

1 **Epigenetic dysregulation of dopaminergic system by maternal cafeteria diet during**
2 **early postnatal development**

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32 **Keywords: EPIGENETIC, DOPAMINE-RELATED GENES, MATERNAL CAFETERIA**
33 **DIET, RAT OFFSPRING.**

34

35 **Abstract:**

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

56

57 **Highlights**

- 58 1. Maternal cafeteria diet decreased the transcription of TH, DRD2 and DAT in the
59 ventral tegmental area of neonatal offspring.
- 60 2. In *nucleus accumbens*, maternal cafeteria diet reduced DRD1, DRD2 and DAT
61 expression at postnatal day 10.
- 62 3. The methylation status of dopaminergic gene promoters was affected by maternal
63 diet.

64 **Abbreviations**

- 65 ACT: activator
66 AP: activator protein
67 C/EBP: CCAAT/enhancer-binding protein
68 CAF: cafeteria
69 CRE: cAMP response element
70 CREB: cAMP response element-binding protein
71 DA: dopamine
72 DAT: dopamine transporter
73 DRD: dopamine receptor
74 E: embryonic day
75 GHSR: ghrelin receptor
76 GRE: glucocorticoid response element
77 HFD: high fat diet
78 INH: inhibitor
79 NAc: *nucleus accumbens*
80 NF-1: nuclear factor 1
81 NF-AT: nuclear factor of activated T cells
82 Sp1: selective promoter factor 1
83 TH:tyrosine hydroxylase
84 VTA: ventral tegmental area

85

86 **Introduction**

87 Obesity represents one of the major public health problems in the world and it is mainly
88 caused by overeating and physical inactivity. Palatable, or high-fat, high-sugar, foods
89 activate the dopaminergic signaling pathways within the mesolimbic reward system
90 (Berthoud, 2006; Fulton, 2010). Dopamine (DA) is a neurotransmitter crucial for motor,
91 motivational, and reward-related functions of the central nervous system(Cragg and Rice,
92 2004)and is also associated with the gratifying effects of sex and drugs of abuse (Nestler
93 and Carlezon, 2006). This neurotransmitter is produced in the dopaminergic neurons of the
94 ventral tegmental area (VTA)by the action of the tyrosine hydroxylase (TH) (Baik, 2013a,

95 2013b). Interestingly, it was shown that there is a high degree of co-expression of TH and
96 ghrelin receptor (GHSR) in VTA in adults (Zigman et al., 2016a, 2016b). It is well known
97 that ghrelin impairs VTA by inducing DA release and stimulating food intake (Fulton,
98 2010); thus, GHSR-TH coexistence suggests a possible coordinated regulation of DA
99 levels. VTA dopaminergic neurons project to the *nucleus accumbens* (NAc), where DA is
100 released and binds to specific DA receptors (DRD1 and DRD2). This nucleus receives
101 sensitive information from various regions, and then projects to the hypothalamic and
102 midbrain areas that contribute to the motor action of food (Valdivia et al., 2014). NAc
103 mediates reward effects in response to natural stimuli and is where termination of DA
104 signaling occurs through reuptake by the active DA transporter (DAT)(Cragg and Rice,
105 2004). Prolonged exposure to palatable food in adult rodents is associated with behavioral
106 and neurophysiological adaptations comparable to those seen in drug addicts. In particular,
107 desensitization of the central reward pathway, which then drives continued
108 overconsumption(Ong and Muhlhausler, 2011).

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

122 Metabolic and eating disorders are associated with alterations in the DNA methylation
123 pattern of particularly genes, such as TH and DAT(Vucetic et al., 2012). DNA
124 methylation represents one of the most important epigenetic mechanisms for blocking gene
125 expression and implicates the addition of methyl groups to CpG dinucleotides. A CpG

126 island is a DNA sequence generally greater than 250 bp that is rich in CpG sites and, thus,
127 it has a key role in transcriptional control (Deaton and Bird, 2011). In this context, DNA
128 methylation provides a mechanism by which maternal diet can modify the predisposition of
129 the offspring to obesity-associated disorders or other pathologies.

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

140

141 **Experimental Procedures**

142

143 **Animals and experimental design**

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

150 Rats were housed two per cage, at 22 ± 2 °C and with a 12-h light–dark cycle, and fed with
151 either standard chow (CON) or a cafeteria diet (CAF) (N= 10/group) from weaning. The
152 standard chow (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina) provided
153 12.55 KJ/g, 5% energy as fat, 23% protein and 72% carbohydrate. The CAF diet was
154 composed of standard chow and food that reflects variety, palatability, and energy density
155 (parmesan cheese, cheese flavored snacks, crackers, sweet biscuits, pudding, and
156 chocolate). This diet provided an average of 20.29 KJ/g, 49% of energy as fat, 7% as

157 protein, and 44% as carbohydrate, in addition to that provided by the standard chow.
158 Dietary composition and treatment procedure are described in Lazzarino et al. (2017).
159 On the 14th week of treatment, when CAF animals were significantly heavier of that than
160 the CON, females were mated with male rats that were housed under standard laboratory
161 conditions during all the experiment (22 ± 2 °C, 12-h light–dark cycle, fed with standard
162 chow). After mating, each dam was single caged and continued with the respectively diet.
163 Five dams per diet group were euthanized on embryonic day 21 (E21). The rest of the
164 animals (N=5/group) were maintained and at day 1 after delivery each litter was adjusted to
165 8 pups per dam. At postnatal day 10 (PND10), pups were euthanized. During all the
166 experimental period, dam body weight and energy intake were recorded weekly and litter
167 weight was measured daily. After euthanization, female fetus and pups brains (named as
168 E21-CAF, E21-CON, PND10-CAF and PND10-CON) were removed, frozen on dry ice
169 and stored at -80 °C until sectioning for RNA and DNA analysis.

170

171 **Micropunches of VTA and NAc**

172 Following the procedure of microdissection technique described by Palkovits (Palkovits
173 and Brownstein, 1988), embryo and pup brains were cut in a cryostat at -12 °C (serial
174 coronal sections of $150\mu\text{m}$). To identify and punch VTA and NAc regions, the atlas of the
175 developing rat nervous system (Paxinos et al. 1994) and atlas of the postnatal rat brain in
176 stereotaxic coordinates (Khazipov et al. 2015) were used. Both areas were removed
177 bilaterally using a 0.5 mm stainless steel micropunch needles and the reproducibility was
178 checked analyzing the topography of the holes under a stereo microscope (Stemi 305,
179 Zeiss, Oberkochen, Germany). Samples were stored at -80 °C until RNA and DNA
180 isolation.

181

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

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

188 Reverse-transcribed products were combined with HOT FIRE Pol Eva Green qPCR Mix
 189 Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer
 190 (Invitrogen) and further amplified in duplicate using Real-Time DNA Step One Cyclor
 191 (Applied Biosystems Inc., Foster City, CA, USA).The primer pairs used are detailed in
 192 Table 1 and the protocol for real-time quantitative PCR is described byRossetti et al.
 193 (2015).

194

195 Table 1. Sequences of primer oligonucleotides for PCR amplification.

Target	Primer sense	Primer antisense	Temperature of annealing (°C)
L19 (housekeeping)	5'-AGCCTGTGACTGTCCATTC -3'	5'-TGGCAGTACCCTTCCTCTTC -3'	60
TH	5'-TACCAAGATCAAACCTACCAGCC-3'	5'-GGTCAAACCTCACAGAGAATGGG-3'	58
DRD1	5'-TCCAAGGTGACCAACTTCTT-3'	5'-GTTACAAAAGGACCCAAAGG-3'	55
DRD2	5'-CCCAGCAGAAGGAGAAGAAA-3'	5'-CAGGATGTGCGTGATGAAGA-3'	55
DAT	5'-CATCACCACTCCATTAACTCC-3'	5'-CATTGTGCTTCTGTGCCATG-3'	56
GHSR	5'-GCTCTGCAAACCTTCCA-3'	5'-AAGCAGATGGCGAAGTAG-3'	56

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197 **Bioinformatics**

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

207

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

Target	Primer sense	Primer antisense	Temperature of annealing (°C)
TH IC	5'-CCATCAGATTTACCTAGAAGC-3'	5'-TGAGACTATGAAGGGACATTG-3'	51.5

TH- <i>MaeII</i>	5'-ACAGCAGGCGTGGAGAGGAT-3'	5'-TGGTGGTCCCGAGTTCTGTC-3'	60
TH- <i>MaeII</i> b	5'-CCTTAGGAAATCCAGCATGG-3'	5'-ATTGCATCCACTGTCACAGG-3'	57.7
TH- <i>MaeII</i> c	5'-CATGTGGCTGCTCCTATGTA-3'	5'-GAGAGAGATTGGCACACACA-3'	52.6
DRD1 IC	5'-GTGGTGAGAATCCCCTCAGG-3'	5'-AGTTCACAGCGGAGAACC-3'	55
DRD1- <i>MaeII</i>	5'-CAGGCAAAGAGGTTACAAG-3'	5'-CCGCCATCTAAACAGTTACC-3'	54.6
DRD1- <i>BstUI</i>	5'-AGCAGGAAACCACAGGCACC-3'	5'-GCTTCTGCGGTCAACTCACG-3'	60
DRD2 IC	5'-AATTCTGTGGTGCCTTCTCCT-3'	5'-ATGGGGTCAATCCAGAGTAGA-3'	55
DRD2- <i>BstUI</i>	5'-AGTGCAGAGATAGTTCTGGG-3'	5'-AGAAGCCACAGACTGTCGTT-3'	63
DAT IC	5'-TTTGGGGTCTCAACTAGAAA-3'	5'-TAAGACCTTTTCAGAACCCA-3'	55
DAT- (a)/ <i>MaeII</i>	5'-CTTCTGACAACCTCGCTGGA-3'	5'-GGGGCTTGACAGGAGTCTTT-3'	60
DAT- (b)/ <i>SmaI</i>	5'-CGTACAACACCGAAGGAAGA-3'	5'-CGAGGTTGTCAGAAGCAGAT-3'	57.7
GHSR IC	5'-TCCAGCATACTCCTTATCCA-3'	5'-TGGCAATCTTAGAACACACC	54.6
GHSR- <i>BstUI</i> (a)/ <i>SmaI</i>	5'-TACGCCACGGTCCTCACCAT-3'	5'-ACGCTGGACACCCACACCAT-3'	61
GHSR- <i>BstUI</i> (b)/ <i>MaeII</i>	5'-TCTCCCTTTCTCTCCAAGC-3'	5'-TTCGTCAGGCAGTGAGTCGT-3'	61

IC: Internal Control.

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212 Methylation-sensitive analysis

213 Genomic DNA from VTA and NAc areas (N= 8/group) was isolated using the
214 phenol/chloroform/isoamyl alcohol extraction and digested with 1 unit of *SmaI/Mae II* or
215 10 units of *BstUI* and 1X enzyme buffer for 1 h at 25°C, 60 °C or 50 °C, respectively. After
216 purification with the Wizard SV gel and PCR Clean-Up System Kit (Promega, Madison,
217 WI), an optimized qRT-PCR protocol was used to analyze the relative methylation levels of
218 various regions of the TH, DRD1, DRD2,DAT and GHSR promoters (Table 2). The
219 procedure for DNA amplification was previously described by our group in several studies
220 (Lazzarino et al., 2017; Rossetti et al., 2018; Rossetti et al., 2016; Rossetti et al., 2015).

221

222 Statistical analysis

223 G Power software (<http://www.gpower.hhu.de/>; RRID:SCR_013726)was used to determine
224 the sample size (Faul et al., 2007). To confirm the normal distribution of the data and
225 variance homogeneity,Shapiro–Wilk test and Levene's test were performed. Weekly body
226 weights,nutrient intake and energy intakewere analyzed using Student's T test; while a two-
227 way ANOVA followed by Bonferroni post-test was implemented to study the ageand diet

228 effects on mRNA and DNA methylation. All the data is expressed as the means \pm SEM and
229 was statistically analyzed using the IBM SPSS Statistics 19 software (IBM Inc.;
230 RRID:SCR_002865), considering significant differences at $p < 0.05$.

231

232 Results

233 Effects of CAF diet on dams' body weight, nutrient intake and energy intake.

234 The body weights of dams fed with CAF diet increased from week 10 of dietary
235 intervention and on the 14th week of treatment (CAF: 266.5 ± 2.92 g; CON: 240.5 ± 3.1 g;
236 $p < 0.05$) females were mated with male rats. During pregnancy, CAF dams significantly
237 increased gestational weight gain in comparison with those fed the control diet (Fig 1a). In
238 addition, energy intakes over the pre-pregnancy (Week 2 to 14) and pregnancy (Week 16 to
239 18) period remained significantly higher in CAF rats when compared with CON animals
240 (Fig. 1b). Moreover, CAF rats consumed a significantly greater percentage of their daily
241 energy intake as fat, and significantly less as protein than CON rats (Fig 1c), during all the
242 experiment.

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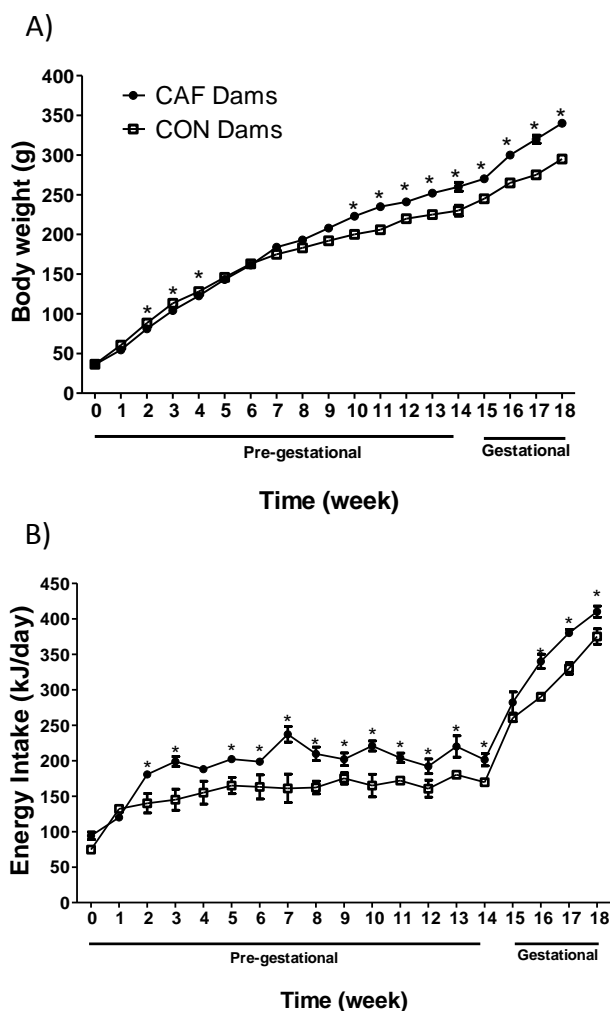
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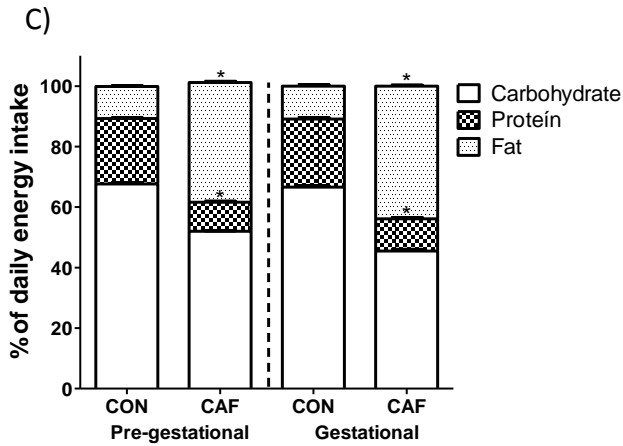
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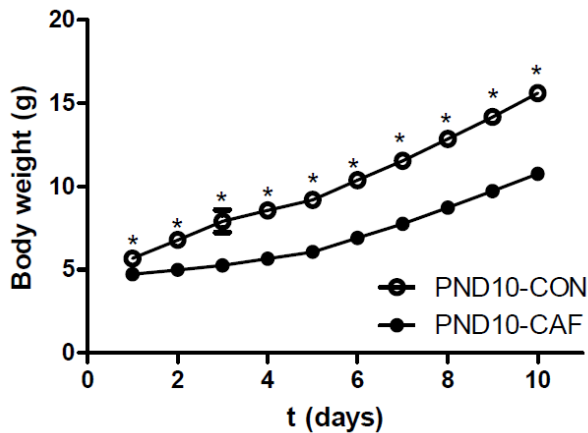
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278 **Fig 1. Body weight (A), energy intake (B) and nutrient intake (C) of dams fed with a control (CON) or a cafeteria**
279 **(CAF) diet during pre-gestation (Week 1 to 14) and gestation (Week 15-18) periods(N=10/Group). * indicates**
280 **significant differences at $p < 0.05$ vs. CONgroupby Student's T test.**
281

282 **Effects of CAF diet on the body weight of the offspring.**

283 Pups of dams fed with a CAF diet had a significantly lower weight ($p < 0.05$) than those
284 from a CON diet from birth to PND10 (Fig. 2). It is interesting to note that during this
285 period no apparent differences were detected in maternal behavior between CON and CAF
286 groups. At E21 no significant differences were detected (data not shown).
287



288 **Fig 2. Body weightof pups from dams fed with a control (PND10-CON) or a cafeteria (PND10-CAF) diet from**
289 **birth up to post-natal day 10 (PND10).Values are means, with standard errors represented by vertical**
290 **bars(N=16/Group). * indicates significant differences at $p < 0.05$ vs. CON groupby Student's T test.**
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Maternal CAF diet modifies the mRNA expression of dopamine-related genes in the reward brain system of the offspring at early postnatal development.

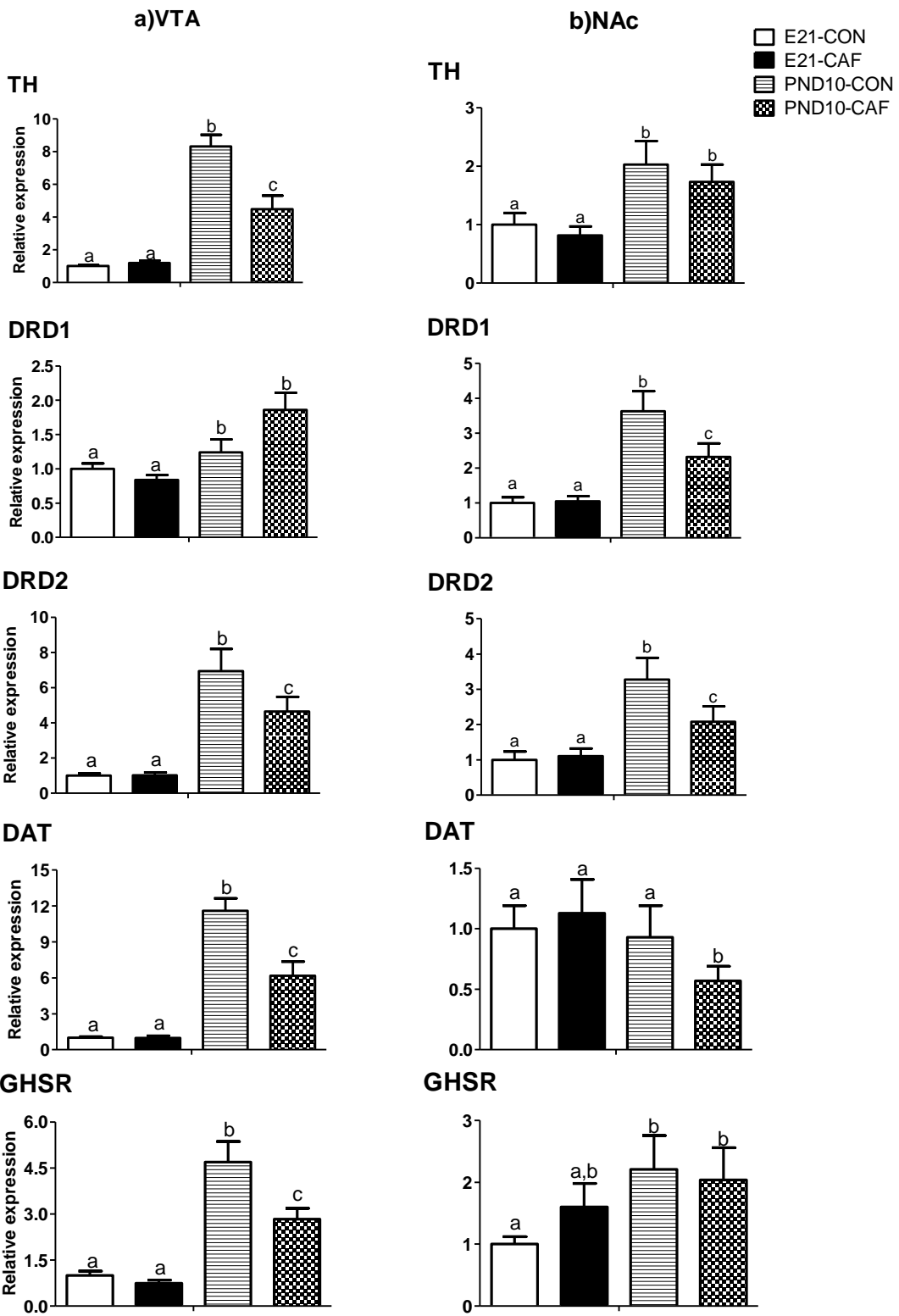
295 To analyze the effect of maternal CAF diet on dopaminergic reward system in the offspring
296 before and during gestation and lactation periods, we analyzed the expression of molecules
297 that are involved in the synthesis, transport and reuptake of DA in two key regions of the
298 reward system,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

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

307 In NAc, the expression of DRD1 ($p < 0.05$, $F = 7.369$), DRD2 ($p < 0.01$, $F = 10.01$) and DAT
308 ($p < 0.01$, $F = 15.29$) was affected by the interactions between age and maternal diet (Fig 3B).
309 Their expression decreased in the offspring of dams fed with CAF diet in PND10 (DPN10-
310 CAF vs DPN10-CON, $p < 0.05$); however, no expression changes were observed in E21.
311 Age generated an increase in the transcription of these genes (E21 vs PND10, $p < 0.05$). TH
312 and GHSR mRNA levels were not found to be modified by maternal diet in NAc, but age
313 improved the expression in the offspring (E21 vs PND10, $p < 0.05$, $F_{TH} = 10.31$,
314 $F_{GHSR} = 5.29$, Fig 3B).

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

379

380 **Transcriptional regulation of dopaminergic-related genes by DNA methylation in the** 381 **reward brain system during development and in response to the maternal diet.**

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

386 In VTA, DNA methylation levels of TH-*MaeII* c ($p < 0.05$, $F = 5.545$, Fig 4A), DAT-*SmaI*
387 ($p < 0.05$, $F = 6.943$, Fig 4D) and GHSR-*SmaI* ($p < 0.05$, $F = 26.91$, Fig 5) sites were affected
388 by the interactions between age and maternal diet. In TH-*MaeII* c and DAT-*SmaI* sites,
389 maternal CAF diet increases methylation in the offspring in PND10 (DPN10-CAF vs
390 DPN10-CON, $p < 0.05$); in GHSR-*SmaI* this occurs in E21 (E21-CAF vs E21-CON, p
391 < 0.05); and in DRD2-*BstUI* this arises at both stages (E21 and PND10, $p < 0.05$, $F = 21.95$,
392 Fig 4C). On the other hand, age decrease methylation levels of TH-*MaeII* c site in the
393 offspring of dams fed with CON diet in PND10 (E21-CON vs DPN10-CON, $p < 0.05$). In
394 addition, age decrease methylation levels of DRD1-*BstUI* and GHSR-*MaeII* sites in the
395 offspring of dams fed with both CON and CAF diet in PND10 (E21 vs DPN10, $p < 0.0001$,
396 $F_{DRD1} = 42.35$ and $F_{GHSR} = 68.51$, Fig 4B and 5). No differences were observed in
397 methylation in the others studied sites (data not shown).

398 In NAc, DNA methylation levels of DAT-*SmaI* ($p < 0.05$, $F = 15.17$), TH-*MaeII* b ($p < 0.05$, F
399 $= 23.66$) and DRD1-*MaeII* ($p < 0.05$, $F = 29.88$) sites were affected by the interactions
400 between age and maternal diet. In the first site, maternal CAF diet increased methylation in
401 the offspring in PND10 (DPN10-CAF vs DPN10-CON, $p < 0.05$). Age also increased
402 methylation levels in this site in the offspring from dams fed with CAF diet (E21-CAF vs
403 DPN10-CAF). Contrary, age decreased DNA methylation levels of TH-*MaeII* b ($p < 0.005$,

404 F = 13.26) and DRD1-*MaeII* ($p < 0.0001$, F = 33.07) sites in the offspring from dams fed
405 with CON diet (E21-CON vs DPN10-CON). No differences were observed in methylation
406 of DRD2, DAT and GHSR sites (data not shown).

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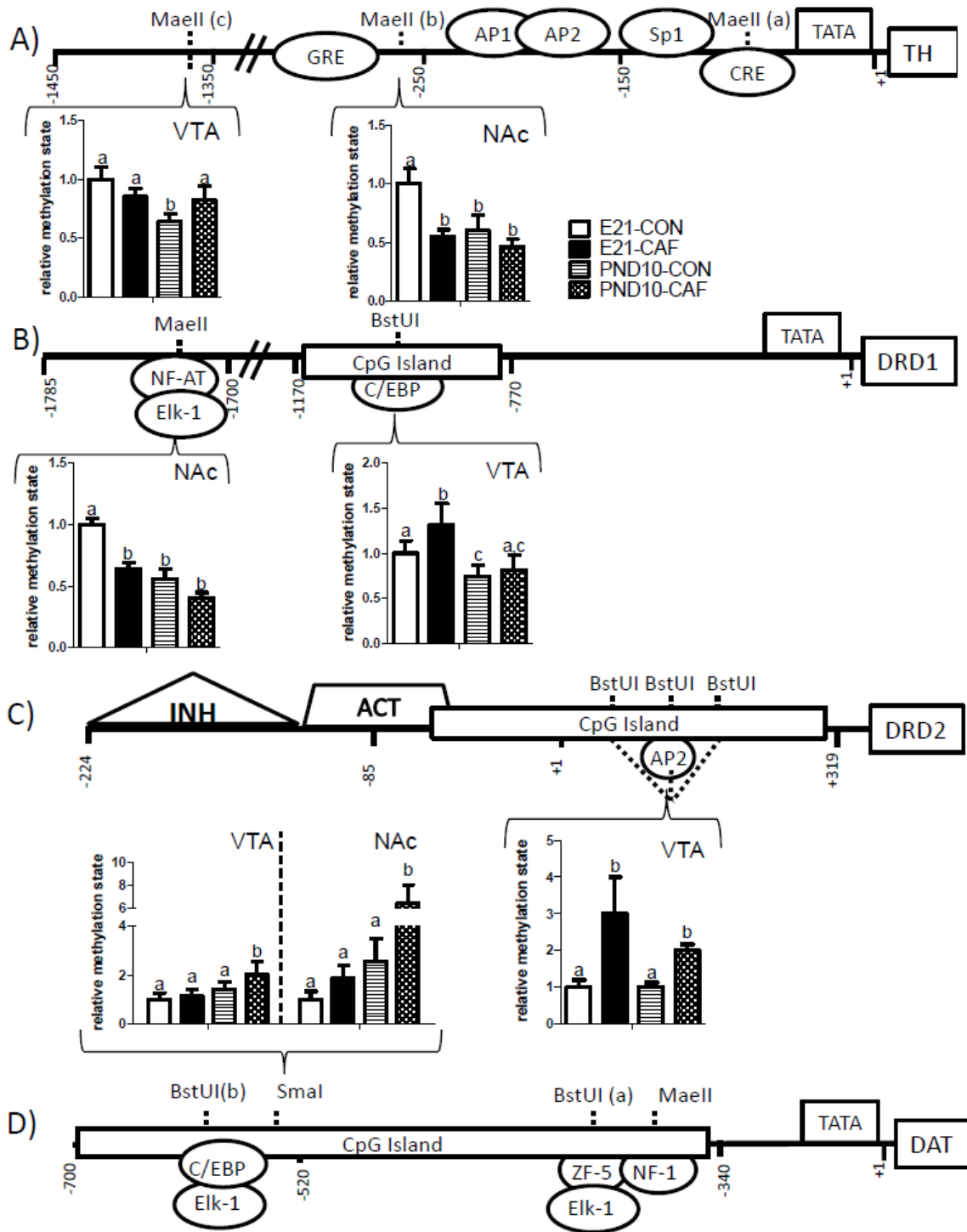
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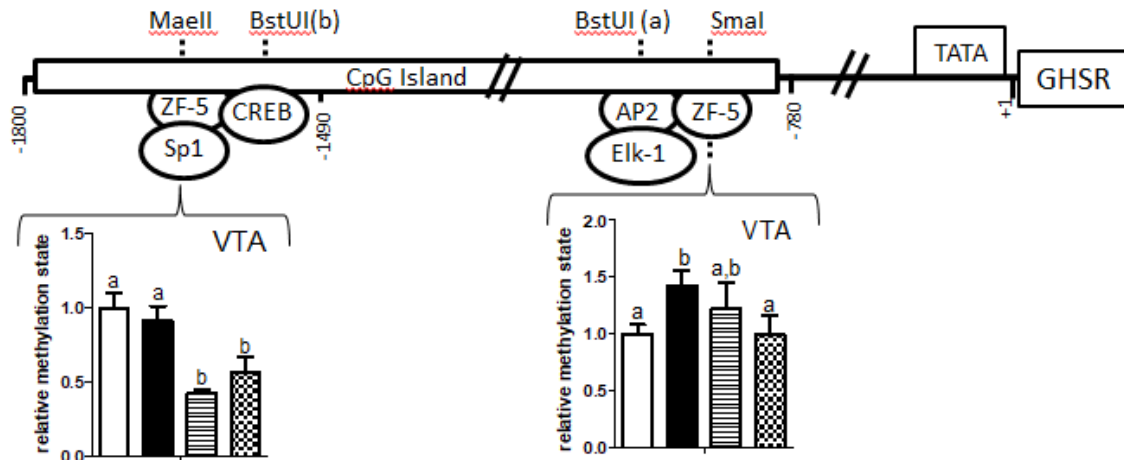


418

419

420 **Fig 4. Methylation analysis of dopaminergic related genes.** Tyrosine hydroxylase (TH, A), dopamine receptor 1
 421 (DRD1, B), dopamine receptor 2 (DRD2, C) and dopamine transporter (DAT, D) promoters were studied in the ventral
 422 tegmental area (VTA) and/or nucleus accumbens (NAc). TATA box, predicted binding sites for transcription factor, CpG
 423 islands and CG target sites for digestion by the methylation-sensitive restriction enzymes are indicated. Offspring on
 424 embryonic day 21 and on post-natal day 10 from dams fed with control (E21-CON and PND10-CON, respectively) or
 425 cafeteria (E21-CAF and PND10, respectively) diet was evaluated. Methylation levels of promoters in E21-CAF, PND10-

426 CON and PND10-CAF are showed as fold changes from those of E21-CON. The means \pm SEM (N=8/group) are
 427 represented by columns and error bars. Significant differences at $p < 0.05$ by Bonferroni's test after two-way ANOVA are
 428 denoted by different letters. ACT: activator, AP: activator protein, C/EBP: CCAAT/enhancer-binding protein, CRE:
 429 cAMP response element-binding protein, CREB: cAMP response element-binding protein, GRE: Glucocorticoid response
 430 element, INH: inhibitor, NF-1: nuclear factor 1, NF-AT: nuclear factor of activated T cells (NF-AT), Sp1: selective
 431 promoter factor 1.
 432
 433



434
 435 **Fig 5. Methylation analysis of ghrelin receptor (GHSR) promoter in the ventral tegmental area (VTA).** The
 436 offspring brain on embryonic day 21 (E21) and on post-natal day 10 (PND10) from dams fed with control (CON) or
 437 cafeteria (CAF) diet was studied. TATA box, activator protein (AP), cAMP response element-binding protein (CREB),
 438 selective promoter factor 1 (Sp1), CpG islands and CG target sites for *BstUI*, *Maell* and *Smal* are described. Methylation
 439 levels of promoters in E21-CAF, PND10-CON and PND10-CAF are indicated as fold changes from those of E21-CON.
 440 The means \pm SEM (N=8/group) are represented by columns and error bars. Significant differences at $p < 0.05$ by
 441 Bonferroni's test after two-way ANOVA are denoted by different letters.
 442

443 DISCUSSION

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

452 and 2) the offspring from dams fed with CAF diet showed alterations in the transcriptional
453 regulation of TH, DRD2 and DAT genes in VTA and NAc at PND10.

454

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

457

458 The development of the dopaminergic system of the striatum in the rat begins during
459 embryonic life and continues up to the 3rd postnatal week (Antonopoulos et al., 2002). The
460 first mesolimbic dopamine neurons can be identified in the rat brain in E12, although
461 dopamine axon innervations are not complete until the 3rd week of postnatal life (Ong et al.,
462 2012). TH mRNA was found in brain tissues on early embryonic development E10-E12 and
463 its specific activity increased from gestation to adulthood (Berger et al., 1985; Burgunder
464 and Young, 1990; Marin et al., 2005). DRD1 and DRD2 receptors were detected in neural
465 tissues on E14 and on E18 their localization was already similar to that observed in the
466 adult brain. At birth, expression of mRNA for both dopamine receptor subtypes in the
467 striatum approximated that seen in mature rats (Ong et al., 2012). In addition, DAT mRNA
468 was first detected in neurons of the ventrocaudal mesencephalon on E14. By E18, intensely
469 expressing neurons in the VTA and substantia nigra resembled the pattern found in adult
470 midbrain (Fujita et al., 1993; Galineau et al., 2004). GHSR mRNA was found in the brain
471 and spinal cord as early as E12 and continued to be expressed in these tissues during
472 postnatal life (Steculorum and Bouret, 2011). Along the same line, we detected the mRNA
473 presence of TH, DRD1, DRD2, DAT and GHSR in VTA and NAc areas at E21. Moreover,
474 we showed that the transcriptional levels of these genes increased from E21 to PND10,
475 suggesting that the development of the dopaminergic system in the rat continues from
476 embryonic stage to the first weeks of life.

477 We found that early changes in gene expression of TH, DRD1 and GHSR in VTA and NAc
478 in female rats are accompanied by alterations in promoter DNA methylation. In PND10-
479 CON, we observed hypomethylation at the TH and DRD1 promoters (in VTA and NAc)
480 and GHSR gene (in VTA), which may explain the increased mRNA expression of these
481 genes, compared to E21-CON. We found that the DRD1 promoter was mostly methylated
482 in two sites, one of it is located in a CpG Island (in VTA), while the other is a potential

483 binding site for the nuclear factor of activated T-cells (NF-AT) and for Elk-1 (in NAc). On
484 the other hand, a potential binding site for ZF5 and forselective promoter factor 1 (Sp1) was
485 predicted in the mostly methylated site within the GHSR promoter. Interestingly, these
486 binding sites have been suggested to have a role in the regulation of dopaminergic related-
487 genes transcription; particularly, DRD1 and DAT (Groth et al., 2008; Lee et al., 2004; J.
488 Wang and Bannon, 2005). Moreover, changes in the methylation patterns of these sites
489 could be related to the brain area involved (as occurs in DRD1 promoter). Although the
490 increase expression of DRD1, GHSR and TH mRNA in the rat brain was previously related
491 to changes in methylation patterns (Gozen et al., 2013; Inoue et al., 2011; Vucetic et al.,
492 2012); we showed for the first time a relation between changes in the expression of these
493 genes and age-associated methylations mechanisms.

494

495 **Maternal CAF diet decreased body weight and affects the transcriptional regulation**
496 **of dopaminergic related-genesin the offspring during perinatal period.**

497

498 *Maternal CAF diet significantly decreased the body weight of the offspring from birth to*
499 *PND10.*

500

501 Dams fed with a palatable diet administered from weaning to adulthood significantly
502 increased energy intakeand body weight gain compared to animals fed with the standard
503 chowduring pre-pregnancy and pregnancy periods, as was previously reported by several
504 authors(Akyol et al., 2009; Goularte et al., 2012; Lalanza et al., 2014; Lazzarino et al.,
505 2017). However, maternal CAF exposure significantly reduced the body weight of the
506 pups, generating a decrease from birth (17%) to PND10 (30%). Bayol et al. (2007)found
507 similar results in the offspring of dams fed with CAF diet at PND1 and PND21; while Ong
508 and Muhlhausler (2011) observed a significant decrease in body weight at PND3. Contrary
509 to our results, the offspring from dams fed with a high fat diet (HFD) showed no body
510 weight differences at birth, while at PND 16 and PND 19 the body weight of the HFD-
511 offspring was 30% higher compared to control animals (Chen et al., 2008; Purcell et al.,
512 2011). These results suggest that the effects of CAF diet on the body weight of the
513 offspring is opposite tothat observed in the offspring from dams fed with other obesogenic

514 diets, such as HFD. However, both low and high birth weights have been associated with
515 the risk of diseases on adult ages, such as glucose intolerance, type II diabetes
516 mellitus, syndrome X, dyslipidemia and obesity (Gluckman et al., 2008; Reyes and
517 Manalich, 2005).

518 The decrease in the body weight of pups from dams fed with a CAF diet could be due to the
519 excess of maternal body weight that acts as a programming agent per se or due to other
520 aspects of the CAF diet that drive the fetal responses as was previously suggested (Akyol et
521 al., 2009). In fact, the increased energy intakes of the CAF-fed dams were accompanied by a
522 significant change in the composition of their intakes: they consumed a much greater
523 proportion of their daily energy consumption from fat and less from protein, as was
524 previously reported (Akyol et al., 2009; Bayol et al., 2007; Esteve et al., 1994; Llado et al.,
525 1995; Shafat et al., 2009). In this sense, Bayol et al. (2007) reported that the reduction in
526 protein intake during gestation and lactation in CAF-fed dams would be a key factor in
527 explaining the reduced birth and weaning weights observed and that maternal protein intake
528 rather than overall energy intake would play a major role in regulating the offspring's body
529 mass at birth and at weaning. Importantly, the effect observed on the body weight of CAF-
530 PND10 pups is similarly to those reported in the offspring of dams fed with a low protein
531 diet model (Bieswal et al., 2006; Langley-Evans and Nwagwu, 1998). On the other hand, it
532 would be possible that the limited protein intake of the CAF diet also affects the production
533 and composition of breast milk. Although this factor was not analyzed here, Rolls et al.
534 (1986) showed that the milk of CAF-fed rats contained more energy, with more fat and
535 long-chain fatty acid content but less protein and medium-chain fatty acid content than that
536 of control rats. Contrary, other authors reported that there were no differences in the protein
537 content of either the early or mid-lactation milk between CON and CAF dams, despite the
538 lower protein intake of the CAF dams during both pregnancy and lactation (Grigor et al.,
539 1987; Pine et al., 1994; Vithayathil et al., 2016). It is important to note that we found no
540 differences in breeding success between the control and CAF dams, as was previously
541 suggested by Akyol et al. (2009).

542

543 ***Maternal CAF diet affects the transcriptional regulation of dopaminergic related-genes***
544 ***in VTA and NAc regions at PND10.***

545

546 We found a decrease in the expression of TH and GHSR in VTA in the offspring of CAF
547 fed-dams at PND10. The diminished expression of TH in VTA has been previously related
548 to a reduced DA production (Naef et al., 2008). In addition, it has been reported that ghrelin
549 impacts in VTA and induces DA release (Fulton, 2010), proposing that GHSR-TH
550 coexistence coordinates regulation of DA levels. Ghrelin is thought to incentivize food
551 intake by increasing acetyl choline levels in the VTA, increasing DA levels in the NAc,
552 activation of dopaminergic projections from the VTA to the NAc, and activation of DRD1
553 and DRD2 in the NAc (Murray et al., 2014). Contrary, in mice, absence of the GHSR gene
554 was associated with lower insulin-like growth factor 1 concentrations and lower body
555 mass, independently of food intake (Chanoine et al., 2009). Although these studies have
556 been performed in adult rats, some works suggest that ghrelin and GHSR have a role in
557 linear growth and development in early life (Chanoine et al., 2009; Steculorum and
558 Bouret, 2011). Interestingly, the decrease in mRNA expression of TH-GHSR in VTA was
559 correlated with a decrease in mRNA levels of DAT and DRD2 in VTA and reduce levels
560 of DRD1, DRD2 and DAT transcripts in NAc. The decrease in the synthesis of DA
561 accompanied by lower levels of its transporter and receptors and therefore, in the actions of
562 the DA, suggests a reduced dopamine signaling in the reward system of these animals.
563 Importantly, during this period, permanent alterations in the function of this pathway could
564 be established and could have a long-lasting effect later in life and in adulthood.

565 The effect of maternal diet on the dopaminergic reward system was not studied in embryos
566 and in early postnatal life; but it was in young and adult rats. Ong and Muhlhausler (2011)
567 reported that the offspring from dams fed with CAF diet decreased DAT expression in NAc
568 in PND42, whereas in adults the expression of DAT increased, compared to control rats.
569 No changes were found in TH, DRD1 and DRD2 expression between groups. Gugusheff et
570 al. (2013) also reported an increase preference for fat, an overall energy intake and bigger
571 fat mass in adult offspring from CAF-fed dams. In the other hand, Vucetic et al.
572 (2010) showed that the offspring from HFD-fed dams have a reduced DA signaling by
573 decreasing the expression of DRD1 and DRD2 receptors and increasing the expression of
574 DAT in adulthood. Contrary, adult offspring from HFD-fed dams displayed increased TH
575 expression in the VTA and NAc and significant increases in DA content in the NAc,

576 suggesting an elevated DA tone in this target field (Naef et al., 2011). Our results together
577 with the previously mentioned works suggest that maternal diets are critical in the
578 development of the dopaminergic pathways and the effect observed during perinatal period
579 could have a long-lasting impact in the offspring and predispose them to certain behaviors,
580 such as those related to food preferences. In this sense, it will be interesting to evaluate in
581 further studies the period (before pregnancy, during pregnancy or during breastfeeding) in
582 which changes associated with the maternal diet are more relevant.

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

601 The reduce dopamine signaling found in the offspring from CAF-fed dams is consistent
602 with changes in the reward pathway observed in adult obese animals and in animals
603 exposed to drugs, such as cocaine or alcohol. Particularly, several studies showed that
604 genetic and functional alterations of the DRD2 have already been linked to the
605 pathophysiology. A reduction in striatal density of DRD2 in overweight individuals (Stice et
606 al., 2008; G. J. Wang et al., 2001) and rodents (Huang et al., 2006; Johnson and Kenny,

607 2010; Thanos et al., 2008)has been reported. Moreover, loss of DRD2 autorreceptors was
608 linked to drug addiction, such as cocaine intake (Bello et al., 2011; Holroyd et al., 2015). In
609 this sense, it has been shown that DRD2 plays an important role in the reward deficiency
610 syndrome, which is related to compulsive and addictive behaviors(Blum et al., 2011). In
611 VTA, a decrease in DRD2 has been linked to a greater motivation for food and the
612 development of obesity(Bello et al., 2011; Koyama et al., 2014). Here, we showed for the
613 first time a downregulation in DRD2 that is correlated with alteration in the methylation
614 levels of it promoter in the offspring of dams fed with a CAF diet at PND10. Considering
615 that these changes could have a long-lasting effect later in life, these results suggest that the
616 epigenetic dysregulation of DRD2 could be an early marker of health diseases related with
617 excessive consumption of food or drugs in adulthood.However, further studies are needed
618 to clarify the cause-effect relationship between early DRD2 dysregulation in response to
619 maternal diet and addictive behaviors in the adult offspring.

620

621 **Declaration of interest**

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

624

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629

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634

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