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# Cafeteria diet differentially alters the expression of feeding-related genes through DNA methylation mechanisms in individual hypothalamic nuclei

Gisela Paola Lazzarino [b], María Florencia Andreoli [a, b], María Florencia Rossetti [a, b], Cora Stoker [a, b], María Virgina Tschopp [b], Enrique Hugo Luque [b], Jorge Guillermo Ramos [a, b, *]

[a] Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina
[b] Instituto de Salud y Ambiente del Litoral (ISAL), Universidad Nacional del Litoral – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

We evaluated the effect of cafeteria diet (CAF) on the mRNA levels and DNA methylation state of feeding-related neuropeptides, and neurosteroidogenic enzymes in discrete hypothalamic nuclei. Besides, the expression of steroid hormone receptors was analyzed. Female rats fed with CAF from weaning increased their energy intake, body weight, and fat depots, but did not develop metabolic syndrome. The increase in energy intake was related to an orexigenic signal of paraventricular (PVN) and ventromedial (VMN) nuclei, given principally by upregulation of AgRP and NPY. This was mildly counteracted by the arcuate nucleus, with decreased AgRP expression and increased POMC and kisspeptin expression. CAF altered the transcription of neurosteroidogenic enzymes in PVN and VMN, and epigenetic mechanisms associated with differential promoter methylation were involved. The changes observed in the hypothalamic nuclei studied could add information about their differential role in food intake control and how their action is disrupted in obesity.



## 1. Introduction

Obesity has become one of the most serious public health problems in the developed world. Although it is a multifactorial metabolic condition, its main cause is presumably the combination of overeating and inactivity. Cafeteria diet (CAF) is an experimental rodent diet model that accurately reflects the variety of highly palatable and energy dense foods that are prevalent in the Western society and that are associated with the current obesity pandemic (Sampey et al., 2011). The interplay of various hypothalamic nuclei with peripheral hormones, neuropeptides, and nuclear receptors represents a critical aspect of the hypothalamic regulation of energy metabolism (Frank et al., 2014). The body weight is regulated by a complex inter-organ circuit connecting the periphery and the brain (Caminos et al., 2008). The hypothalamus is a major hub that integrates nutritionally relevant information originated from all peripheral organs, mediated through circulating metabolites and hormones such as glucose, insulin, ghrelin, and leptin (Lenard and Berthoud, 2008). The central control of energy homeostasis is highly dependent on the activity of peptidergic neuronal circuits located in the hypothalamus (Torri et al., 2002). The arcuate nucleus (ARC) plays a central role in the integration of signals regulating nutritional status and energy homeostasis (Coupe et al., 2010). The ARC contains orexigenic neurons that coexpress neuropeptide Y (NPY) and agouti-related protein (AgRP) and anorexigenic neurons that coexpress proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Coupe et al., 2010). All these neuropeptide circuits project to downstream hypothalamic areas, including the paraventricular (PVN) and the ventromedial (VMN) nuclei, modulating the release of further anorectic or

* Corresponding author. Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina.
*E-mail addresses:* gplazzarino@fbcb.unl.edu.ar (G.P. Lazzarino), mfandreoli@fbcb.unl.edu.ar (M.F. Andreoli), mfrossetti@fbcb.unl.edu.ar (M.F. Rossetti), cstoker@fbcb.unl.edu.ar (C. Stoker), mvtschopp@fbcb.unl.edu.ar (M.V. Tschopp), eluque@fbcb.unl.edu.ar (E.H. Luque), gramos@fbcb.unl.edu.ar (J.G. Ramos).

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**Abbreviations**

| | |
|---|---|
| 17β-HSD | 17β-hydroxysteroid dehydrogenase |
| 3β-HSD | 3β-hydroxysteroid dehydrogenase |
| 3α-HSD | 3α-hydroxysteroid dehydrogenase |
| 5αR-1 | 5α-reductase-1 |
| AgRP | Agouti-related protein |
| ARC | Arcuate nucleus |
| ARO | p450-aromatase |
| AUC | Area under curve |
| CAF | Cafeteria diet |
| CART | Cocaine- and amphetamine-regulated transcript |
| $E_2$ | Estradiol |
| ERα | Estrogen receptor alpha |
| HOMA | Homeostasis model assessment |
| ipGTT | Intraperitoneal glucose tolerance test |
| KISS | Kisspeptin |
| nGRE | negative glucocorticoid-responsive element |
| NPY | Neuropeptide Y |
| Ob-Rb | Long form of leptin receptor |
| P450-17α | Cytochrome P450 17α-hydroxylase |
| P450scc | Cytochrome P450 side-chain cleavage |
| PND | Postnatal day |
| POMC | Proopiomelanocortin |
| PVN | Paraventricular nucleus |
| StAR | Steroidogenic acute regulatory protein |
| VMN | Ventromedial nucleus |

orexigenic peptides that adjust energy intake and expenditure to maintain a stable body weight (Torri et al., 2002). Kisspeptin (KISS) is a neuropeptide synthesized by neurons in the ARC. Although its major role is in reproduction, KISS also has a role in the control of energy homeostasis, as it has the ability to directly excite POMC neurons and indirectly inhibit neurons that express NPY (Fu and van den Pol, 2010).

The brain, like the adrenal glands, gonads, and placenta, is a steroidogenic organ (Mellon and Griffin, 2002). Neurosteroids are endogenous modulators of neuronal function responsible for a broad spectrum of biological and pathophysiological effects (Do Rego et al., 2009). Although it has been recently reported that several neurosteroidogenic enzymes are expressed in the hypothalamus (Munetomo et al., 2015) the relationship between their expression and the metabolic or dietary status of the animals remains unknown. Numerous experiments have shown that the diet and/or nutritional status of the animals affect/s steroidogenesis in different organs including ovary (Newell-Fugate et al., 2015) and adrenal gland (Swierczynska et al., 2015), and in brain regions such as the hippocampus (Ohashi et al., 2015). Our group has recently reported that obese phytoestrogen-deprived rats show alterations in the hypothalamic expression of different neurosteroidogenic enzymes (Andreoli et al., 2016). Numerous experiments have established that steroids may affect food intake. Chronic administration of progesterone by subcutaneous patches increases energy intake in adult female rats (Grueso et al., 2001). Besides, it has been reported that rats fed with standard chow *ad libitum* and treated with allopregnanolone show increased food intake and weight gain (Nakhate et al., 2013). Otherwise, female rats treated with estradiol ($E_2$), administered either centrally or by subcutaneous implants, show decreased food intake and body weight (Dagnault and Richard, 1997; Puerta et al., 1990). Moreover, estrogen signaling potentiates leptin sensitivity, possibly by increasing the expression of the leptin receptor (Ob-Rb) in the hypothalamus (Frank et al., 2014), which is another mechanism through which $E_2$ exerts its anorectic action. We have previously reported that, in phytoestrogen-deprived rats, an alternative mechanism for the induction of obesity is a reduction of circulating $E_2$ levels, which affects hypothalamic estrogen receptor alpha (ERα) signaling (Andreoli et al., 2015).

DNA methylation is a major epigenetic modification that controls gene expression in physiologic and pathologic states. Different metabolic disorders, including obesity, are associated with alterations in gene expression that are caused by genetic and environmental factors, which could modify the DNA methylation pattern in somatic tissues (Barres and Zierath, 2011). DNA methylation at CpG dinucleotides alters gene expression by affecting transcription factor binding activity (Marco et al., 2013). It has been reported that different environmental exposures, including a high caloric environment, could affect DNA epigenetic patterns in hypothalamic feeding control centers resulting in altered gene expression and obesity (Marco et al., 2013). Thus, DNA methylation provides a mechanism by which diet can modify genetic predisposition to disease.

Our hypothesis was that the endogenous synthesis of molecules involved in the homeostatic regulation of food intake may be altered in obese animals fed with CAF, implicating variations in the gene expression of neuropeptides, and receptors involved in food intake control. The expression of neurosteroidogenic enzymes in the rat hippocampus has been widely reported, but more information in discrete hypothalamic nuclei is needed. Thus, the aim of the present study was to evaluate the effect of CAF diet on the mRNA levels of peptides, steroid hormone receptors, and neurosteroidogenic enzymes in individual hypothalamic nuclei obtained using a micropunch dissection technique. Besides, we propose that epigenetic changes, such as altered DNA methylation of genes involved in the regulation of body weight and metabolism, could be involved.

## 2. Materials and methods

### 2.1. Ethics statement

All procedures were approved by the Ethical Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, UNL, Santa Fe, Argentina) and were performed in accordance with the principles and procedures outlined in 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).

### 2.2. Animals, diets, and dietary treatment

Thirty-two female Wistar rats were obtained at the Department of Human Physiology of the School of Biochemistry and Biological Sciences (UNL), weaned at 21 days of age, and randomly divided into two weight-matched groups. Rats were housed two per cage and maintained in controlled conditions (22 ± 2 °C and 12-h light–dark cycle). Animals were fed with either standard chow or a CAF diet (n = 16 per group) from weaning and for 20 weeks. Water was available throughout the experiment. The standard

chow (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina) provided 3 kcal/g, 5% energy as fat, 23% protein and 72% carbohydrate. The CAF diet was composed of food items chosen to reflect the enormous variety, palatability, and energy density of the modern western diet. The CAF diet included standard chow, as well as French fries, parmesan cheese, cheese flavored snacks, crackers, sweet biscuits, cookies, pudding, peanut butter, and chocolate. This diet provided an average of 4.85 kcal/g, 49% of energy as fat, 7% as protein, and 44% as carbohydrate, in addition to that provided by the standard chow. Furthermore, taking into account the caloric content of the standard chow added to the CAF food, the average caloric input of the whole CAF diet is 4.25 kcal/g. Three of the CAF foods were provided in excess quantities and were changed every other day, by replacing all the food with new items, in order to maintain the variety. Therefore, the animals did not receive the same food items for more than two consecutive days. During the experimental period, body weights were recorded weekly, and food intake daily. Food intake was determined by the weight difference between the offered and the remaining food, adjusted to the waste by collecting food spillage. Energy intake was calculated using the energy contents of each food (kcal/g).

On the 19th week of treatment, an intraperitoneal glucose tolerance test (ipGTT) was performed as described later. As an external index of puberty onset, vaginal opening was monitored daily starting on postnatal day (PND) 30 and the estrous cyclicity was supervised from PND 91. Rats were sacrificed by decapitation during the diestrous phase of the estrous cycle to provide stable basal hormone levels (Beale et al., 2014). Trunk blood was collected, samples were centrifuged, and serum was immediately used or frozen and stored at −80 °C until further use. Retroperitoneal and perigonadal fat pads were dissected and weighed. Immediately after euthanization, the brain was removed from the skull, frozen ventral side up on dry ice and stored at −80 °C until sectioning.

### 2.3. Micropunches of individual hypothalamic nuclei

Serial coronal sections of 300 μm were cut from frozen rat brains in a cryostat at −12 °C. By using Palkovits' microdissection technique (Palkovits and Brownstein, 1988), and a rat brain stereotaxic atlas (Paxinos and Watson, 2005), PVN, VMN, and ARC nuclei were identified and punched out with a 1.0 mm stainless steel micropunch needle. Nuclei were removed bilaterally and samples were stored at −80 °C until assayed. To determine the reproducibility of the microdissected areas, the sections were thawed, and the topography of the holes was inspected under a stereo microscope (Stemi 305, Zeiss, Oberkochen, Germany). Each microdissected brain region from an individual animal (between 6 and 10 punches, depending on the brain region) was processed and analyzed as a single data point.

### 2.4. Reverse transcription and real-time quantitative PCR

Eight animals of each experimental group were selected randomly and total RNA was isolated from each hypothalamic region by using TRIzol (Invitrogen) according to the manufacturer's protocol. The concentration and purity of RNA were estimated by measuring the $A_{260}$ and $A_{280}$ in a NanoDrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One microgram of total RNA from each region was reverse-transcribed using 200 pmol of random hexamer primers (Promega, Madison, WI, USA) and Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega). Twenty units of the ribonuclease inhibitor RNAout (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube at a final volume of 30 μl of 1X reverse transcriptase buffer. Reverse transcription was performed at 37 °C for 90 min and at 42 °C for 15 min. Reactions were stopped by heating at 80 °C for 5 min and cooling on ice. Each reverse-transcribed product was diluted with RNAse-free water to a final volume of 60 μl and further amplified in duplicate.

For cDNA amplification, 5 μl of cDNA was mixed with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen Argentina) to a final volume of 20 μl. Primer pairs used for ribosomal protein L19 (housekeeping gene), neuropeptides, neurosteroidogenic enzymes, and receptors are shown in Table 1 cDNA levels were determined by using a real-time PCR system StepOne Cycler (Applied Biosystems Inc., Life Technologies, Carlsbad, CA, USA). Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Negative DNA template controls were included in all the assays, and yielded no consistent amplification. The threshold cycles (Ct) and PCR efficiency were calculated using Step One software (Applied Biosystems Inc.). For each target the fold expression over control values was calculated using the relative standard curve methods designed to analyze data from real-time PCR (Cikos et al., 2007). The relative target quantity for all experimental samples was determined from the standard curve, normalized to the relative quantity of the reference gene, and finally divided by the normalized target value of the control sample. No significant differences in Ct values were observed for L19 between the different experimental groups.

### 2.5. Bioinformatics

The POMC, NPY and AgRP promoters and two different enhancers of the POMC gene (nPE1 and nPE2), previously described by other authors (Plagemann et al., 2009; Melas et al., 2013; Coupe et al., 2010; de Souza et al., 2005) were analyzed for CpG islands using Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA, USA). All of these regions were also checked for restriction sites for *BstUI* (New England BioLabs, Beverly, MA, USA) or *Mae II* (Roche Applied Science, Indianapolis, IN, USA) to evaluate the number of methylation sensitive sites. The bioinformatics tool PROMO was used to recognize the putative binding sites for transcription factors (Messeguer et al., 2002). PCR primers were designed with the software Vector NTI Suite Version 6.0 (Table 2).

### 2.6. DNA methylation-sensitive analysis

Genomic DNA from dissected areas (n = 8/group) was isolated using the phenol/chloroform/isoamyl alcohol extraction. The total concentration of DNA was quantified by $A_{260}$ and stored at 2–8 °C until further use. Equal quantities of DNA (1 μg) were digested with 1 unit of *Mae II* (Roche Applied Science, Indianapolis, IN, USA) or 10 units of *BstUI* (New England BioLab, Beverly, MA, USA) and 1X enzyme buffer for 1 h at 60 °C or 50 °C, respectively. The digestion products were purified with the Wizard SV gel and PCR Clean-Up System Kit according to the manufacturer's protocol (Promega, Madison, WI).

The methylation state of POMC, NPY, AgRP, 5αR-1, 3αHSD, and ARO promoters was analyzed using a combination of single digestions with methylation-sensitive restriction enzymes and subsequently performing real-time PCR analysis (Rossetti et al., 2015). The relative expression level of the different DNA regions was analyzed by real-time PCR (as mentioned above). The 5αR-1, 3αHSD, and ARO promoter regions have been previously studied, and the PCR primers for amplification have been described (Rossetti et al., 2015, 2016). The primer sequences used for POMC, NPY and AgRP are shown in Table 2. Each sample was quantified in

**Table 1**
Oligonucleotide sequences (GenBank/NCBI) used in real-time quantitative PCR.

| Enzyme/protein name | Gene name | Accession number | Primer Sequences | Size (bp) |
|---|---|---|---|---|
| L19 | L19 | NM_031103 | F: 5′- AGCCTGTGACTGTCCATTCC -3′<br>R: 5′- TGGCAGTACCCTTCCTCTTC -3′ | 99 |
| POMC | Pomc | NM_139326.2 | F: 5′-CCTCCTGCTTCAGACCTCCATA-3′<br>R: 5′-TGTTCATCTCCGTTGCCTGG-3′ | 159 |
| CART | Cart | NM_017110.1 | F: 5′-GCTCAAGAGTAAACGCATTCC-3′<br>R: 5′-AAGAATTGCAAGAAGTTCCTCG-3′ | 143 |
| AgRP | Agrp | NM_033650.1 | F: 5′-TTGGCAGAGGTGCTAGATCCA-3<br>R: 5′-AGGACTCGTGCAGCCTTACAC-3′ | 108 |
| NPY | Npy | NM_012614.1 | F: 5′-CTTAATGAGAGAAAGCACAG-3′<br>R: 5′-AACTAGGAAAAGTCAGGAGA-3′ | 101 |
| KISS | Kiss1 | NM_181692.1 | F: 5′- TGCTGCTTCTCCTCTGTG-3′<br>R: 5′- CCAGGCATTAACGAGTTCC-3′ | 116 |
| StAR | Star | NM_031558.3 | F: 5′- GCAAAGCGGTGTCATCAG -3′<br>R: 5′- GGCGAACTCTATCTGGGTCT -3′ | 172 |
| P450scc | Cyp11a1 | NM_017286.2 | F: 5′- AGGGAGAACGGCACACACAG -3′<br>R: 5′- TCGCAGGAGAAGAGAGTCGC -3′ | 143 |
| 3α-HSD | Hsd3a | NM_012596.1 | F: 5′- GCACTCAACTGGACTATGTGGA -3′<br>R: 5′- GCTCATCTCGTGGGAAAAAT -3′ | 87 |
| 3β-HSD | Hsd3b | NM_001007719.3 | F: 5′- AGGCCTGTGTCCAAGCTAGTGT -3′<br>R: 5′- CTCGGCCATCTTTTTGCTGTAT -3′ | 161 |
| P450-17α | Cyp17a1 | NM_012753.2 | F: 5′- GGTGATAAAGGGTTATGCCA -3′<br>R: 5′- GCTTGAATCAGAATGTCCGT -3′ | 117 |
| 17β-HSD | Hsd17b3 | NM_054007.1 | F: 5′- CAACCTGCTCCCAAGTCATT -3′<br>R: 5′- AACCCCTACTCCCGAAGAAA -3′ | 160 |
| ARO | Cyp19a1 | NM_017085 | F: 5′- TGGCAGATTCTTGTGGATGG -3′<br>R: 5′- CGAGGACTTGCTGATGATGAGT -3′ | 118 |
| 5αR-1 | Srd5a1 | NM_017070 | F: 5′- CACCTTCAACGGCTATGTAC -3′<br>R: 5′- AGGATGTGGTCTGAGTGGAT -3′ | 144 |
| IR | InsR | NM_017071.2 | F: 5′-TCTCTCCAGGAAACTACAGTGT-3<br>R: 5′-AAAATAGGTGGGTTCTGTCC-3′ | 80 |
| PR | Pgr | NM_022847.1 | F: 5′- GACCAGTCTCAACCAACTAGGC-3′<br>R: 5′- ACACCATCAGGCTCATCCAG-3′ | 137 |
| ERα | ERα | NM_012689 | F: 5′-ACTACCTGGAGAACGAGCCC-3′<br>R: 5′-CCTTGGCAGACTCCATGATC-3′ | 153 |
| Ob-Rb | ob-Rb | NM_012596.1 | F: 5′-AGGATGAGTGTCAGAGTCAA-3′<br>R: 5′-CTCTTCATCAGTTTCCACTG-3′ | 80 |

L19: ribosomal protein (housekeeping gene); POMC: Proopiomelanocortin; CART: Cocaine- and amphetamine-regulated transcript; AgRP: Agouti-related protein; NPY: Neuropeptide Y; KISS: kisspeptin; StAR: steroidogenic acute regulatory protein; P450scc: cytochrome P450 side chain cleavage; 3α-HSD: 3α-hydroxysteroid dehydrogenase; 3β-HSD: 3β-hydroxysteroid dehydrogenase, P450-17α: cytochrome P450 17α-hydroxylase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; ARO: P450aromatase, 5αR-1: 5α-reductase type 1; IR: insulin receptor; PR: Progesterone receptor; ERα: estrogen receptor alpha; Ob-Rb: leptin receptor.

**Table 2**
Oligonucleotide sequences used in real-time quantitative PCR for DNA methylation-sensitive analysis.

| Gene target | Accession number | Primer forward | Primer reverse |
|---|---|---|---|
| IC POMC | NC_005105.4 | 5′-GGTGAAGGCTGTCAGACAATC-3′ | 5′-CCAAACTCCAAGGGAAAGG-3′ |
| *Mae II* (a) POMC | | 5′-CAGGAAGGTCACGTCCAAGG-3′ | 5′-CCGTTTGGTCCCTGTCACTC-3′ |
| *BstUI* (b) POMC | | 5′-GAGTGACAGGGACCAAACGG-3′ | 5′-CAGGAGCCGAGACACCCTTA-3′ |
| IC nPE1 | | 5′-TAAGGGGCTAAAGGACCAAG-3′ | 5′-CTGCTCCACAACAGAACCAA-3′ |
| *Mae II*/*BstUI* nPE1 | | 5′-TCAGACTGGTGACTGCTGGC-3′ | 5′-GGACTTCCTGGAGGTGCAGT-3′ |
| IC NPY | NC_005103.4 | 5′-GTCCCTTGCTCAAAAGTGGC-3′ | 5′-CATCGGTCAAAACCTGGGTC-3′ |
| *BstUI* (a) NPY | | 5′-TTCCAGAGGCATTAACTCAAGC-3′ | 5′-AAAAGACCAACGCCACTGTG-3′ |
| *BstUI* (b) NPY | | 5′-TCGCAGTTGTCCCAGAGATG-3′ | 5′-GGACCGCTTAGATTGCCTGT-3′ |
| *BstUI* (c) NPY | | 5′-GGAAGTGGCTGTGGGAGTCA-3′ | 5′-CTGCGAGGAATGAGCTCCAC-3′ |
| IC 3α-HSD | NC_005116.4 | 5′-CAGAGAAGGAAGTTTGAATC-3′ | 5′-ATGTCAGATCACTTGGAAGT-3′ |
| *Mae II* (a) 3α-HSD | | 5′-ACTGATTTTTGCTTAGGCTG-3′ | 5′-AAAATTCTGTAGTGAGCCGT-3′ |
| *Mae II* (b) 3α-HSD | | 5′-GGATGTGGCTGGAATACAGA-3′ | 5′-TTCTGTCACTTTGTCTGCCC-3′ |
| *Mae II* (c) 3α-HSD | | 5′-GAAACATTGTGTCTGTATGG-3′ | 5′-GTAAATTGTTAAGGGGAGAC-3′ |
| IC 5αR-1 | NC_005100.4 | 5′-CAACTTTCTGTCCATCTACC-3′ | 5′-CTTACAACTCTCCTCTTTCG-3′ |
| *BstUI* (a) 5αR-1 | | 5′-CACCTTCCCAGCCCTGACAG-3′ | 5′-AGGTGCCAGGAGAGAGGGGT-3′ |
| *Mae II* (b) 5αR-1 | | 5′-AGTCAAGAAATATGCCTGAA-3′ | 5′-AATACGTTCTCGGTATGAAT-3′ |
| *Mae II* (c) 5αR-1 | | 5′-CCACTAAGCGTGAATCTCTC-3′ | 5′-AACACTCCATGACTCTCTGC-3′ |
| *Mae II* (d) 5αR-1 | | 5′-CTGCTGGCTATGTTTCTGAT-3′ | 5′-TGGAATTAAGTCTCTGAGCC-3′ |
| IC ARO | NC_005107.4 | 5′- ACTCAAGGGCAAGATGATAA- 3′ | 5′- AGTGACAAGGCAAGACAAAT- 3′ |
| *Mae II* ARO | | 5′- CTGGAGTAGGAGCCTTTACC- 3′ | 5′- CTTGAGTGGGTAGAGTGACG- 3′ |

duplicate or triplicate. A region without *Mae II* or *BstUI* restriction sites was used as an internal control for DNA quality (IC). The methylation restriction enzymes *Mae II* or *BstUI* are unable to cut at methylated sites, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, *BstUI* or *Mae II* cleaves the DNA and prevents the amplification of the fragment.

The relative degree of promoter methylation was determined by plotting Ct values against the log input (internal control), which yielded standard curves for the quantification of unknown samples, and finally dividing by the normalized target value of the control sample (Cikos et al., 2007).

### 2.7. Intraperitoneal glucose tolerance test (ipGTT)

Rats were fasted overnight and then given an intraperitoneal injection of glucose (2 g/kg). Blood samples were taken from the tail vein, and glucose was measured using an Accu-Chek Performa Nano meter (Roche Diagnostics, Mannheim, Germany) at 0 (fasting), 30, 60, 90, and 120 min after glucose administration (Wang et al., 2011). The total area under curve (AUC) for glucose during the ipGTT (2-h glucose AUC) was calculated using Origin 6.1 software (Origin, Origin Lab Corporation, MA, USA).

### 2.8. Serum assessments

Fasting serum metabolites (glucose, triglycerides, and cholesterol) were determined by a commercially available assay (Wiener Laboratorios, Argentina). Serum insulin levels were assessed by radioimmunoassay (RIA) using an anti-rat insulin antibody (Sigma, St. Louis, Missouri, USA) and standard rat insulin provided by Laboratorios Beta (Buenos Aires, Argentina). The circulating levels of leptin were determined by specific RIA (Giovambattista et al., 2006). Total $E_2$ levels were measured using competitive RIA kits (Immunotech, Marseille, France). To evaluate insulin resistance, the homeostasis model assessment (HOMA-IR) was calculated as [fasting insulin (IU/ml) × fasting glucose (mol/l)]/22.5 (Matthews et al., 1985).

### 2.9. Statistical analysis

An exploratory test was performed to evaluate the distribution of the data (Shapiro-Wilk test) and variance homogeneity (Levene's test) of each variable. For non-parametric variables, data were statistically analyzed by the Mann Whitney test, using IBM SPSS Statistics 19 software (IBM Inc., Armonk, NY, USA) or R software version 3.3.3. Weekly body weights, fat depots and food intake were analyzed using Student's T test. In all cases, the data were expressed as the means ± SEM. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Effects of CAF diet on body weight, adipose tissue and food intake

Animals fed with the CAF diet increased their body weights from week 14 of treatment ($p < 0.01$; Fig. 1). To determine whether there was a region-specific fat gain, perigonadal and retroperitoneal fat depots were excised and weighed. Accordingly with the body weight changes, the CAF diet also increased body fat. Perigonadal as well as retroperitoneal fat pad weights were higher in rats fed with the CAF diet, expressed both in grams (absolute value) and as a percentage of body weight ($p < 0.001$; $p < 0.01$, respectively; Table 3 and Fig. 2). This is consistent with the 21% increase in energy intake observed in CAF-fed rats ($p < 0.001$), which presented a higher daily caloric intake Vs. SC rats throughout the treatment (Fig. 3). However, CAF-fed animals were not hyperphagic, as they markedly decreased their food intake (measured by weight) vs standard chow-fed rats ($p < 0.001$; Table 3). Thus, the increase in energy intake was a consequence of the high-energy content of palatable food.

### 3.2. Metabolic and endocrine effects of CAF diet

Twenty weeks of CAF feeding did not alter fasting serum glucose, cholesterol, triglycerides, or insulin levels. Therefore, the HOMA-IR was unchanged (Table 4). Moreover, the CAF diet did not modify glucose homeostasis, since neither the ipGTT (Fig. 4) nor the AUC were affected by the treatment (Table 4). As expected, leptin was increased four-fold in CAF-fed rats, due to their greater fat depot weights. CAF-fed animals showed no changes in serum $E_2$ levels (Table 4). Dietary treatment did not affect the day of vaginal opening, which was from PND 40 to PND 45 in both groups. Besides, 20 weeks of CAF diet did not affect the estrous cycle, as CAF rats cycled regularly.

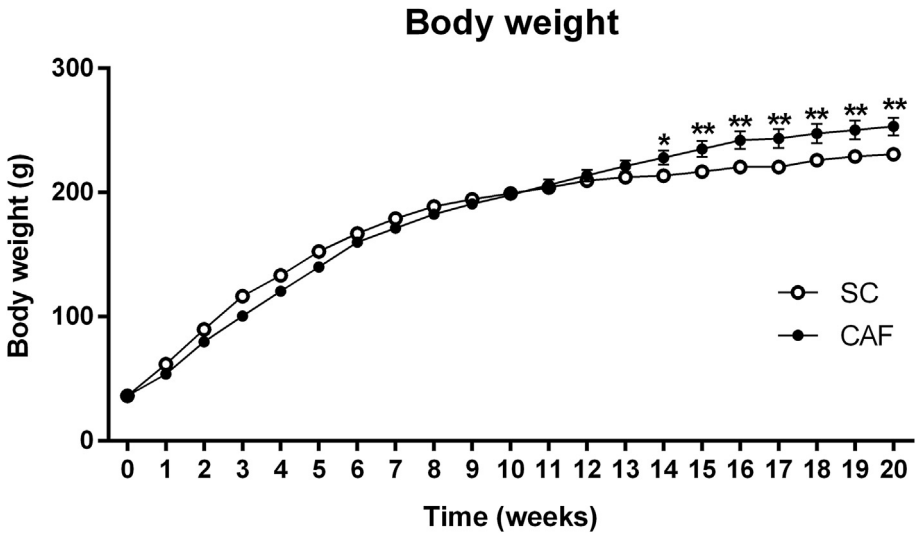



**Fig. 1.** Body weight of rats fed with standard chow (SC) or Cafeteria diet (CAF) for 20 weeks (n = 16/group). * indicates significant differences at $p < 0.05$ and ** indicates significant differences at $p < 0.01$ by Student's T test.

**Table 3**
Adipose tissue weights, food and energy intake.

| Parameter | SC | CAF | p value |
|---|---|---|---|
| Perigonadal fat | | | |
| g | 8.91 ± 0.45 | 14.28 ± 1.05 *** | <0.001 |
| g/100 g of body weight | 3.98 ± 0.16 | 5.76 ± 0.22 *** | <0.001 |
| Retroperitoneal fat | | | |
| g | 6.10 ± 0.42 | 8.76 ± 0.72 ** | <0.01 |
| g/100 g of body weight | 2.72 ± 0.16 | 3.53 ± 0.2 ** | <0.01 |
| Food intake (g/day) | 13.98 ± 0.09 | 11.99 ± 0.14 *** | <0.001 |
| Energy intake (kcal/day) | 41.53 ± 0.27 | 50.87 ± 0.6 *** | <0.001 |

SC: Standard Chow diet; CAF: Cafeteria diet.
Data shown are presented as the mean ± SEM (n = 16/group). ** indicates significant differences at $p < 0.01$ and *** indicates significant differences at $p < 0.001$ by Student's T test.

### 3.3. CAF diet altered the hypothalamic expression of neuropeptides

The expression of neuropeptides was assessed in different hypothalamic nuclei involved in food intake control. In the PVN, AgRP and NPY increased their expression ($p < 0.05$), whereas KISS was not detectable. In the VMN, only AgRP mRNA levels were altered, showing a two-fold higher expression ($p < 0.05$). Meanwhile, in the ARC, the expression of both POMC and KISS was increased by the CAF diet ($p < 0.05$; $p < 0.01$, respectively), while that of AgRP decreased by 50% ($p < 0.05$). CART mRNA expression was not altered in any of the nuclei studied (Fig. 5). It should be noted that, for both SC and CAF animals, the expression of all the neuropeptides measured was 16-fold over in the ARC than in the PVN and VMN (data not shown).

### 3.4. CAF diet altered the expression of different hormone receptors, and neurosteroidogenic enzymes

The relative expression levels of hormone receptors, and neurosteroidogenic enzymes in PVN, VMN, and ARC were evaluated by real-time quantitative PCR (Figs. 6 and 7; respectively). No gene expression of Cytochrome P450 side-chain cleavage (P450scc), 17β-hydroxysteroid dehydrogenase (17β-HSD), and Cytochrome P450 17α-hydroxylase (P450-17α) was detected in any of the samples analyzed. In PVN, CAF did not alter the mRNA expression of any of the receptors studied, however p450-aromatase (ARO) expression was increased ($p < 0.05$), whereas 5α-reductase-1 (5αR-1) expression was decreased ($p < 0.05$). In contrast, in VMN, Ob-Rb and ERα expression were increased by CAF ($p < 0.001$; $p < 0.01$, respectively); 3α-hydroxysteroid dehydrogenase (3α-HSD, $p < 0.01$), 3β-hydroxysteroid dehydrogenase (3β-HSD, $p < 0.05$), and ARO ($p < 0.05$) doubled their expression. In ARC the expression of Ob-Rb, progesterone receptor, and ERα was increased ($p < 0.05$; $p < 0.05$; $p < 0.01$, respectively), despite any of the enzymes or proteins evaluated were altered by CAF. Neither insulin receptor nor Steroidogenic acute regulatory protein (StAR) mRNA levels were altered in any of the nuclei studied (Figs. 4 and 5; respectively).

### 3.5. In silico analysis of candidate sites of DNA methylation and potential transcription binding sites in the rat POMC, NPY, and AgRP genes

Based on the gene expression results, the promoter regions of POMC, NPY, AgRP, 5αR-1, 3αHSD, and ARO were analyzed in order to search for candidate sites for DNA methylation. The 5αR-1, 3αHSD, and ARO promoter regions, their predicted binding proteins and methylation-targeted CG areas have been previously described by our group (Rossetti et al., 2015, 2016). For the POMC gene, two different regions were analyzed: the promoter and the nPE1 enhancer, which is situated 12 kb upstream of the gene and was shown to control the hypothalamus-specific expression of the POMC gene, together with the nPE2 enhancer, which is situated 10 kb upstream of the gene, but does not contain any CpG island (Coupe et al., 2010; Lam et al., 2015). As shown in Fig. 8A, for POMC gene, one *Mae II* site located near the TATA box and one *BstUI* site situated downsteam the promoter were screened, and one *Mae II* and *BstUI* sites on nPE1 were analyzed. For NPY gene, 5 *BstUI* sites were studied (Fig. 8C). The AgRP promoter region does not contain any CpG sites in the 1200 nucleotides upstream of the transcription start site (Coupe et al., 2010). Consequently, methylation analysis could not be performed for this promoter.

### 3.6. CAF diet modifies the DNA methylation pattern of neuropeptides in ARC and PVN

To determine if the changes observed in the POMC and NPY transcript levels are related to DNA methylation modifications, the methylation state of the transcriptionally active promoters of these neuropeptides was determined in ARC and PVN, respectively. For the POMC promoter, a decrease in the methylation state was detected at the *Mae II* (a) site in the ARC of CAF animals ($p < 0.05$; Fig. 8B). Besides, no changes in any methylation-targeted CG site in the nPE1 enhancer were detected (Fig. 8B). Otherwise, the methylation state of a *BstUI* (a) site in the NPY promoter decreased in the PVN of CAF rats ($p < 0.01$; Fig. 8D). Because CAF diet only affectsAgRP mRNA expression (Fig. 5) in VMN, no methylation-

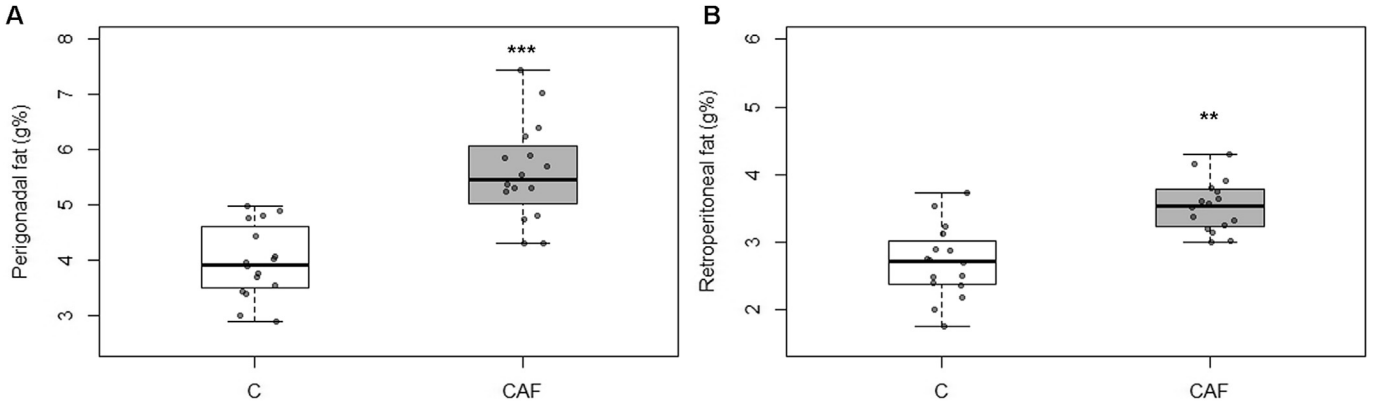



**Fig. 2.** Box-plot of (A) perigonadal and (B) retroperitoneal adipose tissue weights of rats fed with standard chow (SC) or Cafeteria diet (CAF) for 20 weeks (n = 16/group). The values are expressed in g tissue/100 g rat body weight. ** indicates significant differences at $p < 0.01$ and *** indicates significant differences at $p < 0.001$ by Student's T test.

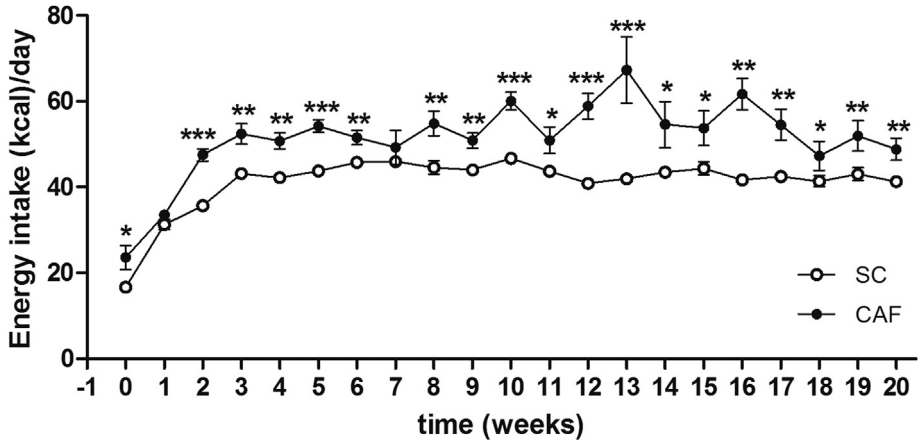



**Fig. 3.** Average daily energy intakes during each week of the dietary treatment in rats fed with Standard Chow (SC) or Cafeteria diet (CAF). Data shown are presented as the mean ± SEM (n = 16/group). * indicates significant differences at $p < 0.05$, ** indicates significant differences at $p < 0.01$ and *** indicates significant differences at $p < 0.001$ by Student's T test.

**Table 4**
Serum parameters and hormones of rats fed with standard chow (SC) or a Cafeteria diet (CAF).

| Parameter | SC | CAF | p value |
|---|---|---|---|
| Triglycerides (mmol/l) | 102.89 ± 11.65 | 82.45 ± 9.96 | 0.25 |
| Cholesterol (mmol/l) | 187.99 ± 7.89 | 196.99 ± 4.82 | 0.48 |
| Glucose (mmol) | 6.42 ± 0.19 | 6.22 ± 0.11 | 0.36 |
| Insulin (mU/l) | 28.92 ± 3.76 | 32.96 ± 6.54 | 0.60 |
| HOMA-IR | 8.22 ± 1.30 | 9.1 ± 1.92 | 0.73 |
| AUC ipGTT | 1410.26 ± 93.14 | 1458.54 ± 118.82 | 0.76 |
| Leptin (ng/ml) | 1.75 ± 0.20 | 6.76 ± 1.22 *** | <0.001 |
| Estradiol (ng/ml) | 0.024 ± 0.004 | 0.019 ± 0.004 | 0.45 |

SC: Standard Chow; CAF: Cafeteria Diet.
Data shown are presented as the mean ± SEM (n = 10/group). *** indicates significant differences at $p < 0.001$ by Student's T test.

sensitive analysis was conducted in this nucleus.

### 3.7. CAF diet modifies the DNA methylation pattern of different neurosteroidogenic enzymes in PVN, but not in VMN

To assess whether CAF diet induced changes in the transcript expression of 5αR-1, 3αHSD, and ARO were associated with differential DNA methylation, the methylation state of the transcriptionally active promoters of these enzymes were analyzed in PVN and VMN. For the ARO promoter, a decrease in the methylation state was detected at the *Mae II* site in the PVN of CAF animals ($p < 0.05$; Fig. 9A). Besides, in the 5αR-1 promoter, the methylation state of the *BstUI* (a) and *MaeII* (c) sites was increased in the PVN of CAF rats ($p < 0.05$; Fig. 9B). When the 5αR-1 exon 1 region was analyzed in the PVN, the methylation state at the *Mae II* (d) site was significantly increased in the CAF rats ($p < 0.05$; Fig. 9B). Otherwise, for the VMN, no changes in any of the methylation-targeted CG sites of the ARO or 3α-HSD studied were detected (Fig. 9C and D).

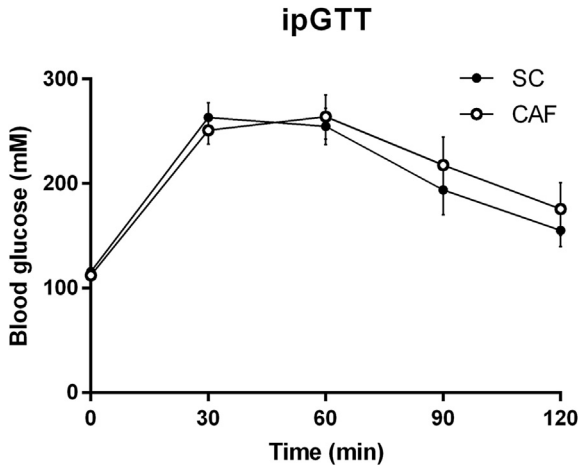



**Fig. 4.** Intraperitoneal glucose tolerance test (ipGTT) of rats fed with a standard chow (SC) or a Cafeteria diet (CAF) for 19 weeks. Data shown are presented as the mean ± SEM (n = 10/group).

## 4. Discussion

In the present study, the effects of CAF diet on the mRNA expression of neuropeptides, hormone receptors, and neurosteroidogenic enzymes in discrete hypothalamic nuclei were evaluated. We hypothesized that epigenetic modifications may be involved in these processes. To the best of our knowledge, this study is the first to evaluate the effects of the CAF diet on the expression of neuropeptides and neurosteroidogenic enzymes, establishing a relation between these changes and alterations in their promoter methylation state in individual isolated hypothalamic nuclei involved in food intake control. The use of the Palkovits' microdissection technique, widely known for being simple, reproducible and accurate (Palkovits, 1973) plus the Paxinos' stereotaxic brain atlas, allowed us to obtain accurate isolated brain nuclei. The CAF diet influenced the mRNA expression of feeding-related neuropeptides and hormone receptors in a different manner, depending on the specific hypothalamic nucleus studied. In agreement with other authors (Goularte et al., 2012; Lalanza et al., 2014; Prats et al., 1989; Sagae et al., 2012), our results showed that a palatable diet administered to female rats from weaning to adulthood significantly increased energy intake, fat depots, and body weight gain compared to animals fed with the standard chow. However, it should be noted that, not in agreement with the report of other authors (Lalanza et al., 2014; Sagae et al., 2012) 20 weeks of CAF feeding did not alter glucose metabolism, triglycerides, or total cholesterol serum levels. Despite the long dietary treatment of CAF-fed animals, they developed slight obesity, given by increased energy intake, body weight, and fat pads, but did not develop metabolic syndrome. We could infer that the homeostatic response of CAF-fed animals to the diet is the

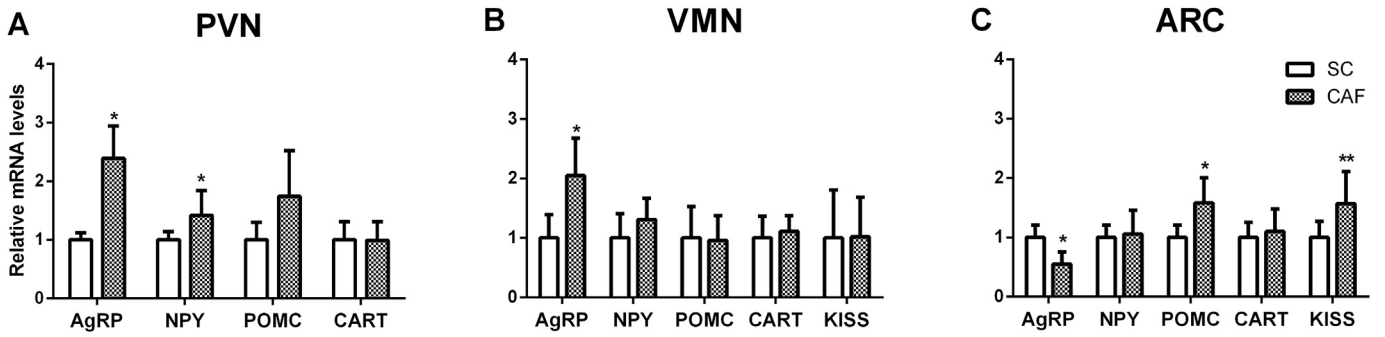



**Fig. 5.** Relative mRNA levels of neuropeptides in different hypothalamic nuclei involved in food intake control in rats fed with Standard Chow (SC) or Cafeteria diet (CAF) in (A) Paraventricular nucleus (PVN); (B) Ventromedial nucleus (VMN); (C) Arcuate nucleus (ARC). AgRP: agouti-related protein, NPY: neuropeptide Y; POMC: proopiomelanocortin; CART: cocaine- and amphetamine-regulated transcript; KISS: kisspeptin. mRNA expression was measured by quantitative real-time RT-PCR and the amounts of mRNA in CAF-fed rats are indicated as fold changes from those in rats fed with SC. Columns and error bars represent the mean ± SEM (n = 6–8/group). * indicates a significant difference at $p < 0.05$ and ** indicates a significant difference at $p < 0.01$ by Mann Whitney test.

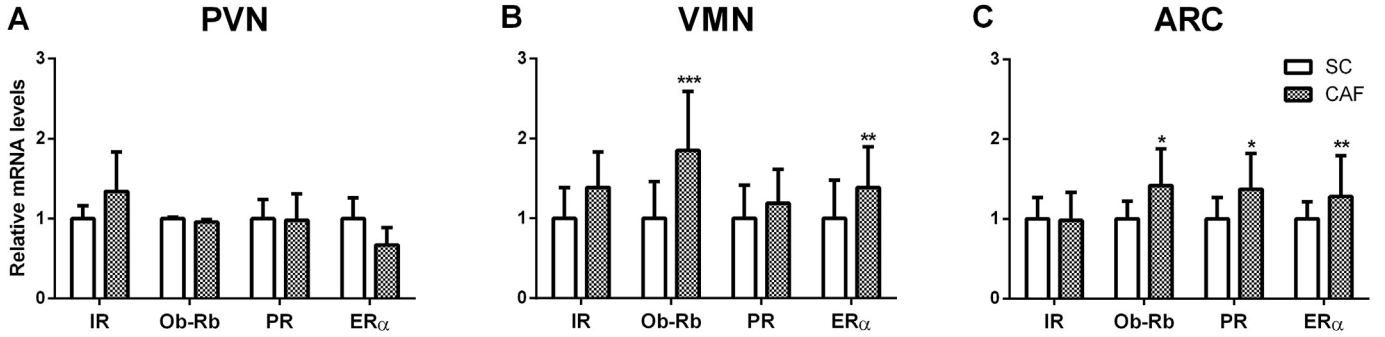



**Fig. 6.** Relative expression levels of hormone receptors in rats fed with standard chow (SC) or Cafeteria diet (CAF) in (A) Paraventricular nucleus (PVN); (B) Ventromedial nucleus (VMN); (C) Arcuate nucleus (ARC). IR: Insulin Receptor; Ob-Rb: Leptin receptor; PR: Progesterone Receptor; ERα: Estrogen Receptor α. mRNA expression was measured by quantitative real-time RT-PCR and the amounts of mRNA in CAF-fed rats are indicated as fold changes from those in rats fed with SC. Columns and error bars represent the mean ± SEM (n = 6–8/group). * indicates a significant difference at $p < 0.05$, ** indicates a significant difference at $p < 0.01$ and *** indicates a significant difference at $p < 0.001$ by Mann Whitney test.

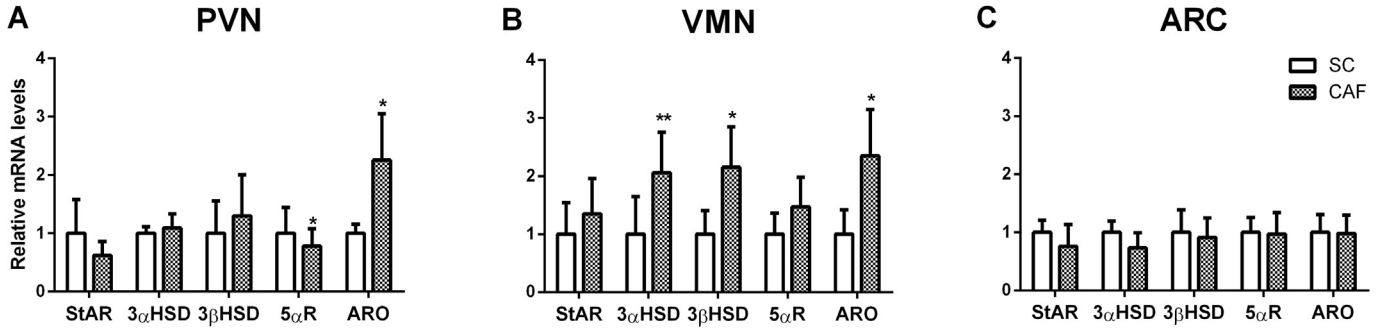
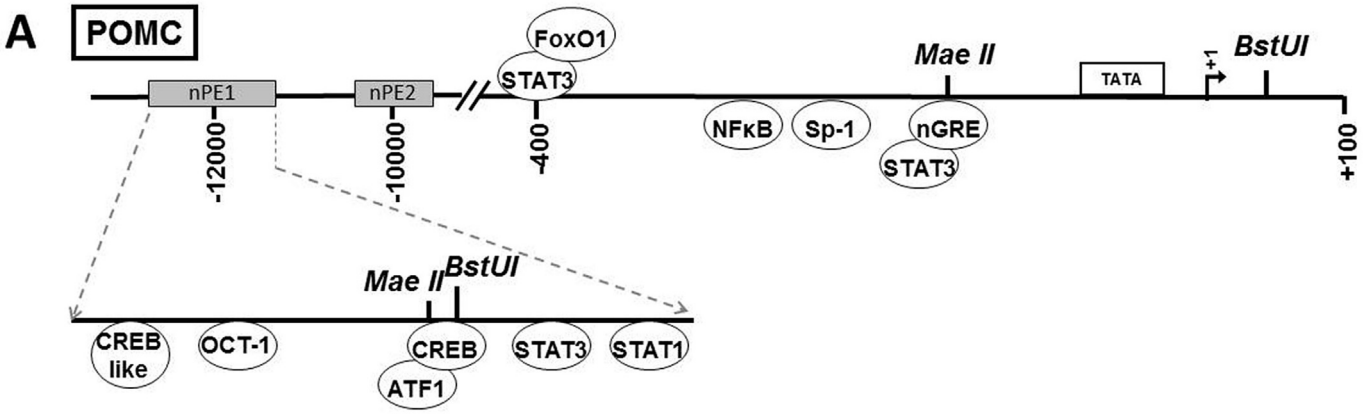
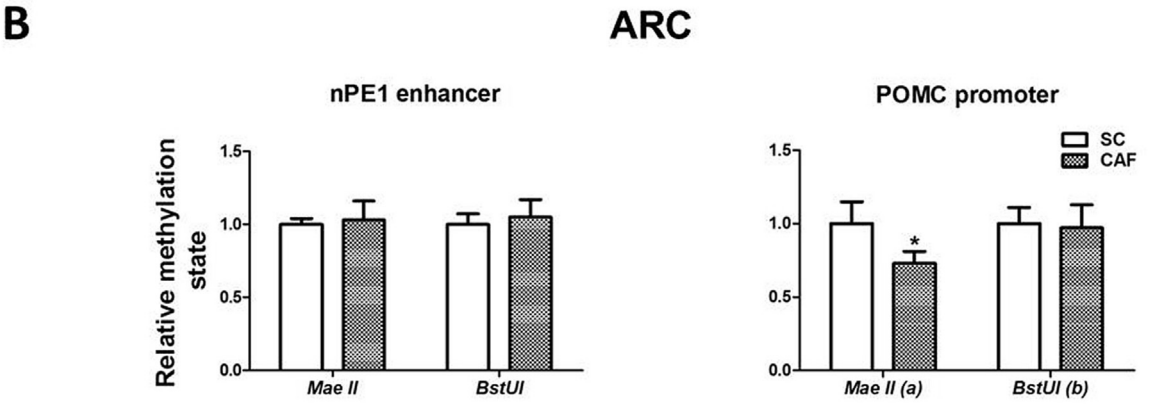


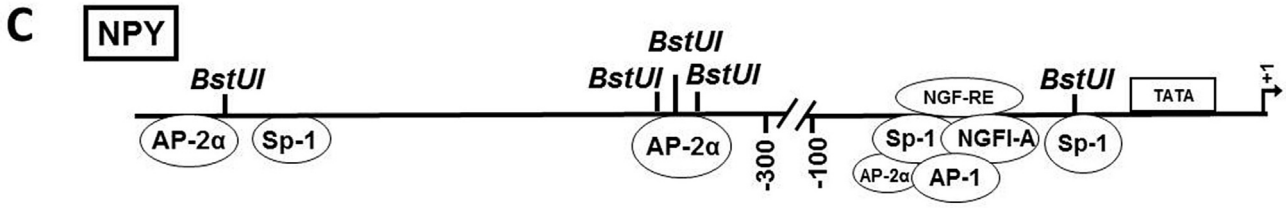

**Fig. 7.** Relative mRNA levels of neurosteroidogenic enzymes in rats fed with standard chow (SC) or Cafeteria diet (CAF) in (A) Paraventricular nucleus (PVN); (B) Ventromedial nucleus (VMN); (C) Arcuate nucleus (ARC). 3α-HSD: 3α-hydroxysteroid dehydrogenase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; ARO: P450aromatase; 5αR-1: 5α-reductase; StAR: steroidogenic acute regulatory protein. mRNA expression was measured by quantitative real-time RT-PCR and the amounts of mRNA in CAF-fed rats are indicated as fold changes from those in rats fed with SC. Columns and error bars represent the mean ± SEM (n = 6–8/group). * indicates a significant difference at $p < 0.05$ and ** indicates a significant difference at $p < 0.01$ by Mann Whitney test.

result of a strong hypothalamic regulation, which protects the animals against uncontrolled food intake and consequent metabolic disorders.

Leptin, is a hormone secreted mainly by adipocytes (Fox, 2006), which promotes negative energy balance by signaling in the brain. This hormone exerts its anorectic effect principally via the ARC, where it inhibits NPY/AgRP and activates POMC and KISS neurons, resulting in reduced food intake (Schwartz et al., 2000; Smith et al., 2006). Disruption of this action leads to a state known as leptin resistance, which is characterized by impaired leptin responses and reduced Ob-Rb expression and/or Ob-Rb protein levels (Zhang and Scarpace, 2006). In the present study, CAF-fed rats developed hyperleptinemia but remained leptin sensitive in the ARC, given by the increased expression of Ob-Rb, POMC and KISS, and the decreased expression of AgRP. However, CAF-fed rats continued with a high energy intake, suggesting that additional brain nuclei would be acting. Orexigenic (NPY/AgRP) and anorexigenic (POMC/CART/KISS) neurons show extensive synaptic reciprocity and



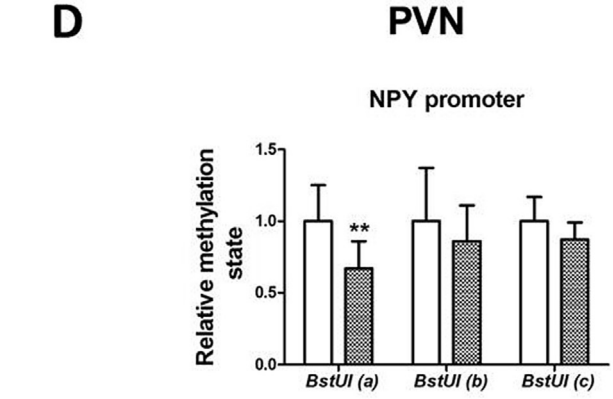




**Fig. 8.** Methylation analysis using methylation-sensitive restriction enzymes followed by real-time PCR in PVN and ARC of rats fed with standard chow (SC) or Cafeteria diet (CAF). Schematic representation of the (A) nPE1 and nPE2 (POMC enhancer) and POMC promoter and (C) NPY promoter, their binding proteins, and methylation targeted CG areas. The positions of the TATA box are indicated. Predicted binding sites for CREB, OCT-1, ATF1, STAT1, STAT3, FoxO1, NFκB, Sp-1, nGRE, AP1, AP-2α, NGF-RE, and NGFI-A binding site are shown. CG target sites for digestion by the methylation-sensitive restriction enzymes *BstUI* (CGCG) or *Mae II* (ACGT) are indicated. (B) methylation-sensitive analysis of POMC enhancer and promoter, and (D) NPY promoter was carried out. Columns and error bars represent the mean ± SEM (n = 6–8/group). * indicates a significant difference at $p < 0.05$ and ** indicates a significant difference at $p < 0.01$ by Mann Whitney test.

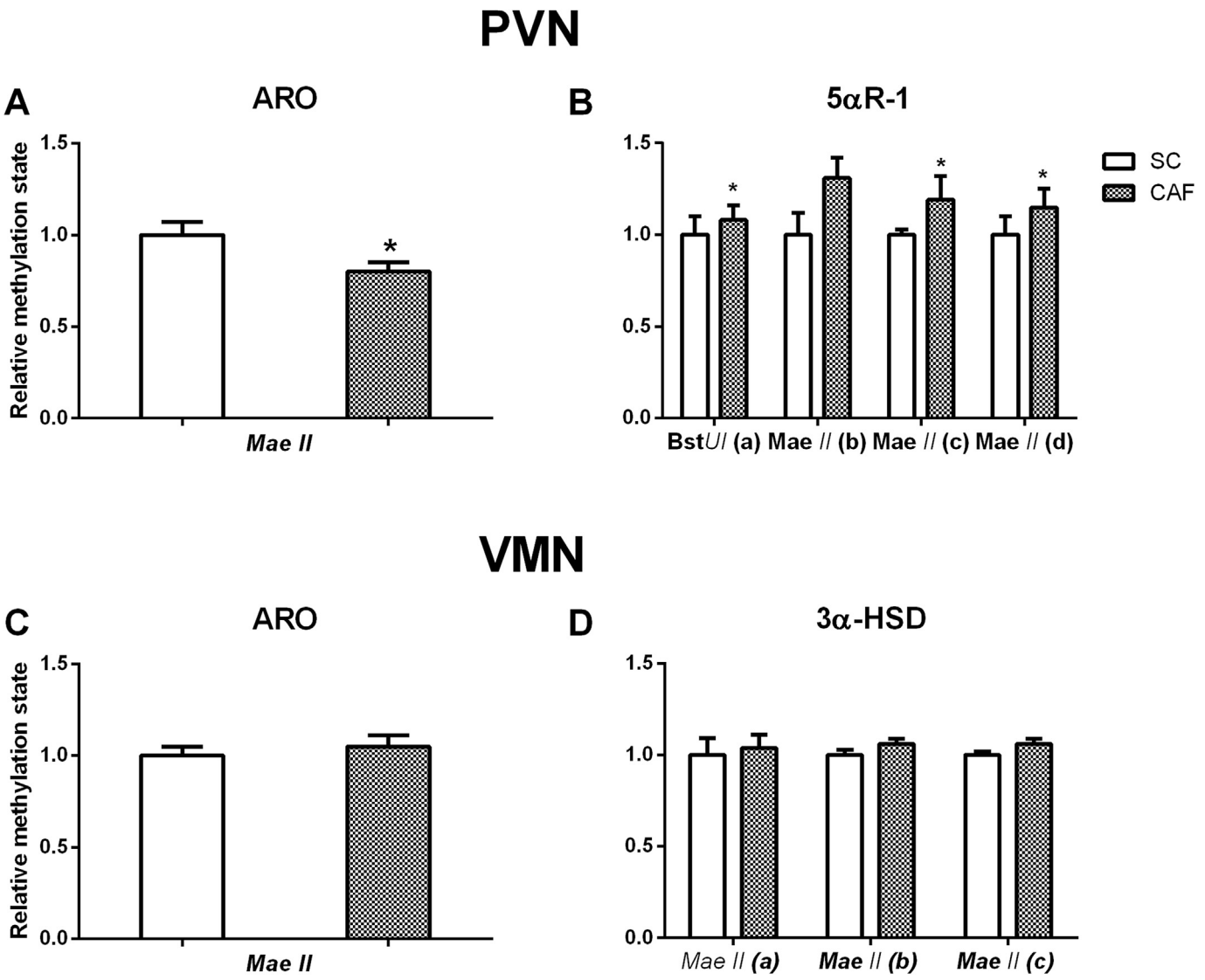



**Fig. 9.** Methylation analysis using methylation-sensitive restriction enzymes followed by real-time PCR in PVN and VMN of rats fed with standard chow (SC) or Cafeteria diet (CAF). Methylation-sensitive restriction sites of the (A, C) ARO, (B) 5αR-1, and (D) 3α-HSD gene promoters were studied. The relative methylation state in the CAF animals are indicated as relative values versus those of the M-SC group. Columns and error bars represent the mean ± SEM (n = 6–8/group). * indicates a significant difference at $p < 0.05$ by Mann Whitney test.

interconnectivity among the hypothalamic nuclei that maintain energy homeostasis through the regulation of food intake and energy expenditure (Millington, 2007; Schwartz et al., 2000; Stanley et al., 2005). In CAF-fed rats, the increased AgRP and NPY mRNA levels in the PVN, together with the overexpression of AgRP in the VMN, and the lack of changes in KISS mRNA in these nuclei could be related to an orexigenic signal that corresponds with the high energy intake observed in these rats. This action was mildly counteracted by the decreased AgRP expression and increased POMC expression in the ARC, presumably along with the higher activity of KISS in this nucleus, perhaps in an attempt to limit energy intake and weight gain. Increased levels of NPY and AgRP have been found in the PVN of genetically obese animals, such as fa/fa Zucker rats, and db/db and ob/ob mice (Dryden et al., 1995; Sanacora et al., 1990; Williams et al., 2001). Besides, it is reported that the overexpression of NPY in the PVN induced obesity via increased food intake (Tiesjema et al., 2009). The present data allow us to hypothesize that, in CAF-fed rats, the orexigenic signal of the PVN and VMN drove the great increase in energy intake, overcame the anorectic trend of the ARC, and triggered obesity. Another possibility would be that the increased caloric intake shifted ARC-dependant regulation into an anorexigenic response, preventing the increment in the body weight. In order to investigate the mechanisms supporting such observation, the methylation pattern of the promoters associated with the POMC and NPY genes was analyzed. POMC expression has been reported to be strongly influenced by promoter methylation in different tissues. Besides, POMC promoter methylation in the hypothalamus has been shown to be sensitive to nutritional conditions (Paternain et al., 2012). Interestingly, in the ARC of CAF-fed rats the POMC promoter was unmethylated in a *Mae II* site, which was found to be adjacent to a potential binding site for the STAT-3 transcription factor. STAT-3 role is critical for mediating genomic effects of leptin to regulate POMC gene expression in the hypothalamus (Munzberg et al., 2003). Moreover, phospho-STAT3 activates POMC promoter in response to leptin signaling through a mechanism that requires an

Sp1-binding site in the POMC promoter (Yang et al., 2009). Thus, the decrease in the methylation pattern of POMC promoter at this point could allow the union of phospho-STAT3, improving leptin action in POMC neurons in ARC, triggering the activation of POMC promoter and the subsequent increment in POMC mRNA expression. These changes in POMC promoter methylation explain, in part, the changes observed in POMC mRNA expression levels in the ARC of CAF animals, and are in line with the increment in Ob-Rb expression as a consequence of the higher levels of circulating leptin. Surprisingly, this demethylated *Mae II* site is a potential binding site for the negative glucocorticoid-responsive element (nGRE) transcription factor. Since nGRE is a DNA element necessary for glucocorticoid repression of the POMC gene (Drouin et al., 1993), it is expected that a decrease in the methylation pattern at this point leads to lower levels of POMC expression, however, this does not occur. In the same manner, other authors (Mahmood et al., 2013; Plagemann et al., 2009) found a lower methylation rate of this site in overfed animals, indicating a possible correlation between the observed changes in the different obesity models. Nevertheless, due to the limitations of the technique used, some methylation-targeted CG sites were not included in the analysis of the POMC promoter; for example the Sp1 binding site, which was reported to be essential for the mediation of leptin effects on POMC expression (Yang et al., 2009). Otherwise, although it is reported that nPE1 and nPE2 are also involved in targeting gene expression to POMC hypothalamic neurons, no differences were found in the methylation pattern of CAF-fed rats compared to the standard chow ones. The same results for these enhancers were reported by other authors (Coupe et al., 2010). On the other hand, a decrease in the methylation pattern of the NPY promoter was found, in a site adjacent to a potential binding site for the AP-2α transcription factor. It is reported that AP-2α is an important transcription factor in promoting NPY gene transcription in the hypothalamus (Li et al., 2000). Thus, demethylation in an adjacent site could modify the ability of this transcription factor to bind to the promoter and thereby contribute to the increased expression of NPY as observed in PVN of CAF animals. This is consistent with the results obtained by other authors (Mahmood et al., 2013), who reported a decrease in the degree of methylation for the NPY promoter in the hypothalamus of adult female rats.

Estrogens and their receptors play a fundamental role in the control of energy homeostasis and glucose metabolism. Estrogen actions in hypothalamic nuclei differentially control food intake, energy expenditure, and white adipose tissue distribution. The effects of $E_2$ on energy balance are mainly mediated by ERα. Increased ERα signaling suppresses energy intake and increases energy expenditure (Mauvais-Jarvis et al., 2013). ERα is extensively expressed in several hypothalamic nuclei, such as ARC, VMN, and PVN. In the ARC, ERα is principally expressed in POMC neurons, where most of the actions of estrogens on food intake occur (de Souza et al., 2011). Indeed, in mice, the deletion of ERα in POMC neurons leads to hyperphagia, without directly influencing energy expenditure or adipose tissue distribution (Mauvais-Jarvis et al., 2013). Thus, in the ARC of CAF-fed rats, the anorectic POMC response is enhanced by the increase in the expression of ERα mRNA, which could restrain the increase in food intake. Besides, the increased expression of ERα and Ob-Rb, which are known to colocalize in POMC neurons (Frank et al., 2014), could indicate that estrogen signaling also potentiates leptin sensitivity (Della Torre et al., 2014).

In female brains, $E_2$ levels depend on the amount of circulating levels of this hormone and on de novo neurosteroidogenesis (Arevalo et al., 2015). ARO is the enzyme responsible for the conversion of testosterone into $E_2$. It has been reported that mice with genetic disruption of ARO (ArKO) develop obesity (Jones et al., 2000). In the PVN and the VMN, the CAF diet doubled the expression of ARO, which could lead to higher synthesis of $E_2$ and a subsequent anorectic action (Brown and Clegg, 2010). On the other hand, progesterone is synthesized by 3β-HSD, which is largely distributed in the rat brain (Schumacher et al., 2014). It has been previously reported that 3β-HSD activity followed by hyperproduction of progesterone is simultaneous to the overexpression of 3β-HSD in the spinal cord (Saredi et al., 2005). Thus, in CAF-fed animals, the increase in VMN 3β-HSD expression might lead to high amounts of progesterone, which could improve energy intake (Grueso et al., 2001). Allopregnanolone is a progesterone metabolite, synthesized first by the enzyme 5αR-1 and subsequently by 3α-HSD. A decrease in the mRNA expression of 5αR-1 has been shown to lead to a decrease in allopregnanolone brain levels (Dong et al., 2001). Our results show that CAF-fed rats doubled the expression of 3α-HSD in the VMN, but decreased 5αR-1 mRNA levels in the PVN, suggesting that the CAF diet could differentially modulate allopregnanolone synthesis in discrete hypothalamic nuclei. Our results provide evidence that CAF diet modifies the expression of several enzymes involved in the de novo neurosteroid synthesis in different hypothalamic nuclei, and may have a significant impact on the control of food intake. However, these effects remain to be determined. In order to determine whether DNA methylation plays a role in the observed altered expression of 5αR-1, 3αHSD, and ARO genes in the PVN and VMN of CAF-Fed animals, the methylation status of CpG dinucleotides in the proximal promoter region of these genes was investigated. In the PVN of CAF-fed rats, hypermethylation at the 5αR-1 promoter was observed, which may explain the decreased mRNA expression of it. The 5αR-1 promoter is methylated in two sites adjacent to potential binding sites for the Sp1 and GATA-1 transcription factors. It is reported that the 5αR-1 promoter activity is dependent on the presence of the transcription factor Sp1 (Blanchard et al., 2007). Moreover, studies have shown that hypermethylation upstream of the Sp1 binding sequence can block its activity (Zhu et al., 2003). These results suggest that the decrease in 5αR-1 expression might be due to methylation of the sites studied, which interfere with the binding of Sp1. The methylation state of ARO promoter was analyzed in PVN and VMN of the animals. In the PVN of CAF-fed rats a reduction in the methylation state of the ARO promoter II was observed. A cAMP-responsive element-like sequence (CLS) has been described in the site studied within the ARO promoter II (Stocco, 2008). This region is recognized by the cAMP responsive element binding protein and the mutation of this element greatly reduces the induction of promoter activity by cAMP (Fitzpatrick and Richards, 1994; Michael et al., 1997). It has been reported that, when a critical CLS within the ARO promoter II were hypermethylated, in adipose fibroblasts, ARO expression levels change, probably by modulating CREB binding to the proximal CRE (Demura and Bulun, 2008). Thus, the hypomethylation of the CLS site could be involved in the increment in mRNA expression of ARO in the PVN of CAF animals. Interestingly, our group has previously reported changes in the methylation state of these sites in the hipoccampus, caused by aging, enriched environments and pregnancy with and without lactation (Rossetti et al., 2015, 2016), suggesting that the methylation sensitive sites studied could be potential transcriptional regulatory sites for the expression of the neurosteroidogenic enzymes. However, no changes in the methylation state of ARO or 3α-HSD were found in VMN. Interestingly, the same results were obtained by other authors (Rossetti et al., 2015), on the methylation state of the 3α-HSD gene in the hippocampus. Nevertheless, the implications of certain transcription factors in the regulation of 3α-HSD expression have been reported. The 5′-flanking regions of the rat and human genes contain consensus sequences for AP-1, Oct-1 and steroid hormone response elements,

which may comprise a steroid response unit (Lin and Penning, 1995). In addition, it was suggested that the trans-acting factors involved in increasing gene expression may include steroid hormone receptors and members of the AP-1 transcription factor family (Lin and Penning, 1995). Moreover, histone modifications have been associated with transcriptional repression or the activity of genes involved in steroid hormone biosynthesis and action (Martinez-Arguelles and Papadopoulos, 2010). Thus, it is an ongoing challenge to study other possible epigenetic modifications or alterations in the action of transcriptions factors that are associated with the regulation of the mRNA expression of the 3α-HSD gene.

## 5. Conclusion

The present study demonstrated that CAF diet administered to female rats from weaning induced differential expression of neuropeptides, hormone receptors, and neurosteroidogenic enzymes in discrete nuclei involved in food intake control. Moreover, the changes in the methylation state of POMC, NPY, 5αR-1, and ARO promoters detected in CAF animals suggests epigenetic control of mRNA expression. Those results indicate that a prolonged intake of CAF diet could result in an altered epigenetic regulation of genes involved in the hypothalamic energy homeostatic mechanism and in certain neurosteroidogenic molecules. Such changes could contribute to the development of obesity. CAF-fed rats increased energy intake, body weight, and fat pads, but did not develop metabolic syndrome. The increase in energy intake was related to an orexigenic signal of the PVN and the VMN, mediated by higher AgRP expression, which was mildly counteracted by the increased POMC and decreased AgRP mRNA levels in the ARC. These counteracted effects of the ARC also included higher ERα expression. The CAF diet affected the expression of neurosteroidogenic enzymes in the PVN and the VMN, a fact that might lead to changes in $E_2$, progesterone, and allopregnanolone synthesis. The changes observed could play a role in the regulation of energy balance, given the influence of $E_2$, progesterone, and allopregnanolone on food intake, although these effects remain to be determined. Further studies are needed to clarify the cause-effect relationship between the synthesis of different neurosteroids and the control of energy intake.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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