Maternal cafeteria diet consumption and the programming of food preferences in the offspring: the role of the mu-opioid receptor

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ABSTRACT

Numerous studies in rodent models have shown that the offspring of dams fed a high-fat high-sugar (cafeteria) diet throughout pregnancy and lactation develop a specific preference for the same kinds of foods in adulthood. Furthermore, studies into potential mechanisms have revealed that the offspring of cafeteria diet fed dams also have altered expression of key components of the mesolimbic reward pathway including the mu-opioid receptor. The current work used a rodent model to look specifically at the role of the mu-opioid receptor in the programming of food preferences and investigated when during development exposure to a maternal cafeteria-style diet could be most harmful.

The first aim of this thesis was to isolate whether exposure to a cafeteria diet before birth or in the pre-weaning period had a greater effect on the adult food preferences of the offspring. Using a cross-fostering method, we demonstrated that the male offspring of control or cafeteria diet fed (JF) dams that were cross-fostered at birth onto JF dams exhibited higher fat intake when challenged with a cafeteria diet at 7 weeks of age than offspring exposed to the cafeteria diet only before birth or not at all. Building on this work, we then investigated the effect of maternal cafeteria diet exposure on the postnatal development of the mu-opioid receptor. Using an in situ hybridisation method, we showed that female offspring of JF dams had reduced expression of the mu-opioid receptor in the ventral tegmental area in late postnatal development (week 3,4) relative to controls but not at the earlier timepoints explored (birth, week 1). The outcomes of the first two chapters of this thesis highlight the importance of the postnatal period in the establishment of offspring food preferences.

The final experiment, which forms the final two chapters of the thesis, used an opioid receptor antagonist to examine in greater detail the potential of the mu-opioid receptor as a mechanism for the programming of food preferences. We demonstrated that whilst the administration of the opioid receptor antagonist naloxone in the fourth week of life significantly reduced fat intake in control offspring given access to a cafeteria diet immediately postweaning, it failed to do so in male JF offspring and was less effective at reducing fat intake in JF females. This outcome provides evidence that changes in mu-opioid receptor expression induced by early life exposure to a cafeteria diet may indeed have functional consequences for the regulation of palatable food by the offspring. We also hypothesised that opioid receptor blockade during the fourth week of life would have long term effects on the food preferences of offspring; this however was not observed in the present study.

Nevertheless, this thesis provides considerable evidence to suggest that alterations in the development of the mu-opioid receptor plays an important role in the programming of food preference in offspring exposed to cafeteria diet in early life. In addition, it also identifies the postnatal period as potentially being 'critical window' during which exposure to cafeteria diet is most harmful to the offspring.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ABBREVIATIONS

ad libitum	to any desired extent
AgRP	agouti-related protein
AMPH	amphetamine
ANOVA	analysis of variance
ARC	arcuate nucleus
BMI	body mass index
BDNF	brain-derived neurotrophic factor
bp	basepairs
CART	cocaine amphetamine related transcript
Cdk5	cyclin-dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoV	coefficient of variance
CRF	corticotrophin releasing factor
D1	dopamine 1 receptor
D2	dopamine 2 receptor
DA	dopamine
DARPP-32	dopamine- and cAMP-regulated neuronal phosphoprotein-32
DAT	dopamine active transporter
E	embryonic day
ELISA	enzyme linked immunosorbent assay
ENK	enkephalin
GABA	gamma-aminobutyric acid
IRS-1	insulin receptor substrate-1
JF	junk food
L-DOPA	L-dihydroxyphenylalanine
LH	lateral hypothalamus
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron

NAc	nucleus accumbens
NEFA	non-esterified fatty acid
NPY	neuropeptide Y
ob/ob	obese
PBN	parabrachial nucleus
PENK	proenkephalin
PFC	prefrontal cortex
PI3K	phosphoinositide 3-kinase
PND	postnatal day
POMC	pro-opiomelanocortin
PPARγ	peroxisome proliferator activated receptor γ
PVN	paraventricular nucleus
PW	postnatal week
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
TH	tyrosine hydroxylase
VTA	ventral tegmental area
WHO	World Health Organisation

PUBLICATIONS ARISING FROM THIS THESIS

1. **Gugusheff, JR**., Ong, ZY., & Muhlhausler, BS. (2014). The early origins of food preferences: targeting the critical windows of development. *The FASEB Journal* In Press

2. **Gugusheff, JR**., Ong, ZY., & Muhlhausler, BS. (2014). Naloxone treatment alters gene expression in the mesolimbic reward system in 'junk food'exposed offspring in a sex-specific manner but does not affect food preferences in adulthood. *Physiology & Behaviour 133* 14-21.

3. **Gugusheff, JR**., Vithayathil, M., Ong, ZY., & Muhlhausler, B. S. (2013). The effects of prenatal exposure to a 'junk food'diet on offspring food preferences and fat deposition can be mitigated by improved nutrition during lactation. *Journal of Developmental Origins of Health and Disease*, *4*(05), 348-357.

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

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6. Muhlhausler, BS., **Gugusheff, JR**., Ong, ZY., & Vithayathil, M. A. (2013). Pregnancy, obesity and insulin resistance: maternal overnutrition and the target windows of fetal development. *Hormone Molecular Biology and Clinical Investigation*, *15*(1), 25-36.

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Chapter 1

General Introduction

Chapter 1: GENERAL INTRODUCTION

1.1 Obesity

Obesity continues to be a growing public health challenge. The prevalence of obesity (as defined by a body mass index (BMI) over 30kg/m^2) has doubled amongst adults in both the United States and Australia between 1980 and 2000 (1,2). Furthermore the World Health Organisation reports that over 300 million people globally are currently obese (3). The number of overweight adults (BMI>25kg/m^2) has also increased, reaching a figure of more than 1 billion worldwide (3). This increase in obesity prevalence has been attributed to a range of causes. Amid these is the increased availability of high-fat high-sugar palatable foods (junk foods) which, together with decreases in levels of physical activity, has led to the creation of an 'obesogenic environment' (4-6). The consequences of obesity are well known and include elevated risks of developing type 2 diabetes and heart disease (7-9). The rise in the occurrence of these disorders has put increased pressure on the healthcare system, with the estimated total cost of obesity in Australia rising from 3.8 billion dollars in 2003 to 8.3 billion dollars in 2008 (10).

The increase in obesity prevalence amongst the general population has coincided with a rise in the number of women entering pregnancy either overweight or obese. A large population-based study in the United States involving over 66,000 mothers across 9 states reported a near 70% increase in the rates of pre-pregnancy obesity between 1993 and 2003 (11). More recently, work by Dodd and colleagues looking at South Australian pregnancies in 2008 stated that almost 50% of women who gave birth in that year were either overweight or obese at their first antenatal visit (12). This increase in obesity prevalence during pregnancy is of particular concern given the association between increasing maternal body weight and many adverse health outcomes, including increased the risk of gestational diabetes and hypertension as well as pre-term and still birth (13-15).

Along with rises in prevalence of maternal obesity, there has also been an increase in the number of infants born large for gestational age (16) as well as an increase in the number of children who are overweight. The World Health Organization estimates that more than 40 million children under 5 are currently overweight or obese (3). The concurrent increases in both maternal and early childhood obesity have led to a number of clinical studies which have demonstrated a clear association between maternal obesity and obesity in the infant/child. A retrospective cohort study involving 8400 participants conducted by Whitaker *et al*, showed

that the children of obese mothers were twice as likely to be obese by age two, than those born to mothers in a healthy weight range (17). Furthermore, a longitudinal cohort study conducted by Boney *et al* suggested that the children of obese mothers who also had gestational diabetes where at a 3.6 times greater risk of being obese at age 11 if they were born large for gestational age (18). Importantly, a study conducted on a northern Finland birth cohort in which data from the mother during pregnancy as well as measurements from the child at birth, at 1, 14 and 31 years of age was collected, identified maternal obesity immediately prior to pregnancy as well as obesity during adolescence as significant predictors of obesity of in adulthood (19). These associations, together with evidence from numerous studies in animal models (20-22) form the basis of the "Barker Hypothesis' or 'Early Origins Hypothesis' which states that maternal obesity and nutritional status can predispose or 'program' the child toward increased fat deposition or food consumption in adulthood (23).

1.1.1 *Maternal overnutrition and offspring obesity*

The exposure of the fetus to an increased nutrient supply as a result of excessive maternal calorie intake during pregnancy has been explored in many animal models as a potential mechanism through which maternal obesity could program obesity in the offspring. Muhlhausler and colleagues demonstrated that lambs of overnourished ewes (fed at 155% of their required energy intake in the last trimester of pregnancy) had higher plasma glucose and higher subcutaneous fat mass at 4 weeks of age (24). This was supported by the work of the Nathanielsz laboratory, also in a sheep model, which showed that exposure to maternal overnutrition *in utero* and during the suckling period resulted in offspring with an increased percentage body fat mass, increased plasma glucose and decreased insulin sensitivity as adults (25). The results of the studies conducted in sheep have also been replicated in the rodent model, with the offspring of dams fed a high- fat diet during pregnancy being heavier at weaning and having an increased fat mass at 90 days compared to offspring of dams fed a control diet (26). In addition, to the increases in fat mass the offspring of dams fed a high-fat diet during pregnancy and lactation have increased concentrations of plasma glucose and leptin at weaning (27) and adipocyte hypertrophy and hypertension by 6 months of age (21). This evidence from animal models provides additional support for the 'Early Origins Hypothesis' of obesity and does indeed suggest that exposure to an increased nutrient supply before birth and whilst suckling increases the offspring's susceptibility to developing diet induced obesity in adulthood.

1.1.2 *Maternal overnutrition and offspring appetite regulation*

The effects of maternal overnutrition appear not to be limited to an increase in fat deposition, with exposure to increased nutrients before birth and through the breastmilk also shown to alter appetite regulating mechanisms and feeding behaviour in the offspring. In rodents, pre-weaning overnutrition induced by reducing litter size shortly after birth results in an increased proportion of total neurons expressing the orexigenic neuropeptide Y (NPY) in the hypothalamic arcuate nucleus of the offspring at 21 days of age (28). In support of this, numerous other studies have also demonstrated alterations in the expression of key appetite regulating neuropeptides, both at weaning and in adulthood, in offspring exposed to increased nutrition across the perinatal period (26,27,29,30). Furthermore, increased maternal calorie intake has also been shown to result in offspring hyperphagia post-weaning (21,22,31). Importantly, similar work has also been performed in sheep, a model in which the timing of the development of these systems is more comparable to humans. A study conducted by Muhlhausler *et al* showed that the offspring of overnourished ewes consume more milk during the first 3 weeks of life and fail to up-regulate the expression of the appetite inhibiting neuropeptide cocaine amphetamine related transcript (CART) in response to increased fat mass (24). The research conducted to date thus provides strong evidence to suggest that the structure and function of the central appetite regulatory system of the fetus and neonate can be altered by maternal overnutrition and that this has long term effects on offspring food intake.

1.1.3 Maternal high-fat diet and offspring food preferences

Given the data from the studies which have demonstrated that perinatal exposure to an increased nutrient supply is associated with altered food intake in the offspring, subsequent investigations have looked to elucidate if there a specific types of foods which exacerbate these effects. In rodents, feeding dams a high-fat, high-sugar diet during across the perinatal period results in altered development of the central appetite regulating neurons in the offspring which is associated with ongoing hyperphagia and increased body weight and fat mass throughout life (26,32,33). Notably, the hyperphagia in the offspring exposed to a high-fat high-sugar diet before weaning is greatly exaggerated when the animals are provided with a similar diet after weaning (26,34). These observations suggest that early life exposure to a high-fat high-sugar diet has the capacity to not only alter the amount of food the offspring consumes in later life but also to increase the propensity of the offspring to overindulge in particular types of foods. Bayol and colleagues directly investigated the link between maternal diet during the perinatal period and offspring food preferences in a rodent model.

The results of this study showed that maternal consumption of high-fat high-sugar junk foods, including biscuits, muffins and jam doughnuts during pregnancy and lactation increased the preference for these same foods in adult offspring (35). In support of this, Teegarden *et al* reported that early life exposure to a diet high in fat, increased the preference for fat in adulthood, but similar exposure to a diet high in carbohydrates had no effect on later food preferences, suggesting this effect may be specific to high-fat foods (36). Furthermore, previous work from our laboratory has shown that a exposure to a cafeteria diet before birth and through the breastmilk have increased fat intake from weaning until 3 months of age when provided with a cafeteria diet throughout this time (37).

There are limited clinical studies looking at the effect of maternal diet during pregnancy and whilst breastfeeding on later food preferences, due to the difficulty of obtaining reliable food intake data and the confounding effects of social influences on feeding behaviour. Nevertheless, a study in 5717 mother-child pairs and 3009 father-child pairs, demonstrated a strong correlation between maternal fat intake during pregnancy and the child's preference for fat at 10 years of age (38). Importantly, the child's food preferences were not related to paternal diet at any time. In support of this, a smaller study involving 428 children from the United Kingdom showed that the children of obese parents have a higher preference for junk food and lower preference for vegetables than those born to lean parents (39). Despite the paucity of studies conducted to date, the available data does appear consistent with the results from animal models, reinforcing the apparent importance of maternal diet for the child's later food preferences.

When considering the effects of a maternal 'junk food' diet on offspring food preferences, it becomes important to define what types of foods are encompassed by the term 'junk food'. The label of 'junk food' can be applied to any food which is high in fat, sugar and/or salt, energy dense, nutrient poor, as well as highly palatable (40). In animal models, 'junk food' diets are often referred to as 'cafeteria diets'. The 'cafeteria diet' that includes a range of human junk foods such as chocolate biscuits, salted snacks and sweetened cereals (41) has long been used in rodents to model the effects overconsumption of junk food may have in humans. Studies which utilise 'junk food' or 'cafeteria diets' over diets simply high in fat (42,43), focus primarily on the ability of these highly palatable foods to act as natural rewards, by activating central reward processing pathways. It is this increased palatability created by combining fat and sugar as well as including a variety of different foods, which

make a cafeteria diet model advantageous to using a diet which is simply high in one macronutrient i.e. fat or sugar (41).

1.1.4 *Summary*

The vast impact of rising obesity rates on the health of the general population is undisputed. What remains unclear however, is the extent to which maternal diet and weight status during pregnancy and whilst breastfeeding facilitates this continuing cycle of obesity. Evidence from animal and clinical studies suggests that both maternal obesity and maternal overnutrition during pregnancy and lactation can program increased fat deposition, glucose intolerance and increased appetite in juvenile and adult offspring. Furthermore, the ability of a high-fat high-sugar maternal diet to program a specific preference for these foods in the offspring, rather than just general hyperphagia suggests that a maternal diet may be capable of altering not only the appetite regulating systems of the offspring but also the areas of the brain which process reward.

1.2 Mesolimbic reward circuitry

The motivation to eat palatable foods has a biological basis that extends beyond the need to satisfy hunger. This is because these foods, in addition to activating appetite regulating mechanisms are also capable of triggering the central neural circuits involved in the regulation of reward (the mesolimbic reward system) in a manner analogous to alcohol and drugs of abuse (44-46). Two of the most important brain areas involved in mediating the response to rewarding stimuli are the nucleus accumbens (NAc) in the forebrain and the ventral tegmental area (VTA) in the midbrain. These brain areas, together with key neurotransmitters which include dopamine (DA), opioid peptides and gamma-amino butyric acid (GABA), all form part of the mesolimbic dopamine pathway. Activation of this pathway by the consumption of palatable foods (or drugs of abuse) results in the release of endogenous opioids that bind to opioid receptors in the VTA and ultimately decreases GABAergic inhibition of dopamine synthesis. These dopaminergic neurons project to the NAc where the dopamine is released (47) (Fig.1.1). It is increases in extracellular dopamine in the NAc that are thought to translate into the acute pleasurable sensation associated with the consumption of rewarding stimuli. The VTA and NAc are not the only brain areas involved in the regulation of reward, indeed numerous other brain regions have been demonstrated to play an important role in reward processing including the prefrontal cortex (PFC) (48), parabrachial nucleus (PBN) (49) and the lateral hypothalamus (LH) (50). However, for the purposes of this review, the focus shall

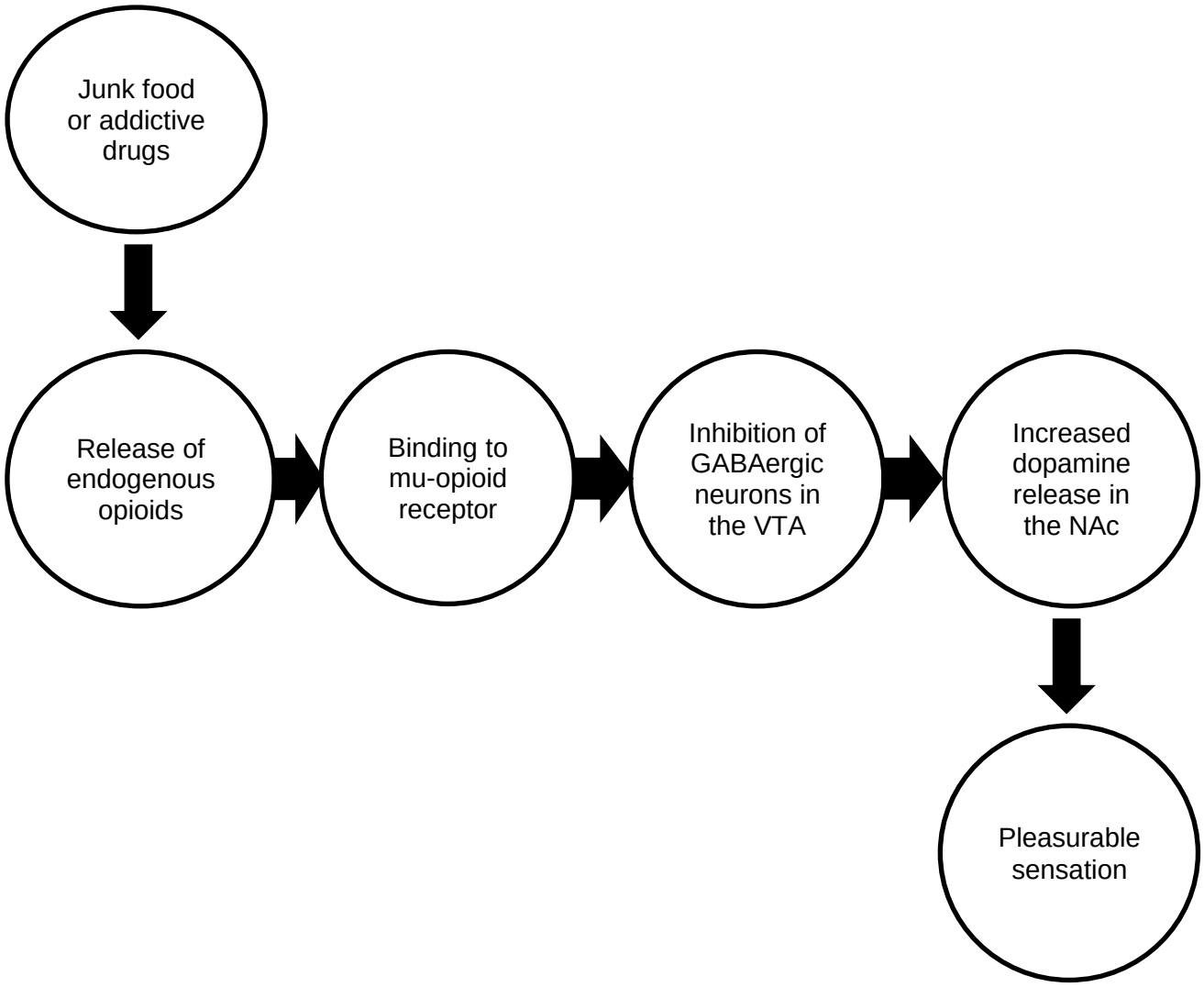
be on opioid and dopamine actions in the VTA and NAc, as their involvement in reward processing have been the best characterised to date.

1.2.1 *Endogenous opioids*

Endogenous opioids have long been associated with the mediation of reward behaviours in the brain. Initial studies linking opioids to reward were a result of investigations into the cause of morphine (an exogenous opioid) addiction, with the understanding that the addiction was caused by the desire to experience the hedonic effects of the drug (51-53). These early studies also demonstrated that morphine injection (at particular dosages), stimulated hunger for palatable food and therefore showed a relationship between the intake of specific foods and reward regulation. Subsequent studies have revealed that endogenous opioids are released in response to a variety of rewarding stimuli including palatable food, alcohol and nicotine and act via the same mechanisms as morphine to elicit the pleasurable effects of the stimulus (54-56)

Both endogenous and exogenous opioids mediate their effects by binding to specific opioid receptors of which there are 3 subtypes, mu, delta and kappa (57-59). Studies administering opioid antagonists specific to each receptor subtype into rats have been performed in order to determine the separate function of each subtype. These studies have shown that whilst activation of both the mu- and kappa-opioid receptors stimulates palatable food consumption, mu-opioid receptor activation is significantly more efficacious (60,61). Further research into mu-opioid receptor induced increases in feeding behaviour has revealed that specific activation of the mu-opioid receptor leads to increased preference for high-fat foods over high-carbohydrate or high-protein foods in addition to the general increase in food intake (62). This together with a study that demonstrated that high-fat feeding leads to decreased expression of the mu-opioid receptor in the VTA, NAc and PFC (63) suggests a possible role for this receptor in mediating the preference for high-fat foods.

Figure 1.1



Simplified representation of the mesolimbic reward pathway**:** Schematic showing the components of the mesolimbic dopamine pathway including the involvement of endogenous opioids. Abbreviations: GABA, gamma amino butyric acid; NAc, nucleus accumbens; VTA, ventral tegmental area.

1.2.2 *Dopamine*

Dopamine (DA) is the most well characterised neurotransmitter involved in reward-associated behaviours. Increased DA release in the shell of the NAc has been observed in response to both addictive drugs and palatable food (64,65). Martel and Fautino demonstrated using microdialysis that during food consumption extracellular DA levels in the NAc increase and that this increase was greater for highly palatable foods compared to foods of lower palatability (66). This DA release in the NAc forms a key part of the mesolimbic dopamine pathway, which involves dopamine projections from the VTA to the PFC and NAc and is a key regulatory pathway involved in the response to reward stimuli (47,62,67-71). Dopamine synthesis occurs within these neurons and begins with the uptake of the amino acid tyrosine into the DA neuron. The tyrosine is then converted into L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH); this is considered to be the rate limiting step in dopamine synthesis (72). L-DOPA is then rapidly converted via decarboxylation to DA by the enzyme aromatic amino acid decarboxylase (AADC) (73). Termination of dopamine signalling occurs through active reuptake of DA through high affinity membrane carriers, known as dopamine active transporters (DAT), which are expressed on DA neurons throughout the striatum (74).

Five DA receptor subtypes have been isolated by examination of ligand binding sites; of these the D1 and D2 receptors are most important in the mesolimbic DA circuitry. In adults, D1 receptors are expressed at higher levels than D2 receptors throughout the brain with the exception of the VTA (75). In the NAc, where both D1 and D2 receptors are abundantly expressed, D1 receptors are thought to be stimulatory, whilst D2 receptors are considered to have a role in inhibitory autoreception, and act as part of a negative feedback system to inhibit dopamine release (76). These proposed differential roles for D1 and D2 receptors in the NAc were demonstrated in a study by Ragnauth and colleagues, which looked at opioid mediated feeding in rats; in this work the D1 receptor antagonist SCH 23390 consistently inhibited feeding, whilst a D2 receptor antagonist was only sporadically effective (77). This suggests that only the D1 receptor is critical in mediating opioid receptor induced feeding.

1.2.3 *Mesolimbic reward pathway and drug addiction*

The effects of nearly all drugs of abuse are mediated in part by activation of the mesolimbic reward pathway. Psychostimulants such as cocaine induce feelings of euphoria, increased alertness and attention focusing by inhibiting the actions of DAT in the NAc, DAT would

normally facilitate dopamine reuptake and thus psychostimulants act to increase levels of extracellular dopamine (78,79). Other more widely used drugs of abuse including alcohol and nicotine, also mediate their hedonic effect by increasing dopamine levels in the NAc however the exact mechanisms of this action are thought to be slightly different. Ethanol is proposed to generate increases in extracellular dopamine in the NAc by increasing the firing rate of the dopaminergic neurons from the VTA (80,81) whereas increases in dopamine in response to nicotine consumption are mediated by nicotine receptor binding on the dopaminergic neurons that project from the VTA to the NAc (82,83). Overconsumption of these substances leads to a reduction in their hedonic value and ultimately to addiction.

Addiction to substances of abuse occurs as the dopamine release in response to the drug stimuli reduces over time, secondary to a decrease in the sensitivity of the dopamine system following repeated exposure to the stimulus, meaning addicted persons have to escalate their intake to get the same hedonic sensations (84,85). As a result, studies in animal models have investigated the transition in reward pathway neuron function after repeated administration of drugs of abuse. An important outcome of this work is the identification of ΔFosB, a protein which is stimulated by D1-receptor binding and modulates the synthesis of the neurotransmitter glutamate, as a significant mediator of the transition to addiction (86,87). Furthermore, rodent models have also begun to identify the reward pathway changes which are responsible for the feelings of withdrawal when the drug stimulus is removed including changes in the expression of brain-derived neurotrophic factor (BDNF) (88,89). The animal evidence highlighting the importance of reduced sensitivity of the mesolimbic dopamine system and subsequent changes in downstream mediators (ΔFosB) in mediating addiction is supported by a small clinical study conducted by Volkow and colleagues. The results of this study showed a reduction in D2 receptor expression in subjects with a history of alcohol abuse compared to those who were mild or moderate drinkers (90). The molecular adaptions that occur in response to drugs of abuse have been reviewed in greater detail elsewhere (91,92). Understanding the mechanisms behind drug addiction may give an insight into the molecular changes that could be induced by overconsumption of palatable food, which is a central focus of the thesis.

1.2.4 *Mesolimbic reward pathway and palatable food intake*

The consumption of highly palatable foods is also thought to involve activation of the mesolimbic reward pathway. Microinjections of exogenous opioids like morphine, (which act through the same opioid receptors as endogenous opioids) into either the NAc or VTA have been demonstrated to enhance the ingestion of foods which are rich in fat and sugar (62,93), whilst injections of opioid receptor antagonists, specifically those which block the mu-opioid receptor have been shown to have the opposite effect (94-96). Studies which have observed increases in the intake of artificial sweeteners in response to opioid agonist injection (97,98) indicate that it is the palatability or 'sweet taste' of the food rather than the energy content, which is likely to be more important for reward pathway activation. Clinically, administration of the opioid receptor antagonist naloxone has been shown to reduce the intake of high-fat/high-sugar snacks including cookies and chocolate bars but not affect the intake of less palatable snacks, such as pretzels (99).

Further evidence to support that intake of palatable foods is regulated by the same brain pathways as drugs of abuse comes from rodent models investigating the effects of chronic exposure and/or withdrawal to a cafeteria diet or sugar. A recent study demonstrated that chronic consumption of a cafeteria diet by adult male rats for 15 weeks resulted in reduced mRNA expression of the mu-opioid receptor in the VTA (100), in line with the desensitisation of the reward pathway observed after chronic drug use. Furthermore, a study conducted by Colantuoni and colleagues, showed that male rodents given daily access to 25% glucose solution had symptoms consistent with withdrawal from drugs of abuse such as teeth chattering, tremors and head shakes when they were administered with the opioid receptor antagonist naloxone or when the sugar solution was removed (101). These studies form part of a growing body of literature suggesting that the reward pathway, in addition to normal appetite regulating mechanisms, plays a critical role in modulating the intake of highly palatable food.

1.2.5 *Summary*

The opioid and dopamine systems in the mesolimbic region of the brain are critical for the processing of rewarding stimuli. Activation of these brain areas results in the hedonic feelings associated with the consumption of both drugs of abuse and palatable foods, whilst it is a reduction in the sensitivity of these systems (due to chronic overstimulation) that can lead to addiction. The role of both opioid and dopamine systems in reward processing in the adult is

supported by a large body of literature, however it is less clear how these system develop in early life and whether exposing the fetus and neonate to high levels of rewarding stimuli could effect this development.

1.3 Reward pathway development

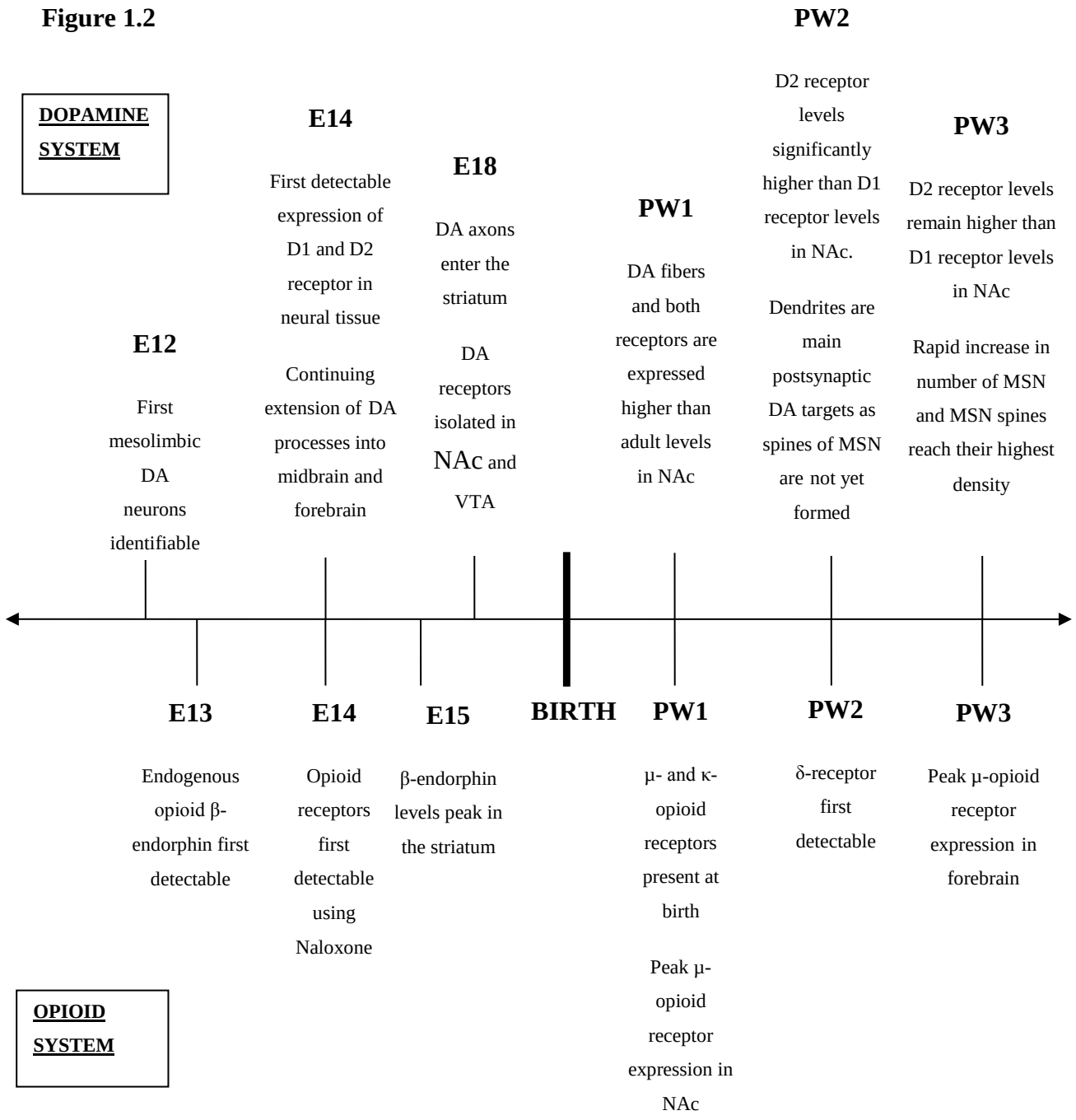
During early life development, the reward pathway is thought be highly plastic and susceptible to alteration by external influences including maternal diet and drug intake. In the rodent model, the development of the mesolimbic reward pathway including the opioid and dopamine systems begins before birth but is not complete to until the fourth week of postnatal life (Fig.1.2). Much less is known about the early life ontogeny of the reward pathway in humans however, as in the rodent model, early life is likely to represent a time of increased reward pathway plasticity.

1.3.1 Opioids-animal models

Components of the opioid system can be detected early in embryonic development in rodents, but opioid signalling is not fully mature until well after birth. The endogenous opioids, including proopiomelanocortin products, proenkephalin products and prodynorphin products, are all present at low levels before birth in the rat brain and increase in concentration with age, generally reaching peak concentrations by the fourth postnatal week (102,103). In line with the increase in the abundance of opioid peptides, opioid receptor binding increases with age to reach adult levels at a similar time.

An elegant study by Spain and colleagues, using specific radiolabelled ligands for each opioid receptor subtype (mu, kappa, delta), demonstrated that the different receptor subtypes each have distinct profiles of expression in the brain during development. The mu-opioid receptor, which is the main receptor subtype involved in the activation of the reward system following the intake of palatable foods, showed an initial decrease in binding during the first four days after birth, followed by a steady increase, reaching adult levels at postnatal day 21 (104). Whilst the kappa-opioid receptor was also present at birth, it exhibited a much slower increase in binding, with only a 2 fold increase by the fourth postnatal week. Unlike the other two opioid receptors, the delta-opioid receptor was not detected at birth. The expression of this receptor could not be detected until in the second week of postnatal life and then rapidly increased in receptor expression reaching adult levels in the fourth week of life. This study is, however, limited by the fact that it did not look at specific brain regions (104).

Figure 1.2



Ontogeny of dopamine and opioid systems**:** Timeline depicting the development of the dopamine (top) and opioid (bottom) systems within the mesolimbic reward system throughout prenatal and first 3 weeks of postnatal life in the rodent. By postnatal week 3, dopamine and opioid systems are similar to that of an adult. Abbreviations: DA, dopamine; E, embryonic day; NAc, nucleus accumbens; MSN; medium spiny neuron; PW, postnatal week; VTA, ventral tegmental area. See text for references. Adapted from (105).

Interestingly, a related study looking at mu-opioid receptor ontogeny specifically in the NAc showed a peak in binding during the first four days after birth, followed by a steady decrease (106) which, is in complete opposition to the forebrain findings presented by Spain and colleagues. *In situ* hybridisation analysis which has expanded on the receptor binding studies and have shown a steady increase in mu-opioid receptor expression from its first detection in the NAc on embryonic day 13 until a peak in expression during the first week of life (107-109), followed by a decrease in mu-opioid receptor expression to adult levels by the third to fourth postnatal week (104,109,110). These studies demonstrate that the development of the mu-opioid receptor is brain region specific and ongoing throughout the postnatal period in the rodent model.

1.3.2 Opioid-human

There are few studies that have investigated the ontogeny of the opioid system in humans, since these studies rely largely on tissues from medical terminations and are difficult to obtain. Nevertheless, the available evidence suggest that opioid system development begins very early in life and is thought to have a role in regulating fetal growth (111). The endogenous opioids, beta-endorphin and enkephalin have both been detected in the fetal striatum by 12 weeks gestation (112). Interestingly, the development of opioid receptors appears to occur well after the detection of endogenous opioids, with specific binding to the mu- and kappa-opioid receptors not observed prior to 20 weeks gestation (113).There clearly remains a need for further clinical studies to better examine the development of this pathway in humans.

1.3.3Dopamine-animal models

The development of the dopamine system in the rat begins during embryonic life but is not complete until approximately the third postnatal week, with the system thought to be highly plastic throughout this time (114). Mesolimbic dopamine neurons have been detected in the rat brain as early as embryonic day 12 and by embryonic day 17 and 18, large dopamine axon bundles have begun to enter the striatum, which includes the NAc, to form a complex network of fibres (115,116). At birth, these dopamine fibres are present at a higher density than in adult rodents, but return to adult levels by the third week of life (114). During this time (postnatal week 3), the number of medium spiny neurons, which are the main neurons found in the NAc, rapidly increases and spine density peaks (117), allowing more synaptic

connections to be made. By the end of the third week, dopamine neuron organisation is much like that observed in the adult rat (118) .

Dopamine receptors are also present before birth in the rat, and development of the dopamine receptor system continues into early neonatal life. Using *in situ* hybridization, Schambra and colleagues showed that dopamine receptor D1 and, at lower levels, the dopamine receptor D2 were both expressed in neural tissues of the fetal rat as early as gestational day 14 (119). From day 7 to day 21 of postnatal life, D2 is expressed at significantly higher levels than the D1 in the NAc and the level of expression of the two receptors equalises during the fourth week after birth (118). The timing of D1 receptor development is thought to be driven predominantly by dopamine levels, whilst dopamine seems to play only a limited role in the development of D2 (118).

1.3.4 Dopamine-human

As with the opioid system, only limited studies have investigated the development of the dopamine system in humans due to the difficulty in obtaining tissue. It has been established, however, that the development of the dopamine pathway in the fetus begins as early as 6-8 weeks gestation (111). By 12 weeks gestation and the fetal striatum exhibits immunoreactivity for TH (the rate limiting enzyme in dopamine synthesis) and D1 dopamine receptor mRNA is expressed at detectable levels (112). At 21 weeks gestation, the D2 dopamine receptor is also detected in the striatum (120), with peak expression being reached 1 month after birth and remaining higher than adult levels until 9-10 years of age (121). The dopamine active transporter (DAT) has been isolated in the striatum at 32 weeks gestation (121), raising the possibility that dopamine signalling may already be functional at this stage of development.

1.3.5 Summary

In the rodent model, the development of opioid and dopamine systems begins as early as gestational day 14 but is not complete to until the 4th week after birth. The evidence from the limited clinical studies available suggests that the majority of reward pathway development in humans occurs before birth. In both rodents and humans, endogenous opioid and dopamine peptides can be detected before their receptors, suggesting receptor development may be peptide meditated. Despite differences in the timing of development, rodent models can still provide valuable information about the ontogeny of both the dopamine and opioid pathways.

The third chapter of this thesis will compare the early and late postnatal development of mu-opioid receptor in a rodent model as part of a larger study investigating the effect of a maternal cafeteria diet on the ontogeny of this receptor.

1.4 The effect of cafeteria diet exposure on reward pathway development and food preferences

Given the role of the reward pathway in regulating palatable food intake; it has been suggested that it is alterations to the development of this pathway, which may be the underlie the preference for palatable food in offspring exposed to a 'junk food' diet in the perinatal period. Indeed several studies have shown that a number of key components of the reward pathway are altered in adolescent and adult offspring of mothers fed a cafeteria/high-fat diet during pregnancy and lactation. Vucetic and colleagues demonstrated that exposure to a high-fat diet before birth and during the suckling period is associated with increased expression of the mu-opioid receptor and DAT and decreased expression of the D1 and D2 dopamine receptor in the NAc at 6 months of age (122). In addition, work by Ong et al, again in a rodent model, has shown that a maternal cafeteria diet across the perinatal period increases the expression of the mu-opioid receptor in the NAc of the offspring at 6 weeks after birth, however in this model, expression of the mu-opioid receptor was decreased compared to controls by 3 months after birth (37). This difference in expression of the mu-opioid receptor at 6 weeks and 3 months demonstrates that the effects of a maternal high-fat diet on the reward pathway of the offspring may not be set at birth, but that reward pathway development could also be susceptible to nutritional/environmental influences in early postnatal life. Furthermore, a very recent study the Reyes laboratory used an embryo transfer experimental design, to demonstrate that exposure to high-diet at conception was also capable of altering the expression of the mu-opioid receptor and the endogenous opioid proenkephalin in adulthood, suggesting the sensitivity of the reward pathway to alteration by maternal diet may begin even earlier than previously thought (123).

Despite the apparent need to identify specifically when during maturation the reward pathway is most vulnerable, there are currently limited studies that have differentiated the effects of exposure to cafeteria or high-fat diets in the prenatal and pre-weaning periods on adult food preferences and fewer still looking at the effects of the immediate post-weaning diet. However, the results from the studies to date do suggest that the timing of palatable diet

exposure plays a critical role in shaping the reward pathway development and food preferences of the adult offspring.

1.4.1 *Before birth*

There are relatively few studies that have attempted to separate the effects of exposure to maternal palatable diets before birth from those of palatable diet exposure in the early neonatal or suckling period. In one elegant study, Chang and colleagues used a cross-fostering approach to evaluate the relative impact of exposure to a high-fat diet before birth and during the suckling period to later reward function and food preference (32). They demonstrated that offspring who had been exposed to a high-fat diet *in utero* exhibited increased body fat mass, an increased body weight as well as an increased preference for dietary fat, independent of whether they were suckled by a dam consuming a control or high-fat diet. Furthermore, they also demonstrated that exposure to a high-fat diet before birth resulted in significant increases in the proliferation of neuronal cells expressing the peptides galanin, enkephalin and dynorphin, all of which are involved in regulating appetite drive (32). Although not the focus of their study, they also reported that these effects were still observed in offspring cross-fostered onto control dams at birth. These results led the authors to conclude that exposure to a high-fat diet before birth was more important than exposure during the suckling for the programming food preferences, the altering of reward function in the offspring as well as for predisposing the offspring to diet-induced obesity (32). However, this finding is not in agreement with similar studies, the results of which have suggested that palatable diet exposure during lactation may be more harmful to the offspring long term than exposure before birth (35,124,125).

1.4.2 *Pre-weaning*

The importance of the suckling period in determining food preferences in adulthood has been investigated in a small number of studies, which either limit maternal cafeteria/high-fat diet access to the lactation period (and not pregnancy) or utilise cross-fostering protocols. Gorski *et al* demonstrated that exposure to a high-fat diet across the lactation period increased the offspring's appetite, specifically for high-energy foods, later in life. In this study, the offspring of obesity resistant dams that were cross-fostered to obesity prone dams fed on a high-energy diet at birth, increased their intake of the high-energy diet in adolescence, in a manner equivalent to those exposed to the high energy diet across the entire perinatal period (124). The outcome of this work suggests that nutritional interventions applied during the

suckling period can reverse the effects of maternal high-fat diet exposure during pregnancy, however there is clearly a need for further studies to support this finding, especially given the evidence that cross-fostering in and of itself can impact on the metabolic profile, subsequent growth and behaviour of the offspring (126).

The apparent importance of the suckling period in determining later feeding behaviour has also been demonstrated studies where rather than cross-fostering, maternal cafeteria diet access was simply limited to either pregnancy or lactation. Bayol and colleagues showed that the offspring of dams who consumed a cafeteria diet during pregnancy but were switched to a control diet at birth had lower body weight and consumed less food when given free access to the cafeteria diet post-weaning than the offspring exposed to the cafeteria diet both *in utero* and during the suckling period (35). Similarly, Wright and colleagues showed that offspring exposed to the cafeteria diet whilst suckling, but not before birth had an increased number of feeding bouts and spent more time feeding when provided with a cafeteria diet in adulthood (125). These results suggest that the suckling period, rather than the prenatal period, is critical for the programming of food preferences. To date however, no studies have directly compared the long term effects of prenatal cafeteria diet exposure to either the effects of exposure during only the early postnatal period or exposure to a maternal cafeteria across the entire perinatal period within the same experiment. A cross-fostering study that enables direct comparisons of the effects of cafeteria diet exposure during different windows of development forms the second chapter of this thesis.

1.4.3 *Immediately post-weaning*

In the rodent model, offspring are capable of consuming solid foods and are no longer dependent on their mother for nutrition by 3 weeks of age (127). However, the development of the reward pathway persists into the fourth postnatal week (102,118), suggesting that dietary treatments applied in the immediate post-weaning period may also shape the development of this pathway and, thus, subsequent food preferences. Teegarden and colleagues have demonstrated that mice offspring exposed to a high-fat diet during only the fourth week of life (22-28 days of age) exhibited an increased preference for this same high-fat diet as adults compared to the rats fed a control diet during this time (36). Importantly, this increased preference for fat was associated with increases in striatal expression of Cdk5 and phosphor-DARPP-32, which are negative regulators of dopamine transmission. The authors proposed that this downregulation of dopamine signalling was the cause of an increased

preference for fat, in an attempt to normalise dopaminergic tone (36). This finding that exposure to a highly palatable diet only during the fourth week of life can program adult food preferences has been replicated in a study which provided access to sugary cereal from postnatal days 22-27 and again showed an increase preference for this food in adulthood (128). While the results from these studies do suggest that the immediate post-weaning period may be important in establishing life-long food preferences, further studies are required to confirm this and to determine the underlying mechanism.

1.4.4 *Summary*

It is clear that maternal palatable diet consumption during pregnancy and lactation is not only capable of programming food preferences in the offspring but also of altering reward pathway development. What remains to be determined is when during development this exposure is most harmful. The evidence from studies in rodent models conducted thus far have highlighted the potential of the suckling period to be the 'critical window' during which maternal diet has the greatest impact on the food choices of the offspring. However, continued investigations, including direct comparisons of the effect of cafeteria diet exposure during different windows of development within the same study, are needed to better understand when during development the reward pathway of the infant is most susceptible to alteration by external influences.

1.5 Mechanisms for the programming of food preferences

Given the evidence to suggest that maternal over-consumption of a palatable diet can alter the development of the reward pathway and program food preferences in the offspring, there has been emerging interest in determining the exact mechanistic causes behind these effects. One potential mechanism is that exposure to increased levels endogenous opioids (as would be released in response to excessive maternal palatable food intake) may alter the reward pathway of the offspring. Alteration of reward pathway development has been observed as a result early life exogenous opioids like morphine (129,130), but whether endogenous opioids can have similar effects is yet to be explored and will be an important area of future investigation. In addition, hormones such as insulin and leptin have also been implicated as potential mediators of these programming effects as they have both been demonstrated to have a neurotrophic role in the development of the hypothalamic appetite regulating system (131,132). Furthermore, there is evidence from clinical studies to suggest that the preference

towards certain foods can also be established by the transmission of flavours into the amniotic fluid and breastmilk (133-135).

1.5.1 Endogenous opioids

Previous studies have shown that exposure to morphine (an exogenous opioid) *in utero* can alter the reward circuitry of fetus and offspring, raising the possibility that increases in endogenous opioids could have similar effects (136,137). Prenatal and early neonatal exposure to morphine have been demonstrated to result in a reduction in mu-opioid receptor expression in early life (129,138) and higher mu-opioid receptor expression in adult life (139,140). However, as the effects of endogenous and exogenous opioids are not always directly comparable (141,142), it remains to be determined whether these effects can be replicated through increased exposure to endogenous opioids. Increases in endogenous opioids, particularly met-enkephalin, have been observed in the NAc of rats consuming a high-fat diet (143) and since opioids readily cross the placenta (144), increases in maternal met-enkephalin would be expected to result in increased concentrations of met-enkephalin in the fetal circulation. Indeed, offspring exposed to a high-fat diet across the perinatal period have been shown to have increased expression of met-enkephalin in reward processing areas of the brain 15 days after birth (32).

The hypothesis that increases in neonatal enkephalin may program changes in reward circuitry development are supported by studies which have examined the reward associated behaviours of adult rodents administered met-enkephalin in early life. Kastin and colleagues demonstrated that adult rats administered met-enkephalin in the first week of life had improved maze performance for a food reward when compared to control animals (145). Similarly, administration of met-enkephalin together with exposure to a specific odour stimulus *in utero* was associated with increased preference for the same odour at 16 days of age (146). Whilst these results suggest that exposure to increased circulating endogenous opioids in early life may potentially alter the developing reward circuitries of the offspring, further work is required to look specifically at the role of endogenous opioids in programming a preference for junk food in offspring exposed to a highly palatable diet early in life. Direct investigations into role of the endogenous opioid system, specifically the mu-opioid receptor, in the programming of food preferences form the basis of the fourth and fifth chapters of this thesis.

1.5.2 Leptin

The most important role of the adipocyte-derived hormone leptin in the fetus and neonate is thought to be in neural development, rather than in appetite or reward regulation as is seen in adults (for reviews see:(56,147-150). Chronic and acute leptin administration into rat pups during the first 9-10 days after birth has no effect on food intake (151). The timing of this apparent insensitivity to the actions of leptin coincides with a distinct surge in circulating leptin levels, which occurs between 7 and 10 days in rodents and is independent of food intake or fat mass (152). Interestingly, the magnitude and duration of the leptin surge in the rat has been shown to be increased in the offspring of high-fat fed dams, which may contribute to the hyperphagia observed in these offspring later in life (26). The role of the neonatal leptin surge is not well understood, one hypothesis is that it functions as a developmental signal for the central appetite regulatory system. Bouret and colleagues showed that leptin plays a neurotrophic role in the development of appetite regulating neurons in the arcuate nucleus (ARC) of the hypothalamus. In this study, leptin deficient mice (Lep^{ob} / Lep^{ob}) had disrupted neuronal pathways in the ARC, which could not be reversed by leptin administration in adulthood. However, leptin administration during the neonatal period (postnatal days 4 to 12) completely normalised ARC neuron development in these leptin deficient animals (131). This, together with the results of *in vitro* studies showing the leptin promotes neurite growth in ARC neurons (131) suggests that the leptin surge may be critical in shaping the appetite regulatory network in the ARC. There are similarities between leptin's regulatory function in the appetite circuitries where in inhibits the release of orexigenic neuropeptides (153,154) and its function in the reward pathway where it inhibits dopamine release (155,156). Given this, it is possible that leptin has a similar developmental role in the reward pathway as it does in the appetite-regulating pathway, although there is currently no direct experimental evidence to support this.

1.5.3 Insulin

Insulin, like leptin, plays an important role in the maturation of neurons within the central nervous system (132,157). Importantly, exposure to maternal overnutrition has been shown to result in increased plasma insulin concentrations in the fetus and neonate (158-160), which may have consequences for brain development. Studies investigating the effect of perinatal hyperinsulinaemia on the hypothalamic appetite regulatory network have shown that exposure to elevated insulin levels during critical windows of development can impair organisation of

the appetite regulatory pathway and that these developmental changes are associated with hyperphagia and the development obesity later in life (161,162). The connection between insulin and the ontogeny of the mesolimbic reward system is, however, less well defined. Nevertheless, in adult rodents, insulin receptors are expressed on dopaminergic neurons in the VTA (163), suggesting that insulin may modulate dopamine activity within the mesolimbic reward pathway. Indeed, insulin has been shown to increase expression and activity of the dopamine active transporter (DAT) (164), whilst hypoinsulinaemia induced by fasting or drugs lowers the rate of dopamine clearance in the striatum by impairing insulin signalling and decreasing DAT function (165-168). Given the role of insulin in neuronal maturation and organisation of the hypothalamic pathways it is therefore possible that perinatal hyperinsulinaemia could alter the development of the mesolimbic reward system in the offspring and result in an increased preference for palatable foods later in life.

1.5.4 Transmission of flavour

The focus of the studies reviewed thus far has been on the potential of endogenous opioid or hormones like insulin and leptin to alter the reward pathway and program food preferences. There is also evidence however that the preferences for certain foods may also be established through the direct transfer of specific flavours from the mother to the fetus/infant via the amniotic fluid or breastmilk. Studies in animal models have demonstrated that offspring actively seek out and prefer familiar flavours, i.e. the flavours they were exposed to either *in utero* or whilst suckling (169,170). Importantly, these findings have been replicated in an elegant series of clinical studies conducted by Menella and colleagues that have shown that exposure to certain flavours (eg garlic, carrots) *in utero* (135) and in the breast milk (135,171,172) resulted in an increased preference towards these specific foods by the child later in life. Furthermore, studies which have compared the flavour preferences of children given milk-based or soy-based formulas as infants, have shown that children fed on soy-based formulas have a greater preference for bitter - and sour- flavoured juices at 4-5 years of age, than those who were fed milk-based formula which lacks the bitter/sour flavours of soy, again suggesting that the preference for certain flavours can be programmed in early life (133). Similar to the reward pathway the systems able to process taste stimuli begin to develop well before birth (For review, see (173)) and are thought to be particularly sensitive to alteration during this time. It therefore appears that in addition to the preference for palatable food being established through alterations to the reward pathway, the preference towards specific

flavours can also be programmed early in life through exposure to certain foods via the amniotic fluid and breast milk.

1.5.5 Summary

Our current understanding of the biological mechanisms that underlie the programming of food preferences is limited; however the studies conducted to date do highlight a critical role for mesolimbic reward system. Whether any changes to mesolimbic reward pathway of offspring are driven by endogenous opioids (which will be investigated as part of this thesis) or maternal hormones like leptin and insulin still requires further study. In addition, the importance of early life flavour exposure in programming food preferences can also not be discounted. There is also the potential that other mechanisms not discussed here, such as epigenetic regulation (For review see: (174)) may also be involved.

1.6 Sex differences

Recently, there has been an emerging view within the literature that sex differences must be considered when interpreting experimental outcomes. However, numerous studies particularly in the field of developmental programming have looked exclusively at male offspring and have then generalised the findings to both sexes. Such an approach is inappropriate in light of evidence suggesting that in many cases males and females respond differently to the same treatment. The importance of sex differences in relation to the regulation of the reward pathway has also been highlighted in research that investigates both drug addiction and fat consumption, however in the majority of studies looking at the effect of maternal diet on the development of the reward pathway in the offspring, the effect of sex has not yet been considered.

1.6.1 Sex differences in drug addiction

Current information from epidemiological studies suggests that there are clear differences in the prevalence and manifestation of addictive behaviours between men and women. In men, the rate of drug addiction is higher than it is in women (175), but women are quicker to escalate drug consumption and find it more difficult to abstain (176). The mechanisms behind these sex- specific behaviours have begun to be explored in rodent models. In such models, female rats tend to self-administer more of the presented drug (For review see:(177)) than their male counterparts and also exhibit a more robust response to stimulants such as cocaine (178,179). Interestingly, the differences in drug taking behaviour appear to be estrogen

mediated, which was highlighted in an elegant study by Lynch and colleagues. These researchers showed that when given a choice between two doses of cocaine, female rats in estrous had a far greater preference for the higher dose than females who were not in estrous and males (180). This finding is also supported by studies looking at ovariectomised females, which have reported that the increased sensitisation to psychostimulant stimulant drugs usually observed in females is ameliorated after ovariectomy (181,182) and can be reinstated by administration of estrogen (183,184). Whilst evidence from these studies indicates a crucial role of estrogen in mediating the sex differences observed in drug addiction, how it facilitates its effects is less clear. The available data suggests that estrogen may have a regulatory effect on the expression of dopamine receptors in the reward pathway (185,186), however more studies are required to confirm this.

Given the wide range of available drugs of abuse and the varying responses of individuals to them, it is important to acknowledge that further studies are required to both identify sex differences in the effects of specific drugs and to determine the underlying mechanisms. Identifying and understanding the mechanisms behind sex differences in drug addiction will ultimately assist in designing improved treatments for males and females and can also provide insights into potential sex differences in response to natural rewards, which forms the primary subject of this thesis.

1.6.2 Sex differences in fat consumption

Similar to what is observed for cases of drug addiction, clear differences between men and women have been reported in the prevalence of obesity and response to high-fat food consumption. Females, whilst more likely to choose a healthy diet than males (187), are also more susceptible to diet-induced obesity and find it more difficult to lose weight (188,189). Understanding sex differences in food choices in a human population is complicated by factors such as social pressures on physical appearance. However, studies in animal model, which remove the confounding social factors present in human studies, have shown clear differences in macronutrient selection, with males were more likely to consume fat and protein and females having a greater preference for carbohydrates (190). The differences in a preference for fat observed between males and females in a rodent model, suggest there sex differences in food choices may be biologically and not just socially driven. Although no study has directly investigated the potential biological mechanisms, feeding behaviour in

females does change across the estrous cycle (191,192), highlighting a possible role of estrogen.

Rodent models have also been used to attempt to identify the mechanisms behind why females appear to have greater weight gain in response to a high-fat diet than males. Medrikova and colleagues demonstrated that when female and male mice were fed on a high-fat diet for 35 weeks post-weaning, females had greater fat accumulation which was associated with larger adipocytes in both gonadal and subcutaneous fat depots (193). A similar study conducted in rats showed that the increased fat deposition in females was also associated with increases in the gene expression of lipoprotein lipase (LPL) and CD36, which both facilitate increased energy influx into adipose tissue (194). Despite the increases in fat deposition in the females, the authors of this study also reported that females had less inflammation (as determined by macrophage infiltration of the adipose tissue) and better insulin sensitivity than males (194). Thus the results of this experiment suggest that, whilst females may be more prone to diet-induced obesity, it is males who are more like to develop metabolic problems such as glucose intolerance. One explanation for the increased susceptibility to inflammation and insulin resistance in males compared to females may be the differences in fat distribution; males are more prone to visceral fat deposition which has been linked adverse metabolic outcomes (195,196) whereas females are more likely to deposit fat subcutaneously (197,198). Nevertheless, it is clear that further investigation is required to understand the specific mechanisms. The evidence presented here highlights that there are significant differences in the both the intake and response to a high-fat diet between males and females, which will need to be considered in any future study looking at appetite, food preference or obesity.

1.6.3 Sex differences in the programming of obesity and food preferences

Despite the evidence from adult rodents showing that the response to fat consumption is different between the sexes, few studies have considered the effects of maternal obesity/ overnutrition on male and female offspring separately. Bayol and colleagues showed that the female offspring of dam's fed a cafeteria diet were more susceptible to increased adiposity than their male counterparts. In addition, the female offspring in this study also demonstrated a more pronounced increase in the expression lipogenic and adipokine genes including peroxisome proliferator activated receptor gamma (PPARgamma), lipoprotein lipase (LPL), adiponectin, leptin and adipsin (199). In line with these results Geary *et al*, demonstrated that

female offspring were more vulnerable to the programming effects of maternal obesity/overnutrition showing that female but not male offspring of lard-fed dams were more likely to be hypertensive as adults than the offspring of control dams (200). In contrary to the evidence suggesting that female offspring are more sensitive to the negative effects of maternal overnutrition/obesity, a study by Samuelsson *et al* demonstrated that male but not female offspring of dams fed a high-fat diet developed glucose intolerance as adults (21). Taken together, these studies show that the programming effects of maternal obesity/overnutrition are sex specific, but also highlight the need for further investigation into the mechanisms behind these differences.

Like studies investigating the programming of obesity in the offspring, the majority of studies conducted focusing on the critical windows of reward pathway development and the programming of food preferences have only considered male offspring or have failed to separate results by sex (32,35,122). Male offspring are often exclusively in used these studies to avoid any possible complications in the interpretation of results introduced by the hormonal fluctuations as part of the estrous cycle in females (201). However, there is emerging evidence to suggest that male and female offspring respond differently to early life nutritional insults, making the extrapolation of male results in these studies to females inaccurate. A previous study by our group, for example, demonstrated that female but not male offspring of junk food fed dams up-regulating the expression of dopamine receptor 1 and 2, TH and DAT, in response to a junk food challenge in adolescence (202). These results suggest that future studies investigating the effects of maternal diet on the food preferences need to consider each sex separately.

1.6.4 Summary

The evidence presented here highlights that the traditional approach of using only males and extrapolating these findings to females is likely to produce inaccurate results or lead to inaccurate conclusions. There are clear sex differences in the both regulation of fat intake and addictive drug, which also extend to differences in the response of offspring to maternal overnutrition/high-fat diet consumption. The differences between sexes appear to be primarily driven by estrogen at least in relation to drugs of abuse but further investigation is required to better understand specific mechanisms. Future studies, including the present work, must consider the sexes separately to ensure that appropriate conclusions are drawn for both males and females

1.7 Research focus

The impact of increased obesity prevalence on the health of the wider community makes designing interventions to prevent a continued rise in obesity rates critical. Maternal overnutrition and obesity during pregnancy are key predictors of obesity in the child, suggesting that poor maternal diet may contribute to the formation of an intergenerational cycle of weight gain and inappropriate food choices. Indeed, both maternal overnutrition and obesity have been shown in animal and clinical studies to program increased fat deposition, glucose intolerance and increased appetite in the offspring (20-22). The ability of maternal diet during pregnancy to predispose the offspring to weight gain appears to extend beyond the programming of simple hyperphagia, with a maternal high-fat high-sugar diet shown to specifically increase the preference for these types of food in the offspring (35,37). Explorations into the mechanisms behind this programming of food preferences have centred on the mesolimbic reward pathway, given the importance of this brain region in regulating the intake of palatable food in adults. Thus the current thesis aims to first, identify whether exposure to a highly palatable cafeteria diet before birth or in the postnatal period has the greatest effect on the food preferences of the offspring and second, to explore the potential of the mu-opioid receptor (a key component of mesolimbic reward pathway) as a possible mechanism. The investigations into the mu-opioid receptor will explore any effects of maternal cafeteria diet exposure on the postnatal ontogeny of this receptor as well as identify any functional consequences of administering an opioid receptor antagonist in early life on later food preferences.

The hypothesis that adult food preferences can be programmed by exposure to a cafeteria diet before birth and during the suckling period has been well tested in the literature (35-37). It is less clear, however, whether it is exposure to a cafeteria diet *in utero* or whilst suckling that has the greatest effect on the offspring. There are currently limited studies that have attempted to separate the effects of prenatal and postnatal exposure to a cafeteria diet on the establishment of food preferences. One study conducted by Bayol and colleagues, showed that offspring that were exposed to a cafeteria diet before birth and then a standard chow diet during lactation did not have an increased preference for fat and sugar as adults, suggesting the lactation period may have a particularly important role in the programming of food preferences (35). However, there are at present no studies that have directly compared the prolonged effects of exposure to a cafeteria diet limited to either the prenatal or the suckling period from those of exposure during the entire perinatal period within the same experiment.

Therefore the **second chapter of thesis** will use a cross-fostering paradigm, where the offspring born to cafeteria diet fed dams are fostered at birth to control dams and vice versa, to investigate the effects of prenatal vs postnatal exposure to a cafeteria diet on adult food preferences and other metabolic outcomes.

In addition to an increased preference for palatable food in adulthood, the offspring of cafeteria diet fed dams have also been demonstrated to have altered expression of key components of the mesolimbic reward pathway, including the mu-opioid receptor. In one such study, Vucetic *et al* demonstrated that exposure to high-fat diet before birth and during the suckling period increased expression of the mu-opioid receptor at 6 months of age (122), whereas a study in our laboratory has shown that the offspring of dams fed a cafeteria diet across the perinatal period have increased expression of the mu-opioid receptor at 6 weeks of age but decreased expression of this receptor compared to controls at 3 months of age (203). In both of these studies, offspring exposed to the high-fat cafeteria diet in early life had a greater preference for these foods as adults. The outcomes of this research suggest that alterations to expression of the mu-opioid receptor in the reward pathway may be a critical mechanism behind the programming of food preferences. However, no studies to date have looked specifically at how early life exposure to a high-fat/cafeteria diet affects the development of this receptor. Thus, the **third chapter of this thesis** will compare the early (birth and week 1) and late (week 3 and 4) postnatal development of the mu-opioid receptor between the offspring of control and cafeteria diet fed dams.

The **fourth and fifth chapters of this thesis** will build on the work of the first two chapters by examining in greater detail the potential of the mu-opioid receptor as a mechanism behind the programming of food preferences as well as further exploring the importance of the timing of cafeteria diet exposure on reward pathway development. While previous studies have identified changes to mu-opioid receptor mRNA expression in the adult offspring of dams fed a cafeteria diet during pregnancy and lactation (37,122) and the third chapter of this thesis investigates mu-opioid expression in early life, it remains unclear whether or not these changes in mRNA expression have functional consequences for the subsequent regulation of food intake in the offspring. Therefore, the aim of **the fourth chapter of this thesis** was to determine whether early life exposure to a cafeteria diet impacted the effectiveness of the opioid receptor blocker naloxone on reducing the intake of palatable food. The **fifth chapter** explores whether blocking endogenous opioids during the fourth week of life (using

naloxone) has any prolonged effect on food preferences in the adult offspring and gene expression of key components of the reward pathway in the fifth week of life.

The overall purpose of this thesis is to provide greater insight into the role of the mu-opioid receptor in the programming of food preferences as well as to identify when during development exposure to cafeteria diet is most harmful. Gaining a better understanding of both the mechanisms behind the programming of food preferences and when these brain pathways are most susceptible to alteration, will ultimately allow the design of targeted interventions to prevent the spread of junk food preference and obesity from generation to generation.

Chapter 2

The effects of prenatal exposure to a ‘junk food’diet on offspring food preferences and fat deposition can be mitigated by improved nutrition during lactation

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Jessica Gugusheff (candidate): Performed all statistical analysis and interpreted data, wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: Date: 9 September 2014

Mini Vithayathil: Collected animal feeding behaviour and fat mass data

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 9 September 2014

Zhi Yi Ong: Established feeding behaviour protocol used in the work

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 July 2014

Beverly Muhlhausler: Assisted in conceptualisation of the work and data interpretation. Contributed to manuscript construction and evaluation.

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 September 2014

Chapter 2: THE EFFECTS OF PRENATAL EXPOSURE TO A 'JUNK FOOD' DIET ON OFFSPRING FOOD PREFERENCES AND FAT DEPOSITION CAN BE MITIGATED BY IMPROVED NUTRITION DURING LACTATION

2.1 Abstract

Exposure to a maternal 'junk food' diet *in utero* and during the suckling period has been demonstrated to increase the preference for palatable food and increase the susceptibility to diet induced obesity in adult offspring. We aimed to determine whether the effects of prenatal exposure to junk food could be ameliorated by cross-fostering offspring onto dams consuming a standard rodent chow during the suckling period. We report here that when all offspring were given free access to the junk food diet for 7 weeks from 10 weeks of age, male offspring of control (C) or junk food (JF) dams that were cross-fostered at birth onto JF dams (C-JF, JF-JF), exhibited higher fat (C-C 12.3±0.34g/kg/d, C-JF 14.7±1.04g/kg/d, JF-C 11.5±0.41g/kg/d, JF-JF 14.0±0.44g/kg/d, $P<0.05$) and overall energy intake (C-C 930.1±18.56kJ/kg/d, C-JF 1029.0±82.9kJ/kg/d, JF-C 878.3±19.5kJ/kg/d, JF-JF 1003.4±25.97kJ/kg/d, $P<0.05$) than offspring exposed to the junk food diet only before birth (JF-C) or not at all (C-C). Female offspring suckled by JF dams, despite no differences in food intake, had increased fat mass as percentage of body weight (C-C 19.9±1.33%, C-JF 22.8±1.57%, JF-C 17.4±1.03%, JF-JF 22.0±1.0%, $P<0.05$) after 3 weeks on the junk food diet. No difference in fat mass was observed in male offspring. These findings suggest that the effects of prenatal exposure to a junk food diet on food preferences in females and susceptibility to diet-induced obesity in males can be prevented by improved nutrition during the suckling period.

2.2 Introduction

The worldwide incidence of obesity has doubled since 1980 (3) and this epidemic has now spread to include women of reproductive age, with greater than 50 percent of women entering pregnancy either overweight or obese (12,204). Whilst the causes of this rise in obesity prevalence are multi-factorial, the ready availability of 'junk foods' is an important contributing factor (205). The term 'junk food' can be applied to a range of foods which are

high in fat, sugar or salt, nutrient poor, as well as highly palatable (40). The consumption of these types of foods during pregnancy and lactation has been shown in animal models to have long term consequences for the food preferences of the offspring. We and others have shown that the offspring of mothers fed a cafeteria diet (a well-established model of junk food feeding in the rodent (41)) during the perinatal period have an increased preference for palatable foods as adults and also exhibit a greater susceptibility to diet-induced obesity when compared to the offspring of mothers fed a standard diet during the same time frame (35,37).

The detrimental effects of early life exposure to a cafeteria diet on the offspring have led to a search for interventions to ameliorate these effects (35,124). There are currently limited studies which have attempted to separate the effects of prenatal and postnatal exposure to high-fat and high-sugar diets on the early life origins of food preferences. However, the results from these studies have provided evidence that nutritional exposures experienced *in utero* are likely to have distinct effects on the long term outcomes in the offspring from those experienced during the early postnatal period. In one such study, providing dams who consumed a cafeteria diet during pregnancy with a standard chow diet during lactation blunted the increased preference for fat and sugar in their adult offspring(35). It has also been demonstrated that providing dams with the cafeteria diet only during lactation resulted in an increased preference for the palatable diet in the adult offspring (36,125). Exposure to a cafeteria diet during lactation has also been associated with increased perirenal fat mass in adult offspring (206), highlighting the importance of this period not only in establishing the regulation of food preferences but also in the programming of increased adiposity.

Despite evidence suggesting that the lactation period has a particularly important role in the programming of future metabolic outcomes, nutritional manipulations during pregnancy alone have also been demonstrated to result in offspring hyperphagia later in life (32,33). There are currently no studies which have directly compared, within the same experiment, the long term effects of exposure to a cafeteria diet exclusively during the prenatal or early postnatal period from those of exposure during the entire perinatal period. A cross-fostering paradigm, in which offspring are switched at birth from a dam consuming a cafeteria diet to a dam consuming a control diet, or vice versa, is the only way to adequately separate the effects of exposure to a cafeteria diet during lactation from the effects of exposure during pregnancy and avoid the carry-over effects on maternal physiology that may exist when a dam consuming a cafeteria diet during pregnancy is switched onto standard rodent feed after the

birth of her pups (207). The ability to clearly delineate the long term effects of junk food exposure in either the pre or postnatal period, and establishing to what extent prenatal exposures can be ameliorated by altering postnatal nutrition, will be critical for determining the optimal timing for intervention.

Therefore, the aim of the current study was to compare the effects of exposure to a cafeteria 'junk food' diet *in utero* or during the suckling period on food preferences and susceptibility to diet-induced obesity in the offspring. Specifically, we aimed to investigate the hypothesis that cross-fostering the offspring of mothers fed a cafeteria diet during pregnancy onto mothers fed a standard diet could prevent the establishment of an increased preference for junk food and decrease the susceptibility to diet induced obesity in the offspring.

2.3 Methods

2.3.1 Animals and feeding regime

This study was approved by the Adelaide University Animal Ethics Committee. 26 female (200-250g) and 4 male (200-300g) Albino Wistar rats were used in this experiment. The animals were individually housed and allowed to acclimatise to the animal housing facility for at least 1 week before initiation of experimental procedure. During this time rats were fed *ad libitum* on standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia) with free access to water. After the acclimatisation period, the female rats were assigned to weight matched groups, designated as either control (control, n=14) or junk food (JF, n=12). Control rats were given free access to standard laboratory rodent feed while JF rats were fed a cafeteria diet comprising of peanut butter, hazelnut spread, chocolate biscuits, savoury snacks, sweetened cereal and a lard and chow mix. Detailed nutritional composition of this cafeteria diet has been published previously (203). Food intake was recorded every 2 days, by subtracting the amount that remained in the cage from the amount initially provided. All rats were individually housed under a 12 hour /12 hour light-dark cycle at a room temperature of 25°C throughout the experiment.

After 4 to 6 weeks on their respective diets, vaginal smears were conducted daily to determine the stage of the estrous cycle. On the evening of diestrous/proestrous, 2 female rats were placed with a male rat for 24 hours. Vaginal smears were performed the following morning. The presence of sperm was used as confirmation of successful mating and designated as

gestation day 0. Female rats were maintained on the same diet as before mating throughout pregnancy and lactation and were weighed once per week throughout the experimental period.

2.3.2 Cross-fostering

Pups were born at day 21-22 of gestation. Within 24 hours of birth, all litters were culled to 8 pups, with 4 males and 4 females where possible. Pups were then cross-fostered to another dam which had given birth within the same 24 hour period from either the same or different dietary treatment group. This resulted in 4 groups of offspring: offspring from a control dam cross-fostered onto another control dam (C-C), offspring from a control dam cross-fostered onto a JF dam (C-JF), offspring from a JF dam cross-fostered onto control dam (JF-C) and offspring from a JF dam cross-fostered onto another JF dam (JF-JF).

Pups remained with their foster mothers until weaning (postnatal day (PND) 21). After weaning, the pups were group housed with same-sex littermates and fed with standard laboratory rat feed until 10 weeks of age (Fig. 2.1). Pups were weighed every second day until weaning and once per week thereafter until the end of the experiment.

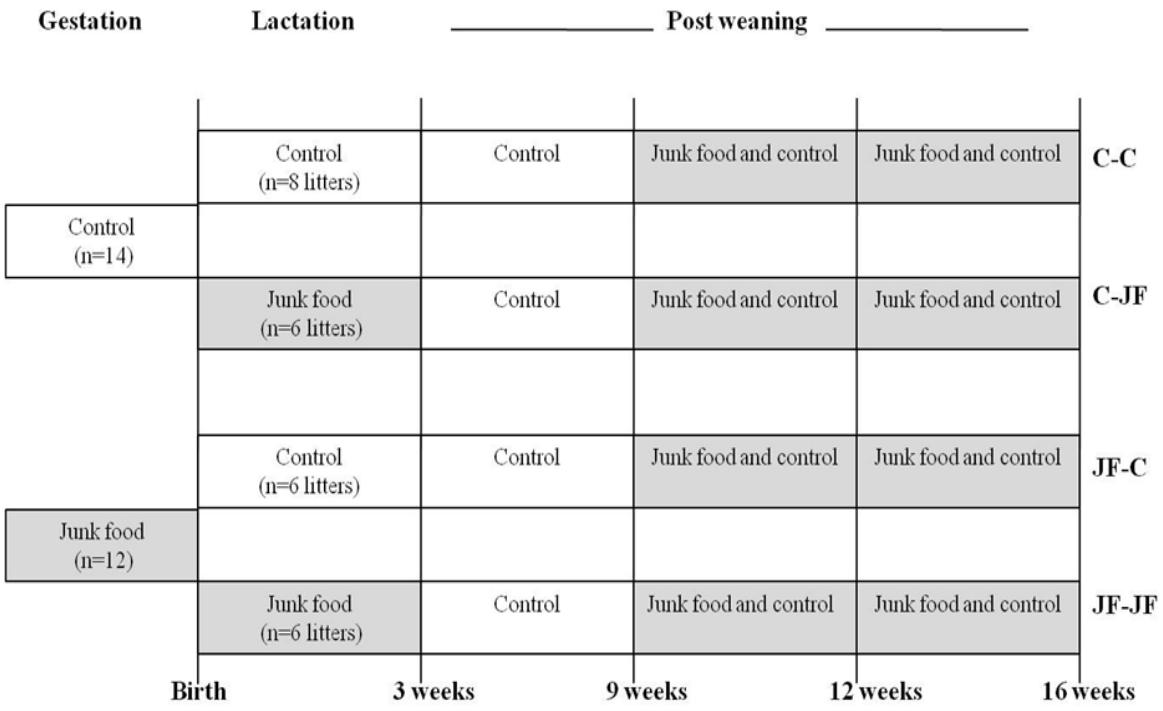
2.3.3 Determination of food preferences

After all offspring had been consuming the control diet for 6 weeks post weaning, up to 2 males and 2 females per litter were randomly selected to study food preferences and susceptibility to diet induced obesity. These offspring were separated from the other offspring, housed with a same sex litter mate and given free access to both the standard chow and cafeteria diet from 10 to 16 weeks (4 months) of age. Food intake was measured every 2 days by subtracting the amount left uneaten in the cage from the amount initially provided. The total intake of each food type was recorded and macronutrient preferences for each cage determined based on the nutritional composition of the foods consumed. The amount of food consumed was normalised to mean body weight. Food intake was divided by the number of offspring in the cage and normalised to the average of their weights.

2.3.4 Post-mortem and tissue collection

At 12 and 16 weeks of age, one male and one female pup from each litter were killed for the determination of body fat mass. The rats were not fasted prior to postmortem and all postmortems were conducted in light phase between 8 and 10 AM. All animals were weighed immediately prior to being killed with an overdose of CO_2. Blood samples were collected by

Figure 2.1



Experimental design: Offspring of control (n=14 litters) and JF dams (n=12 litters) were cross-fostered within 24 hrs of birth to a dam receiving either the same or different diet as their natural mother. Offspring were kept with their foster mother until weaning (PND 21), and then placed on the control diet until 10 weeks of age. From 10-16 weeks of age offspring were given access to both the control and junk food diet for the determination of food preferences**.**

cardiac puncture, and blood was centrifuged at 3,500g, 4˚C for 15 minutes and plasma stored at -20˚C for subsequent analysis of hormone and metabolite concentrations. Individual fat depots including retroperitoneal fat, omental fat, gonadal fat, interscapular fat and subcutaneous fat were isolated and their respective weights recorded. All fat depots were snap frozen in liquid nitrogen and stored at -80°C for future molecular analyses.

2.3.5 Determination of hormone and metabolite concentrations

Plasma concentrations of glucose and non-esterified fatty acids (NEFA) were determined using the Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) and the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan), respectively. Assays were conducted using Konelab 20 (Thermo Scientific, Vantaa, Finland). Plasma insulin and leptin concentrations were measured by immunoassay using the ALPCO Insulin (Rat) Ultrasensitive ELISA kit (ALPCO diagnostics, Salem, NH, USA) and the Crystal Chem Rat Leptin ELISA kit (Crystal Chem INC, Downers Grove, IL, USA). All assays were conducted according to manufacturer's instructions and intra- and inter-assay coefficients of variation were <10%.

2.3.6 Statistical analysis

Comparison of maternal food intake and birth outcomes in the control and JF groups was performed using Student's unpaired *t*-tests. The effect of maternal diet and sex on offspring food intake, body fat mass, plasma insulin, glucose, leptin and NEFA was analysed using three-way ANOVA, with sex, prenatal and postnatal maternal diet as factors. Where there were significant differences between males and females, the data by sex and analysed by two-way ANOVA (prenatal and postnatal maternal diet as factors). Three-way ANOVA and Student's unpaired *t*-tests were conducted using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Offspring body weight gain over time was analysed by two-way repeated measures ANOVA using Stata 11 software (StataCorp.,TX, USA). The litter (mother) was used as the unit of analysis for all statistical tests. All data are presented as mean±SEM with a *P* value of <0.05 deemed statistically significant.

2.4 Results

2.4.1 Body weight and macronutrient intake of dams during pregnancy and lactation

JF dams were heavier than control dams at mating (control 292.1±7.6g, JF 343.4±9.4g, $P<0.01$) and remained heavier until the end of lactation (control 348.4±6.7g, JF 397.3±10.3g, $P<0.01$).

During pregnancy, JF dams consumed significantly more fat (control 3.2±0.2g/kg/d, JF 15.3±0.7g/kg/d, $P<0.01$) than controls, but had lower intakes of protein (control 13.5±0.8g/kg/d, JF 6.6±0.2g/kg/d, $P<0.01$) and carbohydrate (control 41.4±2.4g/kg/d, JF 29.6±1.5g/kg/d, $P<0.01$). Average daily energy intake during pregnancy was not different between groups. During lactation, the higher fat intake (control 6.8±0.4g/kg/d, JF 26.4±1.4g/kg/d, $P<0.01$) the reduced protein (control 28.8±1.6g/kg/d, JF 12.4±0.6g/kg/d, $P<0.01$) and the reduced carbohydrate intake (control 88.8±4.8g/kg/d, JF 49.9±1.8g/kg/d, $P<0.01$) observed in JF dams during pregnancy were maintained. In addition, JF dams also consumed significantly less total energy during the lactation period compared to control dams (control 2643.8±142.6kJ/g/d, JF 2001.6±82.1kJ/g/d, $P<0.01$).

2.4.2 Effect of cross-fostering on birth outcomes and pup growth

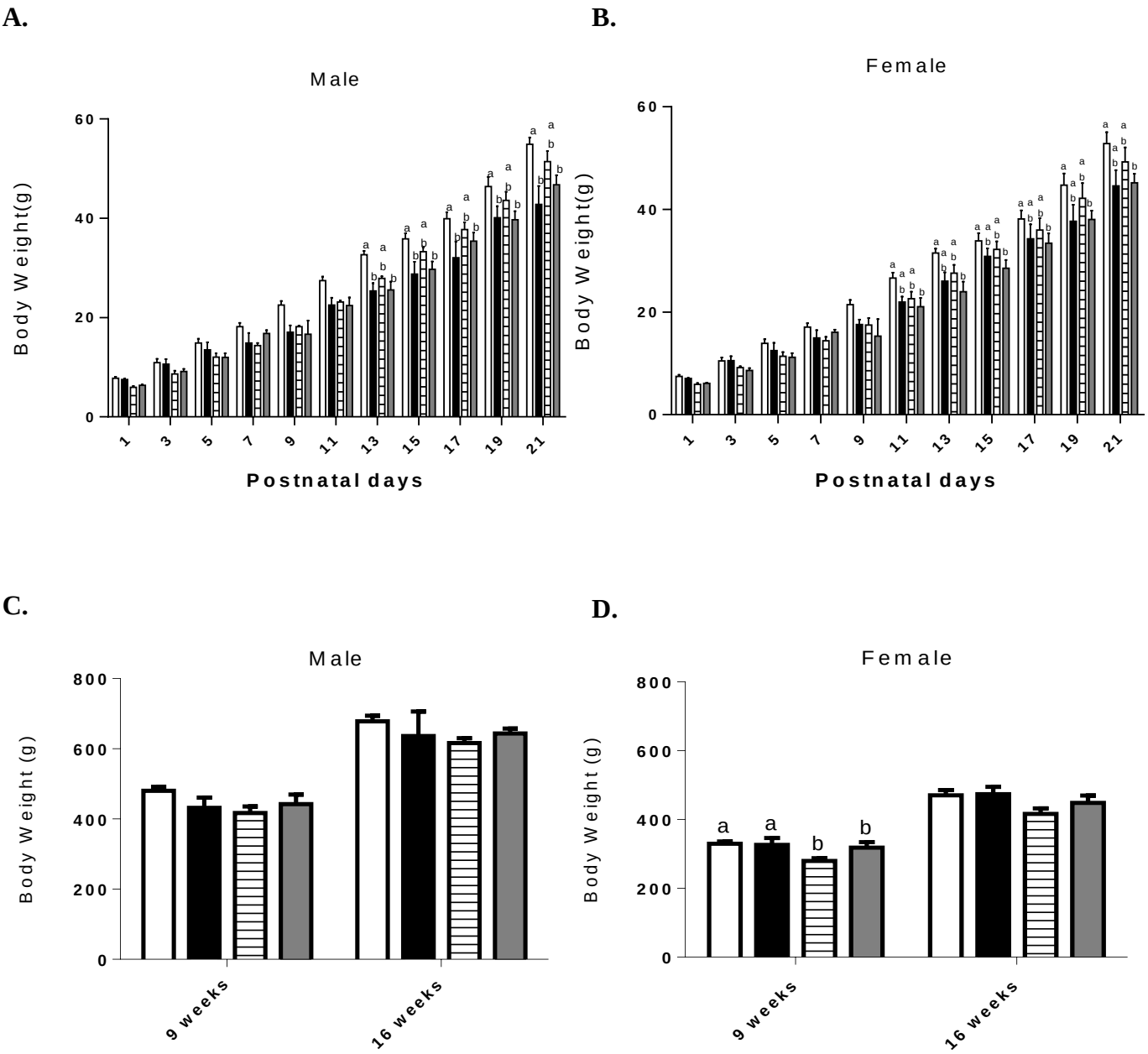
Maternal diet had no effect on litter size (control 13±0.65, JF 13±0.68) or length of gestation (control 22±0.10 d, JF 22±0.00 d). JF litters had increased rates of pup death, with dead pups found in 5 out of 12 JF litters, but no pup deaths were observed in the control litters. All cross-fostered pups survived until weaning.

At birth offspring of JF dams were significantly lighter than offspring of control dams for both males (control 7.7±0.2g, JF 6.2±0.1g, $P<0.01$) and females (control 7.3±0.2g, JF 6.0±0.1g, $P<0.01$). However there was no difference in body weights between groups from PND1 to PND 9. From PND 9 until weaning (PND 21), male offspring suckled by JF dams (C-JF, JF-JF) were lighter than those suckled by control dams (C-C, JF-C), independent of maternal diet during pregnancy. In female offspring, a reduction in bodyweight was observed only in JF-JF offspring compared to controls (C-C) (Fig.2.2A, B).

2.4.3 Offspring growth and food intake during the post-weaning period

In males, there was an interaction between prenatal and postnatal dietary exposure on body weight at 10 weeks of age, such that exposure to junk food diet during lactation decreased the bodyweight of offspring born to control dams but not those born to JF dams (Fig.2.2C, D). In

Figure 2.2



Body weight of male (A, C) and female (B,D) offspring during the suckling period (A,B) and at 9 and 16 weeks of age (C,D) which was immediately prior to and at the conclusion of the determination of food preferences. Offspring of control dams fostered onto control dams (C-C, open bars), offspring of control dams fostered onto JF dams (C-JF, closed bars), offspring of JF dams fostered onto control dams (JF-C, striped bars) and offspring of JF dams fostered onto JF dams (JF-JF, grey bars), n=5-6/group. Results presented as mean±SEM. Different letters above bars denotes means that are significantly different $P<0.05$. Males and females analysed separately.

females, offspring born to JF dams were significantly lighter at 10 weeks compared to those born to control dams, independent of dietary exposure during the suckling period (Fig.2.2).

There was no difference in the intake of the standard rodent feed between groups of offspring from weaning to 10 weeks of age in either males (C-C 1876.7±62.7kJ/kg/d, C-JF 2243.1±34.8kJ/kg/d, JF-C 1863.8±65.5kJ/kg/d, JF-JF 1968.6±50.8kJ/kg/d) or females (C-C 1950.8±68.4kJ/kg/d, C-JF 2170.9±58.2kJ/kg/d, JF-C 2157.9±88.2kJ/kg/d, JF-JF 2058.843.7±kJ/kg/d).

2.4.4 Effect of prenatal and postnatal maternal diet on offspring body composition at 12 and 16 weeks of age

At 12 weeks of age, after 3 weeks on the cafeteria diet, there were no longer any differences in bodyweight between groups in the male offspring (C-C 570.9±11.6g, C-JF 530.3±37.3g, JF-C 512.8±16.5g, JF-JF 539.5±21.3g). In females, however, offspring exposed to the cafeteria diet before birth remained lighter than those born to control dams, independent of dietary exposure during the suckling period (C-C 405.4±6.8g, C-JF 401.2±15.9g, JF-C 354.5±8.7g, JF-JF 388.4±17.7g, $P<0.05$). However, those female offspring who had been exposed to the cafeteria diet during the suckling period had significantly higher omental, epigonadal and total body fat mass as percentage of body weight after 3 weeks of access to the cafeteria diet, independent of dietary exposure before birth (Table 2.1). There were no differences between groups in body fat mass after 3 weeks on the cafeteria diet in the male offspring (Table 2.1).

At 16 weeks of age, after all offspring had been exposed to the cafeteria diet for 7 weeks, there was no difference in bodyweight between groups in either male or female offspring (Fig.2.2C, D). There were also no differences between groups in total body fat mass or the relative weight of any individual fat depot in either males or females (Table 2.1).

2.4.5 Effect of prenatal and postnatal maternal diet on offspring food preferences from 10 to 12 weeks of age

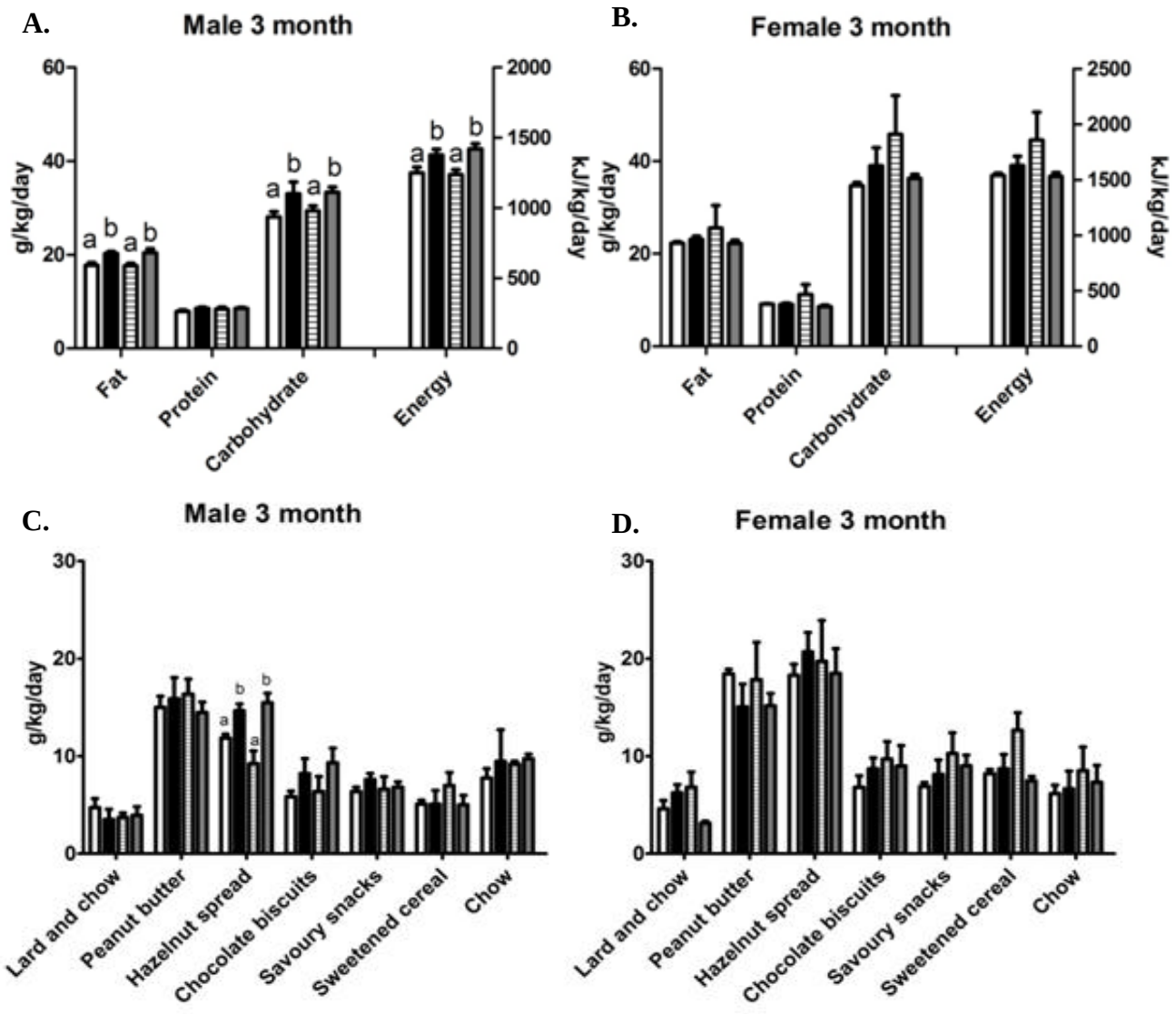
During the first 3 weeks of access to the cafeteria diet, male offspring that were suckled by JF dams had a higher intake of fat, carbohydrate and energy independent of whether they were exposed to the control or JF diet before birth (Fig 2.3A). There was no effect of maternal diet during pregnancy and/or lactation on the intake of fat, protein, carbohydrate or total energy in the female offspring (Fig.2.3B).

Table 2.1 Fat depots as percentage of body weight in male and female offspring at 3 and 4 months of age

		3 months				*4 months*			
Sex	**Parameter**	**C-C**	**C-JF**	**JF-C**	**JF-JF**	**C-C**	**C-JF**	**JF-C**	**JF-JF**
Male	Omental fat	2.1±0.15	2.4±0.18	2.3±0.18	2.5±0.20	3.2±0.22	3.0±0.46	3.1±0.27	3.4±1.90
	Retroperitoneal fat	2.8±0.23	3.1±0.11	3.0±0.13	3.5±0.34	4.5±0.32	3.2±0.60	3.9±0.34	4.6±0.25
	Epigonadal fat	2.4±0.19	2.6±0.14	2.9±0.32	3.2±0.30	3.7±0.25	3.2±0.55	3.5±0.15	4.5±0.19
	Interscapular fat	0.3±0.04	0.5±0.04	0.4±0.04	0.5±0.02	0.3±0.02	0.3±0.06	0.3±0.04	0.4±0.02
	Subcutaneous fat	7.3±0.44	7.8±0.37	7.6±0.58	8.1±0.99	11.0±0.78	10.3±2.15	9.6±1.07	11.6±0.74
	Total fat	14.9±0.95	16.2±0.63	16.2±1.16	17.7±1.77	22.6±1.42	20.9±3.60	20.5±1.70	24.6±1.13
Female	Omental fat	2.7±0.25[a]	3.6±0.31[b]	2.4±0.12[a]	3.3±.16[b]	4.0±0.19	4.3±0.42	3.8±0.31	4.3±0.22
	Retroperitoneal fat	4.2±0.53	4.2±0.54	3.4±0.34	4.6±0.17	4.9±0.24	5.6±0.39	4.9±0.37	5.4±0.35
	Epigonadal fat	3.2±0.43[a]	4.7±0.38[b]	4.0±0.16[a]	5.2±4.9[b]	5.1±0.12	4.9±0.44	5.2±0.20	5.7±0.45
	Interscapular fat	0.6±0.07	0.5±0.13	0.5±0.07	0.6±0.09	0.4±0.03	0.5±0.10	0.5±0.04	0.4±0.02
	Subcutaneous fat	9.3±0.60[a]	9.8±0.67[a]	7.2±0.49[b]	8.3±0.49[b]	10.7±0.29	10.8±1.22	9.6±0.83	10.5±0.53
	Total fat	19.9±1.33[a]	22.8±1.57[b]	17.4±1.03[a]	22.0±1.10[b]	25.1±0.48	26.2±1.88	24.0±1.06	26.4±0.86

Values expressed as mean±SEM, n=5-6/group at 3 months, n=3-6/group at 4 months. Different superscript letters denote values which are significantly different within each timepoint and sex, $P<0.05$.

Figure 2.3



Intake of total energy (A, C) and fat, protein, carbohydrate (B, D) in male (A, B) and female (C, D) offspring during postnatal weeks 10-12. Offspring of control dams fostered onto control dams (C-C, open bars), offspring of control dams fostered onto JF dams (C-JF, closed bars), offspring of JF dams fostered on to control dams (JF-C, striped bars) and offspring of JF dams fostered onto JF dams (JF-JF, grey bars). Results presented as mean±SEM. n=5-6/group Different letters above bars denotes means that are significantly different within each sex, $P<0.05$.

Analysis of the intake of specific components of the cafeteria diet showed that in males, intake of hazelnut spread was significantly higher in offspring suckled by JF dams compared to those suckled by control dams, in line with the results observed for macronutrient intake (Fig.2.3C). Again, this effect was independent of whether they were born to a control or JF dam. There was no effect of nutritional exposure either before birth or during the suckling period on intake of other cafeteria diet components or standard rodent feed in either males or females (Fig.2.3C, D).

2.4.6 Effect of prenatal and postnatal maternal diet on offspring food preferences from 13 to 16 weeks of age

In the final 4 weeks of access to the cafeteria diet, male offspring suckled by JF dams continued to consume significantly more fat and total energy than those suckled by control mothers, independent of nutritional exposure before birth (Fig.2.4A). There was no effect of maternal diet on protein or carbohydrate intake in the male offspring during this 4 week period. There was no difference in macronutrient intake during this period between groups in female offspring (Fig.2.4B).

Examination of the intake of specific foods, showed that male offspring suckled by JF dams consumed more peanut butter and hazelnut spread but less sweetened cereal than those offspring suckled by control dams, independent of nutritional exposure before birth (Fig. 2.4C). In females, offspring exposed to the cafeteria diet during the suckling period exhibited an increased intake of the standard rodent feed and hazelnut spread compared to the offspring suckled by control dams (Fig.2.4D). There was no effect of maternal diet during either pregnancy or lactation on intake of any other components of the cafeteria diet in either males or females or the intake of standard rodent feed in male offspring.

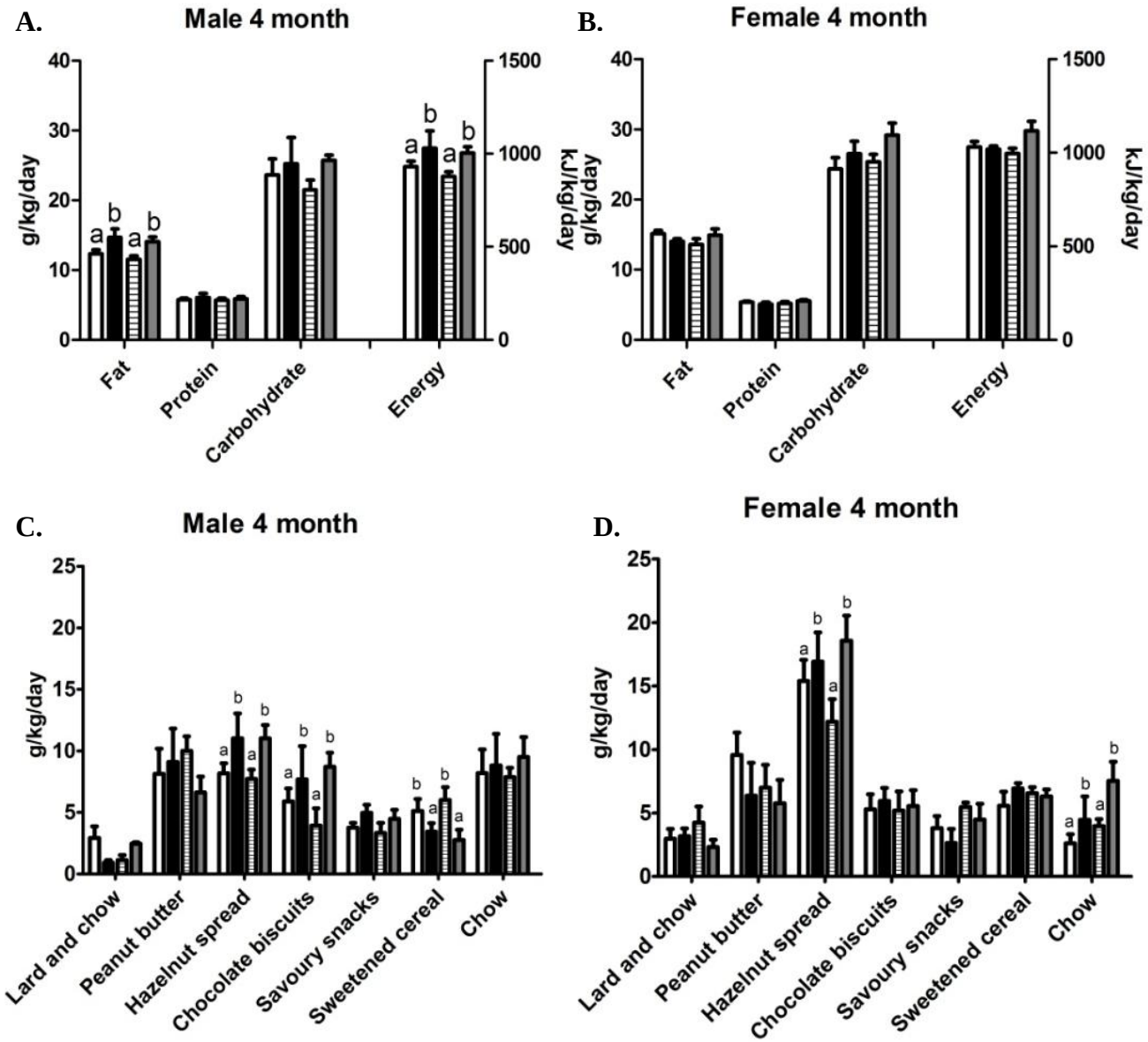
2.4.7 Effect of prenatal and postnatal maternal diet on blood hormones, glucose and NEFA at 12 and 16 weeks of age

At 12 weeks of age, females exposed to the cafeteria diet during the suckling period had increased plasma leptin concentrations (Table 2.2), consistent with the increased fat mass observed in these offspring. Those females who were exposed to the JF diet before birth, however, exhibited higher plasma insulin concentrations and reduced plasma NEFA concentrations at 12 weeks of age, independent of the dietary exposure during the suckling period (Table 2.2). There was no effect of cafeteria diet exposure either before birth or during

the suckling period on plasma concentrations of glucose, NEFA, leptin or insulin in male offspring.

At 16 weeks of age, male offspring suckled by JF dams (C-JF, JF-JF) had increased plasma glucose and insulin concentrations compared to those suckled by control dams, independent of dietary exposure before birth. There was no effect of exposure to the cafeteria diet either before birth and/or during the lactation period on plasma concentrations of glucose and insulin in females and leptin or NEFA in either male or female offspring at 4 months of age (Table 2.2).

Figure 2.4



Intake of fat, protein, carbohydrate and total energy (A, B) and individual components of the cafeteria diet (C, D) in male (A, C) and female (B, D) offspring during postnatal weeks 13-16. Offspring of control dams fostered onto control dams (C-C, open bars), offspring of control dams fostered onto JF dams (C-JF, closed bars), offspring of JF dams fostered on to control dams (JF-C, striped bars) and offspring of JF dams fostered onto JF dams (JF-JF, grey bars). n=3-6/group. Results presented as mean±SEM. Different letters above bars denotes means that are significantly different within each sex, $P<0.05$.

Table 2.2 Plasma concentrations of glucose, NEFA, leptin and insulin in male and female offspring at 3 and 4 months of age

		3 months				*4 months*			
Sex	**Parameter**	**C-C**	**C-JF**	**JF-C**	**JF-JF**	**C-C**	**C-JF**	**JF-C**	**JF-JF**
Male	Glucose (mM)	20.5±1.43	20.9±1.97	21.9±1.57	25.3±2.47	18.3±0.83[a]	25.2±0.75[b]	18.3±1.54[a]	21.8±2.53[b]
	NEFA (mEq/ml)	0.6±0.12	0.8±0.12	0.9±0.22	0.4±0.06	0.4±0.03	0.4±0.06	0.5±0.07	0.4±0.04
	Leptin (µg/ml)	31.2±2.04	28.8±2.34	31.1±2.12	34.7±5.42	34.0±3.30	33.1±6.91	31.1±2.34	38.7±3.96
	Insulin (µU/ml)	2.2±0.81	1.1±0.67	1.1±0.40	3.5±0.89	3.6±0.71[a]	7.8±3.64[b]	1.8±0.50[a]	5.3±0.96[b]
Female	Glucose (mM)	18.5±1.20	20.3±1.36	15.5±0.74	20.1±3.10	19.2±0.74	20.5±1.86	17.9±0.80	18.3±1.59
	NEFA (mEq/ml)	0.6±0.05[a]	0.6±0.09[a]	0.4±0.10[b]	0.4±0.07[b]	0.4±0.03	0.5±0.06	0.4±0.03	0.5±0.06
	Leptin (µg/ml)	29.9±1.68[a]	37.3±6.23[b]	23.5±3.58[a]	31.7±2.67[b]	28.9±2.44	35.6±5.72	31.7±3.31	37.0±5.75
	Insulin (µU/ml)	1.6±0.44[a]	1.1±0.55[a]	2.2±0.48[b]	3.2±0.64[b]	2.5±0.49	2.6±0.51	2.5±0.49	2.7±0.40

Values expressed as mean±SEM, n=5-6/group at 3 months, n=3-6/group at 4 months. Different superscript letters denote values which are significantly different within each timepoint and sex, $P<0.05$.

2.5 Discussion

The findings of this study have demonstrated that there are differing effects of exposure to a high-fat, high-sugar cafeteria diet during the prenatal and early postnatal period on subsequent regulation of palatable food intake, body weight and body fat mass in the adult offspring, and that these effects are sex-specific. Exposure to the cafeteria diet during the suckling period, independent of dietary exposure before birth, was associated with an increased propensity to develop diet-induced obesity in females and an increased preference for palatable foods in male offspring in young adulthood. Importantly, these effects of exposure to a cafeteria diet before birth were ameliorated by cross-fostering offspring to a dam consuming a nutritionally balanced diet. This study is the first to use a cross-fostering approach to isolate the effect of prenatal and early postnatal exposure to a cafeteria diet on the food preferences of the offspring, and adds to the growing body of evidence that there is potential to reverse at least some of the negative effects of inappropriate prenatal nutrition by interventions in the early postnatal period.

2.5.1 Early life exposure to a junk food diet inhibits pup growth pre-weaning

Consistent with previous studies (35,203), we found that both male and female offspring of JF dams were lighter at birth than offspring of control dams. This may be attributed to the reduced protein intake or micronutrient deficiencies in the cafeteria diet compared to the standard chow diet (208). JF offspring cross-fostered onto control dams were no longer lighter than offspring of control dams during the early suckling period, this could suggest that growth deficits in these offspring were overcome by providing access to milk from dams consuming a nutritionally balanced diet. These data suggest that the effect of the maternal diet on milk composition and/or supply plays a central role in the early programming of food preferences, and it will be important in future studies to undertake measurements of milk composition to better explore this. It is also important to note that offspring weights during the suckling period were not recorded separately for individual pups in the current study, and it will be useful to undertake individual assessments in future studies to determine to what extent the growth profiles vary between littermates.

Clear sex differences in the growth profile of the offspring emerged after the first 9 days of postnatal life. In males, offspring suckled by JF dams were lighter at weaning than those suckled by control dams independent of maternal diet before birth. In females, however, weight at weaning was only significantly reduced in offspring exposed to the cafeteria diet

during both the prenatal and suckling periods, suggesting that an improved nutritional environment during the suckling period was not sufficient to overcome the growth deficits induced by maternal junk food intake during pregnancy.

Interestingly, and in contrast to males, female offspring born to JF dams were lighter than those born to control dams after consuming the standard rat chow for 6 weeks after weaning and remained lighter even after 3 weeks of access to the cafeteria diet. It therefore appears that, in females, growth deficits programmed by exposure to a cafeteria diet, which are potentially lacking in protein and key micronutrients, before birth cannot be readily overcome by postnatal nutritional interventions. This result is consistent with the low protein model in which maternal consumption of a low protein diet during pregnancy alone has been demonstrated to impact the growth of female but not male offspring(209,210).

2.5.2 Maternal junk food consumption during lactation increases susceptibility to diet induced obesity in female offspring

In contrast to overall growth, exposure to a maternal junk food diet during the suckling period appeared to play the dominant role in the programming of adipose tissue in female offspring. After 3 weeks of free access to the cafeteria diet, female offspring suckled by JF dams had increased fat mass compared to those offspring suckled by control dams, independent of the diet their mother had consumed during pregnancy. Importantly, this occurred in the absence of a higher food intake, suggesting that these animals had an increased propensity to accumulate body fat. This increased susceptibility to diet-induced obesity was not observed in offspring of JF dams cross-fostered onto a control dam, suggesting that the susceptibility to diet-induced obesity in female offspring exposed to a high-fat, high-sugar diet before birth can be prevented by nutritional interventions in the early postnatal period. Interestingly, there was no longer any difference between groups after the offspring had been exposed to the junk food diet for the full 10 weeks. This suggests that whilst being exposed to an 'optimal' nutritional environment in the perinatal period may render an individual less susceptible to diet induced weight gain and fat deposition, this advantage is negated by persistent overconsumption of a high calorie diet in postnatal life (211) (212).

2.5.3 Maternal junk food consumption during lactation alters the food preferences of male offspring

In males, offspring suckled by JF dams had a greater intake of fat, carbohydrate and total energy compared to offspring suckled by control dams when all offspring were the provided

with the cafeteria diet in adulthood, independent of whether they were born to a control or JF dam. Importantly, there were no differences between groups in the intake of standard rodent feed during this time, indicating that the increased energy intake was the consequence of increased consumption of the cafeteria diet (i.e. an increased preference for this palatable diet). We chose to measure food preferences in the animals' home cage, rather than a metabolic chamber in this study, due to the potential impact of the stress associated with moving the animal to an unfamiliar environment on habitual food intake. However, it will clearly be important in future studies to confirm our findings by conducting more intensive monitoring of metabolic balance in the offspring.

Maternal consumption of a palatable diet throughout both pregnancy and lactation has been shown to induce hyperphagia in the adult offspring (22) and increase offspring preference for a cafeteria diet (35,203). This is the first study, however, to demonstrate that exposure to a maternal junk food diet during the suckling period alone is associated with increases in the preference for a palatable diet equivalent to exposure during the entire perinatal period. The results of the present study are in agreement with the work of Gorski *et al* who also used a cross-fostering approach, and showed that exposure to a high-fat diet during lactation increased offspring consumption of the same high-fat diet in adulthood (124). However, unlike the present study, Gorski and colleagues only provided the offspring with access to a high-fat diet, and therefore were not able to determine food preferences.

There was no significant effect of exposure to a cafeteria diet either before birth or during the suckling period on macronutrient intake in adulthood in female offspring in the present study. This is somewhat different to the results of our previous study, in which both male and female offspring of dams fed on the same cafeteria diet as in the present study exhibited an increased preference for fat intake from weaning until adulthood(203). However, unlike our previous study, the offspring in the current experiment were provided with a standard rodent chow for 3 weeks after weaning, which may have influenced the development of their food preferences. One possibility to explain the sex differences in the programming of food preferences, is that the timing of development of two key systems known to play a central role in the regulation of palatable food intake, i.e. the central appetite-regulating and reward pathways (122,203) (27,32,124), is different in male and female offspring (213). The findings of our study suggest that the suckling period is the critical period for the development of the reward system in males, but not in females. To the best of our knowledge there are no studies which

have directly compared the development of the reward pathway in male and female offspring and this is clearly an important area for future research.

2.5.4 Early life exposure to a junk food diet alters plasma insulin concentrations in adult offspring in a sex specific manner

The effect of maternal cafeteria diet consumption on insulin concentrations in the adult offspring was dependent on both the sex of the offspring and the period of dietary exposure. In females, offspring born to JF dams had higher plasma insulin concentrations, in the absence of higher plasma glucose, after 3 weeks on a cafeteria diet compared to those born to control dams, independent of dietary exposure during the suckling period. The presence of higher insulin concentrations at any given concentration of glucose provides evidence of reduced insulin sensitivity; although this will need to be confirmed by direct assessment of insulin sensitivity in future studies. In males, on the other hand, higher glucose and insulin concentrations were only observed after 7 weeks of exposure to the cafeteria diet in offspring suckled by JF dams, independent of dietary exposure before birth, consistent with previous studies(21,214). These results imply that the impact of cafeteria diet exposure during development on glucose-insulin metabolism is sex-specific. Shelley and colleagues reported that changes to the insulin signalling pathway in skeletal muscle in 3-month old offspring of dams fed a cafeteria diet during pregnancy and lactation, was indeed different in males and females, with male offspring exhibiting increased expression of Akt2 and reduced Akt activity, and female offspring having reduced expression of IRS-1 and P13K (215). It appears that in females, but not in males the effects of exposure to a cafeteria diet before birth on the development of glucose homeostatic pathways cannot be reversed by nutritional interventions applied in the early postnatal period.

2.5.5 Summary and speculation

The present study is the first to show that exposure to a cafeteria diet exclusively during the suckling period is able to program an increased preference for fat and an increased susceptibility to diet induced obesity in the offspring to the same extent as exposure throughout the entire perinatal period. Importantly, these data suggest that the effects of exposure to a high-fat/high-sugar diet before birth on food preferences and susceptibility to diet induced obesity later in life, can be prevented by providing access to a nutritionally balanced diet during the suckling period. Interestingly, the relative contribution of the nutritional environment during the prenatal and suckling periods were different in males and

females, suggesting that the timing of nutritional interventions aimed at ‘reprogramming’ the offspring may need to be sex-specific. We speculate that these sex-differences may be a consequence of differences between sexes in the timing of development of key metabolic systems, and this will be important to further investigate in future studies.

It is important to exercise caution when extrapolating these results to the clinical context, since many of the developmental events which occur during the suckling period in rodents are already complete before birth in the human. Nevertheless, the data from this study provides evidence that there are critical windows of development during which exposure to a junk food diet is most detrimental to long term outcomes, and suggests that there may be an opportunity to prevent at least some of the adverse consequences of prenatal junk food exposure by interventions applied during the lactation period. Gaining a better understanding of the sex specific effect maternal diet has on the long term metabolic outcomes of the offspring will be crucial if targeted and effective interventions to reduce the incidence of overweight and obesity are to be designed.

Chapter 3

A maternal junk food diet alters mu-opioid receptor expression in late but not early postnatal development in female offspring

Prepared Manuscript

Chapter 3: A MATERNAL JUNK FOOD DIET ALTERS MU-OPIOID RECEPTOR EXPRESSION IN LATE BUT NOT EARLY POSTNATAL DEVELOPMENT IN FEMALE OFFSPRING

3.1 Abstract

Exposure to a maternal junk food diet during the perinatal period has been shown to reduce the sensitivity of the opioid pathway and increase the preference for palatable diets in juvenile and adult offspring. We aimed to determine how early in development a maternal junk food diet could affect the opioid pathway by investigating mu-opioid receptor expression during early (at birth and week 1) and late (week 3 and 4) postnatal development in the offspring of dams fed a control or junk food (JF) diet using an *in situ* hybridisation method. We report here that the number of cells with mu-opioid receptor mRNA expression in the ventral tegmental area (VTA) of female JF offspring was 32% (week 3) and 57% (week 4) lower than it was in the female offspring of dams fed a standard rodent diet (control) during late development ($P<0.05$). No effect of maternal diet was observed on mu-opioid receptor expression at the earlier timepoints in female offspring or at any time in male offspring. In addition, we also demonstrated a significant decrease in the number of cells with mu-opioid receptor expression in the nucleus accumbens (NAc) in late postnatal development compared to early postnatal development ($P<0.05$), independent of maternal diet in both male and female offspring. The findings of the current study suggest that exposure to a maternal cafeteria diet throughout the perinatal period, only induces changes in mu-opioid receptor expression in late but not early postnatal development and that this effect is confined to female offspring.

3.2 Introduction

Maternal consumption of high-fat high-sugar highly palatable 'junk foods' during pregnancy and lactation has been demonstrated in numerous studies to program a preference for these types of foods in the adult offspring (32,35-37). The intake of palatable foods is thought to be regulated, at least in part, by the mesolimbic reward pathway, making alterations to this pathway a likely mechanism behind the programming of food preferences (47,62,67-70). Supporting this, studies in our laboratory and others have shown changes in the expression of

key components of the reward pathway including the mu-opioid receptor, in the offspring of dams fed a high-fat or cafeteria diet (a well-established rodent model of a junk food diet (41)). Vucetic and colleagues demonstrated that exposure to a high-fat diet before birth and whilst suckling increased the expression of the mu-opioid receptor in the nucleus accumbens (NAc) at 6 months of age (122). Subsequent studies in our laboratory have highlighted that the effect of a maternal cafeteria diet on mu-opioid receptor expression in the offspring is dependent on the age of the offspring and the length of exposure. We have shown that at 3 weeks of age both male and female offspring of cafeteria diet fed dams have reduced mu-opioid receptor expression in the ventral tegmental area (VTA) (208), whilst at 6 weeks of age these offspring (when maintained on the cafeteria diet) have increased expression of this receptor compared to controls in this same brain region (37). This research highlights a potential critical role of the mu-opioid receptor in the programming of food preferences; however no studies to date have looked specifically at how exposure to a high-fat/cafeteria diet affects the postnatal developmental trajectory of the mu-opioid receptor in the mesolimbic reward pathway.

During postnatal development, the opioid pathway, like many developing neural networks is highly plastic, suggesting that both the fetal and postnatal periods may represent a time when the opioid pathway is particularly susceptible to alteration by nutritional exposures, such as a maternal cafeteria diet. Investigations into the ontogeny of opioid system (including the mu-opioid receptor) in the human fetus and infant have been limited by the scarcity of appropriate tissue samples (102). For this reason, there has been a heavy reliance on animal models, particularly rodents, to provide information on the development of this pathway. In the rat endogenous opioids including enkephalin and endorphin are present before birth peaking in concentration during the fourth postnatal week (216). The mu-opioid receptor has been detected as early as embryonic day 13 in the forebrain (including the NAc) through autoradiographic and *in situ* hybridisation studies and is present at higher than adult levels during the first week of life before decreasing to adult levels by the third to fourth postnatal week (104,109,110). The advantage of using *in situ* hybridisation over methods such as quantitative real-time PCR is that it enables accurate localisation of gene expression to specific brain areas. However, the studies conducted to date have exclusively investigated males and have not looked at mu-opioid receptor expression in brain reward regions outside the forebrain such as the VTA (which also forms part of the mesolimbic reward pathway). Thus, there remains a need for further investigations into the ontogeny of the mu-opioid

receptor as well as how it may be altered by exposure to a maternal cafeteria diet, particularly given that this information is likely provide significant insights into the mechanisms behind the programming of food preferences.

The aim of the current study therefore, was to use *in situ* hybridisation in a rat model to explore in detail the effect of a maternal cafeteria diet during pregnancy and lactation on both the early (birth and week 1) and late (week 3 and 4) postnatal development of the mu-opioid receptor in the offspring. A secondary aim of the study was to investigate any sex differences in the ontogeny of this receptor.

3.3 Methods

3.3.1 Animals and feeding

This study was approved by the Animal Ethics committee of the University of Adelaide. The animals were allowed to acclimatise to the animal housing facility for 1 week before the start of the dietary intervention. During this period, all rats were fed a standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia). Following the acclimatisation period, rats were placed into one of two weight matched groups, the control group (C, n=5) or junk food group (JF, n=5). The control group received a diet of standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia). The junk food group were fed a cafeteria diet which included hazelnut spread, peanut butter, chocolate biscuits, savoury snacks, sweetened cereal and a lard and chow mix. Detailed nutritional information on this diet has been previously published (37). Food intake was determined every 2 days by subtracting the amount left uneaten in the cage from the amount first supplied and rats were weighed weekly throughout the experiment. All animals were individually housed and kept at a room temperature of 25°C in a 12 hour/12 hour light-dark cycle for the duration of the study.

The female rats were provided with their respective diets for 2 weeks prior to mating and throughout pregnancy and lactation. Females were mated with 4 proven males, which were maintained on the standard laboratory rodent feed. The same males were used for mating with both the control and JF groups. In the female rats, vaginal smears were performed daily to determine the stages of the estrous cycle. On the night of di-estrous/pro-estrous, the female rat was placed with a male overnight and vaginal smears were conducted the following morning. The presence of sperm in the vaginal smears was taken as confirmation of successful mating

and was designated as gestation day 0. Pups were born on day 21-22 of gestation. On the day after birth (postnatal day (PND) 1), pups were culled to 10 per litter, 5 males and 5 females where possible. Pups were weighed every 2 days during the suckling period and were weaned on PND 21. Weaned pups were housed with a same sex littermate and fed the same diet as their mothers until the conclusion of the experiment on PND 28. Pups of control and JF dams are referred to as control offspring and JF offspring respectively.

3.3.2 Postmortem and tissue collection

1 male and 1 female pup from each litter was killed at birth, week 1, week 3 and week 4. Postmortems were conducted between 0800 and 1200 with rats weighed immediately prior to euthanasia. Blood samples were collected via cardiac puncture into heparinised tubes and centrifuged at 3,500 g at 4ºC for 15 minutes. Body weight, length (nose to tail) and abdominal circumference were recorded. Brain and liver samples were collected from offspring at birth and week 1, whilst all internal organs and visible fat depots were collected from the pups at week 3 and week 4. All tissues and fat depots were frozen in liquid nitrogen and stored at -80°C for future study.

3.3.3 Tissue sectioning

Brain tissue was collected from the offspring at each postmortem time point. The brain was hemisected and snap frozen before one half was placed face down into a cryostat mould to facilitate the cutting of 15µm sagittal sections. 120 high quality frozen sagittal sections were cut for each animal on a cryostat (Leica Cryostat; Leica, Wetzler, Germany). All sections were mounted on Menzel-Glaser Super frost plus slides (25x75x1.0 mm, Menzel-Glaser, Braunschweig, Germany) and stored at -80˚C in sealed plastic containers. Sections were mounted 3 to a slide and cut in blocks of 8. Every 8^{th} slide was stained with haematoxylin and eosin and the anatomical location of the key regions of interest (VTA and NAc) were identified using specific biomarkers and a neonatal rat brain atlas (217).

3.3.4 Probe synthesis and in situ hybridisation

An antisense probe specific for the mu-opioid receptor was synthesised from PCR primer DNA (5’ CAG AGA TGC AAT TAA CCC TCA CTA AAG GGA GAA CTG GGA GAA CCT GCT CAA A; 3’ CCA AGC CTT CTA ATA CGA CTC ACT ATA GGG AGA TGT GGT TTC TGG AAT CGT GA) (Applied Biosystems, Foster City, CA, USA) before being purified using QIAquick Gel extraction kit (Qiagen, Limburg, Netherlands) and sequenced to confirm its specificity for the gene of interest. A sense probe was also synthesised to act as a

negative control. The probes were labelled using radioactive ^{35}S UTP and activity quantified using a scintillation counter, successful probe synthesis yielded approximately 2.5 million counts per minute per μl.

In situ hybridisation experiments were performed as previously described (218). Briefly sections were fixed in 4% paraformaldehyde, acetylated, dehydrated through an ethanol gradient and hybridised overnight at 55°C using [^{35}S]-labelled cDNA probe. The sections were then treated with RNaseA (Ambion, Texas, USA) to remove unhybridised probe and desalted with a high stringency wash (30 min) in 0.1% saline-sodium citrate (SSC) at 65°C. Following dehydration through an ethanol gradient, slides were allowed to air dry before being exposed under photographic emulsion at 4°C for 4-6 weeks. Under emulsion, silver grain formation was carefully monitored with a series of test slides to ensure that overexposure did not occur and that single cells were distinct. After development, the slides were counterstained with cresyl violet to allow the regions of interest (VTA and NAc) to be accurately located. The number of labelled cells (those containing silver granules) within each of these brain regions were counted at X200 magnification using Image Proplus 5.1 as detailed in (219). In older animals (week 3 and 4) two fields of view were counted for the VTA and three for the NAc, whilst in younger animals (birth and week 1) one field of view was counted for the VTA and two for the NAc, given the much smaller overall brain size.

3.3.5 Statistical analysis

Analysis of maternal food intake and body weight data as well as birth outcomes was conducted using Student's unpaired t-tests. Two-way ANOVA with sex and maternal diet as factors was used to analyse mu-opioid receptor expression at each time point as well as body fat mass at 4 weeks of age. Two-way ANOVA, as well as the Student's unpaired t-tests, were performed using SPSS statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Offspring body weight gain and mu-opioid receptor expression over time was analysed by two-way repeated measures ANOVA, which was performed using Stata 11 software (StataCorp., TX, USA). All data are presented as mean±SEM and a P value of <0.05 was considered statistically significant.

3.4 Results

3.4.1 Dam body weight and nutritional intake during pregnancy and lactation

There was no difference in body weight between the control and JF dams prior to mating (control 224.1±6.8g, JF 210.2±10.4g) or at any point during pregnancy or lactation (data not shown). During pregnancy, the JF dams consumed significantly more fat and significantly less protein than the controls without any differences in carbohydrate, or overall energy intake (Table 3.1). Throughout lactation, in addition to the increased fat and decreased protein intake the JF dams also consumed less carbohydrate and had a lower overall energy intake compared to control dams (Table 3.1).

3.4.2 Effect of maternal diet on birth outcomes

Maternal diet had no effect on litter size (control 15.1±1.0, JF 14.3±1.1) or the percentage of males per litter (control 53.5±3.1%, JF 53.2±5.0%). Offspring of JF dams had a significantly lower birth weight for both the male (control 7.1±0.3g, JF 6.1±0.2g, $P<0.01$) and female pups (control 6.6±0.3g, JF 5.8±0.2g, $P<0.05$).

3.4.3 Effect of maternal diet on body composition and growth

There was no significant difference in bodyweight between control and JF offspring in either males or females throughout the suckling period (to PND 21). However, at the end of the experiment (PND 28) both male and female control offspring were heavier than their JF counterparts (control male 98.9±4.2g, JF male 75.4±4.0g, $P<0.05$; control female 90.2±2.7g, JF female 78.1±7.6g, $P<0.05$). However, the JF offspring of both sexes had a significantly higher total fat mass (relative to bodyweight) than the controls (control male 7.5±0.8%, JF male 12.5±1.8% $P<0.05$; control female 8.3±1.2%, JF female 12.9±0.7% $P<0.05$).

3.4.4 Effect of maternal diet on mu-opioid expression in early postnatal development

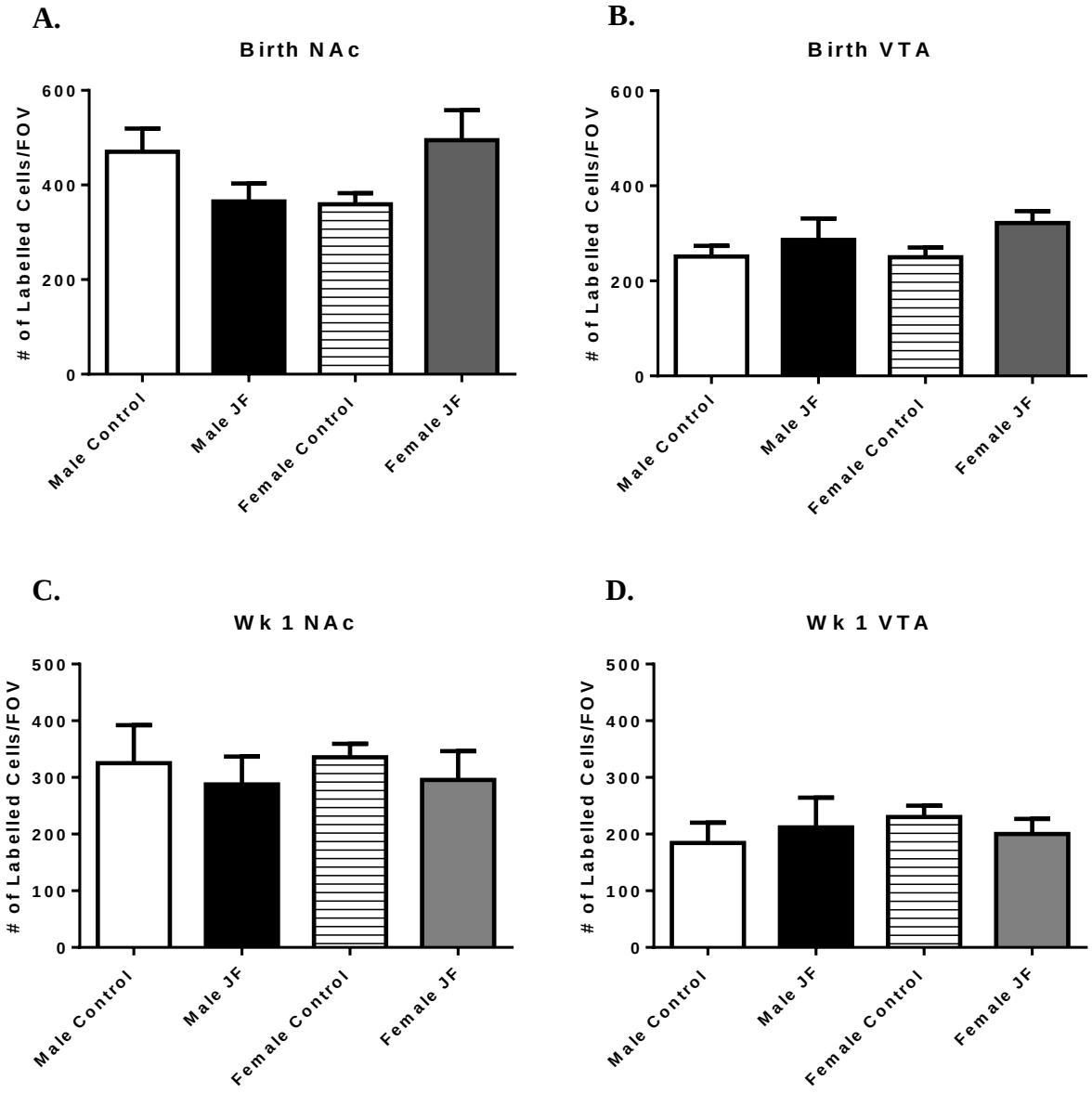
At birth, a significant interaction was present between maternal diet and sex, such that in male offspring being exposed to a junk food diet tended to decrease the number of cells with mu-opioid receptor expression in the NAc ($P=0.08$), whilst in females JF exposure tended to increase the number of cells with mu-opioid receptor expression ($P=0.07$) (Fig.3.1A). However, there were no significant differences in the number of cells expressing the mu-opioid receptor in the NAc in either males or females when the data for each sex was analysed separately. There was no effect of either maternal diet or sex on mu-opioid receptor mRNA expression in the VTA at birth (Fig.3.1B) or in either the VTA or NAc in postnatal week 1 (Fig.3.1C, D).

Table 3.1 Maternal macronutrient intake during pregnancy and lactation in control and JF dams

	Control	JF
Pregnancy		
Fat (g/kg/d)	3.5±0.1	17.2±1.2**
Protein (g/kg/d)	14.8±0.6	9.1±0.4*
Carbohydrate (g/kg/d)	45.4±1.8	43.1±2.3
Energy (kJ/kg/d)	1362.7±55.4	1461.3±72.7
Lactation		
Fat (g/kg/d)	6.6±0.2	23.3±0.8**
Protein (g/kg/d)	28.3±0.6	14.7±0.7*
Carbohydrate (g/kg/d)	86.6±1.9	60.6±2.7*
Energy (kJ/kg/d)	2598.6±57.6	2130.7±80.3*

Data presented as mean±SEM, n=5 for both groups. Significantly different between values between groups are marked as *P<0.05, ** P<0.01.

Figure 3.1



Number of labelled cells expressing the mu-opioid receptor per field of view in the NAc (A, C) and VTA (B, D) of the male and female offspring of control dams and junk food fed dams at birth (A, B) and l week (C, D) of age. An interaction between maternal diet and sex was present in the NAc at birth ($P<0.05$). Results presented as mean±SEM. n=4-5 pups for all groups.

3.4.5 Effect of maternal diet on mu-opioid expression in late postnatal development

At both 3 and 4 weeks of age, the number of cells with mu-opioid receptor mRNA expression in the VTA was significantly reduced in female offspring of JF dams when compared to their control counterparts (Fig.3.2B, 3.3B). No difference in mu-opioid receptor expression was observed in the NAc of female offspring or in the VTA or NAc of male offspring at either postnatal week 3 or 4 (Fig.3.2A, 3.3A). There was no overall effect of sex on mu-opioid receptor expression in either the NAc or VTA at either timepoint.

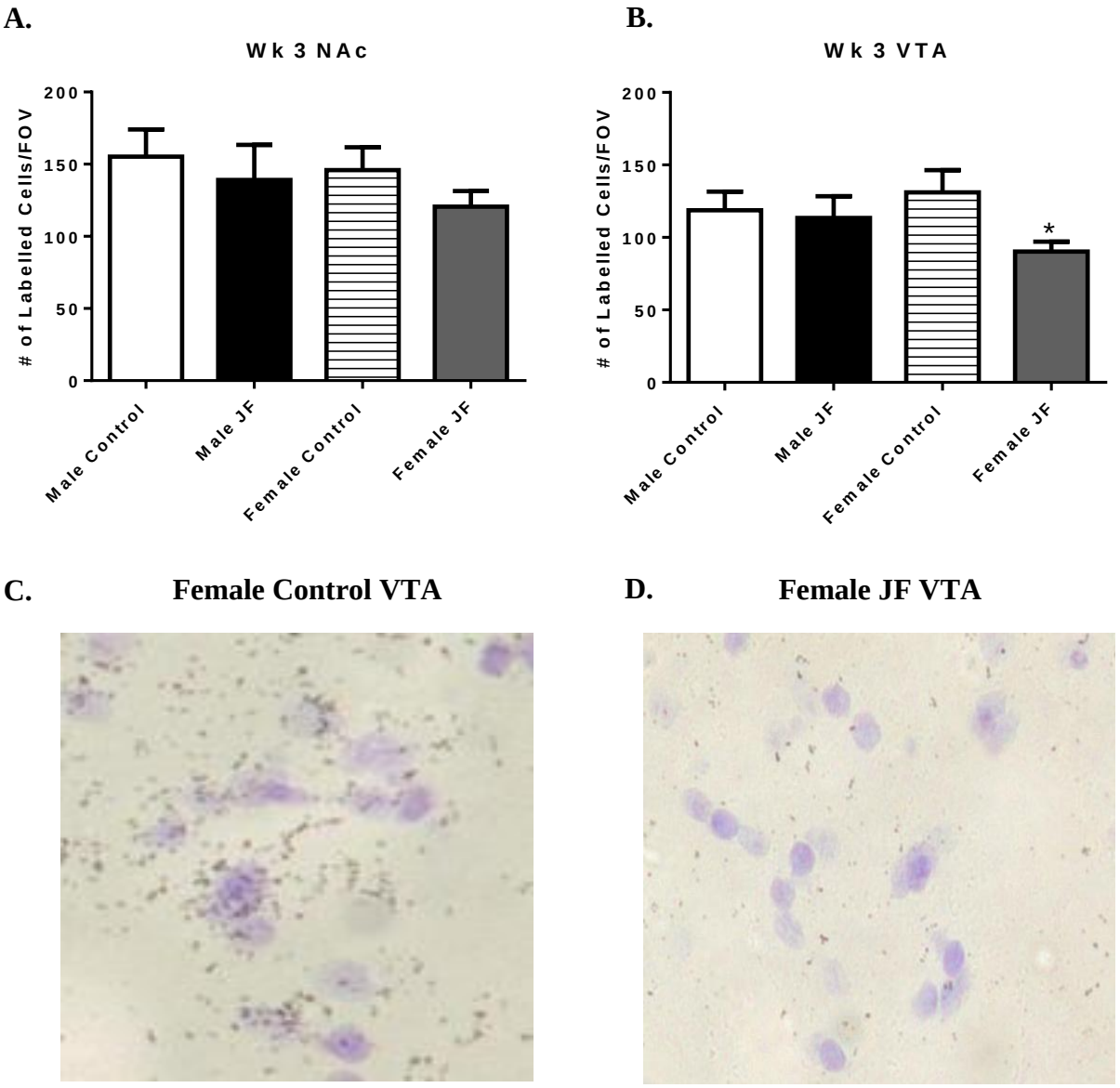
3.4.6 Mu-opioid receptor expression across postnatal development

Analysis of mu-opioid receptor expression over time showed that both male and female offspring had a higher number of cells with mu-opioid receptor expression in the NAc early in development (birth, week 1) than in late postnatal development (week 3, week 4), independent of maternal diet (Fig.3.4, 3.5A). In the VTA, the pattern of expression showed an initially high level of mu-opioid expression early in development, which decreased in the third postnatal week before increasing again in week 4 (Fig.3.5B).

3.5 Discussion

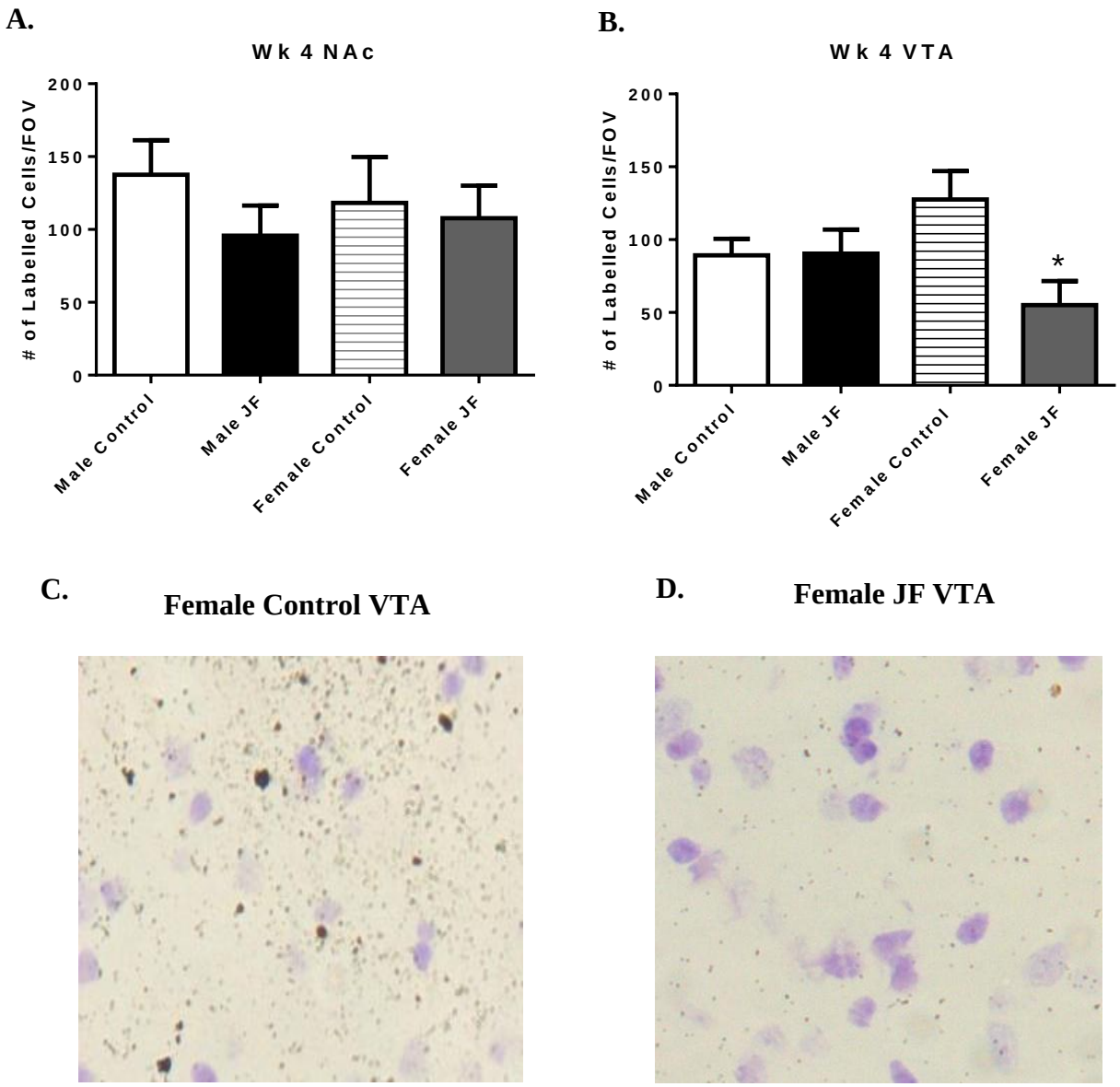
In the current study it was shown that exposure to maternal cafeteria diet alters the late but not early postnatal expression of the mu-opioid receptor in the VTA of female offspring. Furthermore, this work also provides information about the differential response of the mu-opioid receptor to maternal cafeteria diet exposure between the sexes. In addition, we have also shown, in agreement with previous studies, that the number of cells with mu-opioid receptor expression is increased in NAc in early postnatal development compared to later development in males and for the first time that this pattern of expression is also present in female offspring. This study has provided a novel insight into the early life development of the mu-opioid receptor in both the NAc and VTA as well as how that development can be altered by exposure to maternal cafeteria diet at, least in female offspring. The identification of mu-opioid receptor expression changes in the VTA of female offspring in late but not early postnatal development supports previous work by our laboratory and others suggesting that the suckling period may be a ‘critical window’ for opioid pathway development and the programming of food preferences.

Figure 3.2



Number of labelled cells expressing the mu-opioid receptor per field of view in the NAc (A) and VTA (B) of the male and female offspring of control dams and junk food fed dams at 3 weeks of age. * indicates significantly different mean ($P<0.05$). Examples of mu-opioid receptor expression in the VTA of control (C) and junk food (D) female offspring (x400 magnification). Results presented as mean±SEM. *n*=5 pups for all groups.

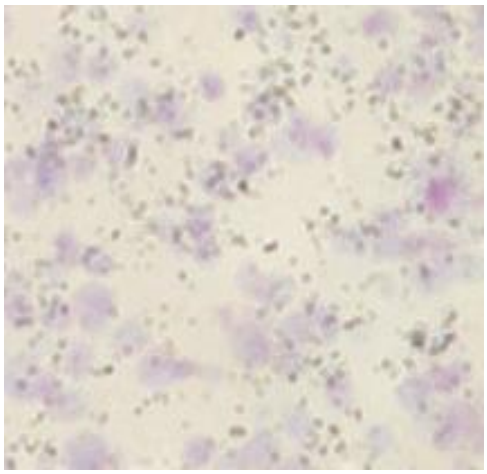
Figure 3.3



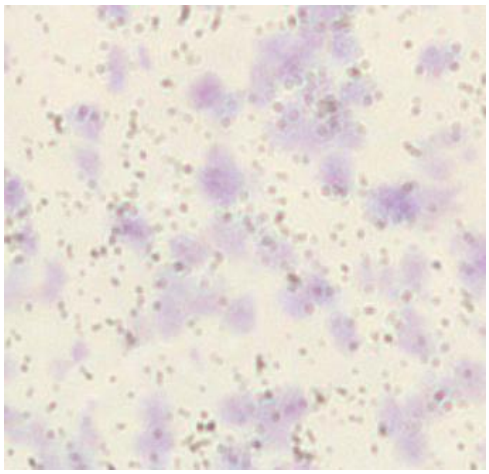
Number of labelled cells expressing the mu-opioid receptor per field of view in the NAc (A) and VTA (B) of the male and female offspring of control dams and junk food fed dams at 4 weeks of age. * indicates significantly different mean ($P<0.05$). Examples of mu-opioid receptor expression in the VTA of control (C) and junk food (D) female offspring (x400 magnification). Results presented as mean±SEM. *n*=5 pups for all groups.

Figure 3.4

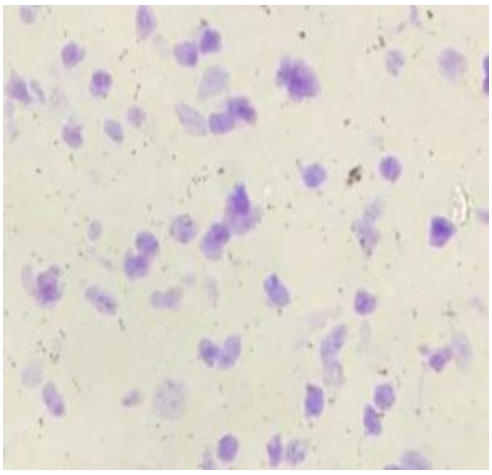
A. **Birth**



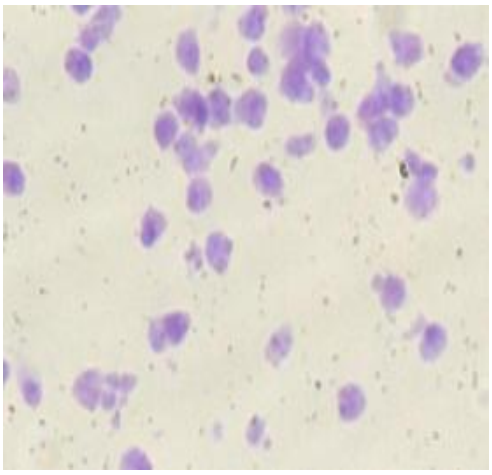
B. **Week 1**



C. **Week 3**

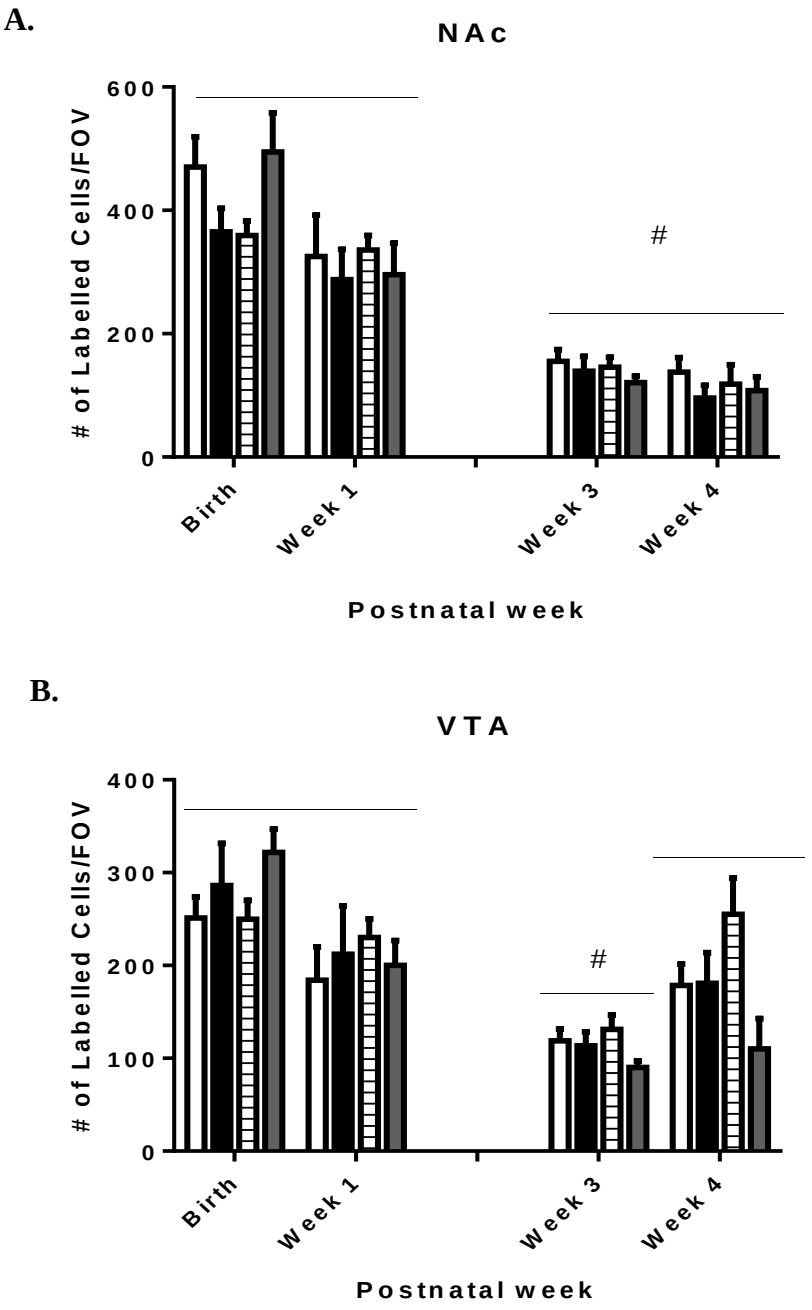


D. **Week 4**



Examples of mu-opioid receptor expression in the NAc of male junk food offspring across postnatal development (x400 magnification). There was a significant effect of time on mu-opioid receptor expression independent of maternal diet or sex, with higher expression observed earlier in development (birth, week 1) compared to later development (week 3, week 4).

Figure 3.5



Number of labelled cells expressing the mu-opioid receptor per field of view in the NAc (A) and VTA (B) of the male (control, open bars, JF, closed bars) and female (control, striped bars, JF, grey bars) offspring of control dams and junk food fed dams across postnatal development. # indicates significant effect of time ($P<0.05$). Results presented as mean±SEM. *n*=4-5 pups for all groups.

3.5.1 Maternal junk food diet decreases the late but not early postnatal expression of the mu-opioid receptor in the VTA

In the present study, a significant reduction in the number of cells with the mRNA expression of mu-opioid receptor in the VTA of the female offspring of junk food fed dams was observed at both postnatal week 3 and 4. This result is consistent with a previous study conducted by our group which utilised quantitative real-time PCR analysis to demonstrate a decrease in mu-opioid receptor expression in VTA of female JF offspring at 3 weeks of age. However, in this previous work the decrease in mu-opioid receptor expression was also observed in male JF offspring (208). This inconsistency between studies can likely be explained by the differences in the sensitivity of the methods used. In our previous work, we observed a 1.9 fold decrease in females and only a 1.4 fold decrease in males (208), creating the possibility that the current *in situ* hybridisation method may not have been sensitive enough to discern the less pronounced change in gene expression that may be present in male JF offspring at this age. The finding that the decrease in mu-opioid expression in female JF offspring extends until the fourth week of life is consistent with our previous work demonstrating that the female reward pathway is particularly sensitive to alteration during this time (220). Notably, no difference in mu-opioid receptor expression was observed in the NAc at this age in either males or females. This is significant given that the majority of studies investigating the adult offspring of JF dams have focused mainly on changes in gene expression in the NAc rather than the VTA (37,122). However, given our current data, it appears that the VTA may also be important and warrant further investigation in adult offspring.

The current study is the first to investigate the effects of maternal cafeteria diet exposure during pregnancy and lactation on mu-opioid receptor expression prior to three weeks of age, however no change in expression was observed the VTA or NAc at birth or in the first week of life. That the reduction in mu-opioid receptor expression observed in the VTA of JF female offspring late in postnatal development was not present at earlier timepoints, could suggest that exposure to a cafeteria diet through the maternal milk supply and in the immediate post-weaning period may have a greater impact on mu-opioid receptor development than exposure *in utero*. Alternatively, it may be that continual exposure to a maternal junk food diet into the fourth postnatal week is necessary to alter mu-opioid receptor development in female offspring, rather than exposure only *in utero* or in very early postnatal period. No studies to date have specifically examined the effects of cafeteria diet exposure isolated to the first four weeks of life on mu-opioid receptor expression at any timepoint, making it an important area

for future investigation. Studies which have isolated cafeteria/high-fat diet access to the suckling period or specifically to the fourth week of life have shown that the offspring develop an increased preference for fat and susceptibility to obesity as adults equivalent to those observed when they have been exposed to the cafeteria diet throughout the entire perinatal period (124,125,221). Given the proposed role of the mu-opioid receptor in the programming of food preferences, it is possible that changes in the late postnatal development of the mu-opioid as demonstrated in the current study could act as the mechanism behind the programming of food preferences. However, this remains to be studied directly.

3.5.2 Sex differences in mu-opioid receptor expression in response to a maternal junk food diet

Contrary to our hypothesis that there would sex differences in the postnatal ontogeny of the mu-opioid receptor, no difference in the expression of this receptor was observed between male and female control offspring in either early or late postnatal development. The current study is the first to compare male and female postnatal mu-opioid receptor ontogeny within the same study. Our findings indicate that the mu-opioid receptor in the NAc and VTA of both sexes has a similar developmental trajectory (under normal physiological conditions), providing some evidence to support the extrapolation of studies conducted only in males to females. Importantly however, we showed sex differences in mu-opioid receptor expression after maternal cafeteria diet exposure, suggesting the similarities between the sexes in mu-opioid receptor development do not extend to the response to reward stimuli.

At birth, the results showed an interaction between maternal diet and sex in the NAc, such that maternal cafeteria diet exposure tended to decrease the number of cells with mu-opioid receptor expression at birth in male offspring and tended to increase the number of cells with mu-opioid receptor expression in female offspring. This interaction, together with the significant decrease in mu-opioid expression in the VTA observed only in female JF offspring in late postnatal development, highlight key differences in the response of males and females to maternal cafeteria diet exposure. We have previously reported sex specific changes in the mu-opioid receptor expression in response to a maternal cafeteria diet at 5 weeks of age, in this previous study (as in the current work) females appeared to be more affected by maternal cafeteria diet consumption than males (220). Interestingly, sex differences in mu-opioid receptor development have also been identified in response to maternal morphine (136,222), indicating that sex differences in response to early life reward stimuli extend beyond maternal

cafeteria diet consumption. Estrogen is a likely mediator behind these sex specific effects, given its known role in opioid pathway regulation (223,224). However, given that there was no difference in mu-opioid receptor ontogeny between the sexes under normal physiological conditions, it suggests that a more complex interaction between maternal cafeteria diet exposure, estrogen and mu-opioid receptor expression might be driving the changes observed. Further investigation is clearly required to better elucidate the mechanism behind the sex differences observed in the current study.

3.5.3 Mu-opioid expression in NAc is higher in early postnatal development than late postnatal development

Consistent with previous studies (104,109,110), the number of cells with mu-opioid receptor expression in the NAc was higher at birth and week 1 than it was in week 3 and 4, independent of maternal diet and offspring sex. Past investigations into the prenatal and postnatal ontogeny of mu-opioid receptor have shown a steady increase in the expression of this receptor from its first detection on embryonic day 13 until a peak in expression during the first week of life (107-109). Following this peak, there is a decrease in mu-opioid receptor expression to adult levels by the third to fourth postnatal week (104,109,110), in line with the results of the current study. The high level of mu-opioid receptor expression before birth and during early postnatal development, together with evidence suggesting that mu-opioid receptors have reduced functionality in the striatum in the neonatal period (225), have led to the hypothesis that the opioid system (including the mu-opioid receptor) is primarily involved in brain development rather than reward processes at this early age. In support of this, numerous studies have implicated endogenous opioids in the regulation of neurogenesis, dendritic growth and spine formation before birth and in the early postnatal period (226-228).

Unlike the development of the mu-opioid receptor in the NAc, which has been well characterised in literature, little is known about mu-opioid receptor ontogeny in VTA. In the current study we observed an initially high level of expression in early development, which was significantly lower in the third postnatal week. However, in contrast to what was observed in the NAc, mu-opioid receptor expression then increased to the levels observed in the early development, in the fourth postnatal week. One other study detected mu-opioid receptor mRNA expression in the VTA in the first week of life in the rodent model however it was not the primary focus of that work (109). There is a clear need for further study into the

ontogeny of the mu-opioid receptor in the VTA, especially given the apparent sensitivity of expression in this brain area to maternal cafeteria diet exposure.

3.5.4 Summary and Speculation

The current study is the first to demonstrate that exposure to maternal cafeteria diet reduces the mRNA expression of the mu-opioid receptor in the VTA of female offspring during late (week 3 and 4) but not early (birth and week 1) postnatal development. These findings suggest that whilst exposure to a maternal cafeteria diet before birth and during the postnatal period, results in changes in mu-opioid receptor expression in late postnatal development, these effects are not present at birth, at least in female offspring. This study also highlighted sex differences in mu-opioid receptor expression in the VTA and NAc in response to maternal cafeteria diet exposure, emphasising the need to consider the sexes separately in studies looking at reward pathway development and the programming of food preferences. We hypothesise that the changes in mu-opioid receptor expression observed in response to a maternal cafeteria diet are driven by postnatal exposure to increased levels of endogenous opioids (as would be created by maternal cafeteria diet consumption), however this remains to be directly investigated. The current work has provided important and novel insights into the impact of maternal cafeteria diet exposure on postnatal mu-opioid receptor development and thus provided valuable information on a potential mechanism behind the programming of food preferences. Understanding such mechanisms will be crucial to stop the transfer of a preference for junk food from mother to child.

Chapter 4

A maternal “junk-food” diet reduces sensitivity to the opioid antagonist naloxone in offspring postweaning

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Jessica Gugusheff (candidate): Performed majority of animal work and sample analysis. Analysed and interpreted data, wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: Date: 9 September 2014

Zhi Yi Ong: Established feeding behaviour protocol used in the work and contributed to sample analysis.

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 July 2014

Beverly Muhlhausler: Assisted in conceptualisation of the work and data interpretation. Contributed to manuscript construction and evaluation.

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 September 2014

Chapter 4: A MATERNAL 'JUNK FOOD' DIET REDUCES SENSITIVITY TO THE OPIOID ANTAGONIST NALOXONE IN OFFSPRING POST-WEANING

4.1 Abstract

Perinatal exposure to a maternal junk food diet has been demonstrated to increase the preference for palatable diets in adult offspring. We aimed to determine whether this increased preference could be attributed to changes in mu-opioid receptor expression within the mesolimbic reward pathway. We report here that mRNA expression of the mu-opioid receptor in the ventral tegmental area (VTA) at weaning was 1.4 fold (males) and 1.9 fold (females) lower in offspring of junk food (JF) fed rat dams than in offspring of dams fed a standard rodent diet (control) ($P<0.05$). Administration of the opioid antagonist naloxone to offspring given a palatable diet post-weaning significantly reduced fat intake in control offspring (males: 7.7±0.7 *vs.* 5.4±0.6 g/kg/d; females: 6.9±0.3 *vs.* 3.9±0.5g/kg/d; $P<0.05$), but not in male JF offspring (8.6±0.6 *vs.* 7.1±0.5g/kg/d) and was less effective at reducing fat intake in JF females (42.2±6.0% *vs.* 23.1±4.1% reduction, $P<0.05$). Similar findings were observed for total energy intake. Naloxone treatment did not affect intake of standard rodent feed in control or JF offspring. These findings suggest that exposure to a maternal junk food diet results in early desensitisation of the opioid system, which may explain the increased preference for junk food in these offspring.

4.2 Introduction

Excessive maternal intake of 'junk foods' during pregnancy and lactation has been shown to program an increased preference for fat and sugar in juvenile and adult offspring (35,37). The term 'junk food' can be used to encompass a wide variety of foods that can be high in fat, sugar or salt as well as energy dense and nutrient poor. The commonality between all junk foods is that they are highly palatable and since the preference for palatable food is thought to be regulated, at least in part, by activation of the mesolimbic reward pathway, this pathway has become the focus of studies attempting to determine the mechanisms underlying the programming effects of maternal junk food consumption on offspring food preference (36,122,229).

Within the mesolimbic reward system, opioid signaling plays a central role in eliciting the pleasurable sensation associated with rewarding stimuli (54,230). The consumption of junk foods is associated with an increased concentration of endogenous opioids within the reward pathway (62,96) that then bind to opioid receptors in the ventral tegmental area (VTA) to stimulate dopamine release (47). Existing investigations into the mesolimbic reward pathway of adult rats, which have been exposed to a cafeteria diet (a well–established rodent model of junk food consumption (41), consisting of a variety of foods that are energy dense, nutrient poor and highly palatable) *in utero* and during the suckling period have highlighted mu-opioid receptor expression within this pathway as being particularly susceptible to alteration. We and others have demonstrated an increased expression of the mu-opioid receptor in the nucleus accumbens (NAc) of adult (122) and juvenile offspring (37) exposed to a cafeteria diet during the perinatal period.

A possible explanation for the effects of a maternal junk food diet on mu-opioid receptor expression in the offspring is that high levels of endogenous opioids, as would be expected in response to a junk food diet, may impact on opioid receptor ontogeny. Chronic consumption of junk food in adult rodents has been demonstrated to reduce the expression of mu-opioid receptor in the NAc (231) whilst excessive sugar intake followed by opioid antagonist administration results in symptoms of opiate withdrawal (101). Importantly, opioids have been previously demonstrated to readily cross the placenta (144) and into breastmilk (232,233), suggesting that increases in maternal opioid levels are likely to result in increased concentrations of opioids in fetal and neonatal circulation. However, the impact of a junk food diet and subsequent increases in endogenous opioids, on the expression of the mu-opioid receptor in the early postnatal period is yet to be adequately explored.

The sensitivity of the developing mu-opioid receptor to the nutritional environment during development, as well as its involvement in the regulation of palatable food intake, has led us to focus on the role of the opioid system in programming of the preference for junk food. Although alterations to mu-opioid receptor expression have been previously observed in adult offspring of dams fed a cafeteria diet during pregnancy and lactation (37,122) it remains unclear whether these changes in expression are present in the early postnatal period, prior to the increase in palatable food intake. It also remains to be determined whether or not changes in mRNA expression of the mu-opioid receptor at weaning in these offspring perinatally exposed to a cafeteria diet have functional consequences for the subsequent regulation of food intake in these offspring. Therefore, the aim of the current study was to determine whether

exposure to a maternal 'junk food' diet during the perinatal period was associated with altered mu-opioid receptor expression in the offspring at weaning and if these changes impacted on the efficacy of the opioid antagonist naloxone in reducing the intake of a cafeteria diet in the immediate post-weaning period.

4.3 Methods

4.3.1 Animals and feeding regime

This study was approved by the Animal Ethics Committee of the University of Adelaide. 17 female and 4 male Albino Wistar rats were used in these experiments. The animals were allowed to acclimatize to the animal housing facility for 1 week prior to the commencement of the dietary intervention. During this period, all rats were fed a standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia). Following the acclimatization period, rats were assigned into weight matched groups designated either control (C, n=8) or junk food (JF, n=9). The control group received a diet consisting of the standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia). The junk food group were fed a cafeteria diet which included hazelnut spread, peanut butter, chocolate biscuits, savoury snacks, sweetened cereal and a lard and chow mix. Detailed nutritional information on this diet has been previously published (37). Food intake was determined every 2 days by subtracting the amount left uneaten in the cage from the amount initially supplied and rats were weighed weekly for the duration of the experiment. All animals were individually housed and kept at a room temperature of 25°C in a 12 hour/12 hour light-dark cycle throughout the experiment.

The female rats were provided with their respective diets for 2 weeks prior to mating and throughout pregnancy and lactation. Females were mated with 4 proven males (same males used for both control and junk food groups), that were maintained on the standard laboratory rodent feed. Vaginal smears were performed to determine the stages of the estrous cycle. On the night of di-estrous/pro-estrous, the female rat was placed with a male overnight and vaginal smears were conducted the following morning. The presence of sperm in the vaginal smears was considered as confirmation of successful mating and was designated as gestation day 0.

Pups were born on day 21-22 of gestation. On the day after birth (postnatal day (PND) 1), pups were culled to 8 per litter, 4 males and 4 females where possible. Pups were weighed every 2 days during the suckling period and were weaned on PND 21. Pups of control and JF dams are referred to as control offspring and JF offspring respectively.

4.3.2 Determination of mu-opioid receptor gene expression in the NAc and VTA

At weaning, a subset of both male (control n=10, JF n=9) and female pups (control n=8, JF n=8) were killed and whole brains removed. The NAc and VTA were isolated using stereotaxic coordinates and microdissection as described previously (37). Total RNA was extracted from these respective brain regions using Trizol reagent (Invitrogen Australia, Mount Waverley, Vic, Australia) and purified with an RNeasy Mini Kit (Qiagen Australia, Doncaster, Vic, Australia). cDNA was synthesized from the purified RNA using Superscript III reverse transcriptase (Invitrogen Australia, Mount Waverley, Vic, Australia) and random hexamers. Real-time qRT-PCR was performed on the LightCycler® 480 Real Time PCR System (Roche Diagnostics, Mannheim, Germany) using the SYBR green system. The primer sequences used for the mu-opioid receptor, have been previously published (37), mRNA expression of the reference gene β-actin was measured using the β-actin Quantitect primer assay (Qiagen Australia, Doncaster, Vic, Australia). The amplification efficiency of the primers was 0.997-0.999 and 2 quality controls were added to each plate to verify interplate consistency. The expression of mu-opioid receptor mRNA relative to β-actin expression was calculated using Q-gene qRT-PCR analysis software (http://www.biotechniques.org).

4.3.3 Naloxone treatment

Pups not used for gene expression analysis were housed with a same sex littermate and were randomly assigned to receive a daily intraperitoneal injection of either naloxone (5mg/kg) or an equivalent volume of saline for 10 days post-weaning. Naloxone hydrochloride dihydrate (5mg, purchased from Sigma Aldrich, St Louis, MO, USA) was dissolved in 10ml of sterile saline (a separate aliquot for each animal) and stored away from light at 4°C for the duration of the experiment. Rat pups were weighed prior to each injection to ensure accurate dosing. Injections were given 30 minutes prior to the onset of the dark cycle (5:30pm). This generated four groups, the offspring exposed to a control diet before weaning and given saline injections (C-C, n=16), the offspring exposed to a control diet before weaning and given naloxone injections (C-N, n=16), the offspring of JF dams given saline injections (JF-C, n=17) and the offspring of JF dams given naloxone injections (JF-N, n=17).

4.3.4 Determination of food preferences

Immediately following the administration of the naloxone/saline injections, the rats were returned to their home cage and provided with free access to both the standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia) and the cafeteria diet until the

time of the next injection 24 hours later. Due to the short half-life of naloxone, food intake of the offspring was measured in the 2 hour period immediately following injection (the period during which naloxone has previously been shown to persist in the brain at concentrations capable of inhibiting food intake (234)), as well as during the entire 24 hour period in all offspring.

Food intake was calculated by subtracting the amount left uneaten after the 2 or 24 hour time points from the amount supplied at the beginning of each period. The amount of standard laboratory rodent feed and each component of the cafeteria diet consumed within the 2 and 24 hour period was recorded and macronutrient preferences were calculated based on the nutritional composition of each food type. The amount of food consumed was normalised to offspring body weight. For all statistical analysis, pups in the same cage were considered as one unit. At the conclusion of the 10 day injection period (PND 31) pups were killed and tissues collected.

4.3.5 Post-mortem and tissue collection

Post-mortems were performed between 0800 and 1200 with rats weighed immediately prior to euthanasia. Blood samples were collected in heparinised tubes via cardiac puncture and centrifuged at 3,500 g at 4ºC for 15 minutes. Body weight, length (nose to tail) and abdominal circumference were determined. All internal organs were weighed and all visible fat depots, including omental fat (which included the mesenteric depot), retroperitoneal fat, gonadal fat, subcutaneous fat and interscapular fat, were dissected to determine the fat mass of individual fat depots and total fat mass. The weight of all internal organs and fat were expressed relative to body weight. All tissues and fat depots were frozen in liquid nitrogen and stored at -80°C for future molecular analyses.

4.3.6 Statistical analysis

Analysis of maternal food intake and body weight data as well as birth outcomes was conducted using Student's unpaired *t*-tests. The effect of maternal diet on mu-opioid receptor mRNA expression was analysed by two-way ANOVA with maternal treatment and sex as factors. The effect of naloxone treatment on the food intake of post-weaning offspring was analysed by one-way ANOVA in each sex, followed by Duncan's post-hoc analysis. Where significant differences in food intake (g/kg/2hr) between the saline and naloxone groups were observed for both control and JF offspring, Student's unpaired *t*-tests were used to compare the magnitude of the change in food intake caused by naloxone treatment between maternal

treatment groups. One and two-way ANOVA, as well as the Student's unpaired *t*-tests, were performed using SPSS statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Offspring body weight gain was analysed by two-way repeated measures ANOVA, which was performed using Stata 11 software (StataCorp., TX, USA). Male and female offspring were analysed separately for all measures except where stated. All data presented as mean±SEM with a *P* value of <0.05 considered statistically significant.

4.4 Results

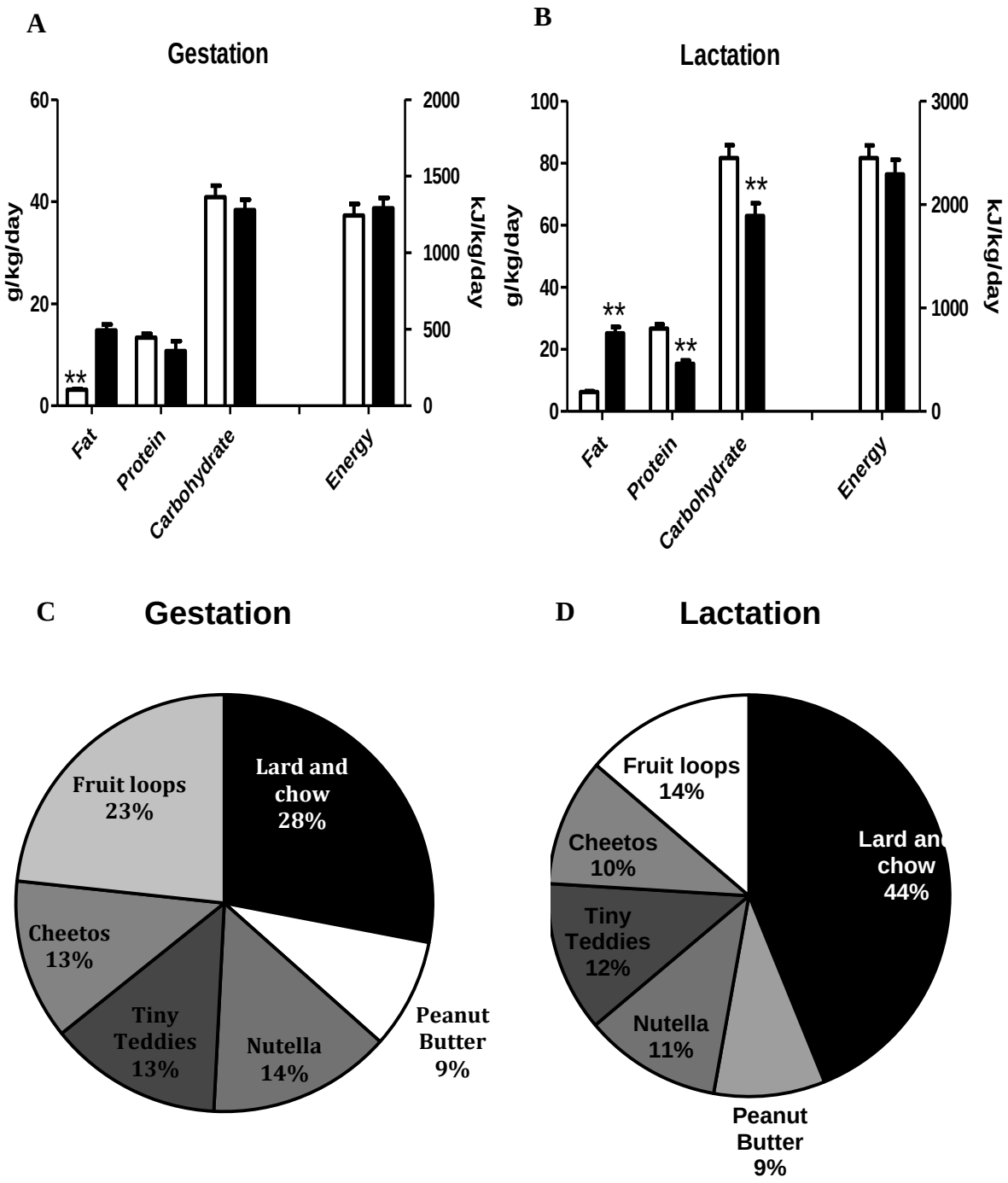
4.4.1 Dam body weight and nutritional intake during pregnancy and lactation

There was no difference in body weight between the control and JF dams prior to mating (control 332.8±9.6g, JF 324.1±9.4g) or throughout pregnancy. During pregnancy, the JF dams consumed significantly more fat than the controls without any differences in protein, carbohydrate, or overall energy intake (Fig.4.1A). Throughout lactation, in addition to increased fat intake the JF dams also consumed less protein and carbohydrate compared to control dams (Fig.4.1B). The composition of the diet of JF dams during gestation and lactation is shown in Fig 4.1C and 4.1D. All dams ate a variety of foods during both these periods, with the main difference being a higher intake of lard and chow mix during lactation compared to intake during gestation

4.4.2 Effect of maternal diet on birth outcomes and pup growth

Maternal diet had no effect on litter size (control 14.6±0.8, JF 13.2±1.2) or the percentage of males per litter (control 53.2±4.0%, JF 60.6±5.1%). Offspring of JF dams had a significantly lower birth weight for both the male (control 7.0±0.2g, JF 6.0±0.1g, $P<0.01$) and female pups (control 6.4±0.2g, JF 5.7±0.2g, $P<0.05$). The offspring of JF dams remained lighter than controls throughout the suckling period and were still significantly lighter than control offspring at weaning (PND 21) in both males and females (male control 53.7±1.7g, male JF 45.0±1.1g; female control 52.3±1.6g, female JF 43.9±0.8g, $P<0.01$).

Figure 4.1



Intake of fat, protein, carbohydrate and total energy of control (open bars, n=8) and JF dams (closed bars, n=9) during gestation (A) and lactation (B). Results presented as mean±SEM, ** indicates $P<0.01$. Intake of individual components of the cafeteria diet as a percentage of total food intake in the JF dams during gestation (C) and lactation (D).

4.4.3 Effect of maternal diet on the expression of the mu-opioid receptor in the NAc and VTA of the offspring at weaning

The mRNA expression of the mu-opioid receptor in the VTA at weaning was lower in offspring of JF dams compared to controls in both males and females (Fig.4.2A $P<0.05$). In the NAc, mu-opioid receptor mRNA expression at weaning was higher in male offspring of JF dams compared to controls (Fig. 4.2B $P<0.05$). There was no effect of maternal diet on mu-opioid receptor expression in the NAc at weaning in female offspring (Fig.4.2B).

4.4.4 Effect of maternal diet and naloxone treatment on offspring growth and body composition

Naloxone treatment had no effect on body weight at any time point during the experiment in either the control or JF offspring. At the end of the injection period (10 days after weaning), both male (C-C 102.6±4.0g, C-N 103.8±3.9g, JF-C 83.3±2.6g, JF-N 88.2±1.8g, $P<0.01$) and female (C-C 98.5±3.7g, C-N 101.7±4.4g, JF-C 82.8±2.3g, JF-N 82.5±2.8g, $P<0.01$) offspring of JF dams were significantly lighter than controls, independent of whether they were treated with saline or naloxone. In males, offspring of JF dams had a higher subcutaneous fat mass compared to control offspring independent of whether they received saline or naloxone (Table 4.1, $P<0.05$); no effect of group was observed on the weight of any other fat depots or total fat mass (Table 4.1). There was no effect of either maternal diet or naloxone treatment on fat deposition in female offspring (Table 4.1).

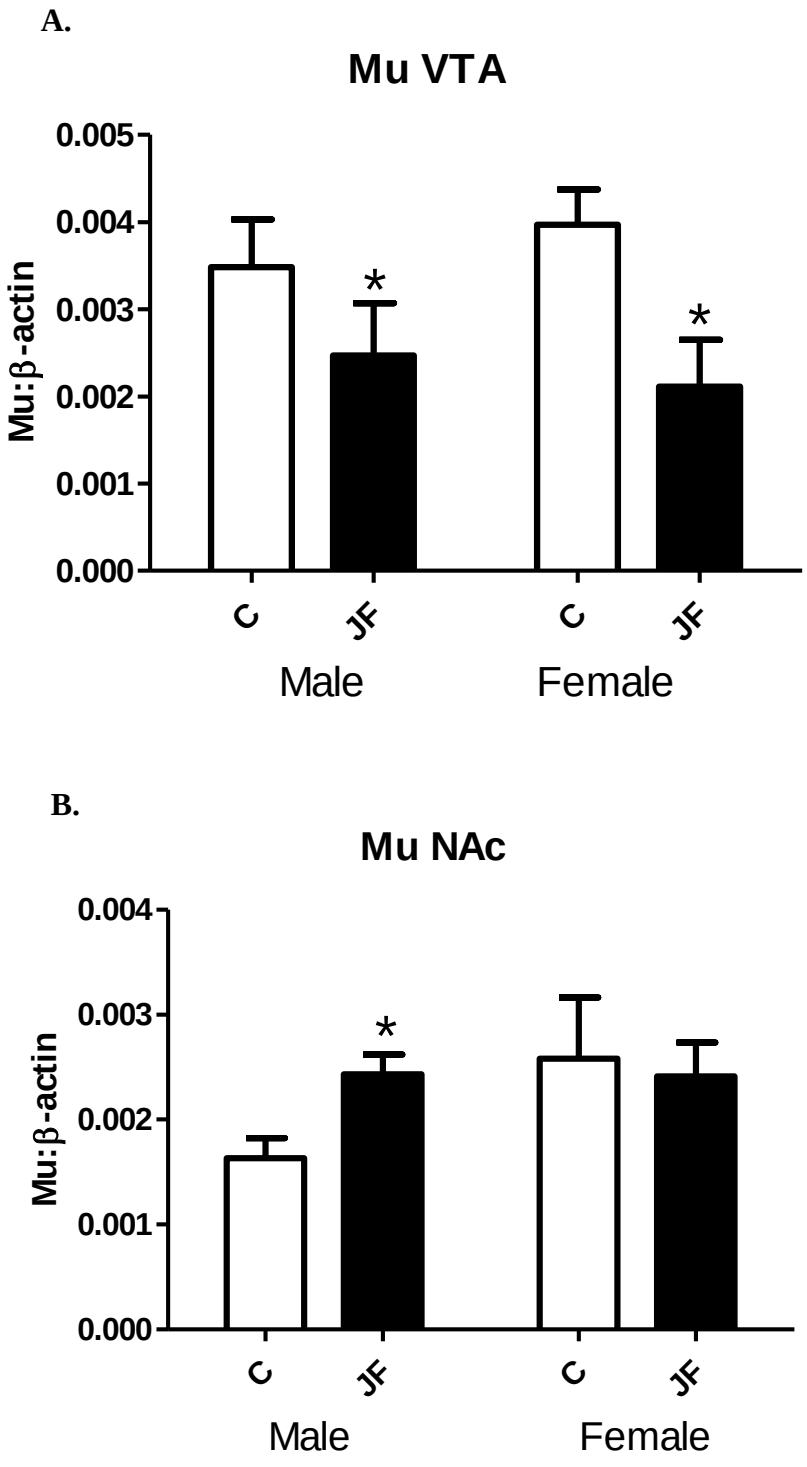
4.4.5 Effect maternal diet and naloxone treatment on offspring food intake

2hrs post injection

In control offspring, the intake of fat, carbohydrate, protein and total energy were all significantly reduced in the 2 hours post injection in those offspring receiving naloxone injections, compared with those administered saline in both males and females (Fig. 4.3A-D, $P<0.05$). In the offspring of JF dams, however, naloxone treatment either had no effect on intake, or supressed food intake to a significantly lesser extent when compared to the effects observed in offspring of control dams.

In the male offspring of JF dams, there was no effect of naloxone treatment on fat intake in the 2 hours post injection (Fig.4.3A). In female JF offspring, the decrease in fat intake in naloxone-treated offspring compared to their saline-treated counterparts was significantly less

Figure 4.2



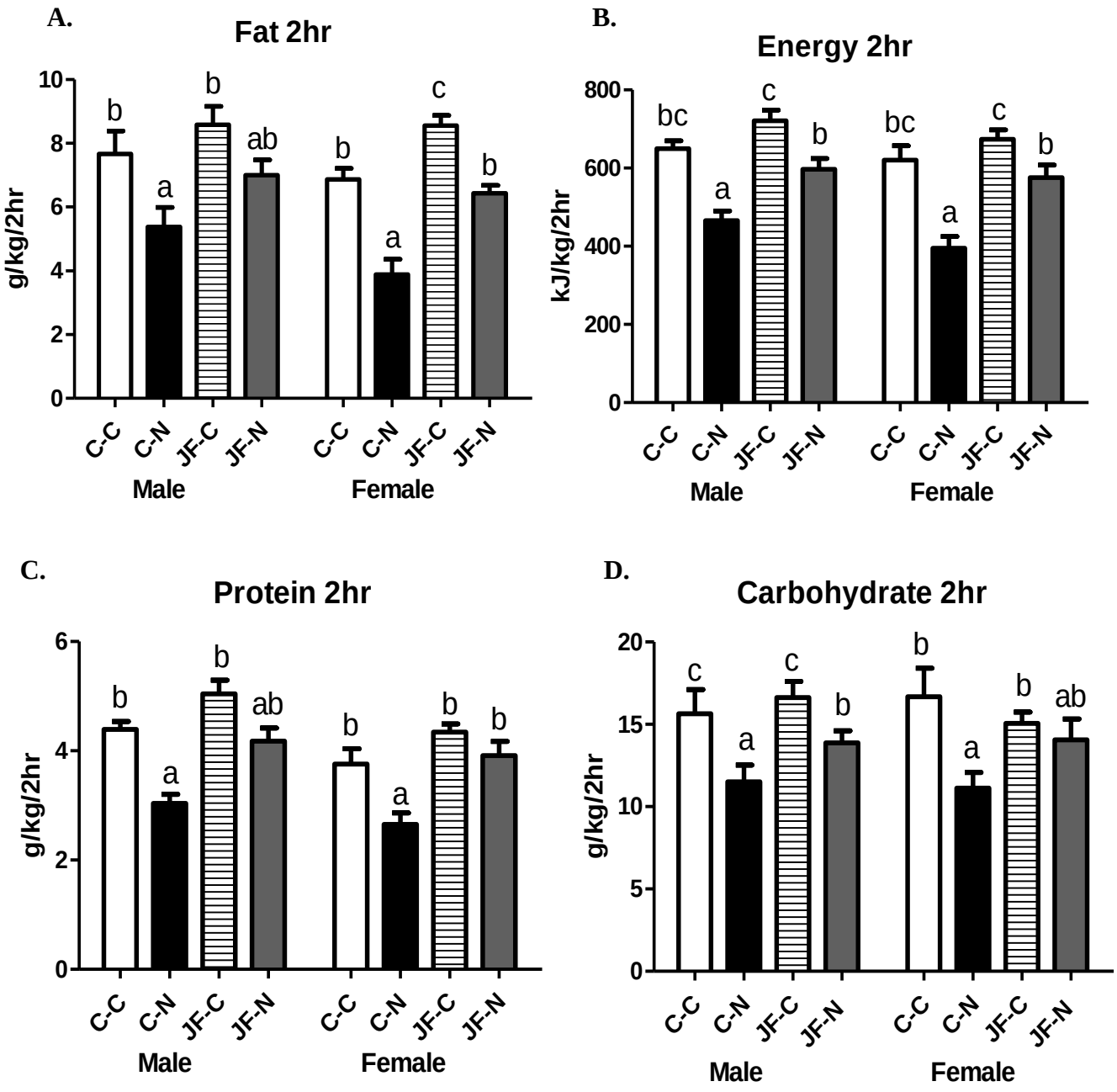
Expression of the mu-opioid receptor in male and female offspring of control (open bars, n=18) and JF dams (closed bars, n=17) in (A) the VTA and (B) the NAc at weaning (PND 21). Results presented as mean±SEM, * indicates $P<0.05$.

Table 4.1.Fat depots as a percentage of body weight in male and female offspring of control or JF dams after repeated daily intraperitoneal injections with saline or naloxone for 10 days post-weaning (PND 31)

	Male				**Female**			
Parameter	C-C	C-N	JF-C	JF-N	C-C	C-N	JF-C	JF-N
Omental fat	0.7±0.05	0.8±0.06	0.9±0.04	0.7±0.04	0.8±0.05	0.8±0.05	0.8±0.04	0.7±0.06
Retroperitoneal fat	1.0±0.13	1.1±0.08	0.9±0.06	1.1±0.04	0.9±0.09	0.9±0.05	0.8±0.02	0.8±0.08
Epigonadal fat	0.7±0.04	0.7±0.03	0.6±0.06	0.6±0.04	0.9±0.05	0.9±0.08	0.7±0.10	0.7±0.06
Interscapular fat	0.6±0.07	0.7±0.08	0.5±0.05	0.6±0.06	0.6±0.04	0.6±0.05	0.5±0.03	0.5±0.04
Subcutaneous fat	6.0 ± 0.41^{a}	6.4 ± 0.27^{ab}	7.2 ± 0.44^{bc}	7.8 ± 0.33^{c}	7.8±0.28	6.9±0.48	7.6±0.46	7.9±0.59
Total fat	9.1±0.52	9.8±0.29	10.1±0.58	10.7±0.42	10.8±0.39	10.0±0.62	10.1±0.48	10.6±0.75

Values are expressed mean ± SEM., n=8 pups for all groups except JF-C and JF-N in the male offspring, where n=9. Different superscript letters denote values that are significantly different, *P<0.05*

Figure 4.3



Intake of fat (A), total energy (B), protein (C) and carbohydrate (D) 2 hours post injection of male and female offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of JF dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars). Results presented as mean±SEM, n=8 pups for all groups except JF-C and JF-N in the male offspring, where n=9. Different letters above the bars denotes mean values which are significantly different $P<0.05$.

than that observed in the offspring of control dams (control 42.2±6.0% reduction, JF 23.1±4.1% reduction, $P<0.05$) (Fig.4.3A).

Total energy intake in both sexes and the intake of carbohydrate in males only, were significantly reduced in the naloxone treated offspring of JF dams compared to their saline treated counterparts during the 2 hours post injection. However, in all cases, the magnitude of these effects was significantly less than observed in the offspring of control dams (Fig.4.3B, D). Naloxone treatment failed to significantly reduce protein intake in the JF offspring in both males and females and carbohydrate intake in female JF offspring was also not different between naloxone and saline treated animals (Fig.4.3C, D).

Analysis of intake of specific components of the cafeteria diet, showed that, in female offspring of both control and JF dams, consumption of hazelnut spread (C-C 5.7±0.8g/kg/d, C-N 2.8±0.5g/kg/d, JF-C 7.1±0.7g/kg/d and JF-N 4.8±0.7g/kg/d, $P<0.01$) and peanut butter (C-C 3.5±0.8g/kg/d, C-N 2.1±0.7g/kg/d, JF-C 5.9±0.6g/kg/d and JF-N 3.6±0.9g/kg/d, $P<0.05$) were significantly inhibited by naloxone. There were no other significant effects of naloxone treatment on the intake of other specific junk foods in either male or female offspring.

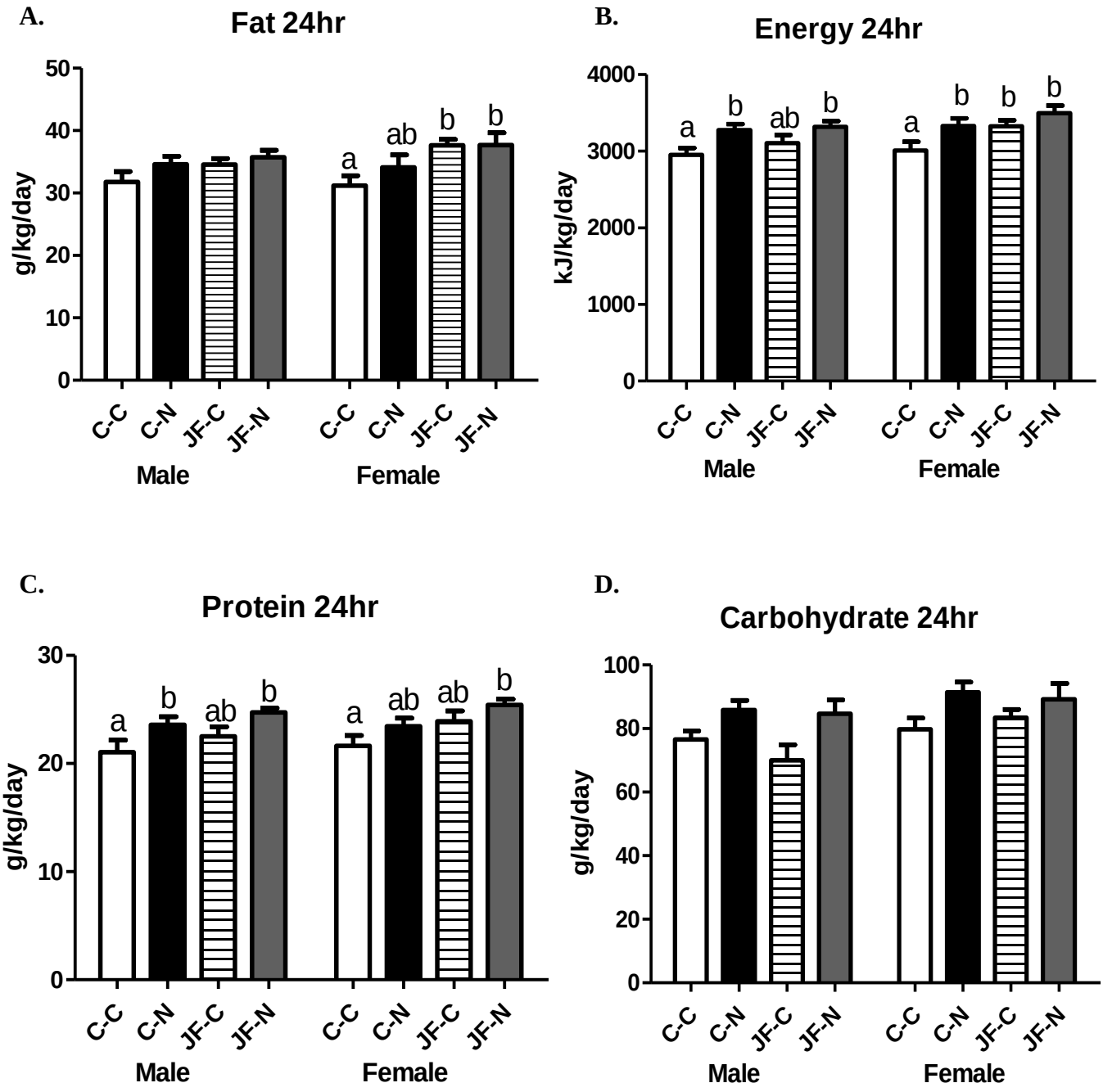
The effects of naloxone on food intake appeared to be specific to the cafeteria diet, since intake of the standard rodent feed offered at the same time was not significantly altered by naloxone treatment in either control or JF offspring in either males (C-C 7.8±1.3g/kg, C-N 5.5±1.2g/kg, JF-C 9.45±1.6g/kg, and JF-N 7.2±1.0g/kg) or females (C-C 6.0±1.3g/kg, C-N 3.4±1.2g/kg, JF-C 6.8±1.0g/kg, and JF-N 8.0±1.8g/kg).

24hrs post injection

In females only, offspring of JF dams consumed significantly more fat than their control counterparts (Fig.4.4A, $P<0.05$). There were no effects of the naloxone treatment on intake of either total energy or any individual macronutrients in either the control or JF offspring (Fig. 4.4B-D). There was also no difference in the intake of either the standard rodent feed or any individual component of the cafeteria diet between saline and naloxone treated offspring.

Investigation into the intake of individual foods revealed that, for females only, the offspring of JF dams consumed significantly less sweetened cereal than controls independent of injection treatment (C-C 32.9±3.9g/kg/d, C-N 38.3±7.2g/kg/d, JF-C 13.9±2.5g/kg/d and JF-N

Figure 4.4



Intake of fat (A), total energy (B), protein (C) and carbohydrate (D) 24 hours post injection of male and female offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of JF dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars). Results presented as mean±SEM, n=8 pups for all groups except JF-C and JF-N in the male offspring, where n=9. Different letters above the bars denote mean values which are significantly different $P<0.05$.

17.5±6.2g/kg/d, $P<0.01$). No difference in intake between groups was observed for the other junk foods or in male offspring. There was no effect of either maternal diet or naloxone treatment on the intake of the standard rodent feed in either male (C-C 74.6±8.8g/kg/d, C-N 89.2±11.4g/kg/d, JF-C 82.2±12.4g/kg/d and JF-N 104.6±21.0g/kg/d) or female (C-C 86.8±8.1g/kg/d, C-N 104.1±11.3g/kg/d, JF-C 67.6±12.4g/kg/d and JF-N 82.5±18.6g/kg/d) offspring.

4.5 Discussion

In the present study, we have shown that exposure to a maternal cafeteria diet during pregnancy and lactation is associated with altered expression of the mu-opioid receptor in both the VTA and NAc at weaning in a region- and sex-specific manner, demonstrating for the first time that the effects of perinatal junk food exposure on the opioid system are already present immediately following the exposure. We have also demonstrated that the opioid receptor antagonist naloxone was less effective at reducing the intake of the cafeteria diet in offspring exposed to the same diet during the perinatal period, consistent with a reduced sensitivity to opioids in these offspring. This study is the first to demonstrate that the changes in mu-opioid receptor expression previously observed in adult offspring of dams fed a cafeteria diet are already present at weaning and that these changes in expression have functional consequences for the regulation of food intake. This work provides important and novel insights into the pathway linking perinatal exposure to a junk food diet with a heightened preference for these foods after birth and adds to the ever growing body of evidence suggesting that a maternal junk food diet can alter the development of the reward pathway of the offspring and that these changes affect food choices from weaning into adulthood.

4.5.1 Maternal junk food consumption decreases rate of postnatal growth of offspring

Consistent with previous studies (35,37), the offspring of JF dams were born smaller and remained smaller than their control counterparts throughout the suckling period. This reduction in body size has been previously attributed to reductions in protein intake; however in the current model, protein intake did not differ between maternal groups during gestation. Thus, the decreased birth weight of the offspring of JF dams appears to be driven by a mechanism other than protein deficiency. Whilst the caloric intake of the JF dams was increased relative to controls, our preliminary analysis of the cafeteria diet provided suggests that it is deficient in a number of key micronutrients including magnesium and calcium,

which have been associated with poor fetal growth outcomes clinically (235,236) and may have contributed to the reduced birthweight of the JF offspring. Whether micronutrient deficiencies also have the potential to impact on the development of the reward system has yet to be defined, and will be an important question to pursue in future studies. During lactation, the protein intake of JF dams was significantly lower than controls, which is likely to have exacerbated the effect of any other nutrient deficiencies present during gestation and contributed to the reduced body weight of the offspring, since exposure to a low-protein diet during the suckling period has been consistently shown to reduce the body weight of offspring (209,210).

4.5.2 Maternal junk food diet decreases expression of mu-opioid receptor in VTA

An important finding of the present study was that alterations to the mRNA expression of the mu-opioid receptor in key regions of the mesolimbic reward system were already present at weaning in the offspring of JF dams. Interestingly, these changes in expression appeared to be dependent on the specific region of the reward pathway examined, with decreased mu-opioid receptor expression observed in the VTA of both sexes and increased expression observed in the NAc of male offspring only. A possible explanation for this disparity of expression between brain areas is their differing rates of development. The appearance and subsequent increase in abundance of the mu-opioid receptor in the brain during development follows a caudal to rostral pattern (108,130), such that mu-opioid receptor proliferation in the VTA occurs earlier in development than that in the NAc. The effects of opioids on receptor development are greatest at times of rapid proliferation (237) and given our hypothesis that the differences in expression in the offspring of junk food fed dams may be driven by increases in maternal endogenous opioids, it may be that the effect on expression is different depending on the stage of development at which the exposure to increased opioid concentrations occurs.

Whilst the current work is the first to investigate the effect of exposure to a cafeteria diet *in utero* and during suckling on mu-opioid receptor expression at weaning, previous studies have reported changes in mRNA expression of the mu-opioid receptor in adult offspring exposed to similar diets perinatally. Vucetic *et al*. demonstrated that adult offspring of dams fed a palatable high-fat diet and weaned onto a standard rodent feed, had an increased expression of the mu-opioid receptor in the NAc at 18-24 weeks of age (122), whereas studies in our own laboratory have shown that offspring of JF dams weaned onto a cafeteria diet exhibited an

increased expression of the mu-opioid receptor mRNA in the NAc at 6 weeks of age and decreased expression in adulthood after being maintained on a cafeteria for 6 weeks post-weaning. Interestingly, we found no changes in the expression of mu-opioid receptors in the VTA in that same study (37). Viewing these results in light of the current findings suggests that, at least in male offspring, a maternal junk food diet increases the expression of the mu-opioid receptor in the NAc at weaning and that this increased expression can persist until adulthood if offspring are weaned onto a standard rodent diet. However, previous work in our own laboratory has revealed that this expression pattern can be reversed by prolonged exposure to a cafeteria diet during adolescence (37). The studies in the adult offspring of JF dams have focused primarily on changes in gene expression in the NAc rather than the VTA, and it is apparent that further studies are required to better elucidate the effect of a maternal cafeteria diet on the VTA in adulthood.

4.5.3 Maternal junk food consumption decreases the effectiveness of naloxone in the offspring

This is the first study to directly demonstrate that changes in mu-opioid receptor expression induced by exposure to a maternal junk food diet have functional consequences for the regulation of palatable food intake in the offspring. We found that the offspring of JF dams were significantly less sensitive to the inhibitory effects of the opioid receptor antagonist naloxone on intake of the cafeteria diet than offspring of control dams. We also observed that the offspring of control dams given naloxone consumed significantly more total energy from 2 to 24 hours post injection compared to those administered saline, in agreement with studies in adult rodents which have also demonstrated an increase in energy intake after the initial suppression of consumption by naloxone injection (238,239). That a compensatory increase in energy intake was observed in the offspring of control dams and not in the offspring of JF dams, supports the finding that naloxone was less effective at inhibiting food intake in these animals.

Naloxone was selected as the antagonist as it only persists at concentrations capable of reducing food intake for two hours post injections (234,240), this acute treatment was selected to minimize the impact prolonged suppression of food intake would have on the growth of the pups. In line with this, we did not observe any difference in body weight between those pups given saline and those given naloxone within the same maternal dietary group. Naloxone or saline injections were given at the onset of the dark cycle for all offspring as it is during this

period that rodents have been demonstrated to consume the most food (241), this allowed for the best observation of the effectiveness of naloxone administration on inhibiting food intake.

Previous studies conducted in adult rodents have demonstrated that the effectiveness of naloxone at inhibiting food intake is dependent on the palatability of the food being consumed (96,242). Consistent with this, we observed no effect of naloxone treatment on the intake of the standard rodent feed in the current study, suggesting that the observed changes in macronutrient intake were specifically due to reduced consumption of the cafeteria diet, rather than an overall decrease in food intake. However, unlike previous studies which have reported the suppressive effect of naloxone to be fat specific (234,243), we observed decreases in the intake of all macronutrients (protein, fat, carbohydrate) in the offspring of control mothers where treatment was most effective. This can most likely be attributed to the fact that a number of the components of the cafeteria diet provided to the offspring were high in both fat and sugar making it difficult to distinguish between the effects on intake of these different macronutrients. Similarly, the effects on protein intake were likely caused by the inhibitory effect of naloxone on peanut butter intake which contains approximately 50% fat and 20% protein.

The reduced sensitivity of the offspring of JF dams to the effects of naloxone on the intake of the cafeteria diet suggests that there was reduced mu-opioid receptor binding in the reward pathway of these offspring. Naloxone is a non-specific opioid receptor antagonist, which binds preferentially to the mu-opioid receptor over kappa or delta receptors (244,245). Naloxone binding to the mu-opioid receptor is thought to inhibit palatable food intake by blocking the binding of endogenous opioids which have been demonstrated to stimulate the intake of junk foods (61,62). Reduced sensitivity to the effects of naloxone in the JF offspring is in agreement with our finding of reduced mu-opioid receptor expression in the VTA of these offspring at weaning and suggests that there was reduced mu-opioid receptor binding in the JF offspring. Mu-opioid receptor binding in the VTA regulates the release of dopamine into the NAc, which is the major terminal area of A10 dopamine neurons (246,247). These results imply that it may be mu-opioid receptor involvement in the control of dopamine release which is the critical mechanism altered by exposure to a cafeteria diet during the perinatal period. That opioid regulation of dopamine is involved in the programming of food preferences is supported by studies looking at adult offspring of dams fed a cafeteria diet

which have demonstrated changes in both the opioid and dopamine systems of these animals (122,229,248).

4.5.4 Sex differences in the programming of food preferences by a maternal junk food diet

We observed that female, but not male, offspring of JF dams had higher fat intake compared to controls during the first 10 days post-weaning. This was in contrast to previous work in adult offspring which reported a higher preference for fat across both sexes in offspring exposed to a cafeteria diet during the perinatal period (35,37). However, in a maternal low-protein diet model, female offspring of the low protein dams had a higher fat intake when compared to their control counterparts, whereas the same effect was not observed in males (210). Therefore, there is some evidence to suggest that the regulation of fat intake in females may be more susceptible to alteration by maternal diet before birth and early in life than that of male offspring. The differences in fat intake between males and females may be attributed to differences in the rate of development of the opioid system between the sexes, however this is poorly explored in the literature and there remains a need for further investigation. Another possible explanation for the sex-specific effect observed is differences in the concentrations of gonadal hormones between the sexes, as estrogen has previously been implicated in the regulation of the endogenous opioid system (223,224).

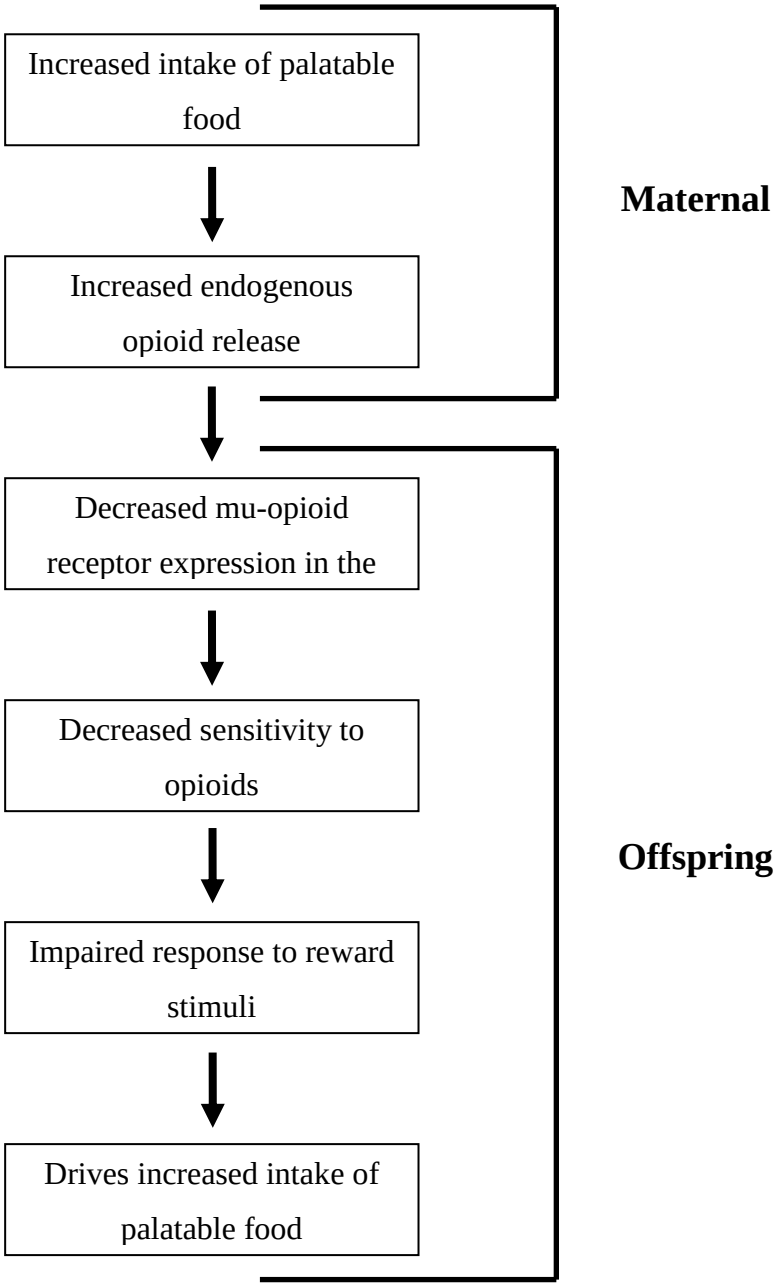
Despite our finding that it was only the female offspring JF dams that had an increased preference for the cafeteria diet in early life; it was the male and not female offspring who had an increase in subcutaneous fat mass. The difference in fat mass in male offspring but not females could be attributed to differences in fat metabolism (199,249). Nevertheless, the lack of differences in total fat deposition observed between the control JF offspring in this study has also been previously reported when both the control and JF offspring were weaned on the cafeteria diet and control diet until 6 weeks and 3 months of age (37).

4.5.5 Summary and Speculation

The present study is the first to demonstrate that a maternal junk food diet during pregnancy and lactation has functional consequences on the reward pathway of the offspring immediately post-weaning, by reducing the ability of an opioid receptor antagonist to suppress the intake of a cafeteria diet. This study also shows that the alterations in opioid receptor expression are already present at weaning and can impact on the regulation of food preferences in the offspring even at this early age. Furthermore, it is likely that decreases in mu-opioid receptor expression and sensitivity present at weaning could have a longer term

impact on the food choices of these offspring, as there would be a need to increase junk food intake to overcome this early desensitisation. We speculate that these changes in the expression and functionality of the opioid system in offspring exposed to a cafeteria diet during the perinatal period may be a result of exposure to high levels of endogenous opioids generated by maternal junk food consumption (Fig.4.5), and it will be important to investigate this hypothesis directly in future studies. This work has provided novel insights into a potential mechanism through which a maternal junk food diet increases the preference for junk food in the offspring. A better understanding of this mechanism is crucial if we are to develop possible strategies for intervention and becomes increasingly important in view of the rapidly rising rates of both childhood and adult obesity.

Figure 4.5



Summary of proposed mechanism through which a maternal junk food diet could establish the preference for palatable food in offspring. We speculate that maternal consumption of a junk food diet throughout pregnancy and lactation acts to increase maternal endogenous opioids levels. These opioids are then transferred to the offspring via the placenta and/or through the breast milk and act on the developing reward pathway to decrease expression of the mu-opioid receptor in the VTA. The resulting densitisation to the effect of endogenous opioids in the offspring of junk food fed mothers would drive an increased intake of palatable foods in order to achieve the same level of stimulation in the opioid pathway.

Chapter 5

Naloxone treatment alters gene expression in the mesolimbic reward system in ‘junk food’ exposed offspring in a sex-specific manner but does not affect food preferences in adulthood.

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Jessica Gugusheff (candidate): Performed majority of animal work and sample analysis. Analysed and interpreted data, wrote the manuscript.

I hereby certify that the statement of contribution is accurate

Signed: Date: 9 September 2014

Zhi Yi Ong: Established feeding behaviour protocol used in the work and contributed to sample analysis.

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 July 2014

Beverly Muhlhausler: Assisted in conceptualisation of the work and data interpretation. Contributed to manuscript construction and evaluation.

I hereby certify that the statement of contribution is accurate and I give my permission for the inclusion of this paper in the thesis.

Signed: Date: 28 September 2014

Chapter 5: NALOXONE TREATMENT ALTERS GENE EXPRESSION IN THE MESOLIMBIC REWARD SYSTEM IN 'JUNK FOOD' EXPOSED OFFSPRING IN A SEX-SPECIFIC MANNER BUT DOES NOT AFFECT FOOD PREFERENCES IN ADULTHOOD

5.1 Abstract

We have previously reported that the opioid receptor blocker, naloxone, is less effective at reducing palatable food intake in offspring exposed to a maternal cafeteria diet during the perinatal period, implicating a desensitisation of the central opioid pathway in the programming of food preferences. The present study aimed to investigate the effect of a maternal cafeteria diet and naloxone treatment on the development of the mesolimbic reward pathway and food choices in adulthood. We measured mRNA expression of key components of the reward pathway (mu-opioid receptor, proenkephalin, tyrosine hydroxlase, D1 and D2 receptors and the dopamine active transporter (DAT)) in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of the offspring of control and cafeteria fed (JF) dams at weaning and after a 10-day naloxone treatment post-weaning; and determined food preferences in adulthood in the remaining offspring. Naloxone treatment decreased the expression of DAT by 8.2 fold in female control offspring but increased it by 4.3 fold in female offspring of JF dams relative to the saline-injected reference groups. Proenkephalin mRNA expression was higher in the NAc of female JF offspring compared to controls, independent of naloxone treatment ($P<0.05$). There was no effect of naloxone treatment on food preferences in adulthood in either control or JF offspring. These data indicate that prenatal exposure to a cafeteria diet alters the impact of opioid signalling blockade in the early post-weaning period on gene expression in the central reward pathway in a sex specific manner, but that these changes in gene expression do not appear to have any persistent impact on food preferences in adulthood.

5.2 Introduction

The increased consumption of junk foods is a major contributing factor to the rise in obesity prevalence (4,250). The term 'junk food' can be used to describe any food that is high in fat, sugar and salt, low in protein and otherwise energy dense and nutrient poor (251). The ready availability of 'junk foods' in modern society makes it important to understand why some people have a greater tendency to over-consume these types of food than others. In this context, it is significant that data from both human and animal studies have shown that food preferences can be established very early in life, and that excessive maternal intake of junk foods during pregnancy and lactation is associated with an increased preference for fat and sugar in juvenile and adult offspring (35,37).

More recently, attempts have been made to understand the mechanisms which contribute to the programming of food preferences, with a particular focus on the impact of perinatal 'junk food' exposure on the mesolimbic reward system. We and others have demonstrated that maternal consumption of high-fat and/or cafeteria diets during pregnancy and lactation induces permanent alterations in the structure and function of this system in juvenile and adult offspring(37,122,248). We have previously shown that the mRNA expression of the mu-opioid receptor in the central reward pathway at weaning is reduced by perinatal exposure to a 'junk food' diet, and that this was associated with a reduced sensitivity to the effect of the opioid antagonist, naloxone, in reducing fat intake after weaning (208). These findings led us to hypothesize that the opioid signalling pathway plays a critical role in the early programming of food preferences, and that exposure to excess endogenous opioids during the perinatal period as a result of maternal junk food consumption (101,231) alters the development of the opioid signalling pathway in the offspring, resulting in persistent effects on the function of the reward system.

In the current study, we sought to extend our previous findings by investigating the impact of naloxone administration for a 10 day period after weaning on gene expression of key components of the mesolimbic reward pathway. Specifically, we aimed to determine the effect opioid receptor blockade on the mRNA expression of mu-opioid receptor and the endogenous opioid proenkephalin as well as elements of the dopamine pathway including tyrosine hydroxylase (TH), dopamine receptors 1 and 2 and the dopamine active transporter (DAT), all of which have been previously implicated in the regulation of palatable food intake(42,202,252-256). We hypothesized that opioid receptor blockade would ameliorate the

changes in gene expression in the dopamine and opioid pathways we have previously reported in offspring exposed to cafeteria diets during the perinatal period (203,208). We also aimed to investigate the hypothesis that opioid antagonist treatment in the immediate post-weaning period would have persistent effects on food preferences in the adult offspring, independent of perinatal dietary exposure.

5. 3 Methods

5.3.1 Animals and feeding

This study was approved by the Animal Ethics committee of the University of Adelaide. Details of the experimental procedure have been published previously (208). Briefly, 37 female and 4 male Albino Wistar rats were allowed to acclimatize to animal housing facility for at least 1 week before the commencement of the dietary intervention. Female rats were divided into either the control (C, *n*=18) or junk food (JF, *n*=19) group, such that the average weight of the animals at the start of the experiment was not different between treatments. The C group received a standard rodent feed (Specialty Feeds, Glen Forrest, WA, Australia), while the JF group received a cafeteria diet that included peanut butter, hazelnut spread, savory snacks, chocolate biscuits, sweetened cereal and a lard and chow mix. Detailed nutritional information on this diet has been published previously (37). The female rats were provided with their respective diets for two weeks prior to mating and throughout pregnancy and lactation.

Females were mated with one of 4 proven males (the same males were used for both groups), that were maintained on a standard rodent feed. Pups were born on day 21-22 of gestation and litters were culled to 8 pups (4 male, 4 female where possible) 24 hours after birth. Pups were housed with their mothers and weighed every second day until weaning at postnatal day 21. The offspring of C and JF dams are referred as C and JF offspring respectively.

5.3.2 Naloxone treatment

At weaning, pups were either killed for gene expression analysis or housed with a same sex littermate and administered the opioid antagonist naloxone or saline (vehicle). Details of this procedure have been published previously (208). Briefly, pups were randomly assigned to receive daily intraperitoneal injection of either naloxone, (5mg/kg, naloxone hydrochloride dihydrate, Sigma Aldrich, St Louis, MO USA) or an equivalent volume of saline (vehicle) 30 minutes before the onset of the dark cycle for 10 days. Naloxone at this dose has been

previously reported to acutely suppress food intake in pre-weaning rat pups (257) and we confirmed in a pilot dose-response study that this dose was the most effective in reducing intake of the cafeteria diet in the immediate post-injection period, without any adverse effects on pup growth/development. All rats were weighed immediately prior to injection to ensure accurate dosage. All offspring were given free access to both the control and cafeteria diet throughout the injection period to allow the determination of food preferences (results previously published (208)). This generated four groups of offspring, offspring of C dams given saline (C-C, *n*=15 male, 16 female), offspring of C dams given naloxone (C-N, *n*=16 male, 16 female), offspring of JF dams given saline (JF-C, *n*=18 male, 14 female) and offspring of JF dams give naloxone (JF-N, *n*=17 male, 14 female).

5.3.3 Determination of gene expression in the NAc and VTA at 3 weeks

Pups not designated to receive the naloxone/saline treatment were killed at weaning and the whole brain was removed (C, *n*=10 male, 8 female and JF, *n*=9 male, 8 female). The nucleus accumbens including both shell and core regions (NAc) and the ventral tegmental area (VTA) were isolated and stored as described previously (203). Total mRNA was extracted using Trizol reagent (Invitrogen Australia, Mount Waverley, Vic, Australia), purified using an RNeasy Mini kit (Qiagen Australia, Doncaster, Vic, Australia) and cDNA synthesized using Superscript III reverse transcriptase (Invitrogen Australia) and random hexamers.

Quantitative real-time RT-PCR was performed on the LightCycler® 480 Real Time PCR System (Roche Diagnostics, Mannheim, Germany) using the SYBR green system. Primer sequences for the mu-opioid receptor and the dopamine related genes: tyrosine hydroxylase (TH), dopamine receptor 1 (D1), dopamine receptor 2 (D2) as well as the dopamine active transporter (DAT) have been previously validated and published (203). mRNA expression of the reference gene β-actin was measured using the β-actin Quantitect primer assay (Qiagen Australia, Doncaster, Vic, Australia). The amplification efficiency of the primers was 0.997-0.999 and 2 quality controls were added to each plate to verify interplate consistency. The expression of target gene mRNA relative to β-actin expression was calculated using Q-gene qRT-PCR analysis software.

5.3.4 Determination of gene expression in the NAc and VTA at 3 weeks and 10 days

At the conclusion of the 10 day injection period during which both the standard rodent feed and cafeteria diet were available, a subset of offspring (C-C, *n*= 8 male, 8 female; C-N, *n*=8 male, 8 female; JF-C, *n*= 9 male, 8 female; JF-N, *n*=9 male, 8 female) were killed and brain

tissue collected. RNA was isolated from the VTA and NAc and cDNA generated as described for the 3wk time point. Quantitative real time PCR was performed using the SYBR green system on the Applied Biosystems ViiA 7 Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA). Target genes were the same as at 3 weeks with the addition of the endogenous opioid proenkephalin. The primer sequences have been previously published (32,203) and the proenkephalin primers were validated for use in our laboratory prior to beginning the experiment. The expression of target genes was quantified relative to three housekeeper genes: β-actin, cyclophilin and GAPDH, using the Applied Biosystems Data Assist software (Applied biosystems, Foster City, CA, USA). This software allows expression of each target gene to be measured against the mean normalised expression of the three housekeepers. Two quality controls as well as a negative RT control were used on each 384-well plate to ensure inter-plate consistency and melt curves were obtained at the end of each run.

5.3.5 Determination of food preferences

Following naloxone/saline treatment, remaining offspring (not used in gene expression analysis) were placed on the standard rodent feed until 10 weeks of age. Offspring (C-C, *n*= 7 male, 8 female; C-N, *n*=8 male, 8 female; JF-C, *n*= 9 male, 6 female; JF-N, *n*=8 male, 6 female) were then given access to the both the standard rodent feed and the cafeteria diet for a further two weeks until 12 weeks age. The amount of standard rodent feed and each component of the cafeteria diet consumed were assessed every two days and macronutrient preference calculated based on the nutritional composition of each food type. Body weight of the offspring was recorded weekly from weaning.

5.3.6 Postmortem

At 3 weeks, 3 weeks +10 days and 12 weeks of age, one male and one female pup from each litter was killed for the determination of body fat mass. The rats were not fasted prior to postmortem and all postmortems were conducted in light phase between 8-10 AM. All animals were weighed immediately prior to being killed with an overdose of CO_2. All internal organs were weighed and individual fat depots including retroperitoneal fat, omental fat, gonadal fat, interscapular fat and subcutaneous fat were isolated to determine the weight of each depot as well as total fat mass. All fat depots were snap frozen in liquid nitrogen and stored at -80°C for future molecular analyses. Blood samples were collected by cardiac

puncture into heparinized tubes, and blood was centrifuged at 3,500g, 4˚C for 15 minutes and plasma stored at -20˚C for subsequent analysis of hormone and metabolite concentrations.

5.3.7 Determination of hormone and metabolite concentrations

Plasma concentrations of glucose and non-esterified fatty acids (NEFA) were analysed using the Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) and the Wako NEFA C kit (Wako Pure Chemical Industries Ltd, Osaka, Japan), respectively. Assays were performed using Konelab 20 (Thermo Scientific, Vantaa, Finland). Plasma leptin and insulin concentrations were measured by immunoassay using the Crystal Chem Rat Leptin ELISA kit (Crystal Chem Inc, Downers Grove, IL, USA) and the ALPCO Insulin (Rat) Ultrasensitive ELISA kit (ALPCO Diagnostics, Salem, NH, USA). All assays were conducted in accordance with manufacturer's instructions and intra- and inter-assay coefficients of variation were <10%.

5.3.8 Statistical Analysis

Analysis of gene expression, plasma hormones, food preference and fat mass was conducted using a two-way ANOVA with maternal diet and naloxone/saline treatment as factors. The two-way ANOVA was performed using SPSS statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Offspring body weight gain and food intake over time was analysed by two-way repeated measures ANOVA, which was performed on Stata 11 software (StataCorp., TX, USA). Male and female offspring were analysed separately for all measures to provide clarity in presentation, as three-way ANOVA analysis using sex as an additional factor revealed a significant interaction between sex and treatment for the majority of outcomes measured. All data are presented as mean±SEM with a P value of <0.05 considered statistically significant.

5.4 Results

5.4.1 Effect of maternal diet on birth outcomes

Birth outcomes including birth weight of these offspring has been previously published (208). Both male (C= 53.7±1.7g, JF=45.0±1.1g, P<0.01) and female offspring (C=52.3±1.6g, JF=43.9±0.8g) of JF dams were significantly lighter at birth than their control counterparts (208).

5.4.2 Effect of maternal diet on target gene expression in the VTA and NAc at weaning

As published previously, mRNA expression of the mu-opioid receptor at weaning was lower in the VTA of both male and female JF offspring, and higher in the NAc of male, but not

female, JF offspring compared to C offspring (208). There was no effect of perinatal diet exposure on DAT expression in the VTA (Table 5.1), whilst in the NAc DAT expression was lower in the offspring of JF dams compared to controls in both males and females ($P<0.05$, Table 5.1). There was no difference in the mRNA expression of TH or the D1 and D2 dopamine receptors in either brain region between the control and JF offspring in either males or females (Table 5.1).

5.4.3 Effect of naloxone treatment on target gene expression in the VTA and NAc in control and JF offspring

Mu-opioid receptor and proenkephalin

There was no effect of naloxone treatment on mu-opioid receptor expression in the VTA of either male or female C or JF offspring (Fig.5.1A). The mRNA expression of the mu-opioid receptor in the NAc was higher in male JF offspring compared to controls ($P<0.05$), and also tended ($P=0.07$) to be higher in female JF offspring, independent of whether they received naloxone or saline (Fig.5.1B).

The effect of naloxone treatment during the post-weaning period on mRNA expression of the endogenous opioid, proenkephalin, in the VTA of male offspring was influenced by perinatal diet, such that mRNA expression was reduced by naloxone treatment only in those offspring exposed to a JF diet before weaning ($P<0.05$, Fig.5.1C). There was no effect of either perinatal diet or naloxone treatment on proenkephalin mRNA expression in the NAc of male offspring (Fig.5.1C). In females, there was no effect of either naloxone treatment or perinatal diet on proenkephalin mRNA expression in the VTA. Proenkephalin expression in the NAc, however, was significantly higher in female JF offspring compared to control offspring independent of naloxone treatment ($P<0.05$, Fig.5.1D).

DAT, TH and the D1 and D2 dopamine receptors

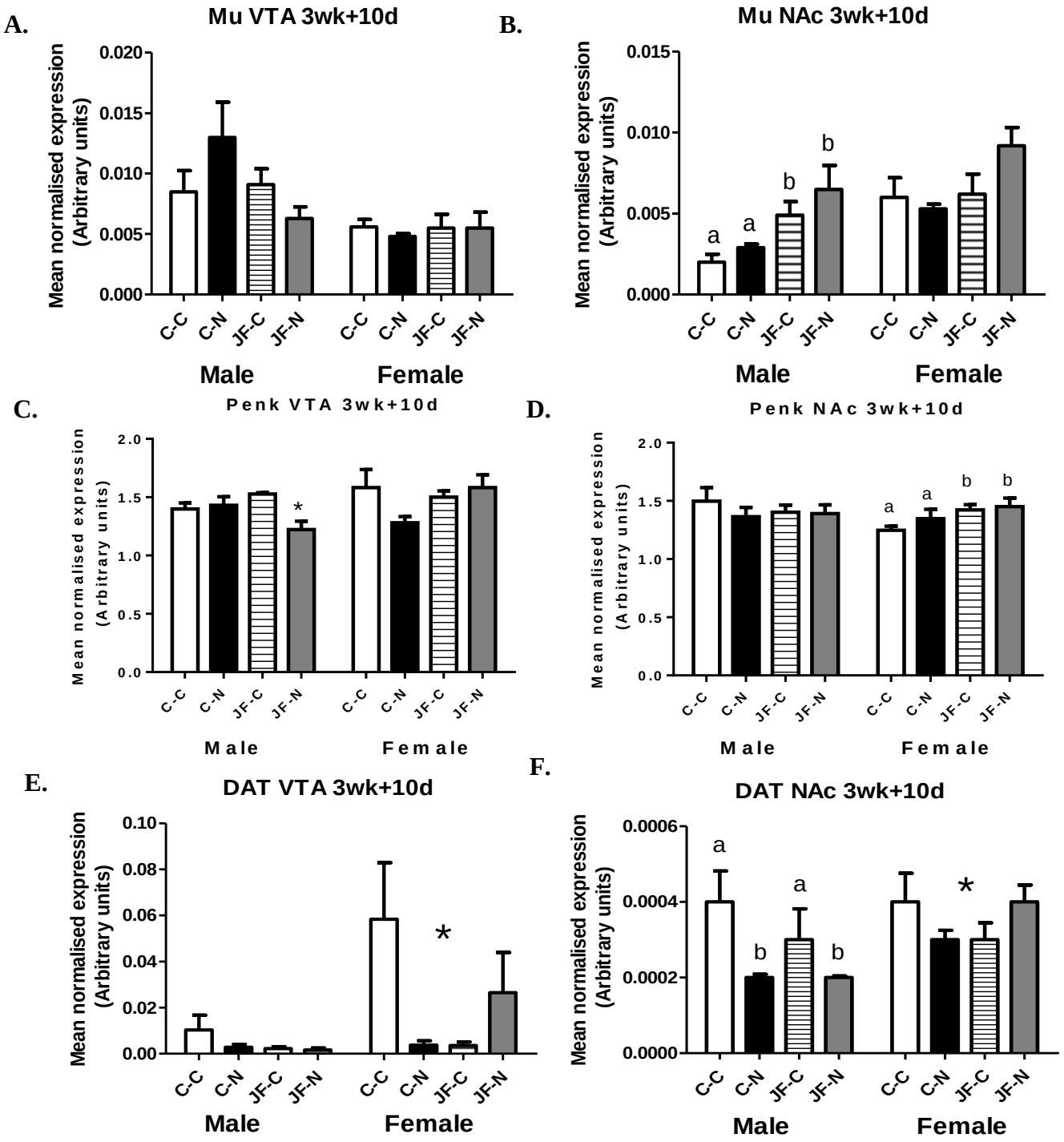
In female offspring, the effects of naloxone treatment on DAT expression in both the VTA and NAc was dependent on perinatal dietary exposure; such that DAT expression was decreased by naloxone treatment in C offspring but increased by naloxone treatment in JF offspring in both brain regions ($P<0.05$, Fig.5.1E, F). In male offspring, naloxone treatment decreased DAT expression in the NAc of both C and JF offspring ($P<0.05$, Fig.5.1F), but there was no effect of either perinatal dietary exposure or naloxone treatment on DAT mRNA expression in the VTA (Fig.5.1E).

Table 5.1 Mean normalised gene expression of dopamine related genes in the VTA and NAc of the male and female offspring of C and JF dams at 3 weeks of age

		Male		***Female***	
Parameter		**C**	**JF**	**C**	**JF**
VTA	TH	480.0±100.0	410.0 ±100.0	530.0±100.0	610.0±200.0
	D1	0.7 ±0.2	0.7 ±0.2	0.7 ±0.3	0.3 ±0.1
	D2	100.0±20.0	100.0±20.0	100.0±20.0	140.0±30.0
	DAT	344.5±70.0	432.9±61.8	441.9±79.0	621.2±125.5
NAc	TH	0.5 ±0.1	0.6 ± 0.1	0.6 ± 0.2	0.4 ±0.1
	D1	30.0±5.0	30.0 ± 4.2	40.0±7.5	40.0 ±7.4
	D2	70.0 ±9.4	70.0 ±10.0	50.0 ±8.5	70.0 ±7.8
	DAT	0.10±0.04	0.04±0.01*	0.20±0.08	0.05±0.02*

Values expressed as mean ± SEM, n=8-10 for all groups. * indicates significantly different values between groups, P<0.05. Values were multiplied by one thousand for ease of presentation.

Figure 5.1



Expression of the mu-opioid receptor, proenkephalin (PENK) and DAT in the VTA (A, C, E) and NAc (B,D, F) in the male and female offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of junk food dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars) at 3 weeks +10 days. Results presented as mean±SEM. *n*=8-9 pups for all groups. Different letters above the bars denote significantly different means $P<0.05$. * indicates a significant interaction between maternal diet and naloxone/saline treatment

There was no effect of either perinatal diet or naloxone treatment on TH, D1 or D2 mRNA expression in male offspring in either the NAc or VTA. In contrast, TH and D2 mRNA expression was decreased in the VTA of female JF offspring compared to C offspring, independent of naloxone treatment (Table 5.2). Naloxone treatment also reduced D1 receptor expression in the VTA, but not NAc, of female JF offspring. In contrast D1 and D2 receptor expression in the NAc was decreased by naloxone treatment in C offspring (Table 5.2).

5.4.4 Effect of maternal diet and naloxone on plasma hormone and metabolite concentrations at 3 weeks +10 days

There was no effect of either perinatal diet or naloxone treatment on plasma concentrations of glucose, NEFA, insulin or leptin at 10 days postweaning in either male or female offspring (Table 5.3).

5.4.5 Effect of maternal diet and naloxone treatment on offspring growth and food intake

From weaning (day 21) until 12 weeks of age (day 90) both male ($P<0.05$, Fig. 5.2A) and female ($P<0.05$, Fig. 5.2B) JF offspring were significantly lighter than C offspring. There was no effect of naloxone treatment on body weight at any time point during the experiment in either C or JF offspring

As previously reported, during the 10 day naloxone treatment post-weaning, when all offspring had free access to both the cafeteria diet and standard laboratory chow female, but not male, JF offspring consumed more fat and energy than their control counterparts (208).

From the end of the 10 day naloxone treatment (3weeks + 10 days of age) until 10 weeks of age all offspring were fed standard laboratory chow. For the first 4 weeks on the chow diet both male and female offspring of JF dams had significantly higher food intake than C offspring independent of whether they had been treated with saline or naloxone ($P<0.05$, Fig.5.3A,B). An interaction was present between perinatal diet and postnatal week, such that the difference in food intake between C and JF offspring decreased with increasing postnatal age. In male offspring only, there were no longer any significant differences in food intake between C and JF offspring by the fifth week on the chow diet (10 postnatal weeks) (Fig. 5.3A). Chow intake in female offspring remained significantly higher than C offspring throughout this period ($P<0.05$, Fig.5.3B).

Table 5.2. Mean normalised gene expression of dopamine related genes in the VTA and NAc of male and female offspring of C and JF dams treated with saline or naloxone at 3 weeks and 10 days

		Male				***Female***			
Parameter		**C-C**	**C-N**	**JF-C**	**JF-N**	**C-C**	**C-N**	**JF-C**	**JF-N**
VTA	TH	20.0±7.1	3.10±1.3	10.0±2.7	8.50±5.3	30.0±9.1[a]	8.6±2.2[a]	1.1±1.1[b]	1.2±0.4[b]
	D1	1.3±0.3	2.1±0.6	1.5±0.2	1.4±0.4	0.6±0.1	0.6±0.2	0.9±0.1	0.5±0.1[*]
	D2	20.0 ±2.7	20.0 ±2.7	20.0 ±3.1	20.0±2.0	20.0±1.3[a]	20.0±2.2[a]	10.0±3.4[b]	10.0±1.5[b]
NAc	TH	0.5±0.1	0.5±0.1	0.4±0.1	0.5±0.1	0.6 ±0.1	0.8 ±0.1	0.5 ±0.1	0.5 ±0.1
	D1	30.0±9.1	30.0±5.3	40.0±8.8	50.0±10.0	30.0 ±3.8	10.0 ±1.8[*]	30.0 ±3.8	30.0±4.0
	D2	50.0±10.0	90.0±20.0	60.0±10.0	50.0±10.0	120.0±10.0	50.0 ±7.6[*]	70.0 ±20.0	80.0 ±8.2

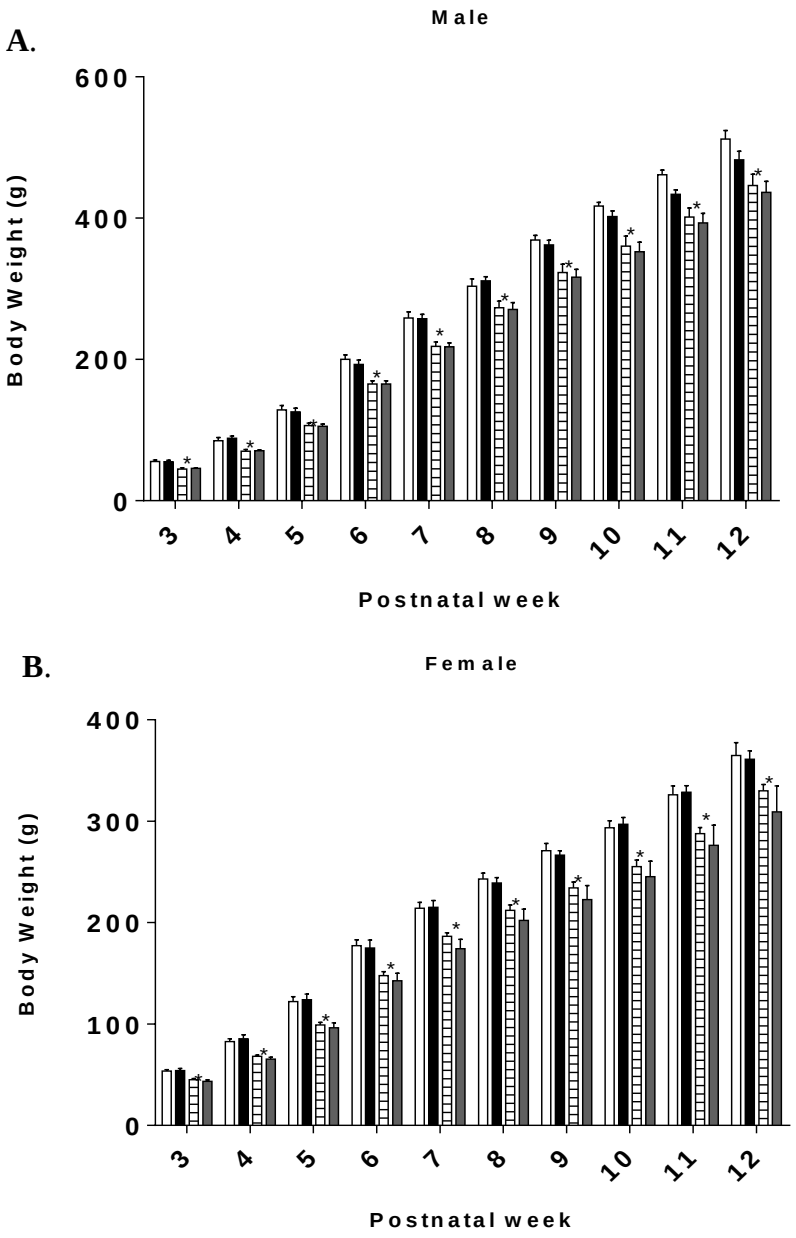
Values are expressed mean ± SEM., different superscript letters denote values that are significantly different, * indicates an interaction between maternal diet and pup treatment. $P<0.05$, n=8-10 for all groups

Table 5.3 Plasma concentrations of glucose, NEFA, insulin and leptin in male and female offspring of C and JF dams treated with either saline or naloxone at 3wk+10days

	Male				***Female***			
Parameter	**C-C**	**C-N**	**JF-C**	**JF-N**	**C-C**	**C-N**	**JF-C**	**JF-N**
Glucose (mM)	7.5±0.52	7.6±0.51	7.9±0.34	8.2±0.42	9.1±1.17	7.8±0.24	7.6±0.48	8.9±1.13
NEFA (mEq/ml)	0.3±0.03	0.3±0.03	0.3±0.04	0.3±0.04	0.4±0.06	0.3±0.05	0.4±0.05	0.3±0.08
Insulin (µU/ml)	0.4±0.10	0.4±0.06	0.4±0.11	0.6±0.13	0.3±0.10	0.7±0.17	0.6±0.16	0.7±0.20
Leptin (µg/L)	9.9±0.93	12.5±1.16	9.4±1.12	10.8±0.65	11.1±0.75	11.7±1.27	10.9±0.90	10.2±1.44

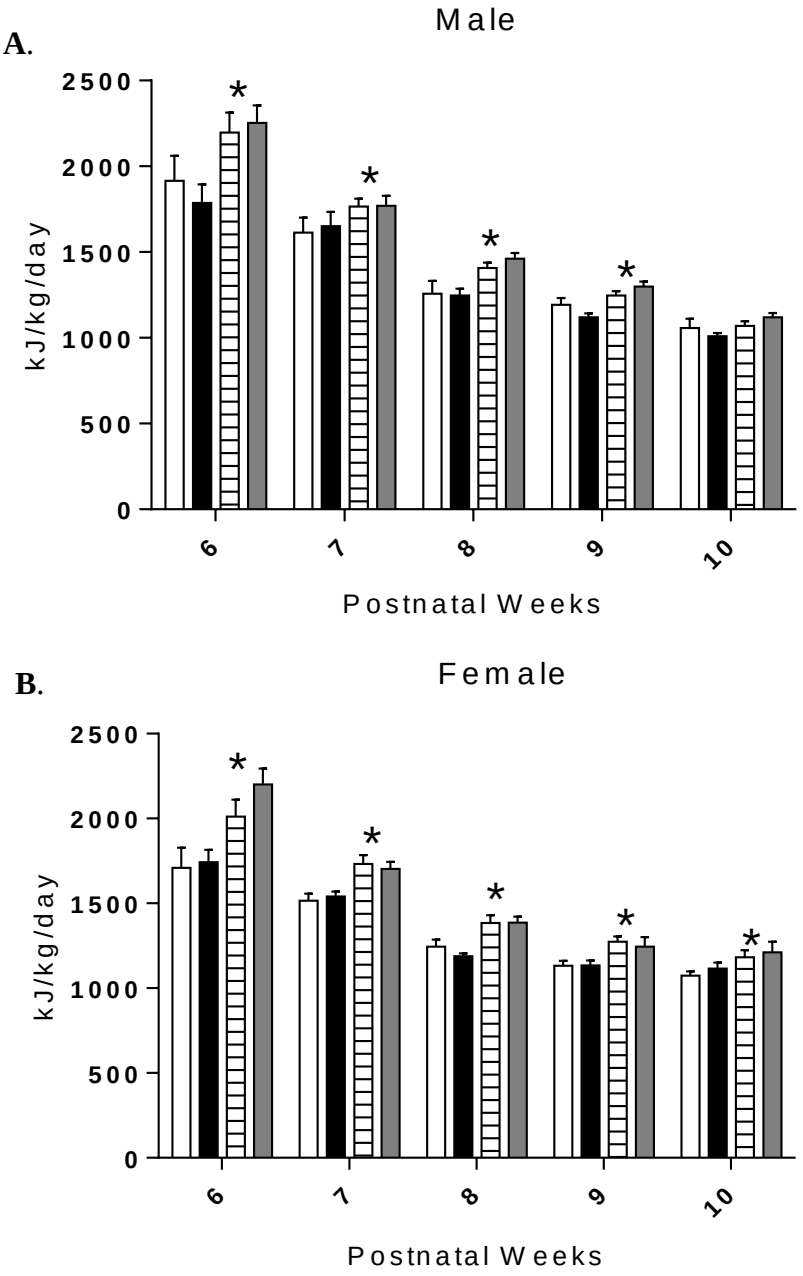
Values expressed as mean±SEM, *n*= 8-9 for all groups.

Figure 5.2



Body weight of male (A) and female (B) offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of junk food dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars) from postnatal week 3 to postnatal week 12. *n*=6-8 animals for all groups, results presented as mean±SEM. * indicates a significant effect of maternal diet on offspring body weight $P<0.05$

Figure 5.3



Intake of total energy from standard laboratory chow of male (A) and female (B) offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of junk food dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars) from postnatal week 6 to postnatal week 10. Results presented as mean±SEM, *n*=6-8 animals for all groups. * indicates a significant effect of maternal diet on offspring energy intake *P*<0. 05.

5.4.6 Effect of maternal diet and naloxone treatment on offspring food preference and body composition

When all offspring were provided with free access to both the control and cafeteria diet for two weeks from 10-12 weeks of age, there was no difference in food intake between groups in either males or females. Thus, neither perinatal diet nor naloxone treatment had any effect on the intake of fat, carbohydrate, protein or total energy during this period (Table 5.4). There was also no difference between groups in the intake of any individual component of the cafeteria diet or the control diet in either males or females (data not shown).

At 12 weeks of age, there was no significant differences in the percentage of total body fat mass between C and JF offspring nor any effect of naloxone treatment in either males (C-C 16.5±0.95%, C-N 16.0±0.64%, JF-C 14.2±0.72%, JF-N 14.8±0.57%) or females (C-C 19.3±0.10%, C-N 19.0±0.78%, JF-C 18.5±0.13%, JF-N 18.7±0.15%).

5.5 Discussion

This study has shown that administration of the opioid antagonist naloxone for 10 days after weaning alters the gene expression of key components of the opioid and dopamine signaling pathways in the mesolimbic reward system in a sex-specific manner. Importantly, the effects of naloxone treatment on a number of these genes were dependent on whether pups had been exposed to a cafeteria ('junk food') diet during the perinatal period. The differences in gene expression at the end of the period of naloxone treatment were not, however, associated with altered food preferences in adulthood in either C or JF offspring. Thus, perinatal junk food exposure alters the short-term response of the reward pathway to opioid receptor blockade in the immediate post-weaning period, but opioid receptor blockade during this time does not appear to cause persistent alterations in food preferences, independent of the perinatal diet.

5.5.1 Maternal JF consumption and naloxone treatment for 10 days postweaning can alter gene expression in the reward pathway of offspring

In the present study, we found no difference in expression of the mu-opioid receptor in the VTA between C and JF offspring at 10 days after weaning, independent of whether the offspring had been treated with naloxone or saline during this period. This is different to the situation at weaning, at which time mu-opioid receptor expression in the VTA is lower in JF offspring than controls (208). In interpreting these findings, it is important to note that all offspring, independent of their perinatal nutrition, were provided with free access to the cafeteria diet during the 10 day period of naloxone/saline treatment. One possible

Table 5.4. Average daily macronutrient intake of control and junk food offspring treated with either saline or naloxone, when given access to both the control and JF diet from 10-12 weeks of age

	Male				***Female***			
Parameter	**C-C**	**C-N**	**JF-C**	**JF-N**	**C-C**	**C-N**	**JF-C**	**JF-N**
Fat (g/kg/day)	16.5±0.47	14.9±1.29	17.1±0.66	16.6±0.55	19.2±0.48	18.4±1.06	19.6±0.62	20.3±0.72
Carbohydrate (g/kg/day)	37.9±0.76	35.4±2.08	38.8±1.69	39.9±1.30	43.7±1.20	43.9±1.91	44.6±3.00	44.8±2.17
Protein (g/kg/day)	9.3±0.32	8.5±0.53	9.0±0.35	9.0±0.36	9.6±0.21	9.8±0.30	9.2±0.50	9.5±0.43
Energy (kJ/kg/day)	1411.0±18.72	1289.2±81.97	1415.1±48.40	1422.1±35.39	1578.9±21.14	1564.2±56.10	1601.5±72.11	1637.8±68.69

Values expressed as mean±SEM, *n*= 6-8 for all groups.

explanation, therefore, is that a 10 day period of junk food exposure after weaning was sufficient to induce a down-regulation of mu-opioid receptor expression to the same level as in offspring who were also exposed to junk food during the perinatal period. In males, mu-opioid receptor expression in the NAc was increased in JF offspring both at weaning (208) and after the 10 day naloxone/saline treatment. Increases in expression of the mu-opioid receptor in the NAc have also been observed in the adult offspring of JF-fed dams, suggesting that this is a persistent consequence of perinatal exposure to a palatable diet (122). These results also highlight that the response to perinatal dietary exposures varies between specific brain regions. It is possible that the increased mu-opioid expression in the NAc is a result of chronic exposure to the cafeteria diet during the development of the reward pathway, since chronic sugar consumption has been previously reported to increase mu-opioid receptor expression in this brain region in adult rats (258).

Interestingly, we found no effect of naloxone treatment on mu-opioid expression in either the NAc or VTA. This was unexpected given that naloxone treatment in rodents prior to weaning has previously been shown to increase mu-opioid receptor levels in the striatum (237,259). However, in these prior studies naloxone treatment was given from birth and receptor levels were measured using radio-labelled binding analysis which may have contributed to the incongruent results. It is also possible, however, that the availability of the cafeteria diet during the period of naloxone exposure, and the associated stimulation of endogenous opioid production, was sufficient to counteract the effect of opioid receptor blockade, thus resulting in a maintenance of receptor expression.

While there were no differences in the expression of the mu-opioid receptor in the NAc between control and JF offspring in females, mRNA expression of the endogenous opioid, proenkephalin, was increased in this brain region in female JF offspring, but was not affected by naloxone treatment. This is consistent with previous studies in which exposure to a high-fat and/or high-sugar diet was reported to increase the release of endogenous opioids (101,231). It is possible that the higher proenkephalin expression in female JF offspring was a consequence of their higher fat intake in the 10 days post-weaning, since previous studies have reported positive associations between proenkephalin expression and fat consumption in adult rodents (143). In male offspring, proenkephalin mRNA expression was reduced by naloxone treatment in offspring of JF dams, but not in offspring of control dams. Given that opioid receptor blockade is typically associated with a compensatory up regulation of

endogenous opioids (260), this result suggests that perinatal junk food exposure alters the subsequent response of the reward pathway to opioid receptor blockade, indicating a potential dysregulation of opioid signaling in these offspring.

5.5.2 Female offspring are more susceptible to the effects of maternal JF diet and naloxone treatment on the dopamine pathway

The majority of the effects of perinatal junk food exposure and naloxone treatment on components of the dopamine signaling pathway were confined to female offspring, with the dopamine active transporter (DAT) being the only gene affected in both sexes.

At weaning (prior to naloxone/saline treatment), DAT mRNA expression in the NAc was decreased in JF offspring compared to controls in both males and females. This result is consistent with previous studies in our laboratory, in which DAT mRNA expression at 6 weeks of age was reduced in offspring exposed to a junk food diet during the perinatal period (203). Since DAT is primarily responsible for the reuptake of dopamine from the synapse, and therefore terminating the dopamine signal, the lower DAT mRNA expression would be expected to result in increased dopamine signaling in the JF group. Interestingly, there were no longer any differences in DAT mRNA expression between the control and JF offspring at the end of the 10 day period of naloxone/saline treatment. Again, it is possible that exposure of the control offspring to junk food during this period could have resulted in reduced DAT mRNA expression. Interestingly, we saw no effect of either naloxone treatment or perinatal junk food exposure on DAT expression in the VTA, which is considered to be the main site of DAT activity (261,262), however the significance of this finding remains unclear.

We found that naloxone treatment reduced expression of DAT in the NAc of male offspring and in the VTA and NAc of female control offspring. Opioid receptor blockade has previously been shown to lower extracellular dopamine concentrations (263,264), which may have elicited a compensatory downregulation of DAT in order to maintain dopamine signaling. Given that the naloxone treatment was applied at a time when the reward pathway is still undergoing development, and that dopamine plays an important role in the ontogenic increase in DAT mRNA expression, an alternate explanation may be that naloxone treatment inhibited this normal developmental process (265,266). Interestingly, in female JF offspring naloxone treatment increased DAT expression in both the VTA and NAc. This unexpected response to opioid receptor blockade may suggest a dysregulation of the reward pathway in these animals as a result of early life exposure to a junk food diet.

In female offspring, but not in males, the expression of TH, D1 and D2 receptor mRNA was decreased by naloxone treatment in offspring exposed to the junk food diet during the perinatal period. Decreases in the expression of elements of the dopamine pathway have been previously associated with chronic cafeteria diet consumption (267,268), whilst opioid antagonism has been shown to reduce extracellular dopamine levels (263,269). Different responses to exogenous opioids between sexes has also been widely reported in adults (270,271) and may be due to differences in levels of gonadal hormones between males and females, as estrogen is known to contribute to the regulation of the endogenous opioid system (223).

5.5.3 Maternal JF consumption increases offspring chow intake during the juvenile period but did not affect palatable food intake in adult hood

In the present study both male and female JF offspring exhibited an increased intake of standard rodent feed throughout the juvenile period (6-9 weeks) compared to controls. This hyperphagia was most marked immediately after weaning, and became less pronounced with increasing postnatal age. The presence of hyperphagia in offspring exposed to an increased supply of fat and/or sugar during the perinatal period has been widely reported in previous studies (21,26), and is thought to be a consequence of programming of the central appetite regulating circuits (32).

However, contrary to our hypothesis, we found no effect of opioid receptor blockade post-weaning on food preferences in adulthood, independent of perinatal junk food exposure. Since the development of the reward circuitry extends into the fourth postnatal week in rodents (125,221), one explanation for the lack of effect observed may be that the junk food exposure after weaning was sufficient to program an increased preference for fat in adult control offspring equivalent to that induced by exposure to the cafeteria diet for the entire perinatal period. This is supported by data from a previous study, in which mice exposed to a palatable diet only during the fourth week of life were found to exhibit a greater preference for palatable foods as adults when compared to animals who had never been exposed to the palatable diet (36). Furthermore, in our study, naloxone treatment was only administered at concentrations (5mg/kg) capable of reducing food intake for two hours post injection (234,240). The acute nature of the treatment may have limited any long term effects, since a previous study has identified changes in feeding behaviour in juvenile rats which were treated

with the opioid antagonist naltrexone (which is capable of reducing food intake for 6hrs post-injection (272) from birth until weaning (273).

5.5.4 Conclusions

We have shown for the first time that opioid receptor blockade induced by naloxone administration immediately post weaning alters gene expression in the reward pathway in a sex-specific manner, and that these effects are altered by perinatal junk food exposure. Contrary to our initial hypothesis, however, opioid receptor blockade in the fourth week of life did not have any long term effects on food preferences. These findings add to the growing body of literature suggesting that the developing opioid and dopamine pathways are susceptible to alteration by palatable food exposure during the perinatal period, but further studies are required in order to determine whether alterations to the opioid signaling system are the biological basis for the changes in food preferences observed. Better understanding the mechanisms behind the programming of food preferences will be vital if we hope to design interventions to prevent the cycle of obesity from mother to child from continuing.

Chapter 6

General Discussion

Chapter 6: GENERAL DISCUSSION

Evidence from clinical trials and studies in animal models suggests that maternal diet during pregnancy and whilst breastfeeding is an important determinant of the offspring's subsequent metabolic health outcomes. Individuals who are exposed to maternal overnutrition before birth and whilst breastfeeding are more likely to have a higher body weight and consume excess amounts of food in both childhood and adult life (17,21,24,26,274,275). Furthermore, investigations into the determinants of food preferences have shown that maternal diet during pregnancy and lactation not only influences the amount of food the child consumes after birth but also their food choices. Studies in rodent models have demonstrated that the offspring of dams fed a cafeteria diet develop a specific preference for these types of highly palatable, high-fat, high-sugar foods and also have altered development of the reward processing centres in the brain, particularly the mesolimbic reward pathway (35-37,122). The current thesis aimed to extend on this previous research by, first, isolating when during development exposure to a cafeteria diet has the greatest effect on the food preferences of the offspring and secondly, by investigating the potential role of altered development/function of the mu-opioid receptor (a key component of mesolimbic reward pathway) in mediating these effects. Specifically, our studies into the mu-opioid receptor focused on whether maternal cafeteria diet exposure affected the ontogeny of this receptor as well as identifying whether these changes translated into functional consequences for opioid-mediated control of palatable food intake.

In the first experimental chapter of this thesis (chapter 2), we aimed to identify the 'critical window' of perinatal development during which cafeteria diet exposure would have the greatest impact on the food preferences of the offspring. Using a cross-fostering methodology, we showed that male offspring exposed to a cafeteria diet during the suckling period consumed more fat at 7 weeks of age when challenged with the cafeteria diet than offspring exposed to the cafeteria diet only before birth or not at all. This finding was in line with the outcomes of a previous study in which offspring of dams given a cafeteria diet before birth but switched to a control diet during lactation did not develop the increased preference for fat and sugar that has been demonstrated in offspring exposed to the cafeteria diet across the entire perinatal period (35).

Based on the outcomes of chapter 2, which suggested that the postnatal period was more important for the programming of food preferences than cafeteria diet exposure before birth,

chapter 3 investigated alterations in the postnatal ontogeny of the mu-opioid receptor as a potential mediator of these effects. The identification of the mu-opioid receptor as a possible mechanism behind the programming of food preferences was based on previous studies which had demonstrated changes in the expression of this receptor in the adult offspring of cafeteria fed dams (37,122). Furthermore, previous studies had shown that the mu-opioid receptor is still developing throughout the postnatal period (not reaching adult levels until the third to fourth postnatal week) (104,109,110) and is thought to be highly susceptible to alterations during this time. Using a radioactive *in situ* hybridisation method, we measured the expression of the mu-opioid receptor in two brain areas known to be involved in the regulation of reward behaviour (the nucleus accumbens (NAc) and the ventral tegmental area (VTA)) in both early (birth, week 1) and late (week 3, 4) postnatal development. We showed that the female offspring of cafeteria fed dams had reduced expression of the mu-opioid receptor in the VTA during late postnatal development (week 3, 4) but not at the earlier timepoints. That this reduction in mu-opioid receptor expression was observed in offspring at the late but not early postnatal/immediate postweaning period timepoints suggests, in support of the findings in chapter 2, that exposure to a cafeteria diet through the maternal milk supply may have a greater impact on mu-opioid receptor development and the programming of food preferences than exposure *in utero*.

Taken together the outcomes of the first two chapters of this thesis suggested that the lactation period as well as the immediate post-weaning period (week 4), represent a critical developmental stage during which exposure to a maternal cafeteria diet can program lifelong food preferences and alter mu-opioid receptor expression. However, from this evidence it remained unclear whether there was a causal relationship between the changes in mu-opioid receptor expression and the increased preference for palatable food; particularly given the sex differences in the two studies showing that male food preferences but female mu-opioid receptor expression is most affected by maternal cafeteria diet exposure. Nevertheless, given that the outcomes of both chapters pointed to the importance of the postnatal period as a critical window of exposure for the programming of food preferences it opened up the exciting possibility that the negative effects of exposure to a cafeteria diet before birth could potentially be avoided by intervening with a more nutritionally appropriate diet during the suckling period. However, there is clearly a need for continued research in this field. An important expansion of this work would be to look more broadly at the effect of maternal cafeteria diet exposure on reward pathway development, particularly on the ontogeny of the

dopamine pathway, as this pathway like the opioid pathway has been demonstrated to play a critical role in reward regulation (42,66,276). Furthermore, when considering the implications of findings in this thesis for clinical practice it must be noted that these studies were conducted in an altricial rodent model, which undergoes a considerable degree of maturation after birth, unlike in human infants where brain development is largely completed before birth (277) . Thus, caution should be exercised when extrapolating the results of these studies to humans.

The fourth and fifth chapters of the thesis extended the findings of the previous two chapters by attempting to demonstrate a functional relationship between changes in the expression of the mu-opioid receptor (along with other components of the mesolimbic reward pathway) and food preferences in offspring. The investigations in the current thesis were the first to show that offspring of dams fed a cafeteria diet throughout pregnancy and lactation are less sensitive to the suppressive effects of the opioid receptor antagonist naloxone on palatable food intake, when this drug is administered during the fourth postnatal week, than their control counterparts. This result in the offspring of cafeteria diet fed dams provides the first evidence to suggest that the reduction in mu-opioid receptor gene expression demonstrated by our laboratory and others (37,122,208), has functional consequences for opioid pathway activity and its regulation of palatable food intake. Furthermore, we also demonstrated that the effect on naloxone on the gene expression of other reward pathway components, such as DAT, was dependent on whether or not the pups had been exposed to the cafeteria diet prior to weaning, thus providing further evidence to suggest that early life exposure to a cafeteria diet has consequences for reward pathway activity, at least in the short term.

As part of the fifth chapter of the thesis we hypothesised that opioid receptor blockade during the fourth week of life would have long term consequences for feeding behaviour in the offspring, however, we did not observe any difference in food preferences in adult offspring. This may be explained by the low dose of naloxone used in the study, which was only given in concentrations capable of reducing food intake for two hours per day (234,240). Interestingly, we also did not observe any difference in the adult food preferences of control and junk food offspring given saline. As all offspring were given access to the cafeteria diet during the injection period (i.e. the fourth postnatal week), it may be that exposure to this diet immediately post-weaning was enough to program an increased preference for fat in adult control offspring comparable to that induced by exposure to the cafeteria diet for the entire

perinatal period. In support of this, a study in a mouse model demonstrated that offspring exposed to a palatable diet during only the fourth week of life, had an increased preference for palatable foods as adults when compared to offspring who had never been exposed to the palatable diet (36). Further evidence that the fourth week of life may represent a critical window for the programming of food preferences is provided by the results of the third chapter of this thesis which showed that cafeteria diet exposure during this time reduced mu-opioid receptor mRNA expression in the VTA, at least in female offspring. While these results do suggest that the fourth week of life may be crucial for the programming of food preferences in the rodent model, further investigations isolating cafeteria diet exposure to this week alone are necessary before definitive conclusions can be drawn.

Another important aspect of this thesis was the identification of clear sex differences in both the programming of food preferences and the development of the reward pathway. In chapter 2, we saw that exposure to a cafeteria diet during the first three weeks of life programmed an increased preference for fat only in male offspring at 7 weeks of age. In chapter 3, we demonstrated that female but not male offspring of cafeteria diet fed dams had reduced expression of the mu-opioid receptor in the third and fourth weeks of life. This complex interaction between maternal cafeteria diet consumption, reward pathway development and the sexes continued to be observed in the studies that comprise the fourth and fifth chapters of the thesis. In these studies, exposure to a cafeteria diet in the perinatal period had a greater effect on the male offspring's response to naloxone than it did in the female offspring suggesting that early life cafeteria diet exposure has a more pronounced effect on mu-opioid receptor function in males. However, when we examined the effect of cafeteria diet exposure and naloxone treatment on the expression of both opioid and dopamine related genes; we observed more pronounced changes in females. Taken together the results presented in this thesis suggest that while exposure to a cafeteria diet has a greater short term impact on reward pathway expression in the postnatal period in females than it does in males, there are more significant effects on mu-receptor function and long term food preferences in males. The mechanism behind these sex differences remain unclear, however as we did not observe any sex differences in postnatal mu-opioid receptor development in the control offspring in chapter 3, it suggests that the sex differences are induced by exposure to a cafeteria diet rather than intrinsic variations in the opioid pathway between the sexes. The sex specific response to a maternal cafeteria diet could potentially be driven by estrogen, which has been

demonstrated to be involved in regulation of the endogenous opioid system in response to drugs of abuse (223,224,278).

Throughout the current thesis we have demonstrated a reduction in the expression of mu-opioid receptor in brain areas involved reward processing as well as functional consequences of this reduced expression for opioid-mediated regulation of palatable food intake. In light of these findings, we propose that the preference for high-fat high-sugar foods in the offspring of mothers who consume these types of foods across the perinatal period (particularly during lactation) may be the result of the offspring developing an opioid pathway that is less sensitive than normal. We further hypothesise that this reduction in the sensitivity of the opioid pathway is driven by overexposure to maternal endogenous opioid. In this proposed model, (as illustrated in Fig. 4.5) maternal consumption of a cafeteria-style diet pregnancy and lactation acts to increase maternal endogenous opioids levels. These opioids are then carried through to the offspring via the placenta and/or through the breast milk and act to decrease expression of the mu-opioid receptor, which is particularly plastic and susceptible to alteration during the first four weeks of life in the rodent model. The subsequent desensitisation of opioid signalling and decrease in responsiveness to endogenous opioids in the offspring of cafeteria diet fed mothers then drives a greater intake of palatable foods in order to achieve the same level of stimulation in the opioid pathway. However, despite the results in the current thesis providing some evidence to support this hypothesis, it is clear that a considerable amount of further investigation into the role of maternal endogenous opioids in shaping reward regulation and food preferences of the offspring is required, before any definitive conclusions about the clinical relevance of the work can be formed.

Future directions to build on the outcomes of the current thesis could include investigations that directly demonstrate a link between increases in maternal endogenous opioids and the programming of food preferences, such as directly exposing the offspring to high levels of opioids in the absence of a maternal cafeteria diet. Furthermore, it would also be interesting to consider the implications of this work for understanding the programming of other addictive behaviours. One study by Bocarsly and colleagues has already demonstrated that offspring exposed to a high-fat diet early in life have an increased preference for alcohol as adults (279). Looking specifically at whether the opioid pathway desensitisation in offspring of mothers fed a cafeteria diet has direct consequences for the consumption of other rewarding

stimuli such as alcohol, nicotine and drugs of abuse would be an important expansion of the outcomes presented in this thesis.

The findings in this thesis add to the existing body of literature which shows a link between maternal cafeteria diet consumption during pregnancy and lactation and the establishment of food preferences in the offspring. This work has highlighted for the first time that the offspring of cafeteria diet fed dams have reduced expression of mu-opioid receptor at weaning and, importantly, that reductions in the expression of this receptor have functional consequences for the regulation of palatable food intake. Furthermore, the studies presented in this thesis have also demonstrated that exposure to a cafeteria diet alters the postnatal development of the mu-opioid receptor and that this period represents a critical window for the programming of food preferences. However, when considering the implications of this work in a clinical setting it is important to note that the stages of brain development that occurs in the early postnatal period in the rodent are thought to occur in approximately the third trimester of the human fetus (280), thus possibly pointing to the importance of late gestation nutrition in pregnant women in programming these pathways. Nevertheless, the identification of the postnatal period as critical window for the programming of food preferences in the rodent model, as well as the improved understanding of the mechanisms driving this programming will ultimately enable the design of targeted interventions to break what has become intergenerational cycle of obesity, poor food choices and metabolic health problems.

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