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A maternal 'junk food' diet reduces sensitivity to the opioid antagonist naloxone in offspring post-weaning

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Short title: Maternal palatable diet and opioid desensitivity

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Abbreviations

C, control diet; C-C, offspring exposed to a control diet before weaning and given saline injections; C-N, offspring exposed to a control diet before weaning and given naloxone injections; JF, junk food diet; JF-C, offspring exposed to a junk food diet before weaning and given saline injections; JF-N, offspring exposed to a junk food diet before weaning and given naloxone injections; NAc, nucleus accumbens; PND, postnatal day; VTA, ventral tegmental area.

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 fat 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 the intake of standard rodent feed in control or JF offspring. These findings suggest that exposure to a maternal junk food diet results in an early desensitization of the opioid system which may therefore explain the increased preference for junk food in these offspring.

Key Words: mu-opioid receptor**,** programming, reward, fat

Introduction

Excessive maternal intake of high-fat and high-sugar 'junk foods' during pregnancy and lactation has been shown to program an increased preference for fat and sugar in juvenile and adult offspring (1-2). 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 palatable diets on offspring food preference (3-5).

Within the mesolimbic reward system, opioid signaling plays a central role in eliciting the pleasurable sensation associated with rewarding stimuli (6-7). The consumption of palatable foods which are high in fat and sugar is associated with an increased concentration of endogenous opioids within the reward pathway (8-9) that then bind to opioid receptors in the ventral tegmental area (VTA) to stimulate dopamine release (10). Existing investigations into the mesolimbic reward pathway of adult rats exposed to a high-fat, high-sugar diet *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 (3) and juvenile offspring (2) exposed to a palatable diet during the perinatal period.

A possible explanation for the effects of a maternal palatable diet on mu-opioid receptor expression in the offspring is that high levels of endogenous opioids, as would be expected in response to a maternal palatable diet, may impact on opioid receptor ontogeny. However, the impact of palatable diets on the expression of the mu-opioid receptor in the early postnatal period is yet to be adequately explored. Previous studies that have examined the effect of maternal intake of the exogenous opioid, morphine, have shown that offspring exposed to morphine during the perinatal period have altered mu-opioid receptor expression, with the direction of this change dependent on the specific timing of the exposure (prenatal vs. postnatal) and the age of the offspring when expression is measured (11-12). Importantly, perinatally morphine-exposed offspring also exhibited an increased preference for sugar in adulthood (13). However, whether exposure to high levels of endogenous opioids during pregnancy and lactation could have similar effects remains to be determined.

The sensitivity of the developing mu-opioid receptor to the nutritional environment and opioid concentrations 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 palatable food. Although alterations to mu-opioid receptor expression have been previously observed in adult offspring of dams fed a palatable diet during pregnancy and lactation (2-3) 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 perinatally junkfood exposed offspring have functional consequences for the subsequent regulation of palatable 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 palatable food in the immediate post-weaning period.

Materials and Methods

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 to receive either a control (C, n=8) or junk food (JF, n=9) diet. The control diet consisted of the standard laboratory rodent feed (Specialty Feeds, Glen Forrest, WA, Australia). The junk food diet 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 (2). 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.

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 (2). 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 (2), 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).

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 exposed to junk food before weaning and given saline injections (JF-C, n=17) and the offspring exposed to junk food diet prior to weaning and given naloxone injections (JF-N, n=17) (Fig. 1).

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 junk food 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 (14)), 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 different type of junk food consumed over each 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 normalized 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.

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, 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.

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.

Results

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 (control 3.14±0.2g/kg/d, JF 14.8±1.12g/kg/d, $P<0.01$) without any differences in protein, carbohydrate, or overall energy intake. Throughout lactation, in addition to increased fat intake (control 6.26±0.3g/kg/d, JF 25.2±1.9g/kg/d, $P<0.01$) the JF dams also consumed less protein (control 26.7±1.3g/kg/d, JF

15.4±1.1g/kg/d, $P<0.05$) and carbohydrate (control 81.7±4.1g/kg/d, JF 63.1±4.1g/kg/d, $P<0.05$) compared to control dams.

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, $P<0.01$).

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. 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. 2B $P<0.05$). There was no effect of maternal junk food feeding on mu-opioid receptor expression in the NAc at weaning in female offspring (Fig.2B).

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 1, $P<0.05$); no effect of group was observed on the weight of any other fat depots or total fat mass (Table 1). There was no effect of either maternal diet or naloxone treatment on fat deposition in female offspring (Table 1).

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. 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. 3A). In female JF offspring, the decrease in fat intake in naloxone-treated offspring compared to their saline-treated counterparts was significantly less than that observed in the offspring of control dams (control 42.2±6.0% reduction, junk food 23.1±4.1% reduction, $P<0.05$) (Fig. 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. 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. 3C-D).

Analysis of intake of specific junk foods, 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 junk food 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. 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. 4B-D). There was also no difference in the intake of either the standard rodent feed or any individual junk food 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 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.

Discussion

In the present study, we have shown that exposure to a maternal palatable 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 palatable food in offspring exposed to a junk food 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 junk food fed

dams are already present at weaning and that these changes in expression have functional consequences for the regulation of palatable food intake. This work provides important and novel insights into the pathway linking perinatal exposure to a palatable 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.

Maternal junk food consumption decreases rate of postnatal growth of offspring

Consistent with previous studies (1-2), the offspring of junk food fed 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 junk food fed dams appears to be driven by a mechanism other than protein deficiency. Clinical studies have suggested that maternal obesity is associated with poor placental function (15-16), which could contribute to growth deficits. In this study, however, maternal body weight was not different between groups; hence this is unlikely to explain the differences in offspring growth. Whilst the caloric intake of the junk food dams was increased relative to controls, our preliminary analysis of the junk food diet suggests that it is deficient in a number of key micronutrients, including magnesium and calcium. Deficiencies of both these micronutrients have been associated with poor fetal growth outcomes clinically (17-18) and therefore may provide an explanation for the reduced birthweight of the junk food offspring.

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 (19-20). In addition to reduced protein intake, junk food fed dams have been reported to have reduced milk production (21) which may explain the reduction of body weight during the last 2 weeks of lactation in junk food dams, since these animals may have to invest greater energy into lactogenesis (21). Difficulties lactating or nutrient deficiency of the milk would also provide an explanation for the lower weight gain of the pups across the suckling period.

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 dams fed a junk food diet during pregnancy and lactation. 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 (22), 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 (23) 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 a maternal junk food diet on mu-opioid receptor expression at weaning, previous studies have reported changes in mRNA expression of the mu-opioid receptor in the adult offspring of junk food fed dams. Vucetic *et al*. demonstrated that adult offspring of dams fed a palatable 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 (3), whereas studies in our own laboratory have shown that offspring of junk food dams weaned onto a junk food 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 junk food diet post-weaning (2). Interestingly, we found no changes in the expression of mu-opioid receptors in the VTA in that same study (2). 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 junk food diet during adolescence (2). 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 junk food diet on the VTA in adulthood

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 junk food fed dams were significantly less sensitive to the inhibitory effects of the opioid receptor antagonist naloxone on palatable food intake than offspring of control dams. Naloxone was selected as the antagonist as it only persists at concentrations capable of reducing food intake for two hours post injections (14), 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.

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 (9, 24). 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 palatable foods, rather than an overall decrease in food intake. However, unlike previous studies which have reported the suppressive effect of naloxone to be fat specific (14, 25), 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 junk foods 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 junk food fed dams to the effects of naloxone on palatable food intake 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 (26).

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 high-fat high-sugar foods (8, 27). Reduced sensitivity to the effects of naloxone in the junk food offspring is in agreement with our finding of reduced mu-opioid receptor expression in the VTA of these offspring at weaning. 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 (28). This may suggest that it is mu-opioid receptor involvement in the control of dopamine release which is the critical mechanism altered by exposure to a junk food 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 junk food fed dams which have demonstrated changes in both the opioid and dopamine systems of these animals (3-4, 29).

One possibility is that the observed decreases in mu-opioid expression and reduced sensitivity to naloxone in the offspring of junk food fed dams may be a result of exposure to elevated levels of endogenous opioids caused by maternal junk food consumption. Opioids have been previously demonstrated to readily cross the placenta (30) and into breastmilk (31-32), suggesting that increases in maternal opioid levels are likely to result in increased concentrations of opioids in fetal and neonatal circulation. Studies looking at the effect of maternal intake of an exogenous opioid (morphine) on mu-opioid receptor expression (as measured by naloxone binding studies post-mortem) have generated conflicting results, with one study reporting increased mu-opioid receptor expression (33) and others decreased expression (12, 34). This variation of results is likely due to differences in brain area explored, sex of animals studied and the timing of exposure. The relevance of these models to the current study must be considered with caution as there is evidence to suggest that exogenous and endogenous opioids may act through different mechanisms (35-36). Investigation into the role of maternal endogenous opioids on mu-opioid receptor expression in the offspring is very limited. Zadina *et al* reported that administration of the endogenous opioid β-endorphin before birth resulted in increased mu-opioid receptor expression in the whole brain of male offspring, whilst postnatal injections caused a decrease in expression (37). The lack of literature exploring the effect of endogenous opioids on the developing reward pathway creates a need for future investigation, particularly as relates to the programming effects of a maternal junk food diet which may be largely opioid mediated.

Temporary inhibition of appetite by naloxone drives compensatory increases in food intake

In the current study, we 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, this effect was not observed in the offspring of junk food fed dams. We suggest that the increased energy consumed by the offspring of control dams was a compensatory mechanism to overcome the time of caloric restriction induced by naloxone. This finding is 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 (38-39). That this compensatory increase in energy intake was not observed in the offspring of junk food fed dams, supports the finding that naloxone was less effective at inhibiting food intake in these animals.

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

We observed that female, but not male, offspring of junk food fed 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 junk food diet during the perinatal period (1-2). 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 (19). 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 previosuly been implicated in the regulation of the endogenous opioid system (40).

Despite our finding that it was only the female offspring junk food fed dams that had an increased preference for junk food 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 (41). Nevertheless, the lack of

differences in total fat deposition observed between the control and junk food offspring in this study has also been previously reported when both the control and junk food offspring were weaned on the junk food and control diet until 6 weeks and 3 months of age (2).

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 palatable food. 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 palatable food intake to overcome this early desensitization. We speculate that these changes in the expression and functionality of the opioid system in offspring exposed to a junk food diet during the perinatal period may be a result of exposure to high levels of endogenous opioids generated by maternal palatable food consumption (Fig. 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.

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Figure 1.

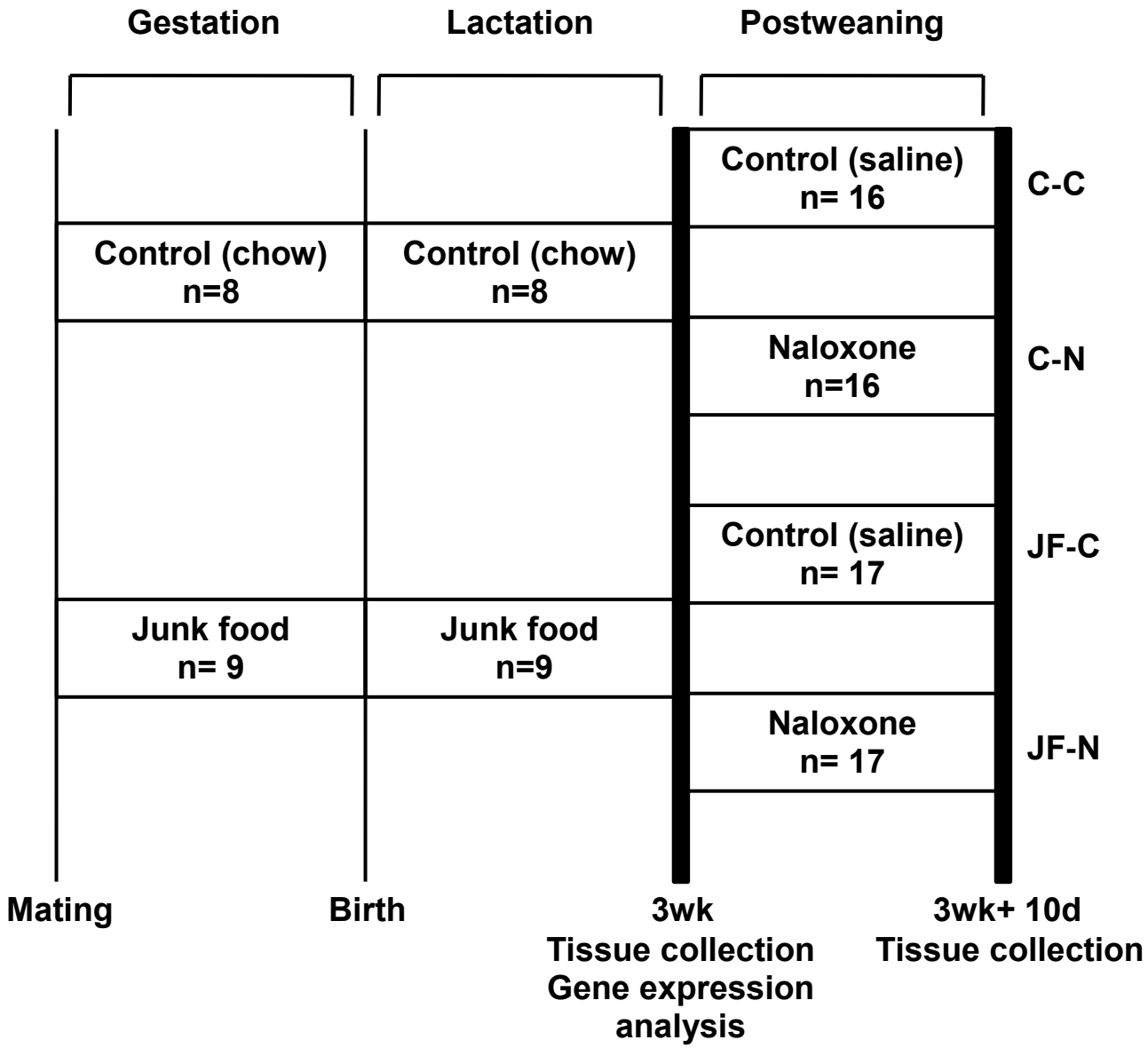
Gestation
Lactation
Postweaning
Control (chow)
n=8
Control (chow)
n=8
Junk food
n= 9
Junk food
n=9
Control (saline)
n= 16
Naloxone
n=16
Control (saline)
n= 17
Naloxone
n= 17
C-C
C-N
JF-C
JF-N
Mating
Birth
3wk
Tissue collection
Gene expression
analysis
3wk+ 10d
Tissue collection

Figure 2.

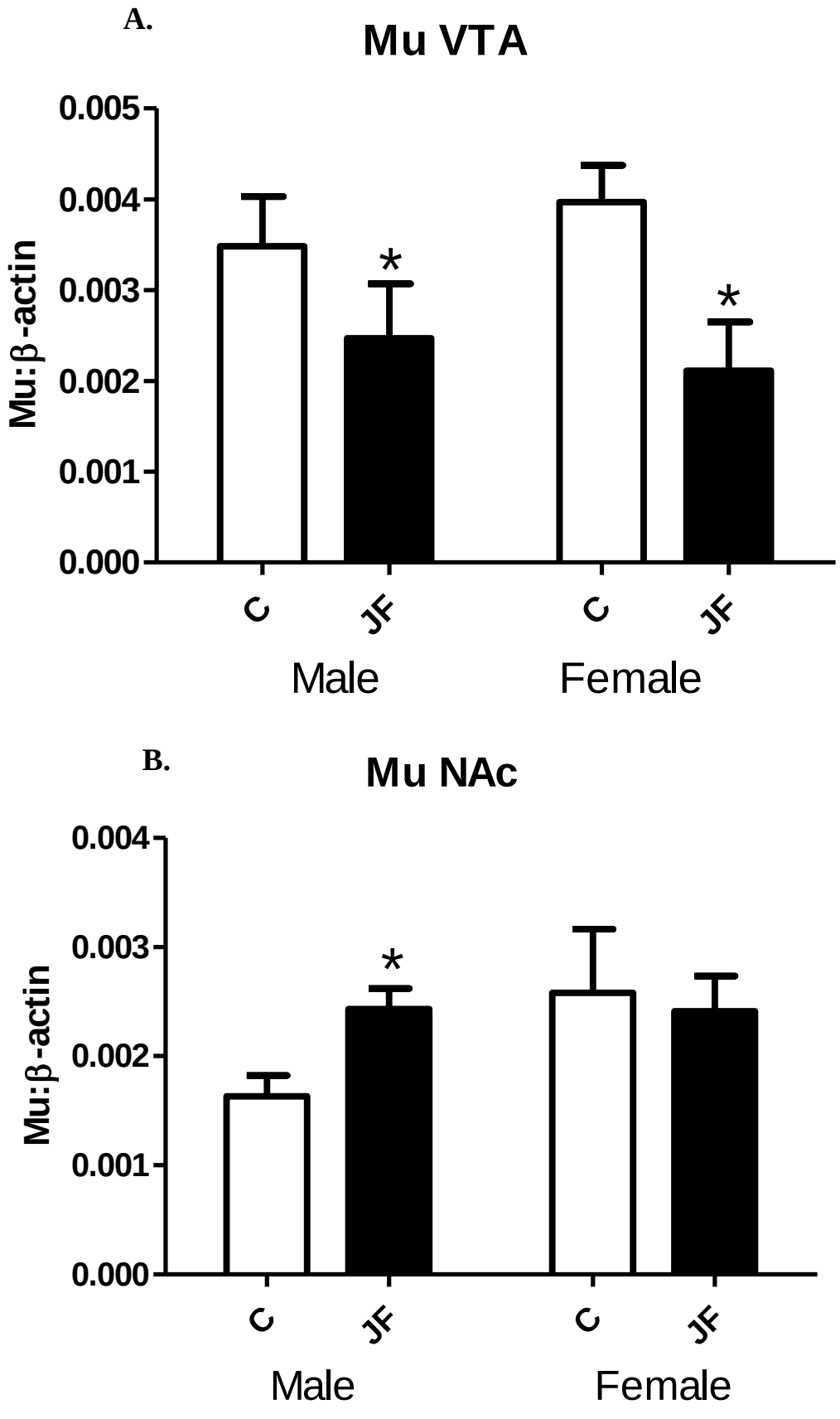
A.
Mu VTA
Mu:β-actin
0.005
0.004
0.003
0.002
0.001
0.000
*
*
C
JF
C
JF
Male
Female
B.
Mu NAc
Mu:β-actin
0.004
0.003
0.002
0.001
0.000
*
C
JF
C
JF
Male
Female

Table 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., different superscript letters denote values that are significantly different, *P<0.05*

Figure 3.

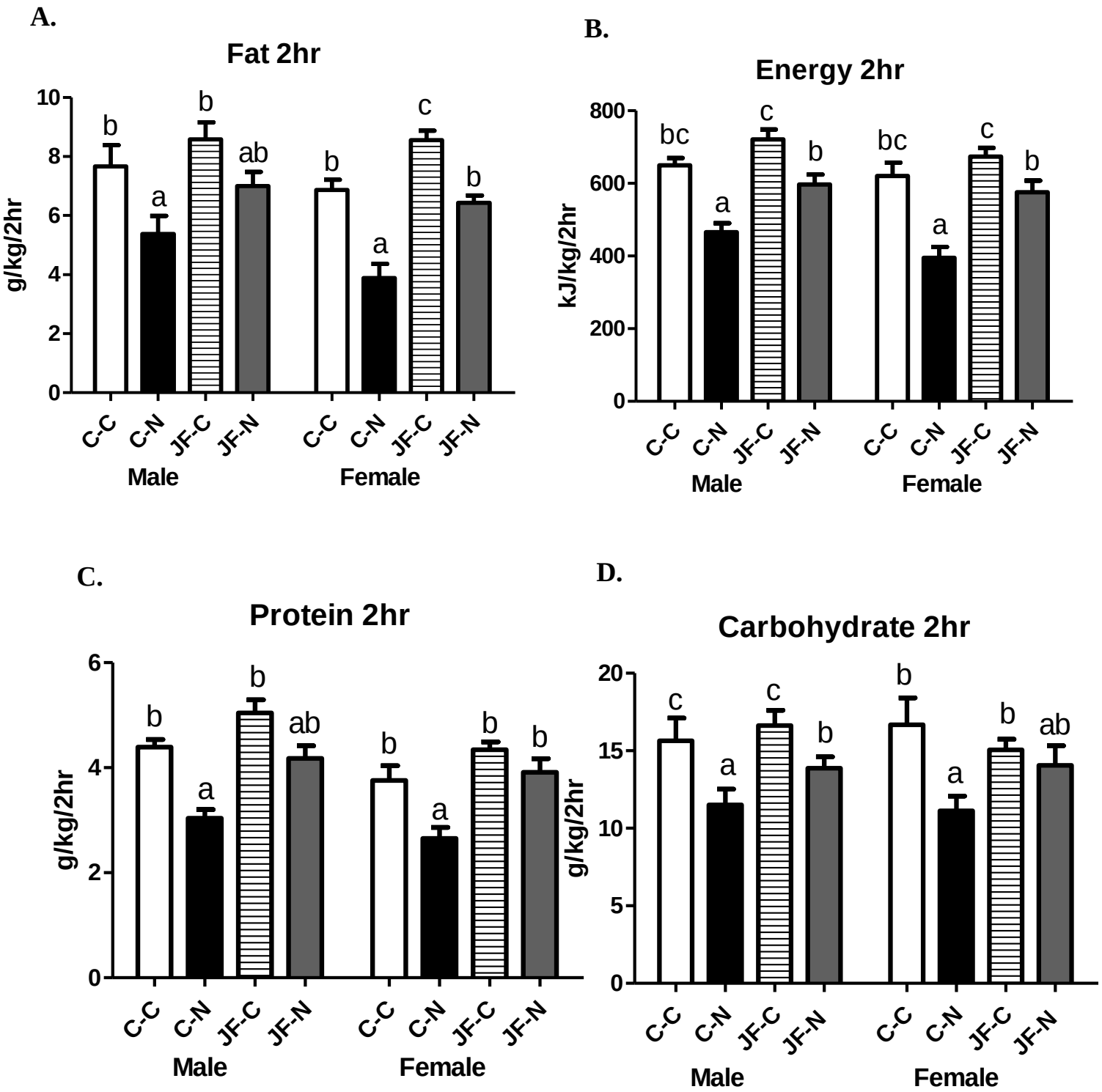
A.
Fat 2hr
g/kg/2hr
10
8
6
4
2
0
b
a
b
ab
b
a
c
b
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female
B.
Energy 2hr
kJ/kg/2hr
800
600
400
200
0
bc
a
c
b
bc
a
c
b
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female
C.
Protein 2hr
g/kg/2hr
6
4
2
0
b
a
b
ab
b
a
b
b
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female
D.
Carbohydrate 2hr
g/kg/2hr
20
15
10
5
0
c
a
c
b
b
a
b
ab
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female

Figure 4.

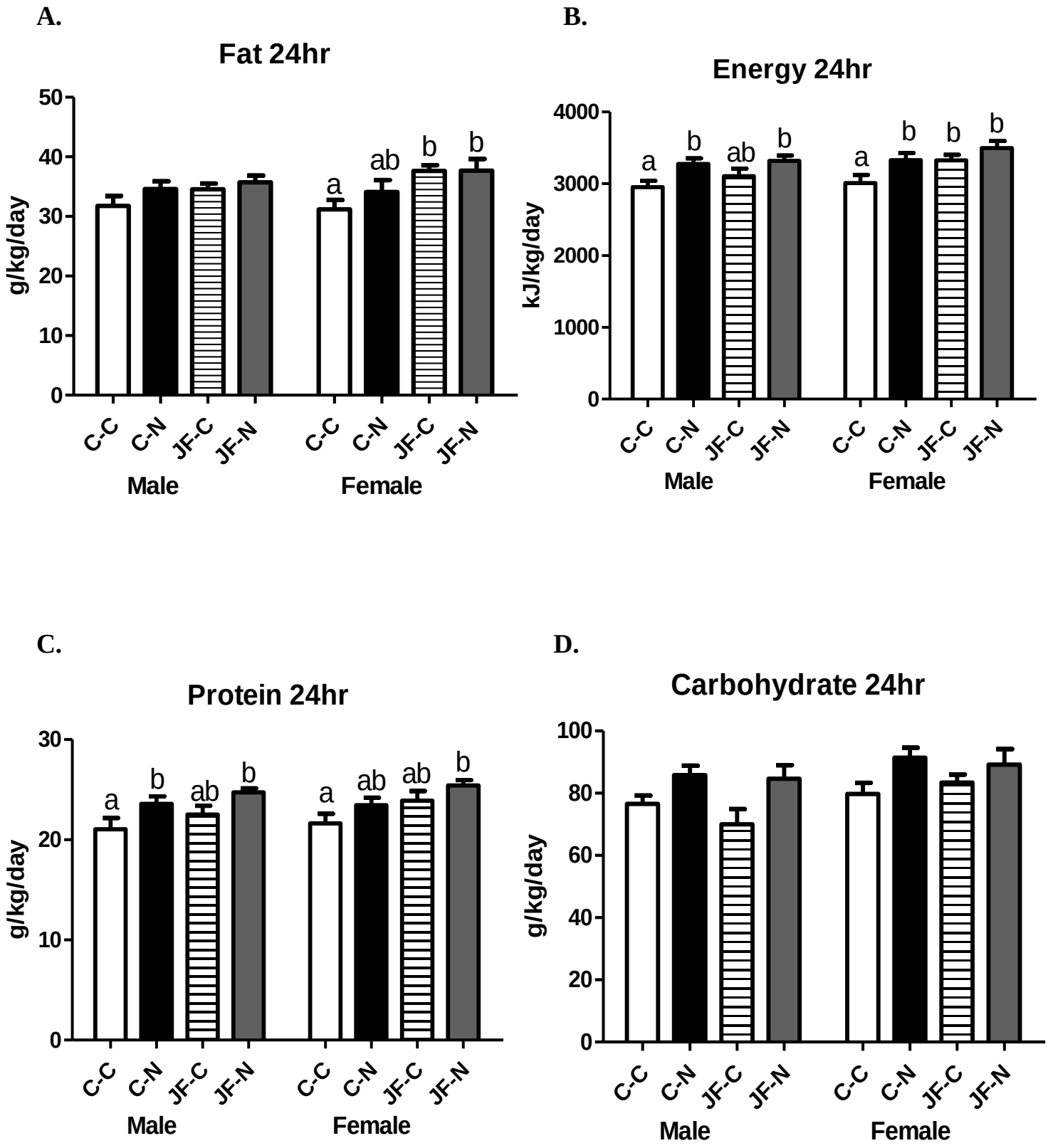
A.
Fat 24hr
g/kg/day
50
40
30
20
10
0
a
ab
b
b
C-C
C-N
JF-C
JF-N
Male
Female
B.
Energy 24hr
kJ/kg/day
4000
3000
2000
1000
0
a
b
ab
b
a
b
b
b
C-C
C-N
JF-C
JF-N
Male
Female
C.
Protein 24hr
g/kg/day
30
20
10
0
a
b
ab
b
a
ab
ab
b
C-C
C-N
JF-C
JF-N
Male
Female
D.
Carbohydrate 24hr
g/kg/day
100
80
60
40
20
0
C-C
C-N
JF-C
JF-N
Male
Female

Figure 5.

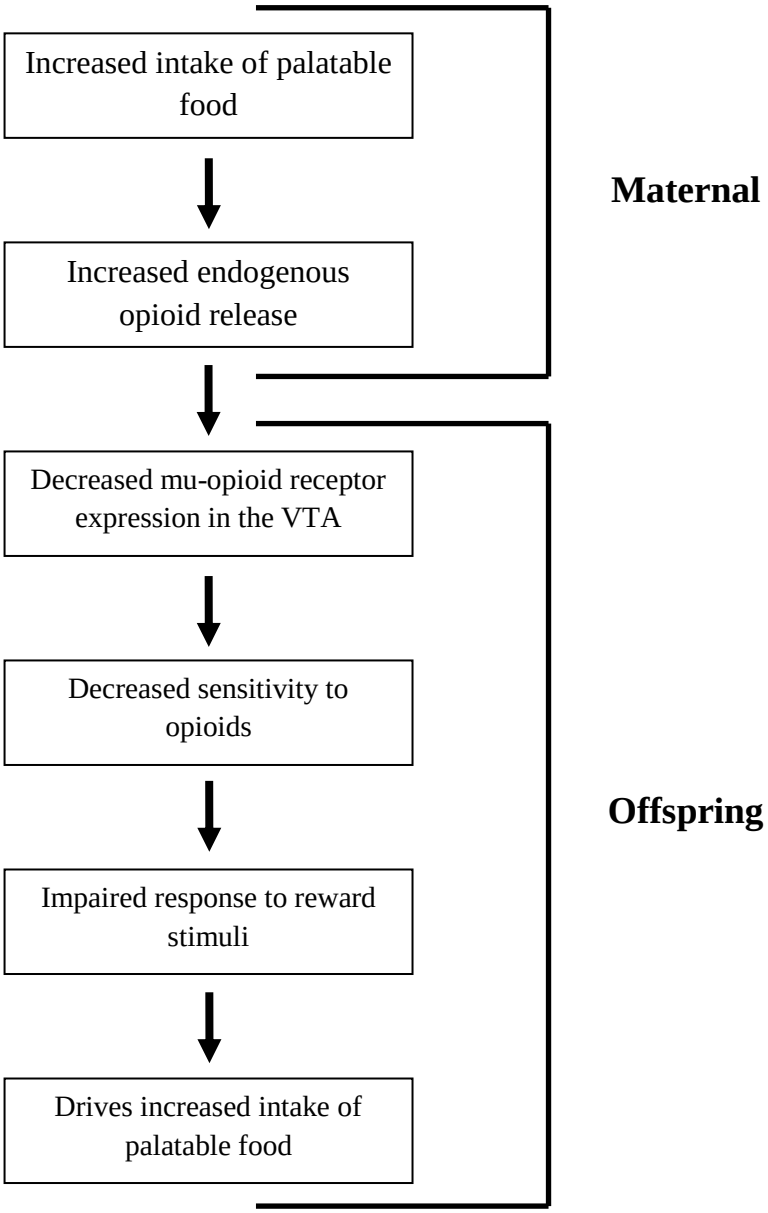
Increased intake of palatable food
Increased endogenous opioid release
Maternal
Decreased mu-opioid receptor expression in the VTA
Decreased sensitivity to opioids
Impaired response to reward stimuli
Drives increased intake of palatable food
Offspring

Figure 1 Experimental design. Dams were given either a control or junk food diet throughout pregnancy and lactation (control n=8, JF n=9). At weaning (3wk) tissue samples were taken and gene expression analysis conducted on a subset of pups (control n=18, JF n=17). Remaining pups, (control n=32, JF n=34) were designated to receive either a saline or naloxone injection daily for 10 days post-weaning.

Figure 2 Expression of the mu-opioid receptor in male and female offspring of control (open bars, n=18) and junk food dams (closed bars, n=17) in (A) the VTA and (B) the NAc at weaning (PND 21). Results presented as mean±SEM, * indicates $P<0.05$.

Figure 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 junk food 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 $P<0.05$.

Figure 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 junk food 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 $P<0.05$.

Figure 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 densitization 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.

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