed as mean ± SEM. *P<0.05, **P<0.01. n = 6 - 10 in each group.

Figure 5.3



Chow intake in male and female Control (open circles) and JF (closed circles) offspring during 4 - 13 weeks of age.

Values are expressed as mean ± SEM. *P<0.05, **P<0.01. n = 6 - 11 for each group.

5.3.2 Food preference study

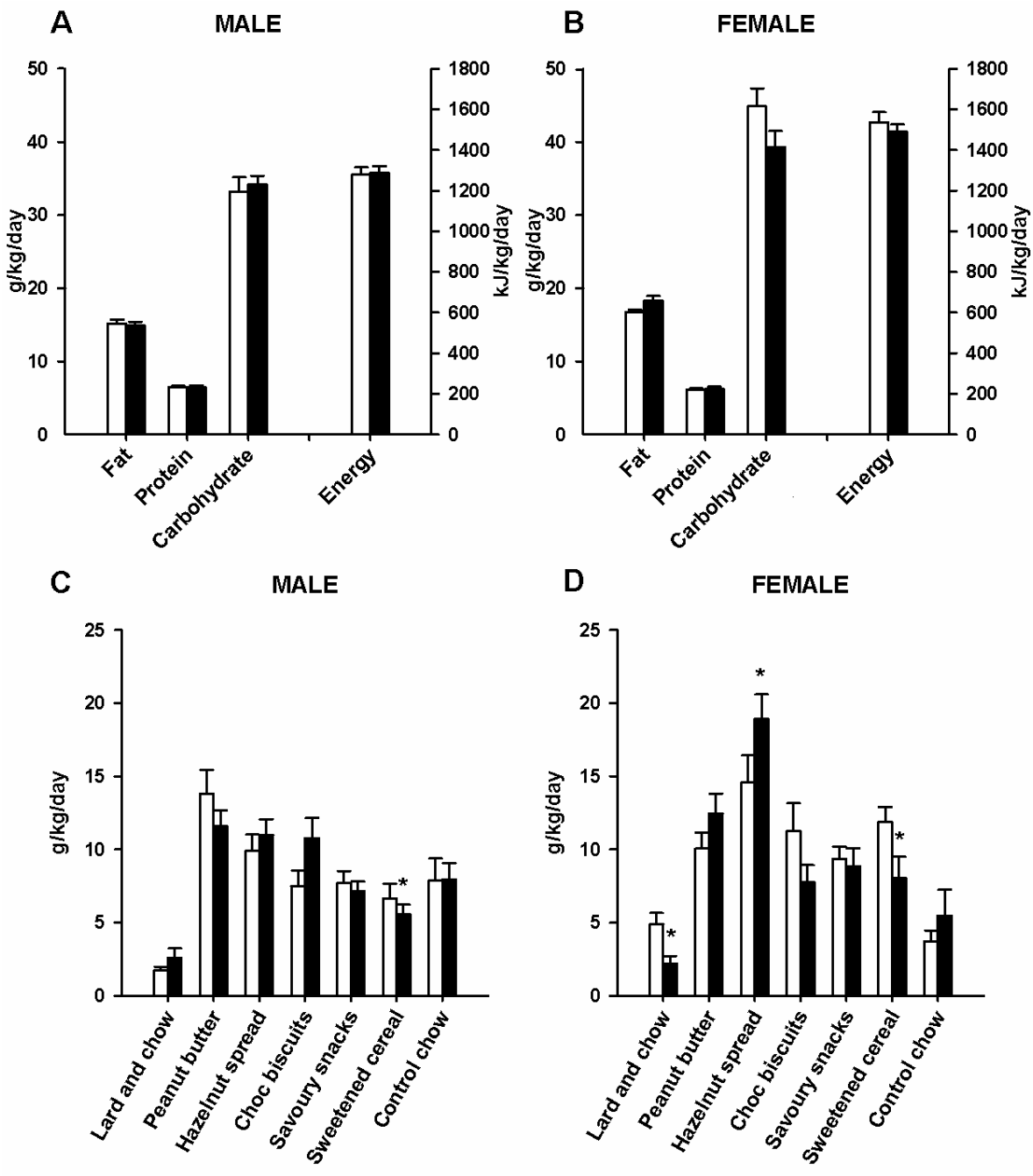
5.3.2.1 Body weight

Male JF offspring were lighter than Control offspring before the start of the food preference study and remained lighter at the end of the 3 week test period (Control: 636.0 ± 13.2g, JF: 573.8 ± 13.6g; $P<0.01$). The percentage weight gain during the first week of the food preferences study tended ($P=0.05$) to be higher in the male Control offspring than the JF offspring (Male Control: 11.1 ± 1.2%, Male Junk Food: 7.6 ± 1.3%). Percentage weight gain during the second week of the study was higher in male JF offspring when compared to Controls (Male Control: 7.6 ± 0.6%, Male JF: 9.9 ± 0.9%; $P<0.05$). There was no difference in percentage weight gain during the third week of the food preference study between groups in the male offspring. In females, there was no difference in body weight gain during the food preference period between the Control and JF groups and body weight at the end of the food preferences study was also not different between groups (Control: 457.8 ± 14.2g, JF: 445.7 ± 13.4g).

5.3.2.2 Food and macronutrient preferences

There was no difference in energy, fat, carbohydrate or protein intake between the Control and JF groups during the food preferences study in either male or female offspring. In females only, JF offspring tended ($P=0.07$) to consume more fat when compared to Controls (Figure 5.4A, B). Both male and female JF offspring consumed less sugary cereal ($P<0.05$) and tended ($P=0.06$) to consume more hazelnut spread when compared to the Control offspring. In females only, JF offspring consumed less lard and chow mix during the food preferences study when compared to Control offspring ($P<0.05$) (Figure 5.4C, D).

Figure 5.4



Intake of fat, protein, carbohydrate and total energy in male (A) and female (B) Control (open bars) and JF (closed bars) offspring and intake of individual junk foods in male (C) and female (D) Control and JF offspring during the food preferences study.

Data analysed by two-way ANOVA and unpaired Student's t-test (when maternal diet x sex interaction is identified). Values expressed as mean ± SEM. Student's t-test, **P*<0.05. n = 9 - 12 in each group.

5.3.3 Immediate effects of junk food removal: after a 3 week food preference study

5.3.3.1 Body weight

At the end of the food preference period, the junk food diet was removed and all offspring were returned to the standard rat chow. In males, neither Control nor JF offspring exhibited any significant change in body weight 1 week after the food preference study. In the female offspring, both Control and JF groups lost weight after the removal of the palatable diet, with an average loss of 5% and 3% of their starting weight respectively. This weight loss was not different between groups.

5.3.3.2 Food intake

Chow intake during the first week following the food preferences study was not different between the JF and Control offspring in either males or females. However, both Control and JF offspring consumed less chow after removal of the junk food diet when compared to the amount of chow consumed before the food preference study. In males, the reduction in chow intake was greater in offspring of JF dams (61% reduction in chow intake/kg body weight) than the Control offspring (45% reduction in chow intake/kg body weight) (P<0.01). In female offspring, there was no difference in the reduction of chow intake/kg body weight between Control and JF groups (51% versus 55% reduction respectively).

5.3.4 Post-food preferences: 18 weeks to 24 weeks of age

Male JF offspring remained lighter than Controls after the food preferences study until 24 weeks of age (P<0.01). In the female offspring, however, there were no differences in body weight between the Control and JF groups after the food preferences study. Total chow intake relative to body weight from the end of the food preferences until post-mortem was not different between Control and JF offspring in either males or females.

5.3.5 Post-mortem: 25 weeks of age (6 months)

At 6 months of age, male JF offspring were lighter than Controls (P<0.01). There was no difference in body weight between female JF and Control offspring at this age. JF offspring were shorter than Controls in both males and females (P<0.01) (Table 5.1). Gonadal fat mass as a percentage of body weight was higher in both male and female JF offspring compared to Controls (P<0.01). In addition, in females only, total percentage fat mass and percentage retroperitoneal fat were higher in the JF offspring compared to Controls (P<0.05) (Table 5.1).

There were no differences in plasma glucose, NEFA and insulin concentrations at 6 months of age between the Control and JF groups in either male or female offspring. There was sex by treatment interaction for plasma leptin concentrations where female JF offspring tended (P=0.07) to have higher plasma leptin concentrations compared to Controls. There was no difference in plasma leptin concentrations between groups in the male offspring (Table 5.1).

5.3.6 Gene expression of TH, D1, D2, DAT and μ-opioid receptors within the NAc and VTA of the offspring

In the NAc, there was a sex by treatment interaction for D2 mRNA expression at 6 months of age. In males, D2 expression was higher in the JF offspring compared to Controls (P<0.05), whilst in the females, D2 expression tended (P=0.07) to be lower in the JF offspring. There were no differences in the expression of TH, D1, DAT and μ-opioid receptor in the NAc between groups in either male or female offspring (Table 5.2).

In the VTA, there was also sex by treatment interaction for D2 mRNA expression. In males, but not in females, D2 mRNA expression were lower in JF offspring when compared to Controls (P<0.05). TH, D1, DAT and μ-opioid receptor mRNA expression was not different between groups in either males or females (Table 5.2).

Table 5.1 Metabolic parameters in Control and JF offspring at 6 months of age

Values are expressed as mean ± SEM. Data analysed by two-way ANOVA and Student's t-test (when maternal diet × sex interaction is identified). - = no difference, M = Maternal diet effect, S = Sex effect, M × S = Maternal diet and sex interaction. Student's t-test, **P*<0.05, ***P*<0.01 between treatment groups, ^*P*<0.05 between sexes. n = 10 - 12 in each group.

	6 months of age				
	Male		Female		
	Control	JF	Control	JF	ANOVA
Body weight (g)	673.7 ± 14.9	605.0 ± 14.4**	424.4 ± 19.0	444.5 ± 9.0	S, M × S
Length (cm)	31.0 ± 0.3	30.0 ± 0.2	27.3 ± 0.2	26.9 ± 0.2	M, S
Abdominal circumference (cm)	25.9 ± 0.5	24.8 ± 0.5	21.2 ± 0.6	21.6 ± 0.5	S
% fat mass					
Gonadal fat	2.8 ± 0.11	3.1 ± 0.11	3.4 ± 0.23	4.4 ± 0.31	M, S
Retroperitoneal fat	3.4 ± 0.20	3.5 ± 0.20	3.0 ± 0.35	4.3 ± 0.25**	M, M × S
Omental fat	2.2 ± 0.09	2.1 ± 0.11	2.6 ± 0.45	3.0 ± 0.18	S
Subcutaneous fat	9.0 ± 0.49	8.1 ± 0.56	6.9 ± 0.80	8.2 ±0.36	-
Interscapular fat	0.3 ± 0.02	0.3 ± 0.02	0.4 ± 0.03	0.4 ± 0.03	S
Total fat	17.6 ± 0.84	17.3 ± 0.86	16.2 ± 1.49	20.3 ± 0.81*	M × S
Plasma hormone & metabolites					
Glucose (mmol/L)	14.7 ± 0.64	16.5 ± 0.76	15.0 ± 0.95	14.5 ± 0.74	-
NEFA (meq/mL)	0.4 ± 0.06	0.4 ± 0.03	0.4 ± 0.04	0.5 ± 0.04	-
Insulin (ng/mL)	3.1 ± 0.49	2.9 ± 0.33	2.4 ± 0.42	3.5 ± 0.55	-
Leptin (ng/mL)	29.3 ± 2.77	26.5 ± 2.28	19.3 ± 3.57^	29.1 ± 2.58	M × S

Table 5.2: mRNA expression of TH, D1, D2, μ-opioid receptor (Mu) and DAT (relative to β actin) in the NAc and VTA of Control and JF offspring at 6 months of age

For ease of presentation, values for all genes in the NAc and D1, Mu in the VTA are multiples of 1000. Values are expressed as mean ± SEM. - = no difference, M = Maternal diet effect, S = Sex effect, M × S = Maternal diet and sex interaction. Student's t-test, **P*<0.05 between treatment groups. n =7 - 11 in each group.

	6 months of age				
	Male		Female		
	Control	JF	Control	JF	ANOVA
NAc					
TH	0.39 ± 0.07	0.36 ± 0.04	0.49 ±0.05	0.34 ± 0.05	-
D1	41.10 ± 7.42	36.69 ± 5.35	36.26 ± 5.18	31.28 ± 2.76	-
D2	68.60 ± 7.22	102.39 ± 14.92*	100.45 ± 10.1	76.70 ± 6.24	M × S
Mu	2.70 ± 0.25	3.18 ± 0.48	2.97 ± 0.36	2.90 ± 0.27	-
DAT	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	-
VTA					
TH	1.09 ± 0.20	0.77 ± 0.10	0.84 ± 0.15	0.82 ± 0.19	-
D1	0.97 ± 0.22	0.66 ± 0.10	0.60 ± 0.06	0.50 ± 0.06	-
D2	0.19 ± 0.03	0.12 ± 0.02*	0.15 ± 0.03	0.20 ± 0.04	M × S
Mu	5.38 ± 0.68	5.59 ± 0.80	5.47 ± 0.50	4.91 ± 0.33	-
DAT	0.47 ± 0.06	0.32 ± 0.03	0.44 ± 0.07	0.41 ± 0.06	-

5.4 Discussion

In Chapter 4, it was demonstrated that providing JF offspring with a low-fat control diet for 3 weeks post-weaning prevented the increased preference for fat in the juvenile JF offspring observed in Chapter 3. However, when these offspring were exposed to a junk food diet during the juvenile period, they were found to be more susceptible to fat accumulation and had increased expression of dopamine-related genes within the reward system. The present Chapter therefore aimed to determine if a longer period of low-fat chow intake would reverse the preference for fat and prevent the development of diet-induced obesity in the offspring of junk food fed dams during adulthood. It was found that providing offspring of JF dams with a low-fat chow diet from weaning until 3 months of age prevented the increased preference for fat and junk foods in both male and female JF offspring and reversed the increased fat deposition in JF males, but not in females. There was no difference in gene expression within the reward system between groups in females but in JF male offspring, D2 was upregulated in the VTA and downregulated in the NAc. Therefore, findings from this study indicated that the effects of a maternal junk food diet was ameliorated, but not completely reversed, even with a long term exposure to a low-fat control diet post-weaning.

5.4.1 Offspring growth

The growth trajectory of the JF offspring was similar to that observed in Chapter 3, even after being weaned on a low-fat chow diet. In males, JF offspring remained lighter and shorter than Control offspring throughout the post-weaning period until 6 months of age. Importantly, this growth deficit in JF offspring appeared to be a consequence of deficits in lean body mass, as total body fat mass was not different between JF and Control offspring. This finding was in contrast to previous studies in which offspring exposed to pre-natal and/or early post-natal overnutrition exhibited accelerated weight gain during post-natal life, regardless of whether they were lighter (40, 44) or of similar body weights (41) to controls at weaning. This lack of post-natal growth in male JF offspring is, however, consistent with previous studies in which exposure to maternal low-protein feeding and global undernutrition in which offspring exposed to undernutrition throughout the pre-natal and suckling period remained lighter than control offspring throughout life (49, 289, 290). In the present study, it is possible that the lower protein intake of the JF dams during pregnancy and lactation (as described in Chapter 3) was the major factor contributing to the poor post-natal growth in the JF offspring. Moreover, the higher chow intake from 9 to

13 weeks of age did not overcome this deficit in lean body mass, suggesting that the amount of lean tissue mass gained during the pre-weaning period is a critical determinant of lean tissue accretion later in life and cannot be compensated for by the introduction of a protein-adequate diet after weaning or increased food intake during adulthood.

The female offspring displayed a different growth pattern to the males. Despite being smaller and lighter at weaning, the body weights of female JF offspring were no longer different from that of Control offspring from 6 weeks of age onwards. Even though female JF offspring consumed more chow from 5 to 7 weeks of age, the rapid weight gain in these offspring appeared to be largely due to an increased accumulation of body fat, since the percentage body fat mass was higher in female JF offspring compared to Control offspring even at 6 months of age, long after differences in body weight between groups had been resolved. The higher fat accumulation in the females compared to males may help explain the ability of female JF offspring, but not in male JF offspring, to catch up to Controls in terms of body weight.

5.4.2 Offspring preference for palatable foods

In Chapter 3, I showed that offspring of JF dams had a higher preference for fat which persisted until at least 3 months of age when these offspring were provided with *ad libitum* access to both a control and palatable diet from weaning. In the present study, it was found that maintaining these offspring on a low-fat chow diet from weaning until 3 months of age reversed this increased preference for fat when the offspring were subsequently provided with access to a palatable diet. This observation is somewhat at odds with the results of previous studies where offspring exposed to high-fat diets before birth and during early life exhibited a higher preference for fat even when provided with a low fat control diet post-weaning (52, 53). Similarly, offspring of dams fed a high-fat diet in late gestation and lactation also exhibited an increased operant responding for a high-fat diet during adulthood, even when they had been consuming a control low-fat diet since weaning (51). These differences between these studies may be due to the nature of the palatable diets used. Previous studies used a high-fat feeding model, where dams were provided with pellets containing approximately 60% fat, whereas the current study utilised a cafeteria diet model, which was high in both fat and sugar. Bayol and colleagues used a similar cafeteria diet model and found that offspring of dams fed on the cafeteria diet throughout pregnancy and lactation consumed more fat, sugar and salt in adulthood, but did not assess food preferences in offspring which had been maintained on a low-fat chow diet after weaning

(40). Since sugar and fat have distinct addictive properties such that removal of sugar but not fat results in the onset of opiate-withdrawal signs, it is possible that the high-fat component and the combination of both fat and sugar/carbohydrate in the maternal diet can differentially impact on subsequent food preferences in the offspring. However, this remains to be fully investigated.

In Chapter 4, it was reported that offspring of junk food fed dams which had been maintained on a low-fat chow for 3 weeks after weaning still exhibited a higher intake of energy, carbohydrate and protein when exposed to a palatable diet during the juvenile period. In the present study, however, we found no differences in energy intake or preference for protein or carbohydrate between JF and Control offspring during the food preferences study after being weaned on a low-fat control diet until 3 months of age. These data therefore suggest that a prolonged exposure to a low-fat control diet is required to reverse the increased appetite for fat and sugar in offspring of junk food fed mothers. Given that the preference for palatable foods was reversed by altering the post-weaning diet to a low-fat control diet from 3 weeks to 3 months of age, it is possible that the reward system post-weaning still retains a high degree of plasticity and is therefore susceptible to nutritional manipulations. This is in line with previous studies which suggest that the rodent brain is highly plastic during the juvenile period. In these studies, leptin administration to ob/ob mice during the juvenile period was shown to have the capacity to rewire the appetite network of these mice (163). Similarly, nicotine administration to rats during the juvenile period, but not adulthood, also caused prolonged neural adaptations in the cholinergic and glutamatergic systems of various regions of the brain including the hippocampus, midbrain and striatum (308, 309). Interestingly, exposure to nicotine during the juvenile period also triggered cocaine self-administration in adulthood (310). It therefore appears that the critical window for programming of food preferences extends beyond the immediate post-natal period and into the juvenile period, and that providing a low-fat control diet beyond the juvenile period is required to overcome programmed changes in food preferences as a result of exposure to a maternal junk food diet early in life.

5.4.3 Offspring chow intake post-food preferences

Whilst chow intake before the food preference experiment was higher in the male JF offspring compared to Controls, there were no longer any differences in chow intake following the removal of the junk food diet at the end of the food preferences study. This appeared to be a consequence of greater reduction of chow intake immediately following

the food preferences study in the JF offspring, which was then maintained throughout the remainder of the study period. The reduction in chow intake after exposure to a palatable diet is a commonly reported symptom of withdrawal in adult rodents when access to palatable foods is removed following a period of chronic exposure to a palatable diet (82, 97). The greater reduction in chow intake after the removal of a junk food diet in the male JF offspring may therefore indicate a more exaggerated withdrawal response in comparison to Controls, which may imply that male JF offspring are more sensitive to the effects of palatable diets on the mesolimbic reward system compared to Controls.

5.4.4 Gene expression in the mesolimbic reward system at 6 months of age

In the present study, peri-natal exposure to a junk food diet was associated with gene expression changes in the male offspring, but not females, at 6 months of age. Male JF offspring exhibited lower D2 expression in the VTA, consistent with the findings of Naef and colleagues on the effects of a maternal high-fat diet in adult offspring (51) and with chronic cocaine administration in adult rodents (311). D2 receptors in the VTA predominantly act as autoreceptors on dopaminergic neurons (68), which are responsible for the negative feedback regulation of dopamine synthesis and release. Therefore, this reduction in VTA D2 expression would be expected to result in a reduced efficacy of dopamine negative feedback in the offspring of JF dams, which could lead to a prolonged dopamine transmission following activation of the reward system.

The lower D2 expression in the VTA occurred in conjunction with an increased D2 mRNA expression in the NAc of male JF offspring at 6 months of age. Since 90% of the overall D2 in the striatum (including the NAc) is located on post-synaptic neurons (312, 313), it is likely that the increased NAc D2 observed in this study would be associated with an enhanced dopamine activity. Similar to findings from the current study, Naef and colleagues also did not observe any differences in D1 and DAT expression in the VTA or NAc in adult offspring of high-fat fed dams (51), which suggests that the negative feedback mechanism or the D2 receptors themselves may be more vulnerable than other components of the dopamine system to high-fat, high-sugar diets during the peri-natal period. The increased NAc D2, together with the desensitised negative feedback mechanism (decreased D2 expression in the VTA) could result in an exaggerated response of the reward system in the presence of a rewarding stimulus, including palatable foods, which could potentially enhance palatable food intake in these JF offspring during subsequent exposure to these diets.

It is important to note that the gene expression data discussed above is not a result of a direct effect of the exposure to the junk food diet at 3 months of age. This is because these offspring had a 9 week washout period where junk food was removed, before post-mortem was conducted and this washout period presumably eliminated any direct effect of 3 week exposure of junk food on gene expression of the reward system. The normalisation of changes within the mesolimbic reward system have been demonstrated in studies of drugs of abuse where the initial increase in D1 density in the NAc associated with cocaine withdrawal was normalised after 45 days in rodents (291) and 90 days in the monkey (314). D2 density, on the other hand remained reduced even after 45 days of cocaine withdrawal (291-293). D2 levels have also been shown to recover to control values but this depends on the period of cocaine exposure, ie the longer the exposure, the longer the recovery period (314, 315). Nevertheless, these studies looked at the protein expression of the receptors in the NAc, whereas in this study, mRNA levels of the receptors both in the NAc and VTA were measured. Since the effects of cocaine on dopamine transmission are more potent than that of palatable foods, the time required for the normalisation of dopamine receptor expression after cessation of palatable food intake would be expected to be less than that of cocaine. This suggests that it is unlikely that the changes in gene expression were due entirely to the direct effects of the 3 week exposure to the junk food diet but rather the adaptation to the short-term junk food exposure. In other words, the mesolimbic reward system of the JF offspring responded and adapted to palatable food intake differently to Control offspring, which is most likely to be due to the programmed effects of an exposure to a high-fat, high-sugar diet before birth and early in life. These adaptations within the mesolimbic reward system may therefore render the male JF offspring more likely to overconsume upon subsequent exposure to palatable foods, although whether these changes in gene expression have functional consequences remains to be determined.

5.5 Conclusion

In summary, this study provided evidence that a prolonged low-fat control diet intake from weaning until adulthood can reverse the preference for junk foods in both male and female JF offspring and normalise the expression in the key components of the mesolimbic reward system in JF females, but not males. The changes within the mesolimbic reward system of male JF offspring indicate a possible increase in dopamine sensitivity towards reward and an increased susceptibility to overeat/develop a higher preference for fat/sugar in the male JF offspring upon subsequent exposure to a highly palatable diet. The higher fat deposition in the female JF offspring suggests that these offspring remained highly susceptible to diet-induced obesity even after being weaned onto a low-fat control diet until adulthood. Therefore, even though the effect of maternal junk food intake on the preference for junk foods was reversed by maintaining the offspring on a post-weaning low-fat control diet until adulthood, these offspring remained susceptible to the effects of subsequent junk food exposure during the post-weaning period. This study indicates that the effects of peri-natal exposure to a junk food diet persist into adulthood and cannot be completely reversed even by introducing a low-fat control diet post-weaning.

CHAPTER 6: GENERAL DISCUSSION

The increasing availability of high-fat, high-sugar foods or 'junk foods' is one major contributor to the growing incidence of obesity worldwide. Whilst it is known that the overconsumption of junk foods and the lack of physical activity can lead to the development of obesity, it is unclear why certain individuals are more likely to overindulge in palatable foods than others. This thesis investigated one of the mechanisms by which food preferences can be determined, that is through nutritional experiences before birth and in early life. These investigations were based on the growing number of human and animal studies demonstrating that maternal nutrient intake of high-fat, high-sugar diets during pregnancy and lactation predicted the offspring's preference for fatty and sugary foods after birth (40, 53, 55), and sought to investigate the mechanisms underlying this association. The mesolimbic reward system, a system in the brain involved in addiction and reward, has been shown to play a key role in the regulation of palatable food intake in adult humans and animals. It was therefore hypothesised that exposure to high-fat, high-sugar foods before birth and during early in life could program an exacerbated preference for these palatable foods after birth through alterations within the mesolimbic reward system in the developing offspring.

The aim of Chapter 2 of the thesis was to investigate the impact of chronic junk food feeding in male and female adult rats on the mesolimbic reward system, which provided the basis for the remaining chapters of this thesis. Results from this study showed that both male and female rats fed on the junk food diet were markedly fatter than their control counterparts with elevated leptin levels. During a 72 hour period of junk food abstinence, both male and female junk food rats lost weight and significantly reduced their standard chow intake, consistent with previous reports indicative of a withdrawal response (82, 97). Despite the similarities in behavioural outcomes between male and female adult rats in response to chronic junk food intake, the effects of the junk food treatment and subsequent junk food abstinence on gene expression within the mesolimbic reward system were distinct between sexes, thus suggesting that the mechanisms underlying the development of junk food dependence are likely to differ between males and females. Nevertheless, in both males and females, the changes in gene expression within the mesolimbic reward system were indicative of a reduced sensitivity of the dopamine system within the reward pathway,. This study in this thesis therefore adds to the growing body of evidence that

chronic junk food intake is associated with changes within the mesolimbic reward system comparable to those of drugs of abuse.

Findings in Chapter 2 supported the role of the mesolimbic reward system in the regulation of food reward in adults. The remaining Chapters of the thesis focused on whether changes in gene expression within the mesolimbic reward system also play a role in the programming of food preferences in the offspring following maternal intake of high-fat, high-sugar diets during pregnancy and lactation. Previous studies using a rodent model of maternal high-fat feeding had shown that offspring of high-fat fed dams exhibited altered expression of key components of the mesolimbic reward system in adult offspring, and that these offspring also exhibited a higher preference for fat (51) and sugar (53). Further studies had shown that offspring of dams fed a cafeteria diet throughout pregnancy and lactation demonstrated an exacerbated preference for the cafeteria diet, when provided with both the standard rat chow and cafeteria diet after weaning (40). However, no prior studies had determined whether peri-natal exposure to high-fat, high-sugar diets were associated with altered gene expression within the mesolimbic reward system of the offspring during the juvenile and young adult periods. Therefore, in Chapter 3, the impact of maternal junk food diet on offspring food preferences and the mesolimbic reward system was investigated. It was demonstrated that offspring of junk food fed dams had a higher preference for fat which persisted from weaning until adulthood. This was associated with increased μ-opioid receptor and reduced DAT expression within the NAc during the juvenile period, suggestive of increased sensitivity of the reward system, which would be expected to promote fat intake. During adulthood however, continuous consumption of junk foods and the specific preference for fat in the junk food offspring led to downregulation of μ-opioid receptor and upregulation of DAT that potentially indicates the desensitisation of the mesolimbic reward system. It is proposed that this reduced reward sensitivity is responsible for driving the preference for fat observed in the adult offspring. This is the first study to demonstrate the impact of maternal junk food diet on offspring mesolimbic reward system and food preferences during the juvenile period and to relate these changes within the reward system to differences in macronutrient preference in the offspring. These findings from Chapter 3 have therefore provided a possible cause and effect relationship between maternal junk food feeding, post-weaning junk food intake and subsequent fat preference in the offspring later on in life.

As mentioned earlier in Chapter 3, the higher intake of fat in offspring is likely due to maternal junk food feeding during pregnancy and lactation. However, given that all offspring were also exposed to the junk food diet post-weaning, it is possible that the preference for fat during the juvenile period was also influenced by the post-weaning diet. The ability of the post-weaning diet to influence the effect of maternal diet on the offspring's post-natal metabolic health had been previously demonstrated; the adverse effects of a maternal high-fat diet on offspring metabolic health outcomes in later life had been shown to be exacerbated by a post-weaning high-fat diet (40, 43, 193), and improved by a low-fat diet (40, 160, 194). Therefore, it had been hypothesised that the preference for fat in the JF offspring could potentially be reversed by introducing a low-fat non-palatable diet after weaning. To date, there is limited evidence as to whether food preferences can be reversed. Given that the brain of the rodent is still relatively plastic during the first few weeks post-weaning, the aim of Chapter 4 of the thesis was to determine if providing the offspring a nutritionally balanced post-weaning diet during the first 3 weeks post-weaning could reverse the fat preference and changes within the mesolimbic reward system

When all offspring were weaned on a control low-fat chow diet, the offspring of junk food fed dams, which had been shown to be fatter at weaning, were no longer fatter than the Control offspring at 6 weeks of age, and this was also reflected by the normalised plasma leptin levels. However, when all offspring were subjected to a food preferences study at 6 weeks of age, the JF offspring did not have a higher fat intake but instead consumed more carbohydrate and total energy. Findings from the food preference study indicate that even though the JF offspring no longer exhibited a higher fat intake, these offspring were hyperphagic when given free access to the palatable foods. The JF offspring also exhibited an increased D1 mRNA expression in the VTA which was positively correlated with carbohydrate intake, suggesting that the upregulation of D1 in the VTA of these offspring may be driving the increase in carbohydrate intake. Furthermore, the re-introduction of the junk food diet during the juvenile period resulted in a higher body fat mass and increased mRNA expression of dopamine genes within the mesolimbic reward system of JF offspring at 3 months of age in comparison to Controls, and this effect was more apparent in females than in males. Findings from Chapter 4 therefore suggested that providing a low-fat diet post-weaning could reverse the effects of maternal junk food diet on fat preference and body fatness in the short term but these offspring remain more susceptible to diet-induced obesity later in life, particularly if being exposed to a junk food diet during the juvenile period.

The ability of a post-weaning low-fat diet to reverse the effects of maternal high-fat diet remains controversial. Whilst some studies have shown that offspring of high-fat dams do not exhibit the obesity phenotype when weaned on a low-fat control diet (40, 160, 194), others have shown that the offspring of overnourished dams were already leptin resistant at weaning (41) and remained fatter than control offspring during adulthood (41, 190). Interestingly, Velkoska and colleagues found that offspring from small sized litters (overnourished during early post-natal life), when weaned on a control diet until the juvenile period, remained fatter and had higher plasma leptin levels. However, there were no longer any differences in body weight, fat deposition, food intake and plasma leptin levels between groups during adulthood, which implies that a longer exposure to the post-weaning low-fat diet may be required to reverse any adverse metabolic outcomes and possibly the preference for fat. Therefore, in Chapter 5, I aimed to determine whether providing offspring of junk food fed dams with a low-fat control diet for a longer period post-weaning (weaning to adulthood) could be effective in reversing the preference for palatable foods and the susceptibility to diet-induced obesity in the offspring. I showed that providing a low-fat chow diet to offspring of junk food fed dams from weaning to adulthood eliminated the preference for palatable foods in both male and female adult offspring. Despite this, however, female JF offspring remained fatter than the Controls and had higher plasma leptin levels at 6 months of age. Male JF offspring did not exhibit any differences in body weight or fat mass, but exhibited increased D2 in the NAc but reduced D2 expression in the VTA. This suggests an increased dopamine sensitivity within the NAc and diminished dopamine negative feedback mechanism within the VTA, both of which could potentially lead to an exaggerated response to rewarding stimuli, including palatable diets. Importantly, these effects were observed even after returning the offspring to a low-fat control diet for 9 weeks following the acute junk food exposure. Therefore, whilst the food preferences of the JF offspring appeared to be normalised by the longer term maintenance of the low-fat diet, there were indications that, particularly in the females, an increased propensity towards diet-induced obesity remained. The male offspring exhibited changes in D2 expression in the absence of any obvious phenotype, so it has yet to be determined if these changes in gene expression have any functional consequences. Although we could not definitively explain the sex differences in response to a low-fat chow diet post-weaning, it is clear that the effects of maternal junk food diet on offspring body composition and changes within the mesolimbic reward system are irreversible, even after being weaned on a low-fat control diet until adulthood.

An important aspect of this thesis was the fact that the responses of both males and females were examined in all experiments, and sex differences within the mesolimbic reward system in response to junk food intake have been consistently demonstrated throughout this thesis. Results from Chapter 2 showed sexual dimorphic changes within the mesolimbic reward system in response to chronic junk food intake. In Chapter 4, female JF offspring exihibited an increase in dopamine gene expression after exposure to a junk food diet during the juvenile period. Female JF offspring were also fatter during adulthood after an acute junk food exposure, as shown in Chapter 5. Unfortunately, very few studies to date have compared the effects of palatable diets on the mesolimbic reward system between males and females and there is currently a strong male bias in biomedical research (316). Despite the fact that females are more prone to addiction and are more likely to have eating disorders and gain weight (209, 222, 317), current understanding of the mechanisms underlying the programming of obesity and the regulation of food intake in females is still unclear. It is speculated that estrogen may be playing a role in the regulation of palatable food intake as estrogen modulates dopamine system by inhibiting GABA-ergic neurons (318). In addition, the development of the gonadal hormone systems may also be altered by *in utero* and early post-natal environment (319), which may subsequently impact on the development of other systems within the brain and how the affected systems respond to nutritional insults in later life. The role of sex hormones in the regulation of food intake and programming of food preferences requires further investigation.

Studies from this thesis have also demonstrated the role of maternal diet on the expression of key genes within the mesolimbic reward system and food preferences in the offspring during post-natal life. An important avenue for future research is to identify the specific components of the maternal diet (sugar, fat, protein) responsible for the alterations within the mesolimbic reward system during offspring development. It is clear from past studies that fat, glucose, leptin and endogenous opioids can cross the placenta and are transferred through breast milk to the offspring (320-323), and are therefore very likely to affect the development of the mesolimbic reward system in the offspring. For example, the absence of leptin during development has been associated with impaired neuronal projections within the appetite neural network (158). High leptin levels have also been shown to disrupt the development of the hypothalamic appetite system, possibly due to leptin resistance (41). Therefore, determination of specific factors influencing the development of the mesolimbic reward system would not only improve our understanding of the

underlying mechanisms but also allow the identification of specific targets for interventions to improve offspring metabolic health outcomes.

The identification of the role of various hormones in modulating the development of the mesolimbic reward system can be achieved through several approaches. The use of the leptin deficient ob/ob mice and pharmacological manipulations such as leptin supplementation during the suckling period would determine the role of leptin during development of the reward systems and on subsequent food choices post-weaning. Similar studies could also be conducted in rodents with the administration of peptides including opioids and insulin.

Although studies in this thesis have developed a rodent model for junk food addiction, maternal junk food feeding, and identified the phenotypes, metabolic parameters, food preferences and changes within the reward system of the offspring of junk food fed dams, there are various aspects of these studies that could be improved. First of all, the use of β-actin was not validated across treatments and ages. Although previous studies have indicated that β-actin expression is not altered with maternal high-fat feeding, whether β-actin expression remains stable with age or with different regions of the brain, is still unclear. Therefore, future studies should validate the use of β-actin as an appropriate housekeeper gene across post-natal ages and include a second housekeeper gene to ensure reliable data interpretation.

Studies in this thesis focussed on the mRNA expression of key genes within the mesolimbic reward system. Another strategy to gain a more in-depth understanding of the present data would be to determine the expression of key proteins using western blots, particularly TH in the NAc. TH mRNA is more abundant in the VTA where it undergoes translation within the cell bodies of the dopaminergic neurons before being transported to the NAc for dopamine synthesis. Measuring TH protein levels at the level of the NAc would therefore provide a better representation of dopamine synthesis. Besides TH, measuring the expression of protein from other key genes within the mesolimbic reward system is also crucial due to potential post-translational modification of these genes, especially the μ-opioid receptor. In addition to protein level measurements, determining dopamine levels/content within the tissue will also allow dopamine quantification to support the mRNA (and protein) data. Another approach to strengthen the current data is to perform ligand binding studies with dopamine and opioids receptors. This will allow

confirmation of receptor availability within the mesolimbic reward system. Pharmacological studies such as central administration of dopamine and opioid agonists and antagonists could also be conducted to investigate the function of the dopamine and opioid receptors, whether or not they were altered in at the level of mRNA or protein.

This thesis has demonstrated a potential mechanism through which maternal junk food diet can program offspring fat preference throughout life and that these pre- and early post-natal dietary manipulations can result in permanent alterations within the reward system. These studies showed that changes within the reward system cannot be reversed by a short term nutritional intervention post-weaning, and that even longer term interventions may not entirely reverse the heightened susceptibility of these offspring to diet-induced obesity, particularly in the females. Studies from this thesis have provided novel and important insights into the mechanisms underlying the early life origins of food preferences and also a basis for future work to further understand the underlying mechanisms of the programming of food preferences. Whilst studies in this thesis were conducted in rodents and have made the majority of references to animal models, it is hoped that these findings can be translated to humans and ultimately develop possible strategies for intervention to counteract the continuing rise in the incidence of obesity in the human population.

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APPENDIX

AUTHORSHIP STATEMENTS FOR PUBLISHED PAPER
(CHAPTER 3 OF THE THESIS)

Maternal "junk-food" feeding of rat dams alters food choices and development of the mesolimbic reward pathway in the offspring.

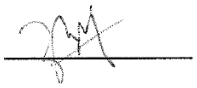
Ong ZY and Muhlhausler BS

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Author Contributions

These experiments were performed in BM's laboratory. BM and ZO conceptualised and designed the experiments. ZO carried out the experiments. BM and ZO analysed and interpreted the data and prepared the first draft of the manuscript. All authors have read and approved the final version of this paper.

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