NEUROSCIENCE
INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION
RESEARCH ARTICLE
M. F. Rossetti et al. / Neuroscience xxx (2018) xxx–xxx

Epigenetic Dysregulation of Dopaminergic System by Maternal Cafeteria Diet During Early Postnatal Development

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Abstract—Dopamine is a neurotransmitter crucial for motor, motivational, and reward-related functions. Our aim was to determine the effect of a palatable maternal diet on the transcriptional regulation of dopaminergic-related genes during perinatal development of the offspring. For that, female offspring from dams fed with a control (CON) or a cafeteria (CAF) diet were sacrificed on embryonic day 21 (E21) and postnatal day 10 (PND10). Using micropunch techniques, ventral tegmental area (VTA) and *nucleus accumbens* (NAc) were isolated from brain's offspring. Bioinformatic analysis of the promoter regions, mRNA quantification and methylation studies were done. The increase in tyroxine hidroxylase (TH), dopamine receptor (DRD) 1 and ghrelin receptor (GHSR) expression in VTA and NAc from E21 to PND10 was correlated with changes in DNA methylation of their promoter regions. Maternal diet did not affect the expression patterns in E21. At PND10, maternal CAF diet decreased the transcription of TH, GHSR, DRD2 and dopamine transporter (DAT) in VTA. Interestingly, the changes in TH, DRD2 and DAT expression were related to the methylation status of their promoters. In NAc, maternal CAF diet reduced DRD1, DRD2 and DAT expression in the offspring at PND10, although alternations in the methylation patterns were only detected in DAT promoter. These results show the importance of maternal nutrition and provide novel insights into the mechanisms through which maternal junk-food feeding can affect reward system during development and early postnatal life. Particularly important is the expression decline of DRD2 given its physiological implication in obesity and addiction.

Key words: epigenetic, dopamine-related genes, maternal cafeteria diet, rat offspring.

INTRODUCTION

Obesity represents one of the major public health problems in the world and it is mainly caused by overeating and physical inactivity. Palatable, or high-fat, high-sugar, foods activate the dopaminergic signaling pathways within the mesolimbic reward system (Berthoud, 2006; Fulton, 2010). Dopamine (DA) is a neurotransmitter crucial for motor, motivational, and reward-related functions of the central nervous system (Cragg and Rice, 2004) and is also associated with the gratifying effects of sex and drugs of abuse (Nestler and Carlezon, 2006). This neurotransmitter is produced in the dopaminergic neurons of the ventral tegmental area (VTA) by the action of the tyrosine hydroxylase (TH) (Baik, 2013a,b). Interestingly, it was shown that there is a high degree of co-expression of TH and ghrelin receptor (GHSR) in VTA in adults (Zigman et al., 2016a,b). It is well known

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Abbreviations: ACT, activator; AP, activator protein; C/EBP, CCAAT/enhancer-binding protein; CAF, cafeteria; CRE, cAMP response element; CREB, cAMP response element-binding protein; DA, dopamine; DAT, dopamine transporter; DRD, dopamine receptor; E, embryonic day; GHSR, ghrelin receptor; GRE, glucocorticoid response element; HFD, high fat diet; INH, inhibitor; NAc, *nucleus accumbens*; NF-1, nuclear factor 1; NF-AT, nuclear factor of activated T cells; Sp1, selective promoter factor 1; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

https://doi.org/10.1016/j.neuroscience.2019.09.016
0306-4522/

Please cite this article in press as: Rossetti MF et al. Epigenetic Dysregulation of Dopaminergic System by Maternal Cafeteria Diet During Early Postnatal Development. Neuroscience (2019), https://doi.org/10.1016/j.neuroscience.2019.09.016

that ghrelin impairs VTA by inducing DA release and stimulating food intake (Fulton, 2010); thus, GHSR-TH coexistence suggests a possible coordinated regulation of DA levels. VTA dopaminergic neurons project to the *nucleus accumbens* (NAc), where DA is released and binds to specific DA receptors (DRD1 and DRD2). This nucleus receives sensitive information from various regions, and then projects to the hypothalamic and midbrain areas that contribute to the motor action of food (Valdivia et al., 2014). NAc mediates reward effects in response to natural stimuli and is where termination of DA signaling occurs through reuptake by the active DA transporter (DAT) (Cragg and Rice, 2004). Prolonged exposure to palatable food in adult rodents is associated with behavioral and neurophysiological adaptations comparable to those seen in drug addicts. In particular, desensitization of the central reward pathway, which then drives continued overconsumption (Ong and Muhlhausler, 2011).

Several epidemiological and experimental studies have demonstrated that susceptibility to obesity can have its origins early in life and can be influenced by the nutritional experience during critical periods of fetal and early postnatal development. In rodents, an experimental model used to reproduce the characteristics of the Western obesogenic food is the cafeteria diet (CAF) (Sampey et al., 2011). Numerous authors reported that exposure to a maternal diet dominated by palatable food and/or high-fat diet before and during pregnancy and throughout lactation, disturb glucose and lipid homeostasis, predispose to adiposity, modify food preference, alter the development of the central reward circuitry and modify the expression of brain genes such as DAT, DRD1 and DRD2 in the offspring after birth and later in life (Akyol et al., 2009; Bayol et al., 2007; Bayol et al., 2008; Chen et al., 2008; Ong et al., 2012; Ong and Muhlhausler, 2011; Sarker et al., 2018; Vucetic et al., 2010). However, there are no prior reports about the effects of these maternal diets on the dopaminergic reward system during perinatal periods.

Metabolic and eating disorders are associated with alterations in the DNA methylation pattern of particularly genes, such as TH and DAT (Vucetic et al., 2012). DNA methylation represents one of the most important epigenetic mechanisms for blocking gene expression and implicates the addition of methyl groups to CpG dinucleotides. A CpG island is a DNA sequence generally greater than 250 bp that is rich in CpG sites and, thus, it has a key role in transcriptional control (Deaton and Bird, 2011). In this context, DNA methylation provides a mechanism by which maternal diet can modify the predisposition of the offspring to obesity-associated disorders or other pathologies.

In the present study, we hypothesized that maternal CAF diet is associated with alterations in the epigenetic control of dopaminergic system of the reward pathway in early postnatal development. Thus, the objective of our work was to analyzed the transcriptional regulation of dopamine-related genes in the brain of female offspring (F1), from dams fed with standard chow and CAF diet, at embryonic day 21 (E21) and postnatal day 10 (PND10). The study was performed in two discrete brain reward areas, NAc and VTA, using microdissection techniques. This experimental model allowed us to study the individual and combined effects of age (E21 vs. PND10) and maternal diet (CON vs. CAF) on the mRNA expression of dopaminergic genes and the DNA methylation mechanisms that are involved in their control.

EXPERIMENTAL PROCEDURES

Animals an experimental design

Wistar female rats were obtained from the Department of Human Physiology of the School of Biochemistry and Sciences (UNL). All animals' procedures were approved by the Ethical Committee of the School of Biochemistry and Biological Sciences (UNL, Santa Fe, Argentina) and designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences (Commission on Life Sciences, National Research Council, Institute of Laboratory Animal Resources, 1996).

Rats were housed two per cage, at 22 ± 2 °C and with a 12-h light–dark cycle, and fed with either standard chow (CON) or a cafeteria diet (CAF) (N = 10/group) from weaning. The standard chow (Cooperación, ACA Nutricion Animal, Buenos Aires, Argentina) provided 12.55 KJ/g, 5% energy as fat, 23% protein and 72% carbohydrate. The CAF diet was composed of standard chow and food that reflects variety, palatability, and energy density (parmesan cheese, cheese flavored snacks, crackers, sweet biscuits, pudding, and chocolate). This diet provided an average of 20.29 KJ/g, 49% of energy as fat, 7% as protein, and 44% as carbohydrate, in addition to that provided by the standard chow. Dietary composition and treatment procedure are described in Lazzarino et al. (2017).

On the 14th week of treatment, when CAF animals were significantly heavier of that than the CON, females were mated with male rats that were housed under standard laboratory conditions during all the experiment (22 ± 2 °C, 12-h light–dark cycle, fed with standard chow). After mating, each dam was single caged and continued with the respectively diet. Five dams per diet group were euthanized on embryonic day 21 (**E21)**. The rest of the animals (N = 5/group) were maintained and at day 1 after delivery each litter was adjusted to 8 pups per dam. At postnatal day 10 (**PND10)**, pups were euthanized. During all the experimental period, dam body weight and energy intake were recorded weekly and litter weight was measured daily. After euthanization, female fetus and pups brains (named as E21-CAF, E21-CON, PND10-CAF and PND10-CON) were removed, frozen on dry ice and stored at −80 °C until sectioning for RNA and DNA analysis.

Micropunches of VTA and NAc

Following the procedure of microdissection technique described by Palkovits (Palkovits and Brownstein, 1988), embryo and pup brains were cut in a cryostat at

−12 °C (serial coronal sections of 150 μm). To identify and punch VTA and NAc regions, the atlas of the developing rat nervous system (Paxinos et al., 1994) and atlas of the postnatal rat brain in stereotaxic coordinates (Khazipov et al., 2015) were used. Both areas were removed bilaterally using a 0.5 mm stainless steel micropunch needles and the reproducibility was checked analyzing the topography of the holes under a stereo microscope (Stemi 305, Zeiss, Oberkochen, Germany). Samples were stored at −80 °C until RNA and DNA isolation.

Reverse transcription and real-time quantitative PCR analysis (qRT-PCR)

VTA and NAc areas (N = 8/group) were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was isolated. 1 μg of RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (10 units; Promega, Madison, WI, USA) as previously described (Rossetti et al., 2015) and final product was diluted with nuclease-free water to a final volume of 60 μl.

Reverse-transcribed products were combined with HOT FIRE Pol Eva Green qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen) and further amplified in duplicate using Real-Time DNA Step One Cycler (Applied Biosystems Inc., Foster City, CA, USA). The primer pairs used are detailed in Table 1 and the protocol for real-time quantitative PCR is described by Rossetti et al. (2015).

Bioinformatics

TH, DRD1, DRD2, DAT and GHSR promoters were analyzed for: a) CpG islands using MethPrimer program (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; RRID: SCR_010269); b) restriction sites for *SmaI* (New England BioLabs, Beverly, MA, USA), BstUI (New England BioLabs) or Mae II (Roche Applied Science, Indianapolis, IN, USA); and c) potential binding sites for transcription factors with the bioinformatic tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3; RRID: SCR_016926) (Messeguer et al., 2002). PCR primers were designed with the online software NCBI Primer-BLAST (National Center for Biotechnology; https://www.ncbi.nlm.nih.gov/tools/primer-blast/; RRID: SCR_003095; Table 2).

Methylation-sensitive analysis

Genomic DNA from VTA and NAc areas (N = 8/group) was isolated using the phenol/chloroform/isoamyl alcohol extraction and digested with 1 unit of *SmaI*/Mae II or 10 units of BstUI and 1X enzyme buffer for 1 h at 25 °C, 60 °C or 50 °C, respectively. After purification with the Wizard SV gel and PCR Clean-Up System Kit (Promega, Madison, WI), an optimized qRT-PCR protocol was used to analyze the relative methylation levels of various regions of the TH, DRD1, DRD2, DAT and GHSR promoters (Table 2). The procedure for DNA amplification was previously described by our group in several studies (Lazzarino et al., 2017; Rossetti et al., 2018; Rossetti et al., 2016; Rossetti et al., 2015).

Table 1. Sequences of primer oligonucleotides for PCR amplification.

Target	Primer sense	Primer antisense	Temperature of annealing (°C)
L19 (*housekeeping*)	5- AGCCTGTGACTGTCCATTCC -3′	5′- TGGCAGTACCCTTCCTCTTC -3′	60
TH	5′-TACCAAGATCAAACCTACCAGCC-3′	5′-GGTCAAACTTCACAGAGAATGGG-3′	58
DRD1	5′-TCCAAGGTGACCAACTTCTT-3′	5′-GTTACAAAAGGACCCAAAGG-3′	55
DRD2	5′-CCCAGCAGAAGGAGAAGAAA-3′	5′-CAGGATGTGCGTGATGAAGA-3′	55
DAT	5′-CATCACCACCTCCATTAACTCC-3′	5′-CATTGTGCTTCTGTGCCATG-3′	56
GHSR	5′-GCTCTGCAAACTCTTCCA-3′	5′-AAGCAGATGGCGAAGTAG-3′	56

Table 2. Sequences of primer oligonucleotides for PCR amplification to evaluate methylation sensitive sites in promoters.

Target	Primer sense	Primer antisense	Temperature of annealing (°C)
TH IC	5′- CCATCAGATTTACCTAGAAGC-3′	5′-TGAGACTATGAAGGGACATTG-3′	51.5
TH-*MaeII*	5′-ACAGCAGGCGTGGAGAGGAT-3′	5′-TGGTGGTCCCGAGTTCTGTC-3′	60
TH-*MaeII* b	5′-CCTTAGGAAATCCAGCATGG-3′	5′-ATTGCATCCACTGTCACAGG-3′	57.7
TH-*MaeII* c	5′-CATGTGGCTGCTCCTATGTA-3′	5′-GAGAGAGATTGGCACACACA-3′	52.6
DRD1 IC	5′-GTGGTGAGAATCCCCTCAGG-3′	5′-AGTTCCACAGGCGGAGAACC-3′	55
DRD1-*MaeII*	5′-CAGGCAAAGAGGTTCACAAG-3′	5′-CCGCCATCTAAACAGTTACC-3′	54.6
DRD1-*BstUI*	5′-AGCAGGAAACCACAGGCACC-3′	5′-GCTTCTGCGGTCAACTCACG-3′	60
DRD2 IC	5′-AATTCTGTGGTGCCTTCTCCT-3′	5′-ATGGGGTCAATCCAGAGTAGA-3′	55
DRD2-*BstUI*	5′-AGTGCAGAGATAGTTCTGGG-3′	5-AGAAGCCACAGACTGTCGTT-3′	63
DAT IC	5′-TTTGGGGTCTCAACTAGAAA-3′	5-TAAGACCTTTTCAGAACCCA-3′	55
DAT- *BstUI* (a)/*MaeII*	5′-CTTCTGACAACCTCGCTGGA-3′	5′-GGGGCTTGACAGGAGTCTTT-3′	60
DAT- *BstUI* (b)/*SmaI*	5′-CGTACAACACCGAAGGAAGA-3′	5-CGAGGTTGTCAGAAGCAGAT-3′	57.7
GHSR IC	5′-TCCAGCATACTCCTTATCCA-3′	5′-TGGCAATCTTAGAACACACC	54.6
GHSR-*BstUI*(a)/*SmaI*	5′-TACGCCACGGTCCTCACCAT-3′′	5′-ACGCTGGACACCCACACCAT-3′	61
GHSR-*BstUI*(b)/*MaeII*	5′-TCTCCCTTTCCTCTCCAAGC-3′	5-TTCGTCAGGCAGTGAGTCGT-3′	61

IC: Internal Control.

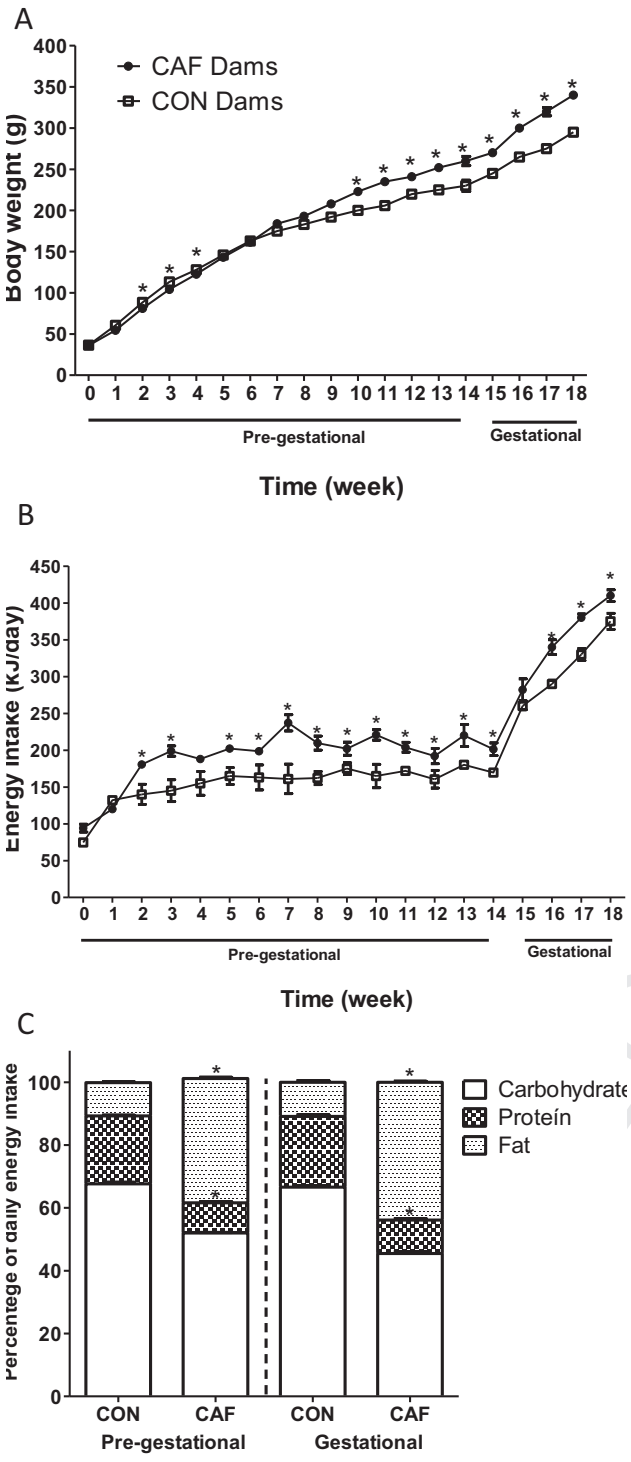

Fig 1. Body weight (A), energy intake (B) and nutrient intake (C) of dams fed with a control (CON) or a cafeteria (CAF) diet during pre-gestation (Week 1 to 14) and gestation (Week 15–18) periods (N = 10/Group). * indicates significant differences at $p < 0.05$ vs. CON group by Student's T test.

Statistical analysis

G Power software (http://www.gpower.hhu.de/; RRID: SCR_013726) was used to determine the sample size (Faul et al., 2007). To confirm the normal distribution of the data and variance homogeneity, Shapiro–Wilk test and Levene's test were performed. Weekly body weights, nutrient intake and energy intake were analyzed using Student's T test; while a two-way ANOVA followed by Bonferroni post-test was implemented to study the age and diet effects on mRNA and DNA methylation. All the data is expressed as the means ± SEM and was statistically analyzed using the IBM SPSS Statistics 19 software (IBM Inc.; RRID: SCR_002865), considering significant differences at $p < 0.05$.

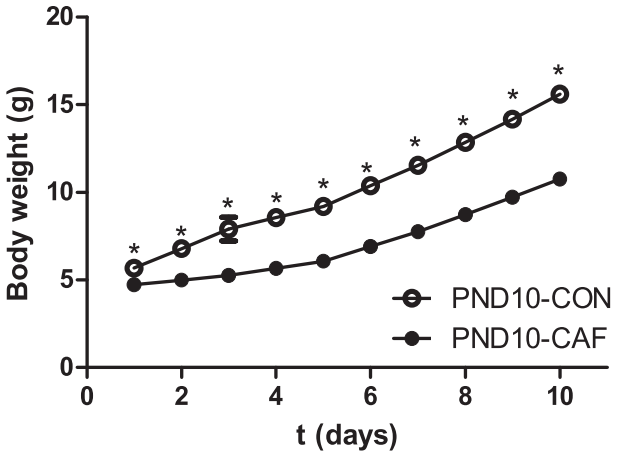

Fig 2. Body weight of pups from dams fed with a control (PND10-CON) or a cafeteria (PND10-CAF) diet from birth up to post-natal day 10 (PND10). Values are means, with standard errors represented by vertical bars (N = 16/Group). * indicates significant differences at $p < 0.05$ vs. CON group by Student's T test.

RESULTS

Effects of CAF diet on dams´ body weight, nutrient intake and energy intake

The body weights of dams fed with CAF diet increased from week 10 of dietary intervention and on the 14th week of treatment (CAF: 266.5 ± 2.92 g; CON: 240.5 ± 3.1 g; $p < 0.05$) females were mated with male rats. During pregnancy, CAF dams significantly increased gestational weight gain in comparison with those fed the control diet (Fig. 1a). In addition, energy intakes over the pre-pregnancy (Week 2 to 14) and pregnancy (Week 16 to 18) period remained significantly higher in CAF rats when compared with CON animals (Fig. 1b). Moreover, CAF rats consumed a significantly greater percentage of their daily energy intake as fat, and significantly less as protein than CON rats (Fig. 1c), during all the experiment.

Effects of CAF diet on the body weight of the offspring

Pups of dams fed with a CAF diet had a significantly lower weight ($p < 0.05$) than those from a CON diet from birth to PND10 (Fig. 2). It is interesting to note that during this period no apparent differences were detected in maternal behavior between CON and CAF groups. At E21 no significant differences were detected (data not shown).

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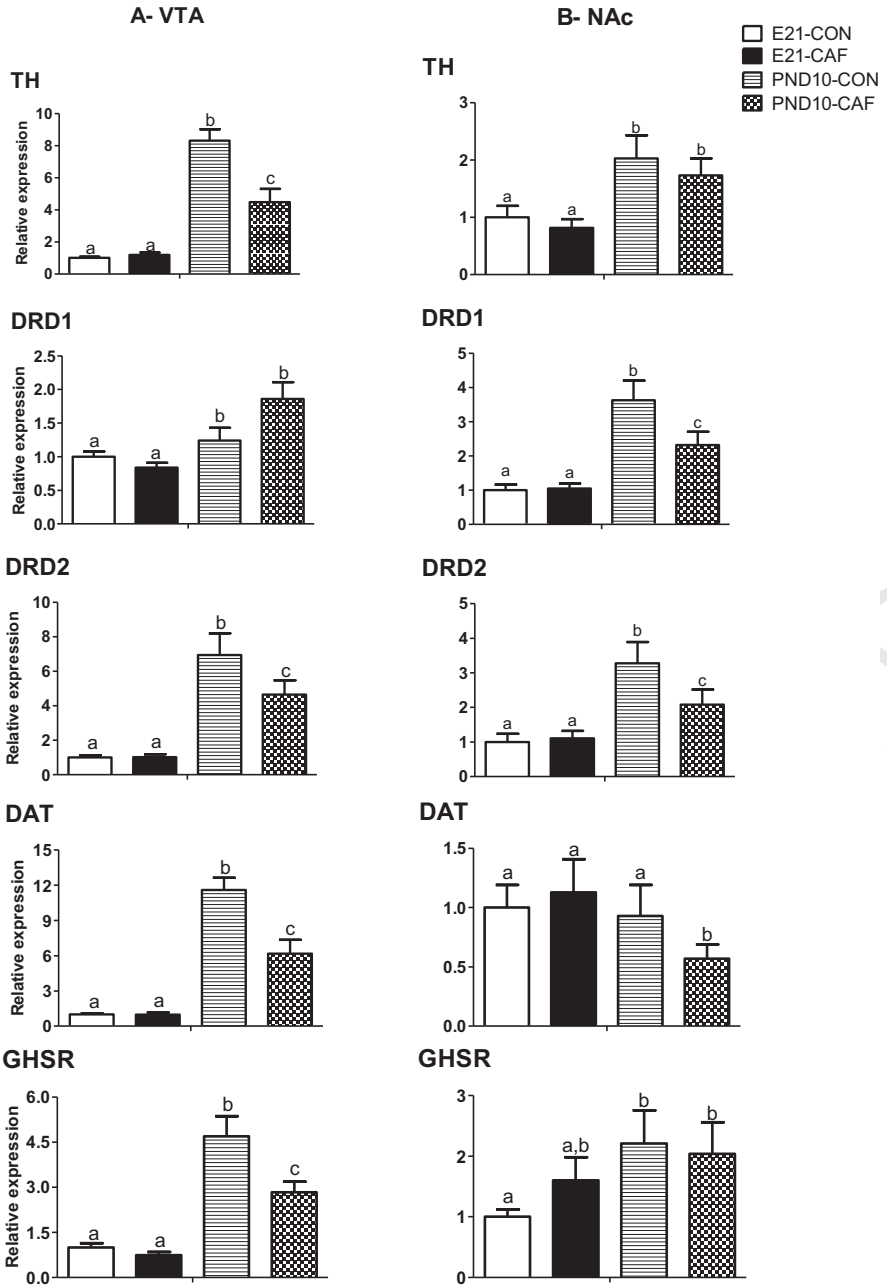


Fig 3. Analysis of relative mRNA levels of dopaminergic related-genes and ghrelin receptor in ventral tegmental area (VTA, A) and nucleus accumbens (NAc, B) of the offspring in embryonic day 21 and on post-natal day 10 from dams fed with control (E21-CON and PND10-CON, respectively) or cafeteria (E21-CAF and PND10-CAF, respectively) diet. Relative amounts of mRNA in E21-CAF, PND10-CON and PND10-CAF are showed as fold changes from those of E21-CON. The means ± SEM (N = 8/group) are represented by columns and error bars. Significant differences at $p < 0.05$ by Bonferroni's test after two-way ANOVA are denoted by different letters. TH: tyrosine hydroxylase, DRD1: dopamine receptor 1, DRD2: dopamine receptor 2, DAT: dopamine transporter, GHSR: ghrelin receptor.

Maternal CAF diet modifies the mRNA expression of dopamine-related genes in the reward brain system of the offspring at early postnatal development

To analyze the effect of maternal CAF diet on dopaminergic reward system in the offspring before and during gestation and lactation periods, we analyzed the expression of molecules that are involved in the synthesis, transport and reuptake of DA in two key regions of the reward system, VTA and NAc, at E21 and PND10.

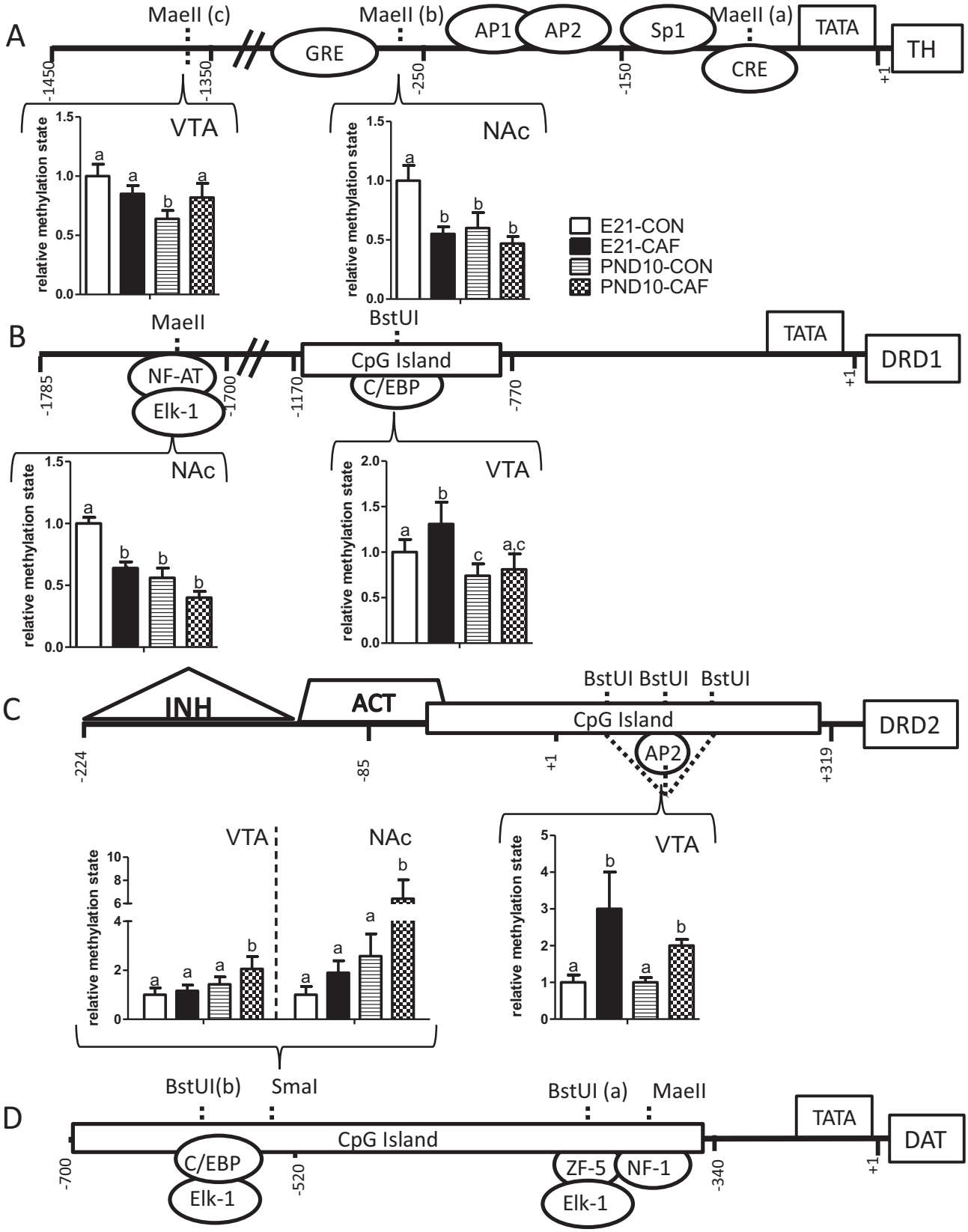
In VTA, the two-way ANOVA revealed interactions between age and maternal diet for the expression of TH ($p < 0.01$, $F = 8{,}804$), DRD2 ($p < 0.05$, $F = 4.875$), DAT ($p < 0.01$, $F = 13.93$) and GHSR ($p < 0.001$, $F = 8.458$) (Fig. 3A). Maternal CAF diet decreased the expression of these genes in the offspring in PND10 (DPN10-CAF vs DPN10-CON, $p < 0.05$), without affecting expression in E21 (E21-CAF vs E21-CON, $p > 0.05$). In addition, the increase in age generated an increase on their transcription (E21 vs DPN10, $p < 0.05$). Related to DRD1, mRNA levels were not found to be modified by maternal diet in VTA, but age increased the expression in the offspring (E21 vs PND10, $p < 0.01$, $F = 48.86$, Fig. 3A).

In NAc, the expression of DRD1 ($p < 0.05$, $F = 7.369$), DRD2 ($p < 0.01$, $F = 10.01$) and DAT ($p < 0.01$, $F = 15.29$) was affected by the interactions between age and maternal diet (Fig. 3B). Their expression decreased in the offspring of dams fed with CAF diet in PND10 (DPN10-CAF vs DPN10-CON, $p < 0.05$); however, no expression changes were observed in E21. Age generated an increase in the transcription of these genes (E21 vs DPN10, $p < 0.05$). TH and GHSR mRNA levels were not found to be modified by maternal diet in NAc, but age improved the expression in the offspring (E21 vs PND10, $p < 0.05$, $F_{TH} = 10.31$, $F_{GHSR} = 5.29$, Fig. 3B).

Transcriptional regulation of dopaminergic-related genes by DNA methylation in the reward brain system during development and in response to the maternal diet

To determine if the changes observed in the transcript levels of those genes are related to DNA methylation modifications, we analyzed *in silico* the promoter regions of TH, DRD1, DRD2, DAT and GHSR and we determined the methylation state in the E21-CON, E21-CAF, PND10-CON and PND10-CAF groups (Figs. 4 and 5).

In VTA, DNA methylation levels of TH-*MaeII* c ($p < 0.05$, $F = 5.545$, Fig. 4A), DAT-*SmaI* ($p < 0.05$, $F = 6.943$, Fig. 4D) and GHSR-*SmaI* ($p < 0.05$,


A
MaeII (c)
MaeII (b)
AP1
AP2
Sp1
MaeII (a)
TATA
TH
GRE
CRE
-1450
-1350
-250
-150
+1
VTA
NAc
relative methylation state
E21-CON
E21-CAF
PND10-CON
PND10-CAF
B
MaeII
BstUI
TATA
DRD1
CpG Island
NF-AT
Elk-1
C/EBP
-1785
-1700
-1170
-770
+1
C
INH
ACT
BstUI BstUI BstUI
CpG Island
AP2
DRD2
-224
-85
+1
+319
D
BstUI(b)
SmaI
BstUI (a)
MaeII
TATA
DAT
CpG Island
C/EBP
Elk-1
ZF-5
NF-1
-700
-520
-340
+1

F = 26.91, Fig. 5) sites were affected by the interactions between age and maternal diet. In TH-*MaeII* c and DAT-*SmaI* sites, maternal CAF diet increases methylation in the offspring in PND10 (DPN10-CAF vs DPN10-CON, $p < 0.05$); in GHSR-*SmaI* this occurs in E21 (E21-CAF vs E21-CON, $p < 0.05$); and in DRD2-*BstUI* this arises at both stages (E21 and PND10, $p < 0.05$, $F = 21.95$, Fig. 4C). On the other hand, age decrease methylation levels of TH-*MaeII* c site in the offspring of dams fed with CON diet in PND10 (E21-CON vs DPN10-CON, $p < 0.05$). In addition, age decrease methylation levels of DRD1-*BstUI* and GHSR-*MaeII* sites in the offspring of dams fed with both CON and CAF diet in PND10 (E21 vs DPN10, $p < 0.0001$, $F_{DRD1} = 42.35$ and $F_{GHSR} = 68.51$, Figs. 4B and 5). No differences were observed in methylation in the others studied sites (data not shown).

In NAc, DNA methylation levels of DAT-*SmaI* ($p < 0.05$, $F = 15.17$), TH-*MaeII* b ($p < 0.05$, $F = 23.66$) and DRD1-*MaeII* ($p < 0.05$, $F = 29.88$) sites were affected by the interactions between age and maternal diet. In the first site, maternal CAF diet increased methylation in the offspring in PND10 (DPN10-CAF vs DPN10-CON, $p < 0.05$). Age also increased methylation levels in this site in the offspring from dams fed with CAF diet (E21-CAF vs DPN10-CAF). Contrary, age decreased DNA methylation levels of TH-*MaeII* b ($p < 0.005$, $F = 13.26$) and DRD1-*MaeII* ($p < 0.0001$, $F = 33.07$) sites in the offspring from dams fed with CON diet (E21-CON vs DPN10-CON). No differences were observed in methylation of DRD2, DAT and GHSR sites (data not shown).

DISCUSSION

The principal aim of the present study was to determine whether exposure to maternal CAF feeding had an impact on dopaminergic reward pathways during perinatal period, selecting a representative point of the embryonic stage (E21) and the lactation period (PND10). Additionally, we analyzed the developmental profile of dopaminergic-related genes between both stages (E21 vs. PND10). We hypothesized that epigenetic modifications may be involved in the transcriptional control of these genes. To our knowledge, this is the first study reporting that: 1)-increase mRNA expression of TH, DRD1 and GHSR genes from E21 to PND10 in females is regulated by methylation mechanisms in VTA and/or NAc; and 2) the offspring from dams fed with CAF diet showed alterations in the transcriptional regulation of TH, DRD2 and DAT genes in VTA and NAc at PND10.

Changes in dopamine-related gene expression in VTA and NAc during early development are regulated by methylation mechanisms

The development of the dopaminergic system of the striatum in the rat begins during embryonic life and continues up to the 3rd postnatal week (Antonopoulos et al., 2002). The first mesolimbic dopamine neurons can be identified in the rat brain in E12, although dopamine axon innervations are not complete until the 3rd week of postnatal life (Ong et al., 2012). TH mRNA was found in brain tissues on early embryonic development E10-E12 and its specific activity increased from gestation to adulthood (Berger et al., 1985; Burgunder and Young, 1990; Marin et al., 2005). DRD1 and DRD2 receptors were detected in neural tissues on E14 and on E18 their localization was already similar to that observed in the adult brain. At birth, expression of mRNA for both dopamine receptor subtypes in the striatum approximated that seen in mature rats (Ong et al., 2012). In addition, DAT mRNA was first detected in neurons of the ventrocaudal mesencephalon on E14. By E18, intensely expressing neurons in the VTA and substantia nigra resembled the pattern found in adult midbrain (Fujita et al., 1993; Galineau et al., 2004). GHSR mRNA was found in the brain and spinal cord as early as E12 and continued to be expressed in these tissues during postnatal life (Steculorum and Bouret, 2011). Along the same line, we detected the mRNA presence of TH, DRD1, DRD2, DAT and GHSR in VTA and NAc areas at E21. Moreover, we showed that the transcriptional levels of these genes increased from E21 to PND10, suggesting that the development of the dopaminergic system in the rat continues from embryonic stage to the first weeks of life.

We found that early changes in gene expression of TH, DRD1 and GHSR in VTA and NAc in female rats are accompanied by alterations in promoter DNA methylation. In PND10-CON, we observed hypomethylation at the TH and DRD1 promoters (in VTA and NAc) and GHSR gene (in VTA), which may explain the increased mRNA expression of these genes, compared to E21-CON. We found that the DRD1 promoter was mostly methylated in two sites, one of it is located in a CpG Island (in VTA), while the other is a potential binding site for the nuclear factor of activated T-cells (NF-AT) and for Elk-1 (in NAc). On the other hand, a potential binding site for ZF5 and for selective

Fig 4. Methylation analysis of dopaminergic related genes. Tyrosine hydroxylase (TH, A), dopamine receptor 1 (DRD1, B), dopamine receptor 2 (DRD2, C) and dopamine transporter (DAT, D) promoters were studied in the ventral tegmental area (VTA) and/or nucleus accumbens (NAc). TATA box, predicted binding sites for transcription factor, CpG islands and CG target sites for digestion by the methylation-sensitive restriction enzymes are indicated. Offspring on embryonic day 21 and on post-natal day 10 from dams fed with control (E21-CON and PND10-CON, respectively) or cafeteria (E21-CAF and PND10, respectively) diet was evaluated. Methylation levels of promoters in E21-CAF, PND10-CON and PND10-CAF are showed as fold changes from those of E21-CON. The means ± SEM (N = 8/group) are represented by columns and error bars. Significant differences at $p < 0.05$ by Bonferroni's test after two-way ANOVA are denoted by different letters. ACT: activator, AP: activator protein, C/EBP: CCAAT/enhancer-binding protein, CRE: cAMP response element-binding protein, CREB: cAMP response element-binding protein, GRE: Glucocorticoid response element, INH: inhibitor, NF-1: nuclear factor 1, NF-AT: nuclear factor of activated T cells (NF-AT), Sp1: selective promoter factor 1.

Please cite this article in press as: Rossetti MF et al. Epigenetic Dysregulation of Dopaminergic System by Maternal Cafeteria Diet During Early Postnatal Development. Neuroscience (2019), https://doi.org/10.1016/j.neuroscience.2019.09.016

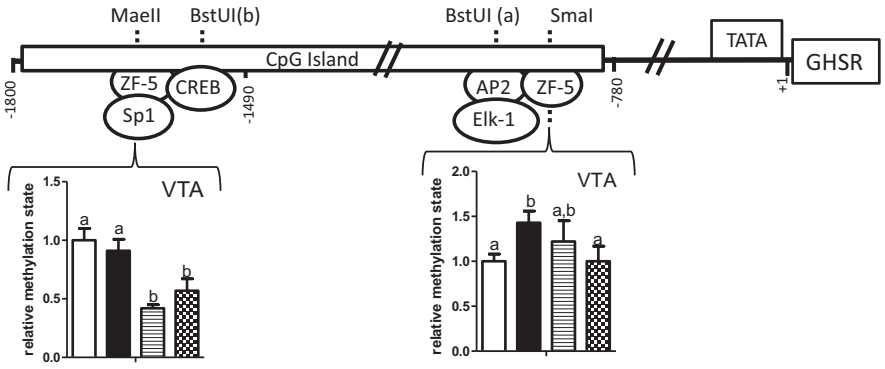


Fig 5. Methylation analysis of ghrelin receptor (GHSR) promoter in the ventral tegmental area (VTA). The offspring brain on embryonic day 21 (E21) and on post-natal day 10 (PND10) from dams fed with control (CON) or cafeteria (CAF) diet was studied. TATA box, activator protein (AP), cAMP response element-binding protein (CREB), selective promoter factor 1 (Sp1), CpG islands and CG target sites for *BstUI, Mae II* and *SmaI* are described. Methylation levels of promoters in E21-CAF, PND10-CON and PND10-CAF are indicated as fold changes from those of E21-CON. The means ± SEM (N = 8/group) are represented by columns and error bars. Significant differences at $p < 0.05$ by Bonferroni's test after two-way ANOVA are denoted by different letters.

promoter factor 1 (Sp1) was predicted in the mostly methylated site within the GHSR promoter. Interestingly, these binding sites have been suggested to have a role in the regulation of dopaminergic related-genes transcription; particularly, DRD1 and DAT (Groth et al., 2008; Lee et al., 2004; Wang and Bannon, 2005). Moreover, changes in the methylation patterns of these sites could be related to the brain area involved (as occurs in DRD1 promoter). Although the increase expression of DRD1, GHSR and TH mRNA in the rat brain was previously related to changes in methylation patterns (Gozen et al., 2013; Inoue et al., 2011; Vucetic et al., 2012); we showed for the first time a relation between changes in the expression of these genes and age-associated methylations mechanisms.

Maternal CAF diet decreased body weight and affects the transcriptional regulation of dopaminergic related-genes in the offspring during perinatal period

Maternal CAF diet significantly decreased the body weight of the offspring from birth to PND10. Dams fed with a palatable diet administered from weaning to adulthood significantly increased energy intake and body weight gain compared to animals fed with the standard chow during pre-pregnancy and pregnancy periods, as was previously reported by several authors (Akyol et al., 2009; Goularte et al., 2012; Lalanza et al., 2014; Lazzarino et al., 2017). However, maternal CAF exposure significantly reduced the body weight of the pups, generating a decrease from birth (17%) to PND10 (30%). Bayol et al. (2007) found similar results in the offspring of dams fed with CAF diet at PND1 and PND21; while Ong and Muhlhausler (2011) observed a significant decrease in body weight at PND3. Contrary to our results, the offspring from dams fed with a high fat diet (HFD) showed no body weight differences at birth, while at PND 16 and PND 19 the body weight of the HFD-offspring was 30% higher compared to control animals (Chen et al., 2008; Purcell et al., 2011). These results suggest that the effects of CAF diet on the body weight of the offspring is opposite to that observed in the offspring from dams fed with other obesogenic diets, such as HFD. However, both low and high birth weights have been associated with the risk of diseases on adult ages, such as glucose intolerance, type II diabetes mellitus, syndrome X, dyslipidemia and obesity (Gluckman et al., 2008; Reyes and Manalich, 2005).

The decrease in the body weight of pups from dams fed with a CAF diet could be due to the excess of maternal body weight that acts as a programming agent per se or due to other aspects of the CAF diet that drive the fetal responses as was previously suggested (Akyol et al., 2009). In fact, the increased energy intakes of the CAF-fed dams were accompanied by a significant change in the composition of their intakes: they consumed a much greater proportion of their daily energy consumption from fat and less from protein, as was previously reported (Akyol et al., 2009; Bayol et al., 2007; Esteve et al., 1994; Llado et al., 1995; Shafat et al., 2009). In this sense, Bayol et al. (2007) reported that the reduction in protein intake during gestation and lactation in CAF-fed dams would be a key factor in explaining the reduced birth and weaning weights observed and that maternal protein intake rather than overall energy intake would play a major role in regulating the offspring's body mass at birth and at weaning. Importantly, the effect observed on the body weight of CAF-PND10 pups is similarly to those reported in the offspring of dams fed with a low protein diet model (Bieswal et al., 2006; Langley-Evans and Nwagwu, 1998). On the other hand, it would be possible that the limited protein intake of the CAF diet also affects the production and composition of breast milk. Although this factor was not analyzed here, Rolls et al. (1986) showed that the milk of CAF-fed rats contained more energy, with more fat and long-chain fatty acid content but less protein and medium-chain fatty acid content than that of control rats. Contrary, other authors reported that there were no differences in the protein content of either the early or mid-lactation milk between CON and CAF dams, despite the lower protein intake of the CAF dams during both pregnancy and lactation (Grigor et al., 1987; Pine et al., 1994; Vithayathil et al., 2016). It is important to note that we found no differences in breeding success between the control and CAF dams, as was previously suggested by Akyol et al. (2009).

Maternal CAF diet affects the transcriptional regulation of dopaminergic related-genes in VTA and NAc regions at PND10. We found a decrease in the expression of TH and GHSR in VTA in the offspring of CAF fed-dams at PND10. The diminished expression of TH in VTA has

been previously related to a reduced DA production (Naef et al., 2008). In addition, it has been reported that ghrelin impacts in VTA and induces DA release (Fulton, 2010), proposing that GHSR-TH coexistence coordinates regulation of DA levels. Ghrelin is thought to incentivize food intake by increasing acetyl choline levels in the VTA, increasing DA levels in the NAc, activation of dopaminergic projections from the VTA to the NAc, and activation of DRD1 and DRD2 in the NAc (Murray et al., 2014). Contrary, in mice, absence of the GHSR gene was associated with lower insulin-like growth factor 1 concentrations and lower body mass, independently of food intake (Chanoine et al., 2009). Although these studies have been performed in adult rats, some works suggest that ghrelin and GHSR have a role in linear growth and development in early life (Chanoine et al., 2009; Steculorum and Bouret, 2011). Interestingly, the decrease in mRNA expression of TH-GHSR in VTA was correlated with a decreased in mRNA levels of DAT and DRD2 in VTA and reduce levels of DRD1, DRD2 and DAT transcripts in NAc. The decrease in the synthesis of DA accompanied by lower levels of it transporter and receptors and therefore, in the actions of the DA, suggests a reduced dopamine signaling in the reward system of these animals. Importantly, during this period, permanent alterations in the function of this pathway could be established and could have a long-lasting effect later in life and in adulthood.

The effect of maternal diet on the dopaminergic reward system was not studied in embryos and in early postnatal life; but it was in young and adult rats. Ong and Muhlhausler (2011) reported that the offspring from dams fed with CAF diet decreased DAT expression in NAc in PND42, whereas in adults the expression of DAT increased, compared to control rats. No changes were found in TH, DRD1 and DRD2 expression between groups. Gugusheff et al. (2013) also reported an increase preference for fat, an overall energy intake and bigger fat mass in adult offspring from CAF-fed dams. In the other hand, Vucetic et al. (2010) showed that the offspring from HFD-fed dams have a reduce DA signaling by decreasing the expression of DRD1 and DRD2 receptors and increasing the expression of DAT in adulthood. Contrary, adult offspring from HFD-fed dams displayed increased TH expression in the VTA and NAc and significant increases in DA content in the NAc, suggesting an elevated DA tone in this target field (Naef et al., 2011). Our results together with the previously mentioned works suggest that maternal diets are critical in the development of the dopaminergic pathways and the effect observed during perinatal period could have a long-lasting impact in the offspring and predispose them to certain behaviors, such as those related to food preferences. Further studies could help to evaluate in which stage the maternal diet produce more changes (before pregnancy, during pregnancy or during breastfeeding), to define the most relevant period.

Some studies showed that the maternal nutritional factors could change the offsprings' epigenetic marks in association with alterations in gene expression (Glendining et al., 2018; Sinclair et al., 2007; Vanhees et al., 2014). Moreover, it was reported that methylation mechanisms are implicated in the transcriptional control of dopamine-related genes. For example, Vucetic et al. (2010) observed global and gene-specific (DAT and Mu opioid receptor) promoter DNA hypomethylation in the brains of offspring from dams that consumed the HFD. In addition, epigenetic dysregulation of TH and DAT genes in a mouse model of HFD-induced obesity was reported (Vucetic et al., 2012). Sanchez-Hernandez et al. (2016) also reported that the male offspring from dams fed with diet with high levels of vitamin A had increased levels of DNA methylation in the DRD2 promoter region compared to control group. Here, we reported for the first time that maternal CAF diet affects the transcriptional regulation of genes TH, DRD2 and DAT involved in dopaminergic reward system by DNA methylation mechanisms in an early stage of development (PND10). The fact that DRD2 and DAT promoters were mostly methylated at two sites located in a CpG Island supports the idea that these methylation-sensitive sites could be potential regulatory sites. To reinforce this hypothesis, it would be interesting to perform further experiments using DNMT inhibitors that block the epigenetic effects of maternal CAF diet in offspring.

The reduce dopamine signaling found in the offspring from CAF-fed dams is consistent with changes in the reward pathway observed in adult obese animals and in animals exposed to drugs, such as cocaine or alcohol. Particularly, several studies showed that genetic and functional alterations of the DRD2 have already been linked to the pathophysiology. A reduction in striatal density of DRD2 in overweight individuals (Stice et al., 2008; Wang et al., 2001) and rodents (Huang et al., 2006; Johnson and Kenny, 2010; Thanos et al., 2008) has been reported. Moreover, loss of DRD2 autorreceptors was linked to drug addiction, such as cocaine intake (Bello et al., 2011; Holroyd et al., 2015). In this sense, it has been shown that DRD2 plays an important role in the reward deficiency syndrome, which is related to compulsive and addictive behaviors (Blum et al., 2011). In VTA, a decrease in DRD2 has been linked to a greater motivation for food and the development of obesity (Bello et al., 2011; Koyama et al., 2014). Here, we showed for the first time a downregulation in DRD2 that is correlated with alteration in the methylation levels of it promoter in the offspring of dams fed with a CAF diet at PND10. Considering that these changes could have a long-lasting effect later in life, these results suggest that the epigenetic dysregulation of DRD2 could be an early marker of health diseases related with excessive consumption of food or drugs in adulthood. However, further studies are needed to clarify the cause-effect relationship between early DRD2 dysregulation in response to maternal diet and addictive behaviors in the adult offspring.

DECLARATION OF COMPETING INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Please cite this article in press as: Rossetti MF et al. Epigenetic Dysregulation of Dopaminergic System by Maternal Cafeteria Diet During Early Postnatal Development. Neuroscience (2019), https://doi.org/10.1016/j.neuroscience.2019.09.016

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Juan Grant, Juan C. Villarreal and Stella Vaira from the Facultad de Bioquímica y Ciencias Biologicas (Universidad Nacional del Litoral, Santa Fe, Argentina).

FUNDING

Grants from the National Scientific and Technical Research Council (CONICET) (PIP 11220150100338CO 2016-2019) and Universidad Nacional del Litoral (CAI +D 2016 No 0420150100085LI) supported this work.

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(Received 14 May 2019, Accepted 11 September 2019)
(Available online xxxx)