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Maternal high-fat diet impairs leptin signaling and up-regulates type-1 cannabinoid receptor with sex-specific epigenetic changes in the hypothalamus of newborn rats

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ABSTRACT

Maternal nutritional imbalances trigger developmental adaptations involving early epigenetic mechanisms associated with adult chronic disease. Maternal high-fat (HF) diet promotes obesity and hypothalamic leptin resistance in male rat offspring at weaning and adulthood. Leptin resistance is associated with over activation of the endocannabinoid system (ECS). The ECS mainly consists of endocannabinoids derived from n-6 fatty acids and cannabinoid receptors (CB1 coded by *Cnr1* and CB2 coded by *Cnr2*). The CB1 activation in hypothalamus stimulates feeding and appetite for fat while CB2 activation seems to play an immunomodulatory role. We demonstrated that maternal HF diet increases hypothalamic CB1 in male offspring while increases CB2 in female offspring at birth, prior to obesity development. However, the molecular mechanisms behind these changes remain unexplored. We hypothesized that maternal HF diet would down-regulate leptin signaling and up-regulate *Cnr1* mRNA levels in the hypothalamus of the offspring at birth, associated with sex-specific changes in epigenetic markers and sex steroid signaling. To test our hypothesis, we used progenitor female rats that received control diet (C, 9% fat) or isocaloric high-fat diet (HF, 28% fat) from 8 weeks before mating until delivery. Blood, hypothalamus and carcass from C and HF male and female offspring were collected for biochemical and molecular analyses at birth. Maternal HF diet down-regulated the transcriptional factor STAT3 in the hypothalamus of male and female offspring, but induced hypoleptinemia only in males and decreased phosphorylated STAT3 only in female offspring. Because leptin acts through STAT3 pathway to inhibit central ECS, our results suggest that leptin pathway impairment might contribute to increased levels of *Crn1* mRNA in hypothalamus of both sex offspring. Besides, maternal HF diet increased the histone acetylation percentage of *Cnr1* promoter in male offspring and increased the androgen receptor binding to the *Cnr1* promoter, which can contribute to higher expression of *Cnr1* in newborn HF offspring. Maternal HF diet increased plasma n6 to n3 fatty acid ratio in male offspring, which is an important risk factor to metabolic diseases and might indicate an over activation of endocannabinoid signaling. Thus, although maternal HF diet programs a similar phenotype in adult offspring of both sexes (obesity, hyperphagia and higher preference for fat), here we showed that molecular mechanisms involving leptin signaling, ECS, epigenetic markers and sex hormone signaling were modified prior to obesity development and can differ between newborn male and female offspring. These observations may provide molecular insights into sex-specific targets for anti-obesity therapies.

1. Introduction

Obesity is a multifactorial metabolic disease of significant public health concern worldwide. Obesity etiology involves nutritional imbalances, sedentarism, behavior disturbances, genetic background and, alterations in early life environment (Lillycrop and Burdge, 2015).

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Maternal nutritional imbalances during the perinatal period are important imprinting factors for metabolic programming and predispose offspring to develop obesity and other chronic diseases throughout life in humans and rodents (Lillycrop and Burdge, 2015). The molecular mechanisms of metabolic programming include epigenetic regulation of gene expression through DNA methylation and histone acetylation (Joss-Moore et al., 2015).

We demonstrated that maternal consumption of high-fat (HF) diet during perinatal life induces early obesity in rat offspring with hypothalamic leptin resistance at weaning and adulthood (Franco et al., 2016, 2012). Leptin is an adipokine that induces anorexigenic effect and increases energy expenditure through binding to its receptor OBRb (long isoform of leptin receptor), which activates the intracellular pathway JAK2 (Janus kinase 2) - STAT3 (Signal transducer and activator of transcription 3). Leptin action leads to phosphorylation of STAT3 (pSTAT3) and is especially critical for hypothalamus maturation (Bouret et al., 2004). Obese subjects frequently present leptin resistance that contributes to positive energy balance (Bjorbaek, 2009).

Leptin resistance and obesity are associated with over activation of the cannabinoid signaling in the central nervous system (CNS) (Jelsing et al., 2009). The endocannabinoid system (ECS) consists of two receptors, the cannabinoid type-1 (CB1 coded by *Cnr1* gene) and the cannabinoid type-2 (CB2 coded by *Cnr2* gene), lipid-derived ligands named endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and ECS-metabolizing enzymes, the fatty acid amide hydrolase (FAAH coded by *Faah* gene) and the monoacylglycerol lipase (MAGL coded by *Mgll* gene) (D'Addario et al., 2014). The ECS components can be modulated by tissue fat composition, since AEA and 2-AG are arachidonic acid-derived active lipids (n-6 fatty acid family). Therefore, the over activation of ECS is triggered by HF western diets, which contains high n-6 to n-3 ratio, and the attenuation of ECS is triggered by n-3 fatty acid-enriched diets (Freitas et al., 2017).

In the CNS, the endocannabinoids act in a retrograde manner. They are synthesized by postsynaptic neurons and bind to CB1 located in presynaptic terminals of GABA or glutamate neurons to inhibit their activity through G protein signaling (Silvestri and Di Marzo, 2013). ECS appropriate regulation of excitatory and inhibitory cell populations generated during brain development is essential for coordinated neuronal activity (Galve-Roperh et al., 2013). The CB1 activation in the hypothalamus increases appetite and stimulates feeding by enhancing orexigenic signals such as orexin A (Koch, 2017). Brain ECS activation also increases appetite for fat and reward to palatable foods, contributing to obesity development (Coccurello and Maccarrone, 2018). On the other hand, the role of CB2 is poorly characterized, but because it is widely expressed in immune cells and glia, this receptor possibly plays an immunomodulatory role at central level (Turcotte et al., 2016).

The ECS has been extensively studied in neurological disorders (Smith et al., 2017) and obesity treatment (D'Addario et al., 2014). However, the role of the ECS in metabolic programming is mostly unknown and the ECS epigenetic regulation in the developmental origins of obesity is unexplored. Recently, we and others have demonstrated that the ECS is a target of maternal nutritional imbalances, programming energy metabolism in the offspring (Almeida et al., 2017; Dias-Rocha et al., 2018; Ramirez-Lopez et al., 2016a, b; Ramirez-Lopez et al., 2015). We have demonstrated that perinatal maternal HF diet programs early obesity in parallel with sex-specific changes in the ECS of hypothalamus and adipose tissue of rat offspring (Almeida et al., 2017; Dias-Rocha et al., 2018). Specifically in the hypothalamus, maternal HF diet increases CB1 protein in male and CB2 in female offspring at birth, before offspring become obese. Although there are sex-specific molecular signatures in the offspring in early life, HF offspring of both sexes develop obesity, hyperphagia and higher preference for fat at adulthood (Dias-Rocha et al., 2018). However, the molecular mechanisms of the sex-specific programming of ECS remain unexplored. In *Cnr1* promoter region, estrogen receptor (ER) (Proto et al., 2012) and androgen receptor (AR) (Lee et al., 2013) response elements have already been reported, suggesting the differential contribution of sex hormones on ECS activation for the obesity development in male and female programmed rats.

In the present study, we hypothesized that maternal HF diet would down-regulate leptin signaling and up-regulate *Cnr1* mRNA levels in the hypothalamus of the offspring at birth, associated with sex-specific changes in epigenetic markers and sex steroid signaling.

2. Materials and methods

2.1. Experimental model

Thirty (n = 30) progenitor female Wistar rats (60 days old), weighing 180–220 g, were obtained from the Center of Reproduction Biology of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. They were kept in a controlled temperature environment (23 ± 2 °C) with a photoperiod of 12 h (7 a.m. to 7 p.m. - light and 7 p.m. to 7 a.m. - dark). Water and the experimental diets were offered *ad libitum* throughout the study. All procedures with animals followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Animal Care and Use Committee of the Health Science Center of the Federal University of Rio de Janeiro (process number 095/17).

2.2. Dietary treatments and experimental design

Progenitor female rats were randomly assigned to two dietary treatments (n = 15/group): Control group (C), which received a standard diet for rodents (9% of the calories as fat), and a high-fat group (HF), which received a high-fat diet (28.6% of the calories as fat) (Supplementary Table 1). In the HF diet, lard was used as fat source, and we also added 1% soy oil to provide the minimal amount of n3 fatty acid for adequate development of rats. Both diets contained approximately 3.9 kcal/g and followed the AIN-93G recommendations for micronutrients (Almeida et al., 2017; Dias-Rocha et al., 2018; Franco et al., 2016, 2012). Female rats were fed these diets during 8 weeks before mating and throughout gestation. After mating in a 3:1 ratio (female: male), pregnant rats were housed in individual standard rat cages. At birth, male and female offspring were euthanized by decapitation. Trunk blood was collected for plasma separation, which was stored at – 80 °C. Hypothalamus was dissected, snap frozen in liquid nitrogen and stored at – 80 °C. For each experimental procedure, rats from at least six different litters per group were used in order to avoid litter effects. Exception was the analysis of plasma and hypothalamus fatty acids when we used animals from 3 to 4 different litters.

2.3. Carcass composition analysis

Body composition (lipid and protein content) was determined by carcass method, as previously described (Franco et al., 2016). Briefly, frozen eviscerated and weighed carcasses were autoclaved and homogenized in distilled water (1:1 w/v). Aliquots of homogenate were used for the measurement of lipid and protein contents. Lipids were extracted from 3 *g* of homogenate and their masses were gravimetrically determined. Samples were hydrolyzed in shaking water bath at 70 °C for 2 h with 30% KOH and ethanol. After the addition of 9 M sulfuric acid, total lipids were extracted by three successive washes with petroleum ether. The samples were dried at room temperature until constant weight was obtained. Proteins were extracted from 1 *g* of homogenate using 0.6 N KOH at 37 °C for 1 h. Then, samples were centrifuged at 800 ×g for 10 min, and the supernatants were collected to measure protein concentration using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Both measurements were expressed as carcass percentage. We used 10 carcasses per group.

2.4. Plasma leptin and sex hormones

Blood samples were collected in heparinized tubes and centrifuged (1233 × *g* for 15 min, 4 °C) for plasma separation. Plasma leptin levels were measured by specific rat Milliplex Adipokine Panel Metabolism Assay (Merck Millipore, USA). Plasma 17β-estradiol and testosterone levels were determined using a radioimmunoassay (RIA) kit (MP Diagnostics, USA) with intra-assay variation of 15.7% and 9.1%, respectively. For each hormone, we used 10 samples per group.

2.5. Western blotting assay

Western blotting assay based primarily on the previously published studies (Almeida et al., 2017; Dias-Rocha et al., 2018) was used to investigate the hypothalamic protein content of OBRb, JAK2, STAT3, pSTAT3, suppressor of cytokine signaling 3 (SOCS3), ER alpha (ERα), AR and the deacetylase sirtuin 1 (SIRT1). The protein content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclophilin (Cyclo) was used as loading control. The hypothalamus samples were homogenized in pH 6.4 lysis buffer (50 mM HEPES, 1 mM MgCl2, 10 mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail Complete (Roche Diagnostics, IN, USA). After centrifugation, the total protein content of supernatant was quantified using a PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). The samples were denatured in sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated at 95 °C for 5 min. Total proteins were analyzed by SDS-PAGE, with a 10% polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes (Hybond-P 0.45 µm PVDF; Amersham Biosciences BKM, ENG). The membranes were incubated with T-TBS containing 5% Bovine Serum Albumin (Sigma Life Science MO, USA) for 90 min to block non-specific binding sites. Then, the membranes were incubated overnight at 4 °C with specific primary antibodies. Membranes were washed and incubated for 2 h at room temperature with peroxidase labeled specific secondary antibodies. All blots were washed and incubated with a luminogen detection reagent (Amersham ECL Prime Western Blotting Detection reagent; Amersham Bioscience, Inc). Information about primary and secondary antibodies is described in Table 1. Chemiluminescent signal was detected by ImageQuant LAS 4000 equipment followed by densitometric analyses (GE Healthcare Life Sciences). Data are expressed as percentage of change compared with control male group. For each protein analyzed by Western blotting, we used 6 or 7 samples per group.

2.6. Quantitative PCR (qPCR) assay

All qPCR reactions based primarily on the previously published study (Dias-Rocha et al., 2018) were used to investigate mRNA relative expression of ECS genes in hypothalamus samples: *Cnr1*, *Cnr2*, *Faah*, and *Mgll*. Total RNA was isolated from hypothalamus using SV Total RNA Isolation System (Promega, WI, USA). Total RNA was reverse transcribed using 1 µg of total RNA and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, CA, USA). The primer sequences used for mRNA quantification were: *Cnr1*: Forward (5′-3′) GAAGCCA CTCACTCCTGATAAA; Reverse (5′-3′) GGCATATAC TAGCTCTCCAC TTC; *Cnr2*: Forward (5′-3′) ATGGACAGACAGGCTTTGG; Reverse (5′-3′) AGGACAAGGCTTCACAAGAC; *Faah*: Forward (5′-3′) GATGCCCAGATG GAACTCTAC; Reverse (5′-3′) CAGGCAGACCGACACTATTT; *Mgll*: Forward (5′-3′) GTCAACCTCCGACTTGTTCC; Reverse (5′-3′) ACCTCTGAT CCTTGCCAATC; *histone deacetylase (Hdac) 6*: Forward (5′-3′) TGGCTA TTGCATGTTCAACCA; Reverse (5′-3′) GTCGAAGGTGAACTGTGTTCCT; *Hdac 7*: Forward (5′-3′) CTGCATTGGAGGAATGAAGCT; Reverse (5′-3′) CTGGCACAGCGGATGTTTG. Products were amplified using Eppendorf Mastercycler RealPlex (Eppendorf HH, GER) and EvaGreen (Solis BioDyne TA, EST). Cycle parameters were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. Samples and negative controls were evaluated in duplicate in the same assay. Efficiency of each reaction was calculated using a serial cDNA dilution. Product purity was confirmed by a single peak in the melting curve analysis. Relative mRNA levels ($\Delta\Delta C_t$) were determined by comparing the PCR cycle threshold (C_t) between groups, after correcting for *Ct* geometric mean of reference genes encoding the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and beta-glucuronidase (*rGUSB*). Primer sequences of reference genes were: *Gapdh*: Forward (5′-3′) GTAACCAGGCGTCCGATA; Reverse (5′-3′) TCTCTGCTCCTCCC TGTTC; *rGUSB*: Forward (5′-3′) GGTCGTGATGTGGTCTGTG; Reverse (5′-3′) TGTCTGCGTCATATCTGGTATTG. Data are expressed as percentage of change compared with control male group. For each mRNA expression analyzed by qPCR, we used 6 or 7 samples per group.

2.7. Gas chromatography–mass spectrometry (GC–MS) analysis

GS-MS assay based primarily on the previously published study (Oliveira et al., 2016) was used to investigate the fatty acid profile in plasma and hypothalamus. The lipid sample was dissolved in a toluene and 1% sulfuric acid in methanol solution. GC–MS analysis was carried out on a Shimadzu GCMS-QP2010 Plus system, using an HP Ultra 2 (5% Phenyl-methylpolysiloxane), Agilent (25 m × 0,20 mm × 0,33 µm). Injector was set at 250 °C. Column temperature was programmed from 40 to 160 °C at 30 °C/min, 160–233 °C at 1 °C/min, 233–300 °C at 30 °C/min and held at 300 °C for 10 min. Electro ionization (EI-70 eV) and a quadruple mass analyzer, operated in scans from 40 to 440 amu. Interface was set at 240 °C and the ion source at 240 °C. The components were identified by comparing their mass spectra with those of the

Table 1
Primary and secondary antibodies used for Western Blot.

Primary Antibody			Secondary Antibody		
Protein	Company	Dilution	Company	Dilution	Specificity
OBRb	Santa Cruz Biotechnology CA, USA	1:200	Invitrogen CA, USA	1:5000	Anti- mouse
JAK2	Santa Cruz Biotechnology CA, USA	1:200	Amersham Bioscience, Inc	1:3000	Anti-rabbit
STAT3	Santa Cruz Biotechnology CA, USA	1:200	Invitrogen CA, USA	1:1000	Anti- mouse
pSTAT3	Santa Cruz Biotechnology CA, USA	1:200	Invitrogen CA, USA	1:1000	Anti-mouse
SOCS3	Santa Cruz Biotechnology CA, USA	1:200	Amersham Bioscience, Inc	1:10000	Anti-rabbit
ERα	Cell Signaling Technology MA, USA	1:1000	Amersham Bioscience, Inc	1:10000	Anti-rabbit
AR	Abcam MA, USA	1:1000	Amersham Bioscience, Inc	1:10000	Anti-rabbit
SIRT1	Cell Signaling Technology MA, USA	1:500	Invitrogen CA, USA	1:4000	Anti- rabbit
GAPDH	Cell Signaling Technology MA, USA	1:1000	Amersham Bioscience, Inc	1:10000	Anti-rabbit
Cyclo	Applied Biosystems™, Thermo Fisher Scientific MA, USA	1:5000	Amersham Bioscience, Inc	1:10000	Anti-rabbit

OBRb: long form of leptin receptor; JAK2: Janus tyrosine kinase 2; STAT3: signal transducer and activator of transcription 3; pSTAT3: phosphorylation of STAT3; SOCS3: suppressor of cytokine signaling 3; ERα: estrogen receptor α; AR: androgen receptor; SIRT1: sirtuin 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Cyclo: cyclophilin

library NIST05 contained in the computer's mass spectrometer. Retention indices were also used to confirm the identity of the peaks in the chromatogram by Supelco 37 Component FAME Mix (Sigma-Aldrich). Fatty acids were quantified by determining peak-area ratios with the 9:0 and 19:0 internal standards. We used 3 or 4 samples per group.

2.8. DNA CpG methylation assay – bisulfite modification

DNA CpG methylation assay based primarily on the previously published study (Fung et al., 2015) was used to investigate the methylation percentage in CpG sites of *Cnr1* gene. Genomic DNA was extracted from hypothalamus samples using the DNeasy Blood and Tissue Kit (Qiagen NRW, GER). The bisulfite conversion was performed using 1 µg of genomic DNA and the EZ DNA Methylation – Gold Kit (Zymo Research, USA). Sequences of *Cnr1* gene containing -78 (promoter region), +7 (exon 1), +146 (intron 1) and +159 (intron 1) CpGs (Fig. 3A) were amplified using the AmpliTaq Gold DNA Polymerase with Gold buffer and $MgCl_2$ kit (Applied Biosystems CA, USA) and the following primers: Forward (5′-3′) TGTAGGTTGTTGGTTTAG GTATA; Reverse (5′-3′) AAAATAAACCATATAACATTATCAC. We explored DNA methylation around exon 1 because it is much more tightly linked to transcriptional silencing than methylation in the upstream promoter region (Brenet et al., 2011; Di Francesco et al., 2015). The PCR products were cloned in pCRII-TOPO vector (Invitrogen CA, USA), inserted into TOP10 competent *Escherichia coli* (Invitrogen CA, USA) using Topo TA Cloning kit (Invitrogen CA, USA). Bacteria were plated on media containing 100 µg/mL of ampicillin (Sigma Life Science MO, USA), and 40 µL of 40 mg/mL X-gal (Bio Vectra, Charlottetown, Canada) and were incubated at 37 °C overnight. Transformants were assayed for the presence of recombinant inserts by the blue/white colony phenotype and the presence of cloned sequence was confirmed by a PCR reaction. The positive transformants to cloned sequence were transferred to liquid media containing 100 µg/mL of ampicillin and incubated at 37 °C overnight. Vectors were extracted with AxyPrep Easy-96 Plasmid DNA kit (Axygen Biosciences CA, USA) and four clones per animal were sequenced using M13 (-20) (Invitrogen CA, USA): Forward (5′-3′) GTAAAACGACGGCCAG. Bisulfite sequence data for CpG methylation analysis in *Cnr1* gene were aligned and quantified using the Quantification tool for Methylation Analysis (QUMA http://quma.cdb.riken.jp). We used 6 hypothalamus samples per group.

2.9. Chromatin immunoprecipitation (ChIP) assay

A revised ChIP protocol, based primarily on the previously published study (Joss-Moore et al., 2011) was used to investigate in *Cnr1* promoter region: 1) the levels of acetylation in histone 3; 2) the ERα relative interaction; 3) the AR relative interaction. Chromatin isolation from male and female whole hypothalamus was performed as follows. Tissue was crushed in liquid nitrogen and fixed in 1% formaldehyde for 10 min and the reaction was stopped by the addition of 125 mM glycine. After centrifuging (5350 xg, 5 min, 4 °C), samples were washed twice with PBS supplemented with protease inhibitor cocktail (PIC) (Thermo Scientific IL, USA) at manufacturer's recommended concentration. After centrifuging (5350 xg, 5 min, 4 °C), cell pellets were resuspended in Lysis Buffer (Thermo Scientific IL, USA) with PIC and homogenized on ice bath using a tight pestle. After centrifuging (5350 xg, 5 min, 4 °C), pelleted nuclei were resuspended in Nuclei Buffer (1% SDS, 10 mM EDTA, pH 8.0, and 50 mM Tris, pH 8.1) with PIC, incubated for 20 min on ice bath and sonicated (12 pulses for 5 rounds) (Fisher Scientific, Pittsburgh, PA). After centrifuging (16,016 xg, 10 min, 4 °C), samples were run on 1.5% agarose gel and the size of chromatin fragments was around 500 bp. Chromatin samples were frozen at −80 °C until immunoprecipitation (IP) reactions were performed.

IP reactions were performed using anti-ERα (#8644, Cell Signaling Technology MA, USA), anti-AR (ab74272, Abcam MA, USA) or anti-acetyl histone H3 (06–599, Millipore MA, USA), 20 µg of chromatin samples and IP Dilution Buffer (0.15 M NaCl, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, 50 mM Tris pH 7.5) supplemented with PIC. As negative control, IP was performed using anti-IgG (Vector Laboratories, Inc. CA, USA) and 20 µg of chromatin samples from pooled samples. IP reactions were incubated overnight at 4 °C with rotation. After centrifuging (16,016 xg, 10 min, 4 °C), the supernatants were transferred to tubes containing 70 µL of protein A/G PLUS-Agarose pretreated with 0.1 mg/mL of salmon sperm DNA (Invitrogen CA, USA) and 1 mg/mL of Bovine Serum Albumin (Sigma Life Science MO, USA), followed by incubation for 1 h at 4 °C with rotation. Samples were washed with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8) and TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8). After washing, 10% Chelex 100 Molecular Biology Grade Resin (Bio-Rad Laboratories, CA, USA), was added followed by incubating for 10 min at 95 °C, adding 20 µg of Proteinase K, incubating for 30 min at 56 °C and for 10 min at 95 °C. After centrifuging (16,016 xg, 1 min, 4 °C), the supernatants containing ChIP DNA samples were used for qPCR analysis. The ChIP-qPCR data were analyzed as relative to input DNA, a non-ChIP genomic DNA. qPCR results were calculated as % Input: % Input = $100*2^{(Ct\ Adjusted\ input\ to\ 100\% - Ct\ ChIP)}$. We used the following primer pair and probe: Forward (5′-3′) CCCCCTCGGCTGAAGCT; Reverse (5′-3′) ACATTTATCACGGCGAATTGC; Probe (5′-3′) ACGCTCG TGTTTAGGG. This primer pair amplifies *Cnr1* promoter between -2881 and -2815 bp. As the chromatin average size was about 500 bp, we mainly monitored by ChIP assay the region between -3381 and -2315 bp of *Cnr1* promoter (Fig. 3A). For each ChIP, we used 6 hypothalamus samples per group.

2.10. Statistical analysis

Sample size was calculated by using the G*Power 3.1.9.2 program and based on the effect of maternal consumption of high-fat diet on plasma and molecular parameters of the newborn and weaning offspring from previous study (Almeida et al., 2017; Dias-Rocha et al., 2018; Franco et al., 2016, 2012). Regarding hormonal parameters, a sample size of 8 animals per group would provide the appropriate power (1-β = 0.8) to identify significant differences (α = 0.05) in the variables analyzed, with an effect size d = 1.33, two-tailed test, and a sample size ratio = 1. For molecular parameters, a sample size of 6 animals per group would provide the appropriate power (1-β = 0.8) to identify significant differences (α = 0.05) in the variables analyzed, with an effect size d = 1.81, two-tailed test, and a sample size ratio = 1.

The statistical comparisons between control and HF groups were performed using the software GraphPad Prism (GraphPad Software Inc., CA, USA). For all analyses, normality was assessed by the Kolmogorov-Smirnov test and Grubb's test was used to detect outliers.

Data were analyzed employing two-way ANOVA with maternal diet and offspring sex as main factors, followed by within sex pairwise comparisons using Bonferroni post hoc test, considering $p < 0.05$ statistically significant. Body weight of progenitors was analyzed by Student's unpaired *t*-test. Results are shown as mean ± SEM, except for western blotting and real time PCR assays in which data are expressed as percentage of change compared with control male group (% C male).

3. Results

Before mating, C and HF progenitors presented similar body weight (C = 281.1 ± 3.85 and HF = 290.2 ± 3.92). Regarding offspring body weight, male offspring had increased body weight compared to female offspring at birth (+ 4.6%, $p < 0.05$) (Fig. 1A). At weaning, the offspring sex effect remains, but there is also a maternal diet effect,

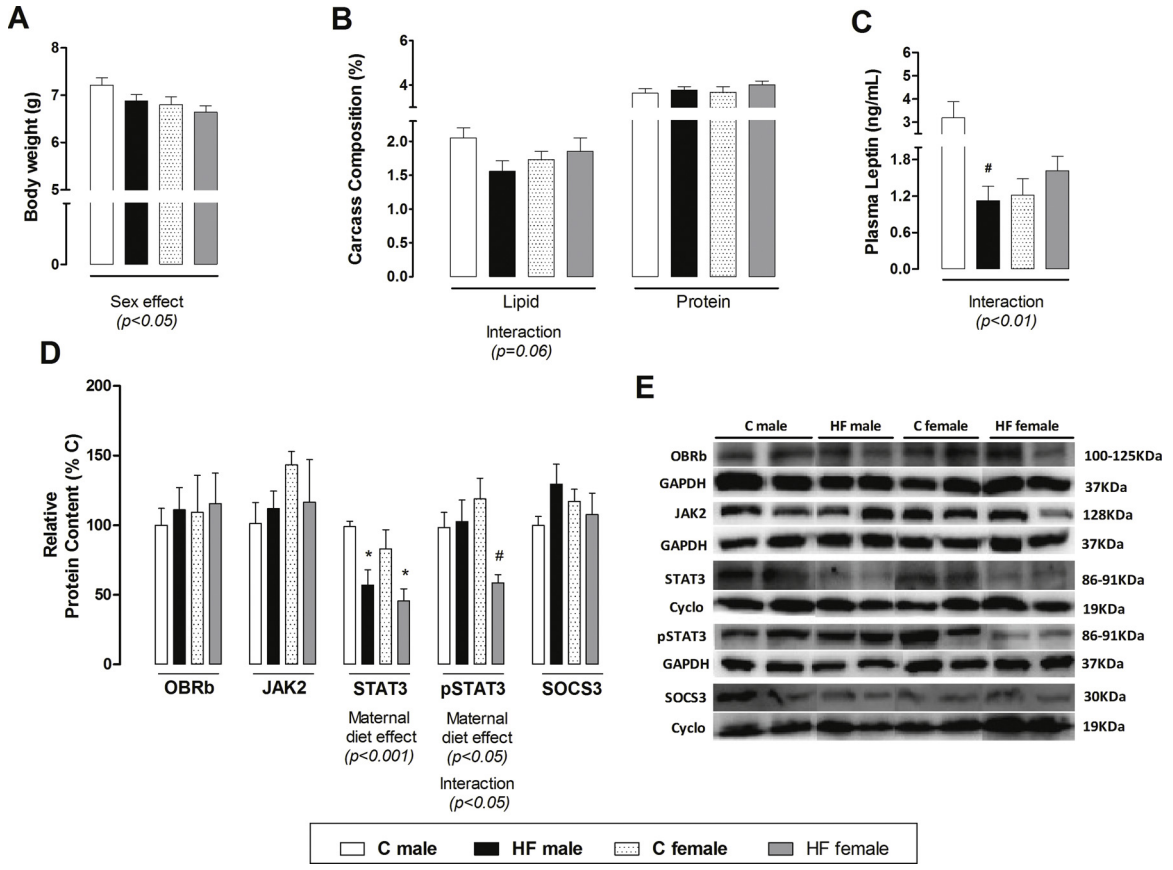

Fig. 1. Effect of maternal HF diet on body weight, carcass composition, leptinemia and hypothalamic leptin signaling of rat offspring at birth. (**A**) Body weight, (**B**) carcass composition, (**C**) leptinemia (**D**) hypothalamic protein content of long form of leptin receptor (OBRb), janus tyrosine kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 (pSTAT), suppressor of cytokine signaling 3 (SOCS3) and (*E*) representative bands from westernblots of control (C) and high-fat (HF) male and female offspring at birth; (n = 6–10 pups from different litters /group). Statistically significant differences were determined by two-way ANOVA (factors: maternal diet and sex) followed by within sex pairwise comparisons using Bonferroni post hoc test: $*p < 0.05$, $\# p < 0.01$.

in which maternal HF diet increases body weight in male (+13.7%, $p < 0.0001$) and female offspring (+17.5%, $p < 0.0001$) (Almeida et al., 2017). Here, we showed that there was an interaction effect trend between maternal diet and sex factors in carcass lipid content ($p = 0.06$), while there was no effect in carcass protein content of offspring (Fig. 1B). Maternal HF diet decreased leptinemia only in male offspring (- 64.6%, $p < 0.01$) (Fig. 1C). Regarding hypothalamic leptin signaling, maternal HF diet did not change OBRb, JAK2 or SOCS3 protein content, while it decreased STAT3 protein content in male (- 42.9%, $p < 0.05$) and female (- 44.9%, $p < 0.05$) offspring (Fig. 1D). Besides, maternal HF diet decreased hypothalamic pSTAT3 protein content only in female offspring at birth (- 50.7%, $p < 0.05$) (Fig. 1D).

In the hypothalamus, maternal HF diet increased the *Cnr1* mRNA levels compared to C groups (+ 22.8%, $p < 0.01$) (Fig. 2A). Male offspring presented lower levels of *Cnr2* mRNA than female offspring (+ 2.4-fold, $p < 0.0001$) (Fig. 2A). Maternal HF diet decreased *Faah* mRNA only in male offspring (- 33.7%, $p < 0.01$) (Fig. 2A). On the other hand, maternal HF diet or offspring sex did not change *Mgll* mRNA levels (Fig. 2A). Regarding n-6 to n-3 fatty acids, maternal HF diet increased plasma n-6 to n-3 fatty acid ratio only in male offspring (+ 92.8%, $p < 0.05$) while it did not change hypothalamic n-6 to n-3 fatty acid ratio (Fig. 2B).

We investigated whether maternal HF diet changed the *Cnr1* mRNA levels parallel to epigenetic mechanisms in the hypothalamus of offspring at birth. There were no effect on methylation percentage in the -78, + 7 and + 146 CpGs while female offspring had lower methylation percentage in the + 159 CpG site of *Cnr1* intron compared to male offspring (- 35.5%, $p < 0.05$) (Fig. 3B). Furthermore, female offspring had higher H3 acetylation percentage in *Cnr1* promoter than male offspring (+ 42.3%, $p < 0.001$) (Fig. 3C). In addition, maternal HF diet increased H3 acetylation in *Cnr1* promoter only in male offspring (+ 40.8%, $p < 0.05$) (Fig. 3C). Concerning epigenetic regulators, female offspring present higher deacetylase SIRT1 content (+ 43.8%, $p < 0.01$) (Fig. 4A) and higher mRNA levels of *Hdac* 6 (+ 2.4-fold, $p < 0.05$), *Hdac* 7 (+ 2.4-fold, $p < 0.05$) in hypothalamus compared to male offspring at birth (Fig. 4C). Furthermore, maternal HF diet increased hypothalamic SIRT1 protein content (+ 73.1%, $p < 0.05$) only in male offspring (Fig. 4A).

In addition, changes of the hypothalamic *Crn1* mRNA levels were associated with alterations in sex hormone signaling. Maternal HF diet increased estradiol plasma levels only in male offspring (+ 89.9%, $p < 0.05$) (Fig. 5A). Male offspring had higher plasma testosterone levels compared to female offspring (+ 267-fold, $p < 0.05$) (Fig. 5A). Similar to estradiol plasma levels, there was an interaction effect between maternal diet and sex offspring factors in hypothalamic content of ERα ($p < 0.01$) (Fig. 5B). Maternal HF diet increased hypothalamic content of AR only in male offspring (+ 87.7%, $p < 0.05$) (Fig. 5B). Chromatin immunoprecipitation assay showed that maternal HF diet or offspring sex did not change ERα binding to *Cnr1* promoter. However, maternal HF diet increased AR binding to *Cnr1* promoter in the offspring compared to C groups (+ 42.1%, $p < 0.05$) (Fig. 5D).

4. Discussion

Maternal HF diet induced hypoleptinemia only in newborn male offspring, which may be associated with the carcass lipid profile, since

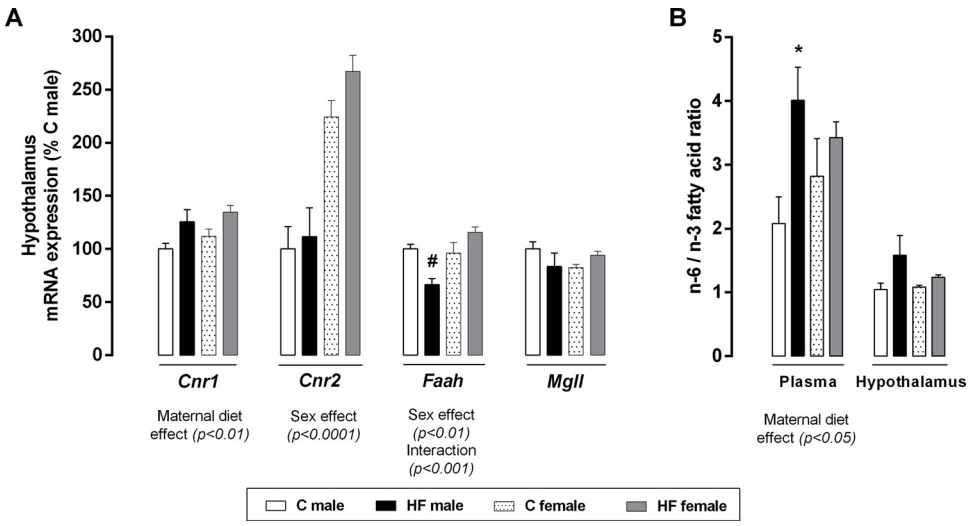

Fig. 2. Effect of maternal HF diet on expression of endocannabinoid system components and n-6 to n-3 fatty acid ratio of rat offspring at birth. (**A**) Type 1 cannabinoid receptor (*Cnr1*), type-2 cannabinoid receptor (*Cnr2*), fatty acid amide hydrolase (*Faah*) and monoacylglycerol lipase (*Mgll*) mRNAs in the hypothalamus of control (C) and high-fat (HF) male and female offspring (n = 6 or 7 pup from different litters /group). (**B**) n-6 to n-3 fatty acid ratio in the plasma and hypothalamus of C and HF male and female offspring (n = 3 or 4 pups from different litters /group). Statistically significant differences were determined by two-way ANOVA (factors: maternal diet and sex) followed by within sex pairwise comparisons using Bonferroni post hoc test: $*p < 0.05$, $\#$ $p < 0.01$.

leptin is mainly produced by white adipocytes and its serum levels are directly correlated with body fat (Ahima and Osei, 2008). Sex differences in fat deposition, and consequently changes in leptinemia, might arise from sex-specific changes in placenta function induced by maternal HF diet, which can impact differentially the energy substrate transfer to male and female fetuses (Rosenfeld, 2015). Here, we also demonstrated a sex-specific impairment on hypothalamic leptin signaling, in which maternal HF diet decreased STAT3 protein in both male and female offspring, but reduced pSTAT3 only in female offspring. Our results partially corroborate previous data in a similar experimental model, in which maternal HF diet induced hypoleptinemia and decreased hypothalamic mRNA levels of OBRb in newborn offspring of both sexes, while reduced hypothalamic mRNA levels of STAT3 and SOCS3 only in male offspring (Morris and Chen, 2009).

Downregulated leptin signaling has been associated with over activation of the central ECS and contributes to obesity development (Cristino et al., 2016). In fact, we have recently demonstrated that maternal HF diet increased hypothalamic CB1 protein content in newborn male offspring, while increased CB2 in female offspring, and this profile was associated with higher preference for HF diet in both sexes in adulthood (Dias-Rocha et al., 2018). Intending to further investigate the molecular mechanisms involved in the hypothalamic ECS alterations, here we analyzed the effect of maternal HF diet on mRNA expression of the ECS components and markers of gene expression regulation in the hypothalamus of male and female offspring at birth. We observed that maternal HF diet increased *Cnr1* mRNA levels in the offspring, independently of offspring sex. In males, increased *Cnr1* mRNA agrees with increased CB1 (Di Francesco et al., 2015; Dias-Rocha et al., 2018), but in females we observed a discordance between mRNA and protein levels. We speculate that this discordance might be related

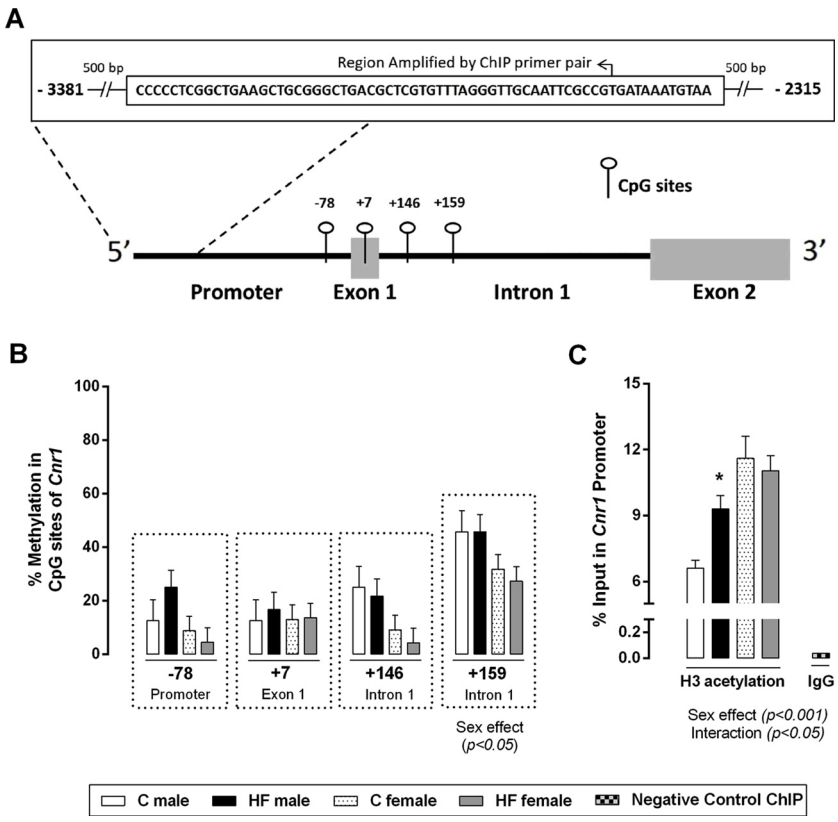

Fig. 3. Effect of maternal HF diet on epigenetic marks of type 1 cannabinoid receptor (*Cnr1*) gene in the hypothalamus of rat offspring at birth. (**A**) Scheme of *Cnr1* gene showing the promoter investigated by chromatin immunoprecipitation assay and CpG sites investigated for DNA methylation, (**B**) Percentage of methylation and (**C**) histone 3 acetylation of control (C) and high-fat (HF) male and female offspring (n = 6 pups from different litters /group). Statistically significant differences were determined by two-way ANOVA (factors: maternal diet and sex) followed by within sex pairwise comparisons using Bonferroni post hoc test: $*p < 0.05$.

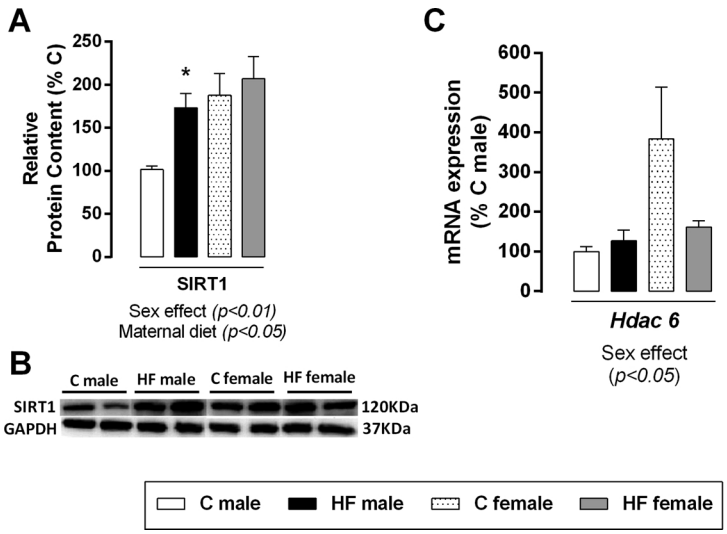

Fig. 4. Effect of maternal HF diet on expression of epigenetic regulatory factors in the hypothalamus of rat offspring at birth. (**A**) Protein content of the deacetylase sirtuin 1 (SIRT1), (**B**) representative bands from westernblots and (C) mRNA levels of the histone deacetylase 6 (*Hdac6*) and 7 (*Hdac7*) of control (C) and high-fat (HF) male and female offspring (n = 6 or 7 pups from different litters /group). Statistically significant differences were determined by two-way ANOVA (factors: maternal diet and sex) followed by within sex pairwise comparisons using Bonferroni post hoc test: $^{*}p < 0.05$.

to interferences in multiple regulatory mechanisms involving micro RNAs (Chiarlone et al., 2016).

Maternal HF diet decreased hypothalamic levels of *Faah* mRNA only in male offspring. However, in a previous study, we observed that maternal HF diet did not change hypothalamic FAAH protein content in the offspring at birth (Dias-Rocha et al., 2018), possibly with no effect on local AEA levels since FAAH metabolizes preferentially this endocannabinoid (Henry et al., 2017). *Faah* mRNA down-regulation might be related to changes in estrogen signaling in newborn male offspring, since a previous study showed the presence of estrogen response elements in *Faah* promoter (Waleh et al., 2002). To the best of our knowledge, androgen response elements in *Faah* promoter were not described yet, but we speculate they might also be present in this region.

The endocannabinoids, AEA and 2-AG, are produced mainly from n-6 polyunsaturated fatty acid (PUFA) pathways, and n-6 PUFA can also regulate CB1 function and expression (Freitas et al., 2017). We found that maternal HF diet increased the plasma n-6 to n-3 PUFA ratio only in male newborn offspring, which is an important risk factor for the development of obesity, cardiovascular disease, cancer, inflammatory and autoimmune disorders (Freitas et al., 2017). Concerning ECS, increased plasma n-6 to n-3 PUFA ratio can contribute to higher

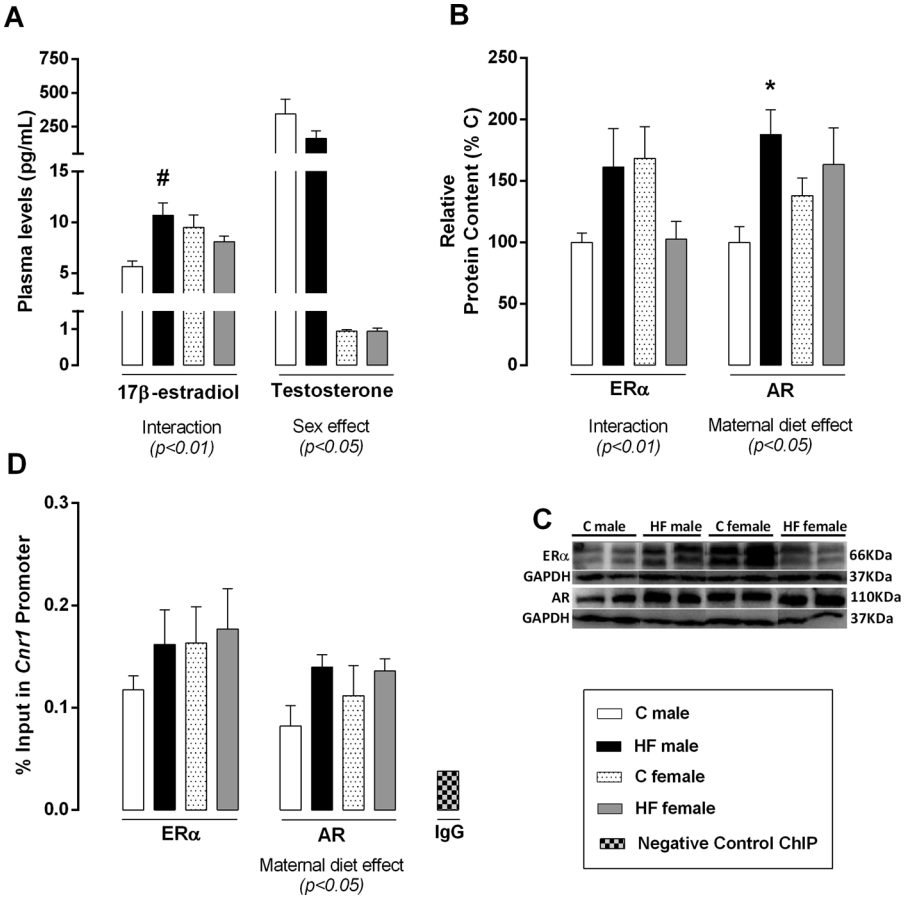

Fig. 5. Effect of maternal HF diet on signaling of sex hormones. (**A**) Plasma levels of 17β-estradiol and testosterone, (B) hypothalamic protein content of estrogen receptor alfa (ERα) and androgen receptor (AR), (**C**) representative bands from westernblots and (**D**) percentage of ERα and AR binding to type 1 cannabinoid receptor (*Cnr1*) promoter of control (C) and high-fat (HF) male and female offspring (n = 6 or 10 pups from different litters /group). Statistically significant differences were determined by two-way ANOVA (factors: maternal diet and sex) followed by within sex pairwise comparisons using Bonferroni post hoc test: $^{*}p < 0.05$, $\#\ p < 0.01$.

circulating levels of AEA and 2-AG (Freitas et al., 2017), indicating an over activation of endocannabinoid signaling.

Interestingly, it was demonstrated that different n-6 to n-3 PUFA dietary ratios induce distinct epigenetic marks in genes from mice brain (Chakraborty et al., 2017). Epigenetic mechanisms, such as DNA methylation and histone acetylation, are associated with gene expression regulation and metabolic programming. In the present study, we observed that hypothalamic *Cnr1* promoter was relatively low methylated, ranging from 5% to 25%, which is in agreement with other studies showing low DNA methylation levels in *Cnr1* promoter in the brain (D'Addario et al., 2017). We did not observe effect of maternal HF diet on the DNA methylation profile of the four CpG sites analyzed in the *Cnr1* gene. However, other *Cnr1* CpGs might be regulated by maternal HF diet. We choose these CpGs because they have shown inverse correlation with mRNA levels in other experimental models (Di Francesco et al., 2015; Santoro et al., 2017). However, this was not true for our model or we could not observe methylation changes at the same time point we analyzed mRNA levels, since DNA methylation is a highly dynamic process. It is possible that DNA methylation changes had occurred before birth and we observed the *Cnr1* mRNA levels changes in neonate pups as a consequence. On the other hand, we showed that maternal HF diet increased the histone acetylation percentage of the *Cnr1* promoter only in male offspring, which can contribute to increased hypothalamic levels of *Cnr1* mRNA. To the best of our knowledge, we were the pioneers on the demonstration of epigenetic dysregulation of *Cnr1* gene in the developmental origins of obesity, although it has been reported in animal and in vitro models from other experimental contexts. For instance, chronic unpredictable stress decreases CB1 expression in cingulate cortex of mice associated with reduced histone acetylation in *Cnr1* gene (Lomazzo et al., 2017). Extravirgin olive oil up-regulates CB1 associated with reduced methylation in *Cnr1* promoter in human colon cancer cells and rat colon (Di Francesco et al., 2015).

Recently, it was demonstrated that maternal HF diet can impact long-term health by disrupting epigenetic regulatory factors during offspring brain development at late gestation in mice in a sex-specific manner (Glendining et al., 2018). Therefore, we also investigated the expression of epigenetic regulatory factors, such as SIRT1 and *Hdac*, which deacetylase transcription factors, histones and co-factors (Nillni, 2016). We showed that maternal HF diet increased hypothalamic SIRT1 protein in newborn male offspring, which could not explain the increased histone acetylation of the *Cnr1* promoter, but might contribute to early obesity development, since hypothalamic SIRT1 induces positive energy balance by increasing orexigenic and decreasing anorexigenic peptides (Nillni, 2016). In fact, we previously showed that HF male offspring has increased content of orexin at birth (Dias-Rocha et al., 2018). On the other hand, maternal HF diet did not alter *Hdac6* or *Hdac7*. We speculated that epigenetic regulatory factors that we did not assess or enzymatic activity might explain increased histone acetylation percentage in female *Cnr1* promoter, regardless maternal diet.

Epigenetic marks affect gene transcription in part by changing the binding of regulatory factors. It is well known that estrogens and androgens mediate most of their effects via nuclear receptors acting as transcription factors. The brain is a crucial site of estrogen and androgen actions, where these sex hormones have strong modulatory effect on physiology and behavior (Abi Ghanem et al., 2017; Balthazart and Ball, 2006; Juntti et al., 2010). Therefore, in the present study, we investigated whether maternal HF diet would regulate *Cnr1* gene in hypothalamus of newborn offspring through mechanisms involving sex steroid signaling.

Plasma levels of estradiol and hypothalamic ERα protein in newborn offspring suggest that estrogen signaling is upregulated in HF male offspring and downregulated in HF female offspring. The modified estrogen signaling might be deleterious since estrogen regulates the endocrine system of developing fetus (Kaludjerovic and Ward, 2012). Regarding energy homeostasis, decreased hypothalamic ERα signaling contributes to weight gain, since ERα deletion in pro-opimelanocortin neurons from hypothalamic arcuate nuclei results in hyperphagia while ERα silencing in the ventromedial hypothalamus leads to obesity as a consequence of reduced energy expenditure (Xu et al., 2011). Although ERα has been considered the most relevant estrogen receptor for regulating energy homeostasis (Fuente-Martin et al., 2013) and is widely expressed in hypothalamus (Mitra et al., 2003), the estrogen receptor β is the predominant isoform in hypothalamic paraventricular nucleus (Mitra et al., 2003). Interestingly, the inhibitory effect of central estradiol on food intake and body weight is blocked by ERβ silencing in periventricular region in rats, suggesting that the central anorexic effects of estradiol may involve ERβ signaling (Liang et al., 2002). Concerning ERα binding to hypothalamic *Cnr1* promoter, we showed no effect of maternal HF diet. It is possible that an initial modulation of estrogen signaling occurs around birth and that *Cnr1* binding alterations occur later in life. The interaction of ECS and steroid hormones is reported in colon cancer cell growth, in which estradiol up-regulates CB1 (Proto et al., 2012).

Regarding androgen signaling, newborn male offspring had high levels of plasma testosterone, as expected. This profile reflects the testis secretion that peaks at birth and then declines to infant levels. Maternal HF diet increased hypothalamic AR protein content only in newborn male offspring, which suggests an upregulation of androgen signaling pathway. Maternal HF diet also increased AR binding to *Cnr1* promoter in newborn offspring. In trigeminal ganglia neurons of rats, AR binding to *Cnr1* promoter activates transcription and increases *Cnr1* mRNA (Lee et al., 2013). Thus, we suggest that in our model, increased AR binding to *Cnr1* promoter might contribute to high levels of *Cnr1* mRNA in hypothalamus of newborn HF offspring.

The main limitation of the present study is the lack of measurement of serum and tissue endocannabinoid (AEA and 2-AG) levels due to methodological difficulties. In addition, in the present study, we decided to perform the experiments using whole hypothalamus instead of isolated hypothalamic nuclei, in line with our previous study with hypothalamus at birth (Dias-Rocha et al., 2018). Also, future experiments are needed to explore whether epigenetic mechanisms are associated with changes in the *mRNA* levels of the other ECS components in hypothalamus of newborn offspring. Furthermore, it will be interesting to assess whether maternal HF diet changes micro RNAs that target cannabinoid receptor genes.

In conclusion, maternal HF diet down-regulated leptin signaling and up-regulated *Cnr1* mRNA levels in the hypothalamus of newborn rat offspring. In parallel, maternal HF diet increased the histone acetylation and the AR binding in the *Cnr1* promoter only in male offspring, which may contribute to higher expression of *Cnr1* gene in newborn HF offspring. Maternal HF diet increased plasma n6 to n3 fatty acid ratio in male offspring, which is an important risk factor for metabolic diseases and might indicate an over activation of endocannabinoid signaling. Thus, although maternal HF diet programs a similar phenotype in adult offspring of both sexes, such as obesity, hyperphagia and higher preference for fat, here we demonstrated that molecular mechanisms involving leptin signaling, ECS, epigenetic marks and sex hormone signaling were modified prior to obesity development and can differ between male and female offspring. These observations may provide molecular insights into sex-specific targets for anti-obesity therapies.

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M. M. A., C. P. D.-R. and C. F. R.-G. have participated in animal experiments, collection and interpretation of data. M.M.A., H.W. and L.

J–M contributed with the DNA CpG methylation and chromatin immunoprecipitation assays. G. C. A. contributed with the gas chromatography–mass spectrometry analysis. I. H. T., C. C. P.-M., A. C. delineated the experimental design and supervised the study. M. M. A., L J–M and I. H. T. have written the manuscript.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.02.004.

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