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Abstract- Mesolimbic dopaminergic circuit is essential for food reward and motivational behaviors, and can contribute to weight gain and obesity. Litter reduction is a classical model for studying the effects of neonatal overfeeding and overweight. Litters of Wistar rats were reduced to 4 pups/dam for Small Litter (SL) and 10 pups/dam for normal litter at postnatal day (PND) 4. Immediately after performing the feeding behavior tests, the animals were sacrificed in PND21 and PND90. The ventral tegmental area (VTA), Nucleus Accumbens Core (NAcC) and Shell (NAcSh) were isolated from frozen brain sections using the Palkovits micro-punch technique. RNA and DNA were extracted from these areas, gene expression was measured by RT-qPCR and DNA methylation levels were measured by MSRM-qPCR technique. SL-PND21 animals presented increased expression levels of Tyrosine Hydroxylase and Dopamine Receptor D2 in VTA, decreased expression levels of Dopamine Active Transporter (DAT) in VTA, and higher expression levels of DAT in NAcC. On the other hand, SL-PND90 animals showed decreased expression levels of Dopamine Receptor D1 and higher expression of DAT in NAcSh. These animals also evidenced impaired sensory-specific satiety. In addition, altered promoter methylation was observed at weaning,

and remained in adulthood. This work demonstrates that neonatal overfeeding induces disruptions in the mesolimbic dopaminergic circuitry and cause alterations in feeding behavior from weaning to adulthood, suggesting that the neonatal period is critical for the normal development of dopaminergic circuit that impact on feeding behavior.

Keywords: Small litter, postnatal programming, dopaminergic mesolimbic circuit, DNA methylation, behavior.

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

1. Introduction

Excess in body weight occurs as a result of positive energy balance, when energy intake exceeds energy expenditure [1]. Energy balance is dynamically regulated by two key brain systems. The homeostatic system is involved in basic metabolic processes and survival, while the hedonic (or reward) system is driven by sensory perception or pleasure. Reward circuit can promote food intake even when energy requirements have been met, contributing to weight gain and obesity [2]. The neurotransmitter dopamine (DA) has emerged as a major hedonic influence over food intake, since playing a critical role in feeding behavior [3]. The mesolimbic dopaminergic circuit, essential for food reward and motivational behaviors, begins in dopaminergic neurons of Ventral Tegmental Area (VTA) that innervate to Nucleus Accumbens (NAc) [1,4]. The tyrosine hydroxylase (TH) enzyme present in the soma of VTA neurons synthesized DA, TH is rate-limiting enzyme in its biosynthesis [5]. Although five variants of DA receptors have been identified, DRD1 and DRD2 are the most abundantly subtypes expressed in the brain [6] and their role mediating food reinforcement is still not clear. DRD1 is important in reward related learning including translation of motivation into action [7]. DRD2 can act as a somatodendritic autoreceptor, modulating DA neurotransmission and inhibiting impulse-dependent DA release [6]. The termination of the DA signaling occurs in the synaptic space through re-uptake via DA active transporter (DAT) [1]. Therefore, there are two mechanisms that control DA neurotransmission: inhibition of release via presynaptic DRD2 autoreceptor and DA uptake via DAT. NAc is subdivided in two compartments, a medioventral shell (NAcSh) and a laterodorsal core (NAcC) [8]. Consumption of palatable food stimulates DA transmission preferentially in NAcSh compared to NAcC [4]. On the other hand, DA projections to NAcC

provide a generic motivation value associated with a possible reward that allows it to be translated into concrete actions, leading to the development of motivated behaviors [9]. However, the contribution of each compartment to the regulation of feeding remains scarcely investigated. Furthermore, DA neurons in VTA express receptors for circulating hormones that, as regulators of energy homeostasis, may act modulating dopaminergic function [10]. Specifically, insulin and leptin can decrease food reward behaviors and modulate the function of neurotransmitter systems and neural circuitry that mediate food reward, such as the midbrain DA and opioidergic pathways [11,12]. Besides, ghrelin can increase food reward behaviors, and support midbrain DA neuronal function [13].

The potential role of epigenetics in developmental programming has often been discussed in the context of the fetal period, during which DNA methylation patterning is known to be particularly active and dynamic to allow normal tissue development [14,15]. Evidence shows that changes in early postnatal nutrition quality and/or intake can influence the epigenetic and expression profiles of various organs involved in body weight regulation [14]. However, the idea that nutrition-induced epigenetic programming is a true cause of the onset of obesity. Gene-environment interactions are essential for understanding the regulation of food intake and the etiology of obesity in this developmental programming. This is particularly relevant during the perinatal period when the reward circuitry is still maturing, and permanent alterations in its function may occur [16]. There are several possible mechanisms that could cause food-reward disruptions, including epigenetic modifications that lead to altered gene expression [14]. DNA methylation is an epigenetic modification that regulates gene expression without altering the underlying DNA sequence. This process occurs mostly at CG dinucleotides in CpG islands that, in general, co-localize with promoter regions and transcription factors' (TFs) binding sites, which regulate transcription of specific genes. A higher frequency of hypomethylated DNA regions was observed in transcriptionally active chromatin, therefore it is thought that the degree of gene transcription is inversely proportional to the level of DNA methylation (canonical form) [17]. Our group and others have reported that several genes involved in food intake control are regulated through this mechanism [18–21]. Moreover, several studies showed that environmental manipulations could induce epigenetic changes in dopaminergic circuitry, thus reprogramming neurobehavioral substrates related to food intake and food reward [1,20]. It has been shown that several TFs regulate the expression of TH, DAT, vesicular monoamine transporter 2, and L-aromatic amino acid decarboxylase, all of which are critical for DA synthesis and transport [22].

A classic model to study the effect of neonatal overfeeding is reduction in litter size, which decreases competition for milk during the lactation period and leads to overfeeding and weight gain. The early postnatal period is a critical developmental window with

significant epigenetic plasticity. In mammals, this period is considered a continuation of the fetal phase that enables full maturation of the organism. Key organs and hormonal axes governing metabolism and energy homeostasis, such as the hypothalamus, adipose tissue, liver, and gut, undergo structural and functional development after birth. Some species, including rodents, have a shorter gestation time and deliver fragile and immature newborns in litters, requiring constant maternal care during early life [14]. Neonatal overfeeding contributes, at least in part, to altered metabolic phenotype and early programming of brain circuitry, which may persist into adulthood [20,23]. However, the molecular mechanism of this "programming" has not yet been clarified. Therefore, the aim of the present study was to determine how neonatal overfeeding affects the expression of molecules involved in mesolimbic dopaminergic circuit at weaning and adulthood. Also, we assessed whether changes in DNA methylation status could be involved in genes with impaired expression. Furthermore, we evaluated whether this brain programming could affect feeding behavior through different behavioral tests that evaluate dopamine circuit performance.

2. Materials and Methods

2.1 Animals and experimental design

To carry out this experiment, 17 Wistar rats (females, 75 days old) were acquired from the Department of Human Physiology of the School of Biochemistry and Biological Sciences (National University of Littoral, Santa Fe, Argentina). The animals were housed under controlled conditions (22±2°C and 12h light-dark cycle) with free access to water and standard chow (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina). After mating with males of proven fertility, the females were placed in individual cages until delivery. Only litters with 10–12 pups per dam were included. At postnatal day four (PND 4), when lactation was fully established [23], litters from 10 dams were reduced to 4 pups (3 males and 1 female) to generate the small litter group (SL) and litters from 7 dams were reduced to 10 pups (7–8 males and 3–2 females) to create the normal litter group (NL). Body weights were recorded weekly during the lactation period. At weaning, one or two male pups from each dam were sacrificed by decapitation as described below, conforming SL-PND21 (n=7) and NL-PND21 (n=7) groups. The rest of the male rats were housed under controlled conditions with free access to water and standard chow. Body weight and food intake were recorded weekly until adulthood. Behavioral tests were carried out with at least one week apart to let the animals rest and to avoid the influence of one test over the other. At PND90, the animals were fasted for 12 hours and sacrificed by decapitation as described below, conforming SL-PND90 (n=14) and NL-PND90 (n=14) groups. Only male rats were used to minimize the effects of female sex hormones on metabolism, therefore all female rats were culled at

weaning. The animals were sacrificed, trunk blood was collected, and the brain was removed from the skull. Half of the brain samples were frozen ventral side up on dry ice and stored at -80°C until sectioning, and the rest were fixed with paraformaldehyde for immunohistochemistry. Also, the epididymal adipose tissue was dissected and weighed.

All the procedures carried out in this study were approved by the Ethical Committee of the School of Biochemistry and Biological Sciences (National University of Litoral, Santa Fe, Argentina) and performed in accordance with the principles and procedures outlined in the Guide for Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council-2010).

2.2 Micropunches of individual brain regions and nucleic acids isolation

Serial coronal sections of 200 µm for PND21 and 300 µm for PND90 were cut from frozen rat brains in a cryostat at -15°C. In these sections, NAcC, NAcSh and VTA enriched regions were removed using Palkovits' microdissection technique [24] with a 1.0 mm stainless steel micropunch needle. To identify these regions, the Atlas of the Postnatal Rat Brain in Stereotaxic Coordinates P21 [25], Atlas of the Developing Rat Nervous System 2nd edition [26] and The Rat Brain 6th edition [27] were used. The tissues were removed bilaterally and stored in TRIzol at -80°C. The topography of the holes was inspected under a stereo microscope (Stemi 305, Zeiss, Oberkochen, Germany) to determine the correct location of the microdissected nuclei. Total RNA and DNA were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and purity of nucleic acids were evaluated by measuring the absorbance at 260 in a NanoDrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA samples were stored at −80°C and DNA samples at -20°C.

2.3 Reverse transcription and real-time quantitative PCR

Reverse Transcription (RT) was carried out using 0.5 µg of total RNA and Moloney Murine Leukemia Virus reverse transcriptase (200 units; Promega, Madison, WI, USA), as previously described by our group [28]. Each RT product was diluted in RNAse free water to a final volume of 60 µl and stored at -20°C. For amplification and quantification of the cDNA, a quantitative polymerase chain reaction (qPCR) was performed using HOTFIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Estonia) in a Real-Time PCR Step One Cycler (Applied Biosystems Inc., Life Technologies, CA, USA) as described by our group [21,28]. The primer pairs used for the amplification were designed in our laboratory [18,21] and are detailed in Table 1. Ribosomal protein L19 was selected as the housekeeping gene because it is highly stable and does not evidence expression changes between experimental groups [29]. Product purity was confirmed by dissociation curves, and random samples were

subjected to agarose gel electrophoresis. cDNA amplification was carried out in duplicate, and negative DNA template controls were included, which always yielded no consistent amplification. The threshold cycle (Ct) and qPCR efficiency were calculated using Step One software (Applied Biosystems Inc., Foster City, CA, USA). The fold expression over control values was calculated for each target gene by the relative standard curve method [30]. The target's relative quantity for all experimental samples was determined with the standard curve, normalized to the relative quantity of the reference gene L19, and divided by the normalized target value of the control sample. No significant differences in Ct values were observed for L19 between experimental groups (Data not shown).

Table 1. Sequences of primers used in qPCR for mRNA analysis.

Protein name	Gene name	Accession number	Primer Sequences	Size (bp)
L19	*L19*	NM_031103.1	F: 5'-AGCCTGTGACTGTCCATTCC-3' R: 5'-TGGCAGTACCCTTCCTCTTC-3'	99
TH	*Th*	NM_012740.3	F: 5´-TACCAAGATCAAACCTACCAGCC-3´ R: 5´-GGTCAAACTTCACAGAGAATGGG-3´	118
DRD1	*Drd1*	NM_012546.3	F: 5´-TCCAAGGTGACCAACTTCTT-3' R: 5´-GTTACAAAAGGACCCAAAGG-3´	126
DRD2	*Drd2*	NM_012547.1	F: 5´-CCCAGCAGAAGGAGAAGAAA-3´ R: 5´-CAGGATGTGCGTGATGAAGA-3´	95
DAT	*Slc6a3*	NM_012694.2	F: 5´-CATCACCACCTCCATTAACTCC-3´ R: 5´-CATTGTGCTTCTGTGCCATG-3´	89
Ob-Rb	*Ob-Rb*	NM_012596.1	F: 5'-AGGATGAGTGTCAGAGTCAA-3' R: 5'-CTCTTCATCAGTTTCCACTG-3'	80
GHSR	*Ghsr*	NM_032075.3	F: 5'-GCTCTGCAAACTCTTCCA-3' R: 5´-AAGCAGATGGCGAAGTAG-3´	99
IR	*InsR*	NM_017071.2	F: 5'-TCTCTCCAGGAAACTACAGTGT-3' R: 5'-AAAATAGGTGGGTTCTGTCC-3'	80

L19: Ribosomal protein (housekeeping gene); TH: Tyrosine hydroxylase; DRD1: DA receptor D1; DRD2: DA receptor D2; DAT: DA active transporter; Ob-Rb: Leptin receptor; GHSR: ghrelin receptor; IR: insulin receptor.

2.4 Analysis of DNA methylation

Methylation state of gene promoters was studied using methylation-sensitive restriction enzymes (MSREs) and qPCR analysis, as previously described by our group [18,21,28]. Therefore, we only studied promoters that could be analyzed by this methodology. DNA isolated from different brain regions was digested using *Mae* II (Roche Applied Science, Indianapolis, IN, USA) and *BstU* I (New England BioLab, Beverly, MA, USA). Then, relative degree of promoter methylation was analyzed by qPCR and determined

by the relative standard curve method [30]. A region without restriction sites was used as internal control (IC) for DNA quality. The primer sequences used to study the different restriction sites and the IC in each gene promoter are shown in Table 2. The *in silico* study of candidate sites for DNA methylation and potential transcription binding sites used to design the primers for the analysis of *Th, Drd1* and *Slc6a3* promoters were previously published by our group [18,21]. For the *Th* promoter, two *Mae* II sites [Th *Mae* II (a), in the cAMP response element (CRE) site, and (b), near the binding site of AP1 and AP2 transcription factors] were studied, as we could not identify CpG islands in the *Th* gene (Fig. 1 A). In *Drd1* promoter, a *BstU* I site was analyzed (Fig. 1 B). Regarding the *Slc6a3* promoter, two *BstU* I sites [Slc6a3 *BstU* I (a) and (b)] were evaluated (Fig. 1 C).

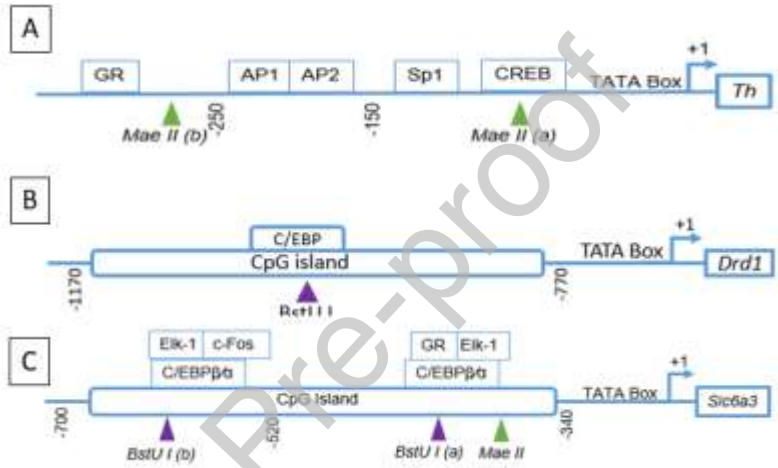

Fig. 1. Scheme of *Th* (A), *Drd1* (B) and *Slc6a3* (C) promoter regions, with CpG islands, restriction sites for methylation-sensitive restriction enzymes and putative binding sites for transcription factors. AP: the activator protein; GR: glucocorticoid receptor; Sp1: specificity protein 1. CREB: family of cAMP response element-binding protein; C/EBP: family of CCAAT/enhancer binding protein; ElK-1: Ets LiKe gene1. Violet triangles show the *BstU* I restriction sites, and green triangles show the *Mae* II restriction sites. Modified from [21].

Table 2. Primer Sequences used in qPCR for DNA methylation-sensitive analysis.

Gene target	Primer forward	Primer reverse
Th IC	5´- CCATCAGATTTACCTAGAAGC-3´	5´-TGAGACTATGAAGGGACATTG-3´
Th *Mae* II (a)	5´-ACAGCAGGCGTGGAGAGGAT-3´	5´-TGGTGGTCCCGAGTTCTGTC-3´
Th *Mae* II (b)	5´-CCTTAGGAAATCCAGCATGG-3´	5´-ATTGCATCCACTGTCACAGG-3´
Drd1 IC	5´-GTGGTGAGAATCCCCTCAGG-3´	5´-AGTTCCACAGGCGGAGAACC-3´
Drd1-*BstU* I	5´-AGCAGGAAACCACAGGCACC-3´	5´-GCTTCTGCGGTCAACTCACG-3´
Slc6a3 IC	5´-TTTGGGGTCTCAACTAGAAA-3´	5´-TAAGACCTTTTCAGAACCCA-3´
Slc6a3 *BstU* I(a)	5´-CTTCTGACAACCTCGCTGGA-3´	5´-GGGGCTTGACAGGAGTCTTT-3´
Slc6a3 *BstU* I(b)	5´-CGTACAACACCGAAGGAAGA-3´	5´-CGAGGTTGTCAGAAGCAGAT-3´

Th: Tyrosine hydroxylase gene; *Drd1*: Dopamine receptor 1 gene; *Slc6a3*: Dopamine active transporter gene; IC: Internal control.

2.5 Immunohistochemistry

Immunohistochemistry was performed as previously described [31]. Briefly, brains were fixed with paraformaldehyde, dehydrated in a graded series of ethanol, then the tissue was cleared with Bioclear (Biopack) and embedded in paraffin for subsequent microtome (Leica biosystem) sectioning. Coronal sections of 5 µm were cut, and the sections containing VTA, identified with the help of the atlas mentioned in section 2.2, were reserved to perform this technique. Again, these sections were placed in Bioclear for paraffin removal and later in ethanol for tissue dehydration. Microwave treatment was performed for antigen retrieval. The tissue was incubated in a mixture of hydrogen peroxide and methanol to block endogenous peroxidase activity, and then in non-fat dry milk (Sigma–Aldrich) with 5% normal goat serum, to block nonspecific protein binding sites. TH primary antibodies (Millipore monoclonal antibody) were used at 1/2000 and incubated overnight at 4° C. After incubation with anti-mouse biotin conjugated secondary antibodies (1/250) for 1 h, reactions were developed using a streptavidin-biotin peroxidase method with diaminobenzidine (Sigma–Aldrich) as the chromogen substrate. For the negative controls, the primary antibody was omitted. Images were obtained using a SPOT color video camera (Diagnostic Instruments, Inc., Arnold, MD) connected to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan), and processed with ImageJ 1.49v software.

2.6 Behavioral tests

The animals were subjected to behavioral tests to assess their responses to different sweet-tasting foods. At weaning, the Sensory-Specific Satiety *(SSS)* test was performed, and in the adulthood, the animals were evaluated with the SSS and Conditioned-Place Preference (CPP) tests, as detailed below.

2.6.1 Sensory-Specific Satiety test (SSS test)

This test evaluates if the animal presents "sensory-specific satiety" when eating. This refers to a sensory hedonic phenomenon that influences food intake through decreased satisfaction in the consumption of the same food, and the consequent renewal of appetite when exposed to a new flavor [32]. This test was performed according and adapted from Reichelt et al [33]. Two solutions were used: 10% sucrose (S) solution dissolved in tap water (pink) and 10% maltodextrin (M) solution dissolved in tap water (blue). These solutions had the same energy content (1680 kJ per 100 ml) and were equally preferred and discriminable [33]. The test protocol consisted of two stages (Fig. 2): a familiarization stage where the animals recognized the two solutions individually in successive exposures for two days, and a pre-exposure and choice stage, where the animals were exposed to one solution and then

to both solutions in successive sessions for two days. The volume of liquid consumed in each session was recorded by weight difference of the bottles with solution before and after offering them to the animals at each stage of the test. The test was always carried out between 9 and 12 am. During these days, the animals were kept without water except for a period of 2 h after the end of the test (13 to 15 pm), when they were given free access to it.

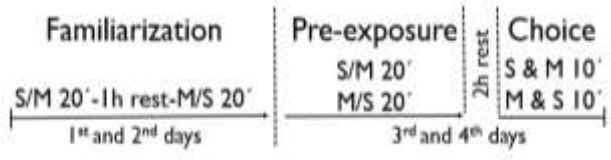

Fig. 2. Scheme of the protocol of sensory-specific satiety test. S: sucrose. M: maltodextrin.

2.6.2 Conditioned Place Preference test (CPP) to sweet food

This test assesses the preference or aversion towards an environment previously conditioned with a certain food. The protocol is described in Portella et al. [19]. The test was carried out in a cage (30 cm x 40 cm x 20 cm) equally divided in two compartments connected by a small opening. One compartment was fully illuminated (lit side), while the other was darkened to receive only 40% of light (dark side). The test consisted in studying if the animals reduced their natural preference for dark areas, given that they are nocturnal, by receiving a palatable food on the lit side. On day 1, the baseline time of permanence in the lit side without food was recorded. During the following six days, the training with food was carried out. For this, the rats were placed in the cage for 20 min with free access to both sides. In the lit side a palatable food (chocolate flavored wafers) was placed, in order to generate association and preference. During these days, the animals were kept in a mild food restriction, receiving only 85% of their usual standard food consumption. On day 8, the time spent in the lit side without food was recorded. The time difference recorded on the lit side between day 1 (baseline) and day 8 (post-conditioning with palatable food) was indicative of the conditioning of a place-preference generated by the palatable food.

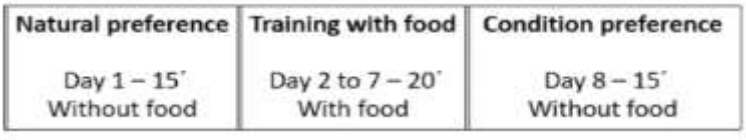

Fig. 3. Scheme of the protocol of "Conditioned place preference test".

2.7 Statistical analysis

An exploratory test was performed to evaluate the normal distribution (Shapiro-Wilk test) and variance homogeneity (Levene's test) of each variable. For the analysis of SL-PND21 vs NL-PND21 and SL-PND90 vs NL-PND90, parametric data were analyzed using Student's T test, while non-parametric data were analyzed with Mann Whitney test. For the SSS test, data analysis of the familiarization and pre-exposure stages was performed with a

two-way ANOVA test followed by the Bonferroni multiple comparison post-test, using the experimental groups (NL and SL) and the solutions offered (S and M) as factors. For the choice stage in SSS test, ANCOVA test was performed followed by the Bonferroni multiple comparison post-test, using the experimental groups and exposure to the solution [Pre-exposed (P) and Non Pre-exposed (NP)] as factors, and the volume of consumed solution during pre-exposure as co-variable. A one-way ANOVA test was carried out when the ANCOVA test showed significant interaction between factors. To analyze the data of the CPP test, a two-way ANOVA test was performed followed by the Bonferroni post-test, using the experimental groups and the time of permanence in the lit side before (Baseline) or after (Post-condition test) conditioning with sweet food as factors. Analyses were performed using IBM SPSS Statistics 19 software (IBM Inc., Armonk, NY, USA). In all cases, the data were expressed as the mean ± standard error of the mean (SEM). Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1 Neonatal overfeeding altered gene expression in VTA and NAcC at PND21

SL-PND21 group presented higher mRNA levels of TH and DRD2 (p=0.0035 and p=0.0002, respectively) and lower expression levels of DAT (p=0.02) in VTA (Fig. 4 A). This group also showed higher transcript expression of DAT in NAcC (Fig. 4 B, p=0.0001), and no changes in gene expression in NAcSh (Fig. 4 C, NS).

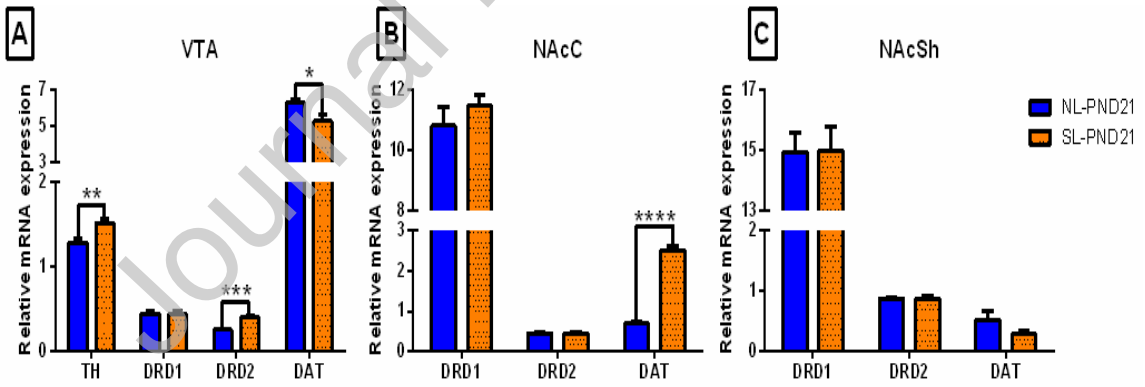

Fig. 4. Relative mRNA levels of genes of interest in male rats raised in normal (NL) or small (SL) litters at PND21. TH: tyrosine hydroxylase; DRD1: Dopamine receptor D1; DRD2: Dopamine receptor D2; DAT: Dopamine active transporter. VTA: Ventral tegmental area region; NAcC: Accumbens nucleus Core region; NAcSh: Accumbens nucleus Shell region. Values are means ± SEM (n=7/group), ****p <0.0001, ***p <0.001, **p <0.01 and *p <0.05 by Student's T test or Mann Whitney test.

3.2 Neonatal overfeeding altered DNA methylation patterns of *Th* and *Slc6a3* promoters at PND21

DNA methylation patterns were analyzed in promoter regions of *Th* in VTA and *Slc6a3* in VTA and NacC. SL-PND21 group showed increased methylation levels in the *Mae*

II (b) site of the *Th* promoter (Fig. 5 A, p=0.01). Regarding *Slc6a3* promoter, SL-PND21 group presented lower methylation levels in the *BstU* I (b) site in NAcC (Fig. 5 C, p=0.02), while no methylation differences were observed in VTA (Fig. 5 B, NS).

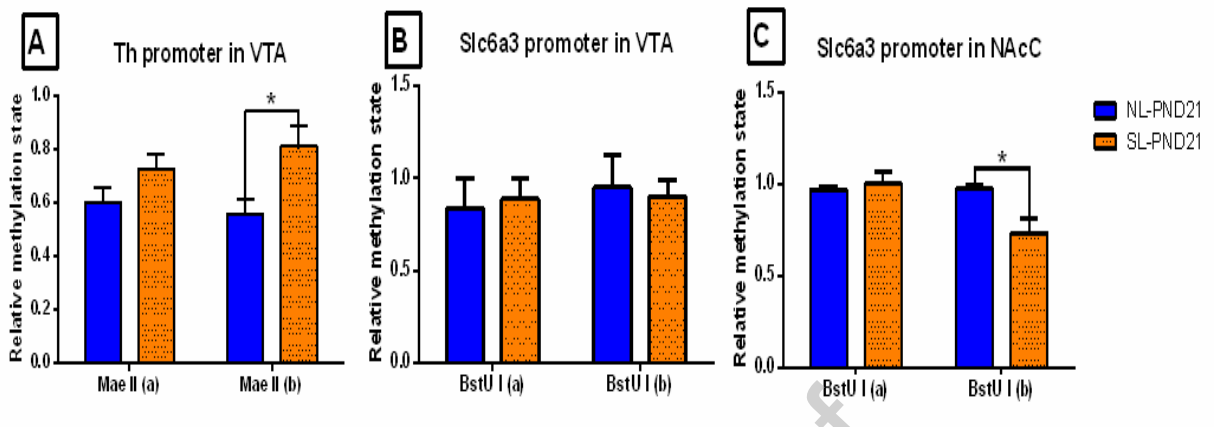

Fig. 5. Relative methylation state of promoters of interest in male rats raised in normal (NL) or small (SL) litter at PND21. *Th*: Tyrosine hydroxylase gene; *Slc6a3*: Dopamine active transporter gene. VTA: Ventral tegmental area region; NAcC: Accumbens nucleus core region. Values are means ± SEM (n=7/group), *p <0.05 by Student's T test or Mann Whitney test.

3.3 Immunohistochemistry of TH protein in VTA at PND21

Immunostaining of TH protein and Nissl staining were performed in VTA of one animal from NL-PND21 and one from SL-PND21 (Fig. 6). Nissl staining allowed us to anatomically identify the VTA nucleus, confirming that the TH protein assessed by immunohistochemistry was present in this region. In addition, as Nissl staining is used to observe cellular structures within neurons and also to understand neuronal damage through observation of Nissl bodies, we were able to qualitatively assess that there were no signs of damage and no morphological differences between the neurons in the sections of NL-PND21 and SL-PND21 brains evaluated. Microphotography of the SL-PND21 animal seems to show greater labeling of the TH enzyme in axons and dendrites, which would correlate with the higher expression levels of TH observed in this group. However, other more precise techniques regarding protein quantification are needed to further evaluate this association.

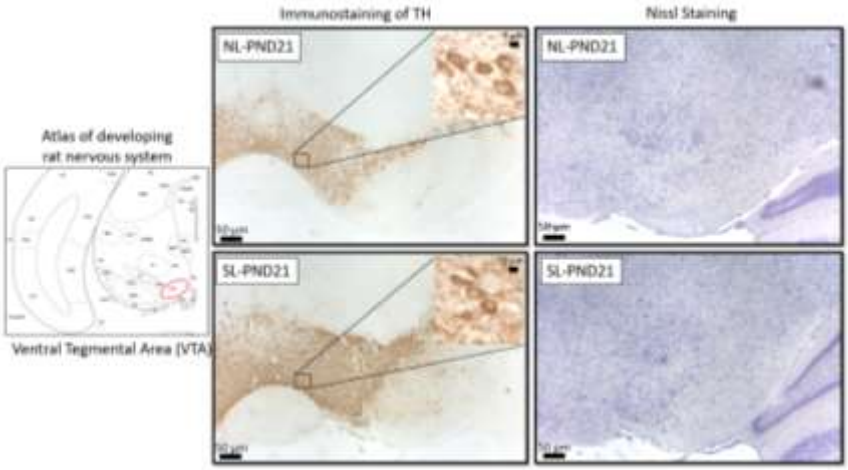

Fig. 6. Representative photomicrographs showing immunohistochemistry of TH protein and Nissl staining in VTA of male rats raised in normal (NL) or small (SL) litters at PND21. TH: tyrosine hydroxylase. VTA: Ventral tegmental area.

3.4 Neonatal overfeeding did not alter sensory-specific satiety of rats at PND21

The volume consumed of the S and M solutions, at different stages of the test is shown in Fig. 7. SL-PND21 group showed no significant differences in the volume consumed of the solutions, indicating that SL-PND21 animals presented a similar behavior in SSS test than the control group.

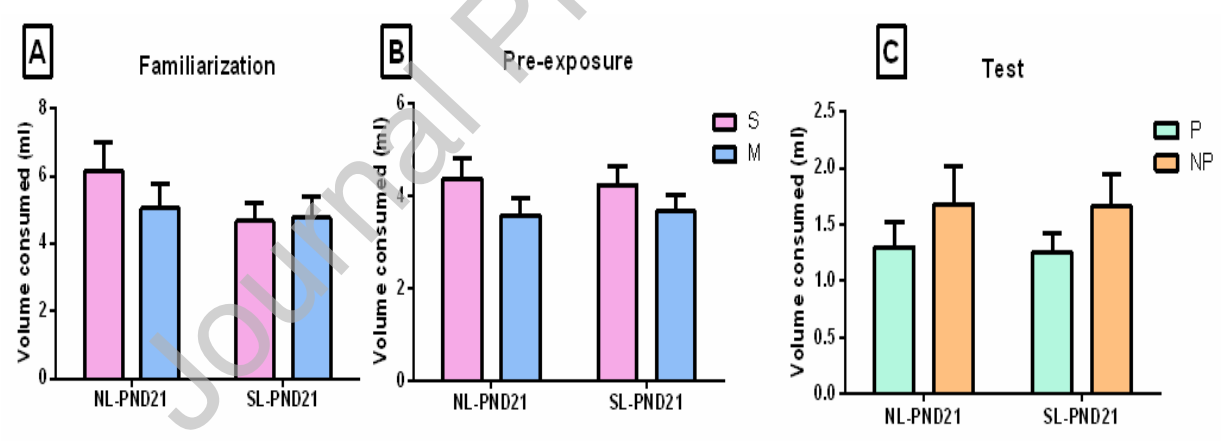

Fig. 7. Volume of consumed solution measured in milliliters (ml) in each stage of SSS test for male rats raised in normal (NL) or small (SL) litter at PND21. A) Familiarization stage; B) Pre-exposure stage: C) Choice stage. S: sucrose solution; M: maltodextrin solution; P: pre-exposed solution; NP: non pre-exposed solution. Values are expressed as mean ± SEM (n=14/group), p=NS by ANOVA or ANCOVA test with a Bonferroni post-test.

3.5 Neonatal overfeeding altered gene expression in NAcSh at PND90

The SL-PND90 group showed lower expression levels of DRD1 (p=0.03) and higher expression levels of DAT (p=0.03) in NAcSh (Fig.8 C) compared to NL-PND90. On the other hand, this group showed no changes in expression of the studied genes in both VTA and NAcC (Fig.8 A and B, NS).

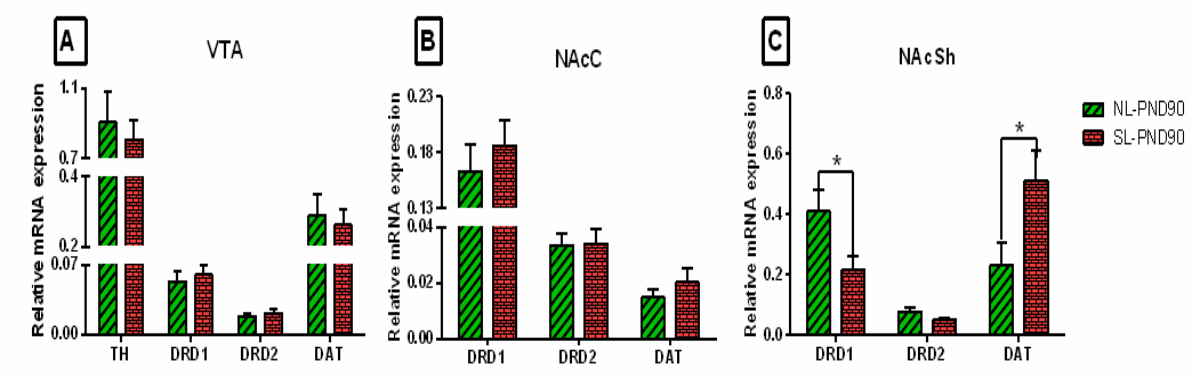

Fig. 8. Relative mRNA levels of genes of interest in male rats raised in normal (NL) or small (SL) litters at PND90. TH: Tyrosine hydroxylase; DRD1: Dopamine receptor D1; DRD2: Dopamine receptor D2; DAT: Dopamine active transporter. VTA: Ventral tegmental area region; NAcC: Accumbens nucleus core region; NAcSh: Accumbens nucleus Shell region. Values are expressed as mean ± SEM (n=7/group), *p <0.05 by Student's T test or Mann Whitney test.

3.6 Neonatal overfeeding altered DNA methylation patterns of *Th* and *Slc6a3* promoters at PND90

DNA methylation patterns were analyzed for promoter regions of *Th* in VTA, *Drd1* in NAcSh and *Slc6a3* in VTA, NAcC and NAcSh at PND90. For the *Th* promoter in VTA (Fig. 9 A), SL-PND90 group showed increased methylation levels in *Mae* II (a) and (b) sites (Fig. 9 A, $p_{(a)}$=0.0079 and $p_{(b)}$=0.01). In *Drd1* promoter no alterations were observed (Fig. 9 B). Regarding *Slc6a3* promoter, SL-PND90 group showed decreased methylation levels in the *BstU* I (a) site in VTA (Fig. 9 C, p=0.0003) and increased methylation levels in *BstU* I (a) and (b) sites in NAcSh (Fig. 9 E, $p_{(a)}$=0.0014 and $p_{(b)}$=0.0017). On the other hand, SL-PND90 animals did not show differences in methylation levels of the promoter sites studied in NAcC (Fig. 9 D).

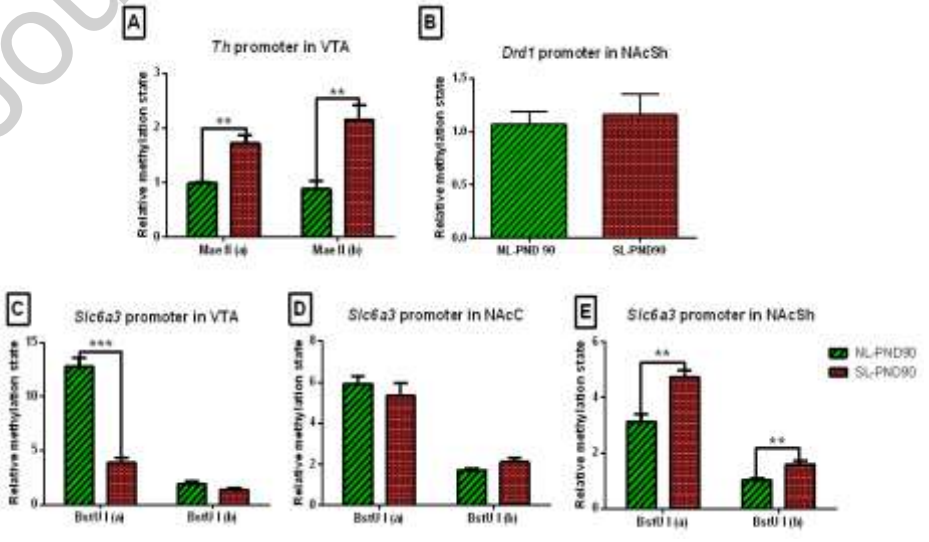

Fig. 9. Relative methylation state of promoters of interest in male rats raised in normal (NL) or small (SL) litter at PND90. *Th*: Tyrosine hydroxylase gene; *Drd1*: Dopamine receptor 1 gene; *Slc6a3*: Dopamine active transporter

gene. VTA: Ventral tegmental area region; NAcC: Accumbens nucleus core region; NAcSh: Accumbens nucleus Shell region. Values are expressed as mean ± SEM (n=7/group), ***p <0.001 and **p <0.01 by Student's T test or Mann Whitney test.

3.7 Immunohistochemistry of TH protein at PND90

Immunostaining of TH protein and Nissl staining were performed in VTA of one animal from NL-PND90 and one from SL-PND90 groups, as shown in Fig. 10. Here again, Nissl staining allowed us to anatomically identify the VTA region. Histologically, we were able to observe that the neuronal bodies of the VTA region presented the characteristic structures of normal cells: clear nucleus with its prominent nucleolus and Nissl perinuclear bodies representative of the basophilic substance. Qualitatively, no differences were observed between neurons in the studied sections of the NL-PND90 and SL-PND90 brains. In line with TH expression results, no significant differences were observed in enzyme staining. Other more accurate protein quantification methods should be used in order to perform a correct quantification.

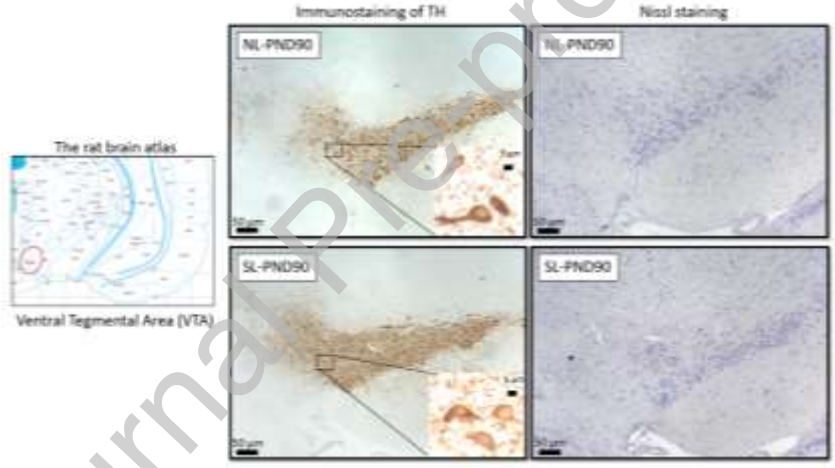

Fig. 10. Representative photomicrographs showing immunohistochemistry of TH protein and Nissl staining in VTA of male rats raised in normal (NL) or small (SL) litters at PND90. TH: tyrosine hydroxylase. VTA: Ventral tegmental area.

3.8 Neonatal overfeeding impaired sensory-specific satiety of rats at PND90

The volume of the two solutions consumed during the different stages of the SSS test is shown in Fig. 11. In the familiarization stage (Fig. 11 A), SL-PND90 and NL-PND90 animals consumed both solutions equally. However, during the pre-exposure and choice stage, SL-PND90 group showed a significant difference in S intake (Fig. 11 B, p=0.0094) and pre-exposed solution consumption (Fig. 11 C, p=0.0183), respectively. This indicates impaired sensory-specific satiety in SL-PND90, as this group chose to consume the pre-exposed solution rather than the novel, non pre-exposed solution. This behavior was not observed in NL-PND90 group (Fig. 11 B and C).

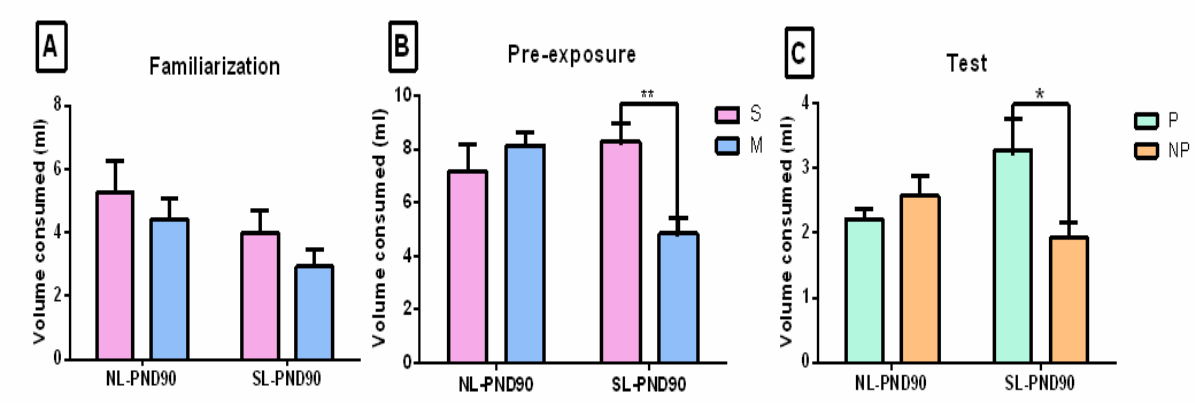

Fig. 11. Volume of consumed solution measured in milliliters (ml) in each stage of SSS test for male rats raised in normal (NL) or small (SL) litter at PND90. A) Familiarization stage; B) Pre-exposure stage: C) Choice stage. S: sucrose solution; M: maltodextrin solution; P: pre-exposed solution; NP: non pre-exposed solution. Values are expressed as mean ± SEM (n=14/group), **p <0.01 and *p <0.05 by two-way or one-way ANOVA test with a Bonferroni post-test.

3.9 Neonatal overfeeding did not change condition place preference at PND90

Time spent on the food paired side (lit side) for the two animal groups at the beginning of the test (Baseline) and after the training with a sweet food (Post-conditioning time) is shown in Fig. 12. Both groups showed a natural preference for darkness, spending more time in the dark side (Data not shown). No differences in preference were found after training with palatable food, as both, SL-PND90 and NL-PND90 groups spent an equal amount of time in the lit side than before training (Fig. 12, p=NS).

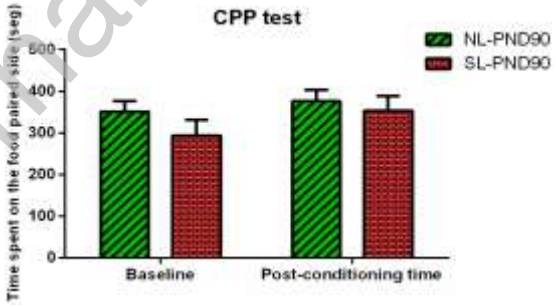

Fig. 12 Conditioned place preference test. Time spent on the food paired side at baseline or post-conditioning time for male rats raised in normal (NL) or small (SL) litter at PND90. Values are expressed as mean ± SEM (n=14/group), p=NS by ANOVA test with a Bonferroni post-test.

3.10 Neonatal overfeeding did not alter the expression of hormone receptors

SL group presented similar expression levels of Ob-Rb, GHSR and IR receptors in VTA than NL group at PND21 (Fig. 13 A, p=NS) and PND90 (Fig. 13 B, p=NS).

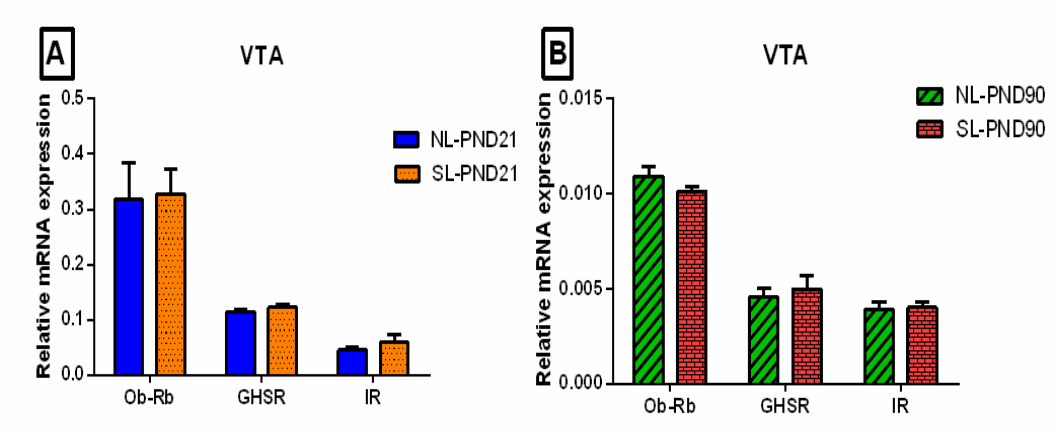

Fig. 13. Relative mRNA levels of receptors in VTA of male rats raised in normal (NL) or small (SL) litters at PND21 (A) and PND90 (B). Ob-Rb: leptin receptor; GHSR: ghrelin receptor; IR: insulin receptor. VTA: Ventral tegmental area region. Values are means ± SEM (n=7 or 14/group), p=NS by Student's T test or Mann Whitney test.

4. Discussion

Previous studies from our group [28] and others [34,35] demonstrated that raising rats in small litters induces neonatal overfeeding, leading to early development of obesity, increasing body weight, and affecting homeostatic control of food intake at weaning. However, those alterations are restored in adulthood. The present results complement our previous work [28], showing the effects of neonatal overfeeding on the hedonic system that controls food intake in the same animal model. For this, we analyzed the mesolimbic dopaminergic circuit, and evaluated feeding behavior at weaning and in adulthood. To our knowledge, little has been studied about the molecular changes and functioning of brain mesolimbic dopaminergic circuit in a SL model. Our study is the first to analyze the effects of a SL in a timepoint as early as weaning, showing significant alterations in gene expression and DNA methylation patterns in DA-related genes of rats raised in small litters. These observations help us understand how neonatal feeding can influence mesolimbic dopaminergic circuit, feeding behavior and susceptibility to obesity at weaning and in adulthood.

SL-PND21 animals showed higher levels of TH expression in VTA, which is consistent with TH protein immunostaining in the VTA region. Similar results have been observed in studies using a high fat diet and obesity prone rats model, which has been related to increased DA production [36,37]. SL animals also presented decreased expression levels of DAT in VTA, which modulates synaptic DA concentration [38]. Therefore, SL-PND21 group could have increased DA synthesis and reduced re-uptake in VTA, which could generate a higher DA concentration in synaptic space. This could motivate the animal to engage in food reward-seeking behaviors and overconsumption [1,39]. SL animals also presented increased expression levels of DRD2 in VTA. This autoreceptor

plays a key role in regulating the DA system by providing feedback inhibition that controls cell firing and synthesis, release, and uptake of DA [6]. Consequently, increased expression of DRD2 could act as a compensatory mechanism to regulate DA levels in the synaptic space. On the other hand, SL-PND21 group showed a higher expression of DAT in NAcC in what could be an attempt to increase DA reuptake, in order to reduce the amount and signaling of DA in the synaptic space [40,41]. Besides, no modifications in gene expression were detected in NAcSh of SL animals. Despite the alterations observed in gene expression in SL-PND21 animals, no behavioral changes were detected, as the SSS test and the preference for a novel solution were not affected.

In SL-PND90 animals, the alterations in the expression levels of various genes observed at PND21 in VTA and NAcC were restored. In contrast with the increased TH gene and protein expression in the VTA at PND21, at PND 90 no differences were observed as a consequence of neonatal overfeeding. We believe that the switch from breastfeeding to standard chow might reduce the stimulation of the dopamine circuit, thus dopamine synthesis and reuptake might be restored in VTA and NAcC. Furthermore, specific alterations in gene expression, DNA methylation and feeding behavior changes not observed at weaning emerged in adulthood, showing the long-term consequences of early overfeeding. SL-PND90 animals showed increased DAT expression, which could lead to a higher DA reuptake [38], and reduced DRD1 expression in NAcSh, which has also been observed in rodents chronically fed with a high-fat diet [20,42]. These alterations could represent a molecular mechanism related with changes in feeding behavior such as decreased preference for novelty and increased sucrose intake on the SSS test. It was reported that DRD1 in NAcSh regulates the response to novel stimuli [43] and that intra-cisternal infusions of DRD1 antagonists blunt the rewarding effects [44,45] and modulate the intake [46,47] of palatable food. Other authors have also reported a greater preference for sweet foods in adult SL animals [48]. Unfortunately, they did not show molecular changes that might be involved in this preference. It is important to highlight that the alterations in PND90 occur in the Shell region of the NAc. The role of NAc in mesolimbic-mediated appetite regulation has been widely studied [1], but each region of this nucleus has different implications in dopaminergic circuits that remain to be completely elucidated. Some authors [4,8,9] suggest that DA signaling in NAcC is related to the development of motivated behaviors by learning and predicting generic motivational values of food. In contrast, NAcSh is stimulated by the release of DA during the initial exposure to novelty. No differences were observed in the CPP test, probably because the palatable food stimulus was not strong enough to alter the rodents' natural preference for dark rooms. Our results demonstrate the long-term effects of neonatal overfeeding on the mesolimbic dopaminergic circuit and the control of motivated behaviour towards novel food.

Several studies show that the effects of overstimulating the dopaminergic circuit persist even after removing the stimulus [36,42] as we observed in adult animals. In addition, some researchers have suggested that changes in the expression of molecules of the dopaminergic circuit could be related to epigenetic alterations [20]. It has been well established that TH is regulated at multiple levels, from gene transcription to control of enzymatic activity [49], in a complex modular way by both, positive and negative regulatory elements [50]. The current results show that neonatal overfeeding affects the methylation levels of *Th* promoter regions in VTA, but the observed changes are not in line with gene expression changes. The SL-PND21 group showed higher methylation levels in regions near the binding site of AP1 and AP2 transcription factors, and normal methylation levels in CRE site. We believe that increased methylation only in the AP1/AP2 binding region was not sufficient to reduce TH expression. On the other hand, SL-PND90 group presented increased methylation in the two studied sites without significant changes in gene expression. It has been reported that the regions of the promoter that we studied and the TF AP1/AP2 and CREB are important modulators of TH expression [49,51–53]. However, in this case it is possible that methylation changes in regions that were not evaluated in this study, or others epigenetics mechanisms, may be regulating *Th* gene expression. In spite of this, epigenetic changes in the *Th* gene in the early postnatal period and in adulthood represent a stable mark that may allow the organism to adapt and respond to different rewarding stimuli. The regulation of *Drd1* expression by epigenetic mechanisms, such as DNA promoter methylation, has not been well described. We analyzed methylation levels of one restriction site on the CpG island in *Drd1* promoter and we did not find differences between SL-PND90 and NL-PND90. Therefore, we presume that this regulatory mechanism, or at least this promoter region, is not involved in gene expression control associated with neonatal overfeeding. DAT expression in VTA was reduced in SL-PND21 without methylation changes in *Slc6a3* promoter, while at SL-PND90, methylation levels were reduced without changes in gene expression, indicating long lasting epigenetic effects not exclusively involved in gene expression regulation. Instead, in NAcC, SL-PND21 group showed increased expression of DAT and reduced methylation levels in a CpG Island of the *Slc6a3* promoter. However, in NAcSh, increased DAT expression was associated to increased methylation levels in studied regions of *Slc6a3* promoter in SL-PND90 animals. This may indicate that epigenetic regulation of the Slc6a3 promoter in NAcSh may not follow the canonical association of DNA methylation with the gene repression pathway. Similarly, Cortes et al. [54] found that DNA methylation and demethylation regulate the number of ERα cells in the ARC, and methylation correlates with activation of the Esr1 gene in this region. Although contrary to the canonical pathway, there are a growing number of cases where DNA methylation of specific gene regions promotes transcription [55]. Further studies are

needed to understand which methylation sites are positively correlated with gene expression. This will help us to determine whether the methylation changes we have seen in SL animals are long-term genetic markers as a result of neonatal overfeeding.

It has been previously described by our group [28] and others [56,57] that neonatal overfeeding affects leptin, insulin and ghrelin hypothalamic signaling pathways. These hormones are highly involved in dopaminergic mesolimbic circuit function, influencing physiological mechanisms related to eating and pleasure [58–60]. However, in SL animals we did not find changes in the expression of these hormone receptors in VTA. Therefore, it seems plausible that the effects of neonatal overfeeding observed in the dopaminergic mesolimbic circuit do not involve the signaling pathways of those hormones.

Conclusion

The present work demonstrates that neonatal overfeeding generated by the small litter (SL) model affects dopaminergic mesolimbic circuit function both at weaning and in adulthood. SL animals showed disruption in both gene expression and methylation status of molecules involved in the dopaminergic mesolimbic system, as well as alterations in feeding behavior. These results agree with other studies [33,42], suggesting that excessive stimulation of the dopaminergic mesolimbic circuit is responsible for altering its correct functioning. Furthermore, these alterations are manifested even in short periods of stimulation, are independent of weight gain and could be maintained after a period without stimulation. Therefore, the neonatal period seems to be critical for the normal development of dopaminergic circuit and could influence feeding behavior throughout life.

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Declaration of interest

None.

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