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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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Short title: Naloxone and postweaning reward system

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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; D1, dopamine receptor 1 ; D2, dopamine receptor 2 ; DAT, dopamine active transporter ; 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; TH, tyrosine hydroxylase ; VTA, ventral tegmental area.

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 desensitization 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 signaling 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.

Key Words: opioids, high-fat diet, programming

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1. Introduction

The increased consumption of junk foods is a major contributing factor to the rise in obesity prevalence [1, 2]. 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 [3]. 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 [4, 5].

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 [4, 6, 7]. 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 [8]. These findings led us to hypothesize that the opioid signaling 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 [9, 10] alters the development of the opioid signaling 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 hydroxlase (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[11-17]. 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 [4, 8]. 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.

2. Methods

2.1 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 [8]. 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 [4]. 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.

2.2 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 [8]. 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 [18] 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 [8]). 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).

2.3 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 [4]. 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 [4]. 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.

2.4 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 [4, 19] 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 normalized 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.

2.5 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.

2.6 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.

2.7 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%.

2.8 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.

3. Results

3.1 Effect of maternal diet on birth outcomes

Birth outcomes including birth weight of these offspring has been previously published [8]. 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 [8].

3.2 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 [8]. There was no effect of perinatal diet exposure on DAT expression in the VTA (Table 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 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 1).

230 **Table 1**. Mean normalized 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 significant differences between groups, P<0.05. Values were multiplied by one thousand for ease of presentation

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

3.3.1 Mu-opioid receptor and proenkephalin

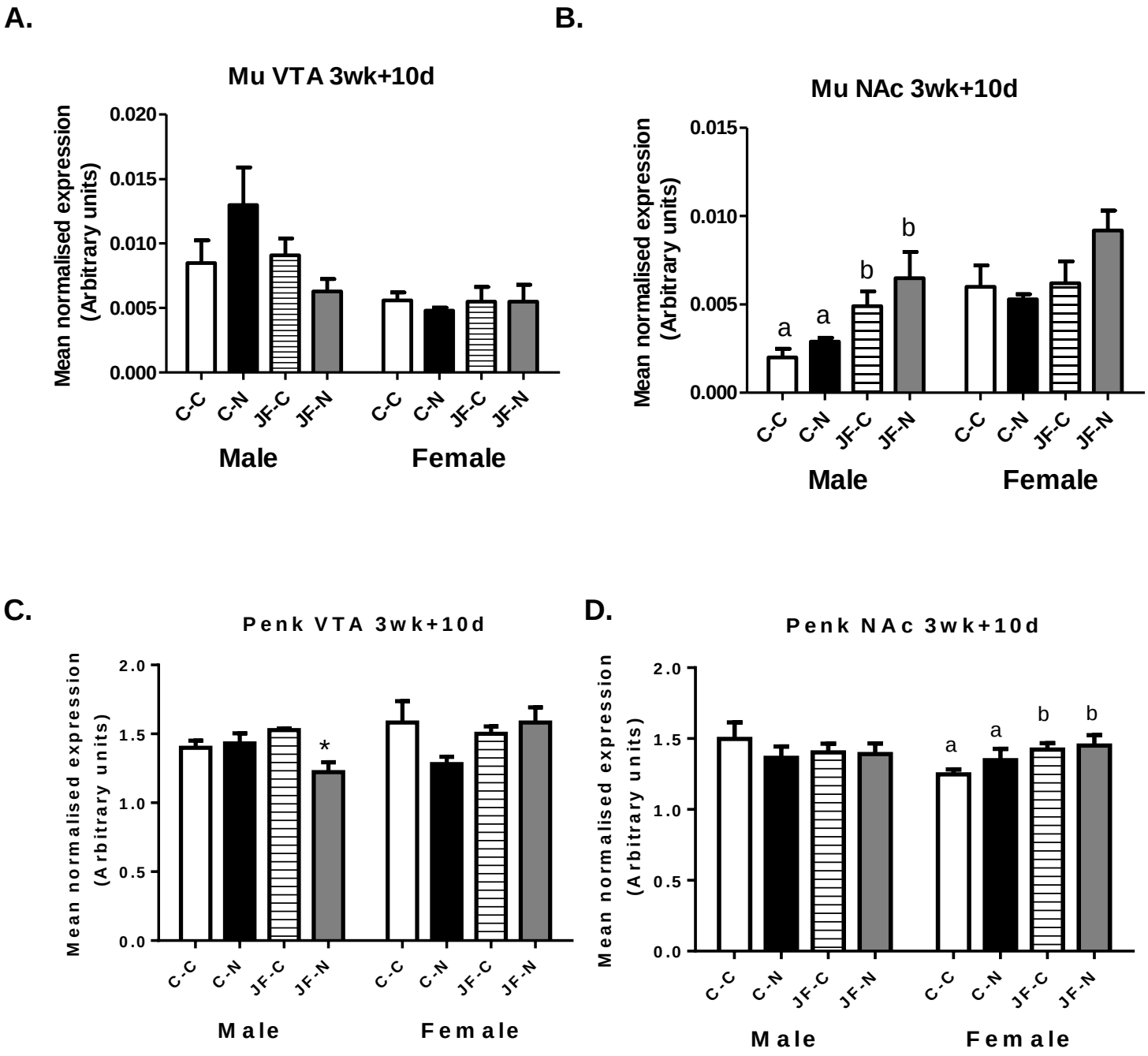
There was no effect of naloxone treatment on mu-opioid receptor expression in the VTA of either male or female C or JF offspring (Fig. 1A). The mRNA expression of the mu-opioid receptor in the NAc was higher in male JF offspring compared to controls ($P<0.05$), and also tended ($P=0.07$) to be higher in female JF offspring, independent of whether they received naloxone or saline (Fig. 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 1C). There was no effect of either perinatal diet or naloxone treatment on proenkephalin mRNA expression in the NAc of male offspring (Fig. 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. 1D).

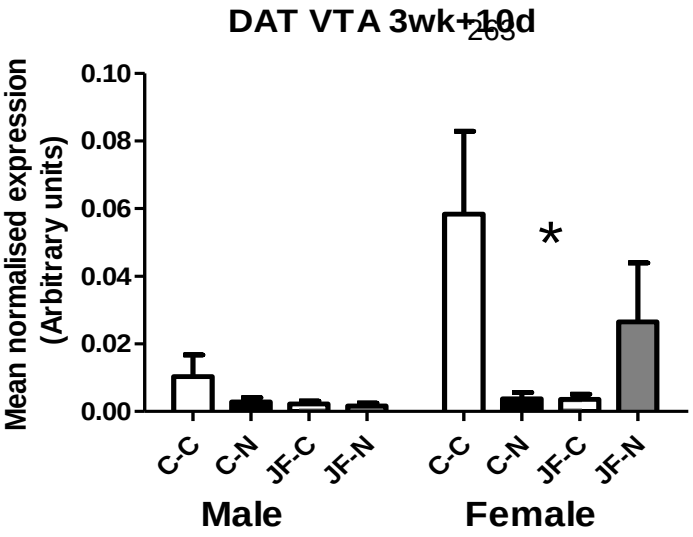
3.3.2 DAT, TH and the D1 and D2 dopamine receptors

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. 1E, F). In male offspring, naloxone treatment decreased DAT expression in the NAc of both C and JF offspring ($P<0.05$, Fig. 1F), but there was no effect of either perinatal dietary exposure or naloxone treatment on DAT mRNA expression in the VTA (Fig. 1E).

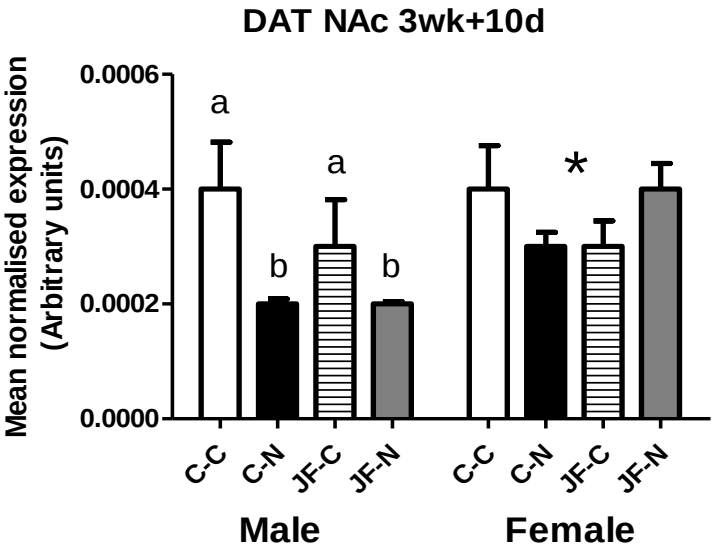
Figure 1 Expression of the mu-opioid receptor, proenkephalin (Penk) and DAT in the VTA (A, C, E) and NAc (B,D, F) in the male and female offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of junk food dams given saline (JF-C, striped bars) or naloxone (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 significant differences between groups $P<0.05$. * indicates a significant interaction between maternal diet and naloxone/saline treatment.



E.


DAT VTA 3wk+10d
Mean normalised expression (Arbitrary units)
0.10
0.08
0.06
0.04
0.02
0.00
*
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female

F.


DAT NAc 3wk+10d
Mean normalised expression (Arbitrary units)
0.0006
0.0004
0.0002
0.0000
a
b
a
b
*
C-C
C-N
JF-C
JF-N
C-C
C-N
JF-C
JF-N
Male
Female

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 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 2).

3.4 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 3).

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. 2A) and female ($P<0.05$, Fig. 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 [8].

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, 4A,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. 3A). Chow intake in female offspring remained significantly higher than C offspring throughout this period ($P<0.05$, Fig. 3B).

294 **Table 2**. Mean normalized 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 3weeks and10 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 expressed as mean±SEM, *n*= 8-9 for all groups. Values have been multiplied by one thousand for ease of presentation. Different superscript letters denote significant differences between groups within each sex * indicates interaction between maternal diet and naloxone treatment, $P<0.05$

295 **Table 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

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	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 2 Body weight of male (A) 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) 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 $P<0.05$

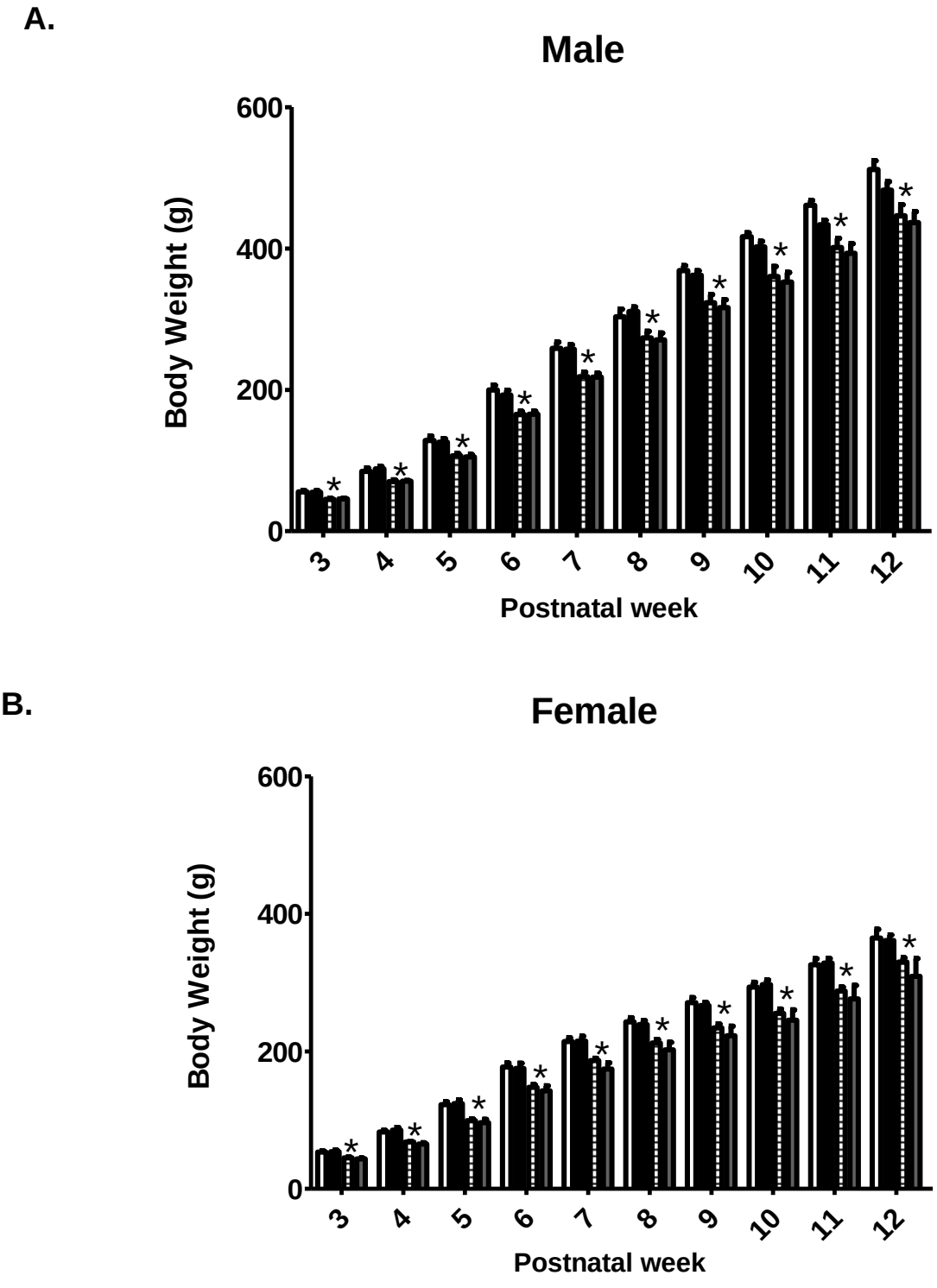
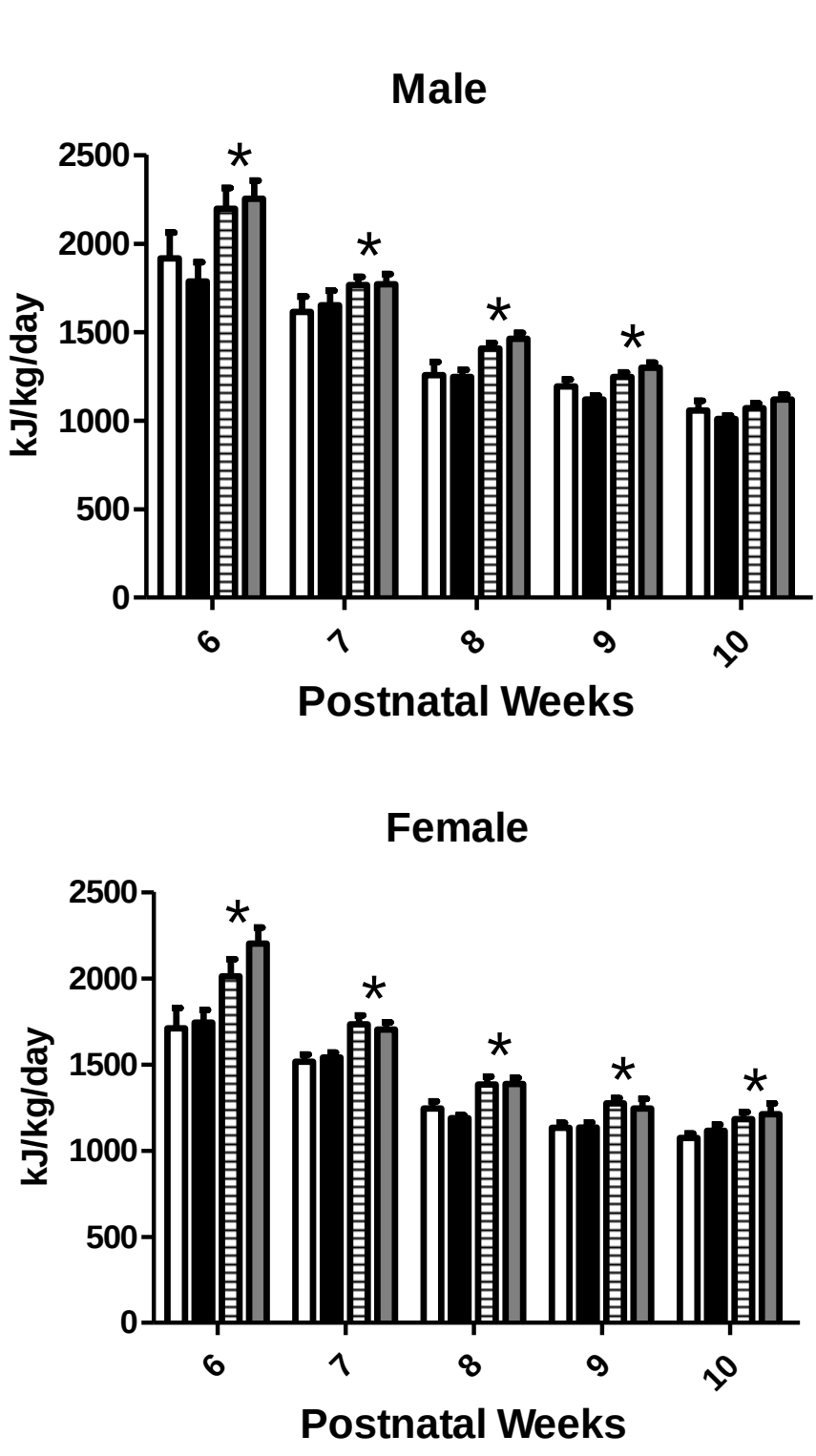


Figure 3 Intake of total energy from standard laboratory chow of male (A) and female (B) offspring of control dams given saline (C-C, open bars) or naloxone (C-N, closed bars) and offspring of junk food dams given saline (JF-C, striped bars) or naloxone (JF-N, grey shaded bars) from postnatal week 6 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 $P<0.05$.



3.5 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 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%).

323 **Table 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.

4. 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.

4.1 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 [8]. 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 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 [8] 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 [6]. 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 [20].

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 [21, 22]. 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 [9, 10]. 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 [23]. 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 [24], 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.

4.2 Female offspring more 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 [4]. 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 [25, 26], 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 [27, 28], 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 [29, 30]. 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 [31, 32], whilst opioid antagonism has been shown to reduce extracellular dopamine levels [27, 33]. Different responses to exogenous opioids between sexes has also been widely reported in adults [34, 35] 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 [36].

4.3 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 [37, 38], and is thought to be a consequence of programming of the central appetite regulating circuits [19].

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 [39, 40], 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 [41]. Furthermore, in our study, naloxone treatment was only administered at concentrations (5mg/kg) capable of reducing food intake for two hours post injection [42, 43]. The acute nature of the treatment may have limited any long term effects, since a previous study has identified changes in feeding behavior in juvenile rats which were treated with the opioid antagonist naltrexone (which is capable of reducing food intake for 6hrs post-injection [44])) from birth until weaning [45].

4.4 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.

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