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Early weaning leads to disruption of homeostatic and hedonic eating behaviors and modulates Serotonin (5HT) and Dopamine (DA) systems in male adult rats

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Abstract

Early weaning is associated with disruption of eating behavior. However, little is known about the mechanisms behind it. 5HT and DA systems are key regulators of homeostatic and hedonic eating behaviors, respectively. Thus, this study aims to evaluate the effects of early weaning on feeding behavior and 5HT and DA systems. For this, rats were submitted to regular (PND30) or early weaning (PND15) and between PND250 and PND300 were evaluated food intake of standard diet in response to 4h food deprivation, during the 24h period and per phase of the circadian cycle, in addition to the palatable food intake. Additionally, body mass and mRNA expression of 5HT1B, 5HT2C, SERT, DRD1 and DRD2 were evaluated in the hypothalamus and brainstem. The results demonstrate that early weaning promoted an increase in standard food intake in response to a 4h food deprivation in the 24h period and in the dark phase of the circadian cycle, in addition to an increased palatable food intake. No differences in body mass between regular or early weaning were observed. In the hypothalamus, increased mRNA expression of SERT and DRD1 was observed, but decreased 5HT1B mRNA expression. In the brainstem, the expression of 5HT1B, SERT, 5HT2C, DRD1 and DRD2 was increased in early weaned rats. In a nutshell, the stress promoted by early weaning has programmed the animals to be hyperphagic and to increase their palatable food intake, which was associated with modulation of 5HT and DA systems.

Keywords: Eating behavior; Early weaning; Serotonin; Dopamine; Hypothalamus; Brainstem.

1. INTRODUCTION

During the early stages of life mammals are quite susceptible to environmental influences [1–3]. Several studies show that during pregnancy and lactation, environmental stimuli can even permanently affect the growth and development of individuals [4–8]. One of the main systems affected by these stimuli is the central nervous system, precisely because it is in an intense process of development in the early stages of life [9,10]. As this system is responsible for the formation of neurobehavioral patterns in individuals, disturbances in their development can modulate the development of these patterns even permanently.

Among the environmental factors that can modulate the development of the central nervous system are stressors. Several rodent studies have shown that adverse conditions such as malnutrition or even changes in maternal care during pregnancy and lactation may impair neurobehavioral development [11–15]. In animals submitted to neonatal stress due to maternal separation, several neurobehavioral alterations are observed, such as increased anxiety-like behavior [16], alterations of the hypothalamus-pituitary-adrenal (HPA) axis [14,17], and changes in feeding behavior [8,18,19]. When submitted to early weaning, rodents show increased anxiety-like behavior, changes in eating behavior, increased response to stressors agents and decreased social interaction [20–26].

Early weaning is an experimental model consisting of abrupt and early interruption of the lactation in rats [23,25,27]. Thus, rats are precociously deprived of two important factors in modulating proper development, maternal care and lactation. Regarding feeding behavior, it is already observed that animals of both sexes submitted to early weaning have hyperphagia and males have increased palatable food intake [24–26]. However, little is known about the neurophysiological mechanisms behind these changes in these animals. What is currently known is that young females present modifications of gene expression in the serotonergic system, which seems to be related to the hyperphagia observed in these animals [26].

The serotoninergic system is precisely related to the control of food intake, as it signals satiety in the hypothalamus [28–30]. Also, the dopaminergic system is closely related to hedonic eating behavior, which is related to food reward, and is even associated with obesity when altered [31–33]. Due to this, our hypothesis is that the

changes on feeding behavior observed in early weaned animals are due to disruption of 5HT and DA systems. Thus, this study aims to investigate the effects of early weaning on feeding behavior as well as mRNA expression of key compounds of 5HT and DA systems in rats.

2. MATERIAL AND METHODS

2.1 Animals

Female *Wistar* rats (n=10) were obtained from the colony of the Department of Nutrition of the Federal University of Pernambuco. When pregnant, rats were kept individually in cages, in facilities with constant conditions of temperature (20ºC), luminosity (12-12h cycle, lights on at 6 pm) and free access to water and commercial laboratory chow (Presence®). At PND01, the litters were culled to 8 pups (4 males and 4 females, always as possible). Animal care comply with the ARRIVE guidelines and followed the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978), being conducted in agreement of the National Council of Control of Animal Experimentation (CONCEA), with the approval of the Animal Ethical Committee of the Federal University of Pernambuco (nº: 0020/2018).

2.2 Early weaning and experimental groups

From each litter, a maximum of 3 male pups was used to compose the experimental groups, established according to the age of weaning. The regular weaning group (RW) was composed of pups weaned at PND30. RW litters were kept with the dams until PND30, when they were separated from the dams and allocated in cages in groups of 02 or 03 [27,34]. The early weaning group (EW) was composed of pups weaned at PND15 [27,34]. At PND15, EW litters were separated from the dams and kept together until PND30, when they were separated in cages in groups of 02 or 03 rats per cage. During this period (PND15 - PND30) EW litters had access to crushed commercial laboratory chow in a recipient on the cage floor, and normal chow on cage dispenser, and RW litters had access to breastmilk from the dam and commercial laboratory chow available on cage dispenser. At least 03 days before the beginning of the tests, the rats were individually separated in cages.

2.3 Food intake after food deprivation

When rats were aged between PND250 and PND300, they were food deprived for a 4h-period, between 12:00 pm and 04:00 pm, during the dark phase, while individually allocated in cages (n = 07 – 08 per group). At 04:00 pm a known amount of commercial laboratory chow was offered to the rats and at 05:00 pm the remaining amount of food on cage dispenser was measured, and the food intake was assessed through the difference between offered and remaining food.

2.4 Food intake per phases of the circadian cycle

Between PND250 and PND300, while individually located in cages, rats had their food intake in the dark and light phases measured (n = 08 – 09 per group). Food intake was measured by weighing food available on cage dispenser at 06:00 am (lights off) and 06:00 pm (lights on). The difference of chow weight between 06:00 am and 06:00 pm was considered food intake in the dark phase, as well as the difference between 06:00 pm and 06:00 am was considered food intake in the light phase. To measure the food intake during the day (24h – period), we summed the food intake in dark and light phases.

2.5 Palatable food intake

Between PND250 and PND300, the animals (n = 08) were deprived of the commercial laboratory chow for 3 consecutive days between 13h and 14h, during which time they had access to chocolate cookies for habituation to the new food. 24h after the last day of habituation, the animals were again deprived of the standard food at the same time, when a known amount of chocolate cookies were offered, with the food weighing at the end of the period. The difference between the food offered and the rest in the cage dispenser was considered as palatable food intake.

2.6 Body mass

Rats aged between PND250 and PND300 and individually located in cages had their body mass accessed during the dark phase and were immediately returned to their home cages (n = 10 per group).

2.7 mRNA expression using real-time PCR

At the dark phase, within the time interval from 04:00 pm to 05:00 pm, without food deprivation or any stimulus, rats aged between PND250 and PND300 were euthanized by decapitation (n = 3 – 6 per group). After decapitation, the skulls were immediately dissected, and the brainstem and hypothalamus were collected and stored at -20°C. The total RNA of the samples was obtained by the guanidine isothiocyanate extraction method using the TRIzol reagent. Initially the tissues were lysed using TRIzol. After 5min incubation at room temperature, chloroform was added to the samples and then centrifuged at 10,625g for 15min. The aqueous phase obtained was transferred to another tube, incubated in ice-cold for 10min and then centrifuged at 10,625g for 10min. The formed RNA was then washed in 75% ethanol and centrifuged at 7,378g for 5min. The RNA pellet was then resuspended in RNAse free-water and stored. RNA quantification was performed in duplicate by diluting the samples in a ratio of 1:50 in RNAse free-water. The absorbance of the samples was determined by spectrophotometry at 260nm (corresponding to the peak of RNA uptake) and 280nm (corresponding to the peak of protein absorption). For RNA purity analysis, the absorbance value obtained at 260nm was divided by the value obtained at 280nm and samples with a 260/280 ratio equal to or greater than 1.8 were used (indicative of high purity).

Real-time PCR was performed with GoTaq® 1-Step RT-qPCR System kit, (Promega, USA), following the manufacturer's recommendations. Briefly, concentrations of 900ng of RNA, 10μmol of primer and two units of Taq polymerase were used, with a final reaction volume of 15μl [35]. The analyzes were performed on a Rotor-Gene Q thermocycler (Qiagen, USA). The sequences of primers used are shown in table 1.

All quantifications were normalized to the housekeeping gene as an internal control (β2M). Relative quantification of all target genes was analyzed using a comparative CT method [36].

Table 1. Sequence of primers designed for the study of *5ht1b*, *5ht2c*, *Sert*, and the reference constitutive gene *β2M.*

Primer	Forward (5' - 3')	Reverse (5' – 3')	Ref.*
5ht1b	AGAAGAAACTCATGGCCGCT	GGGGAGCCAGCACACAATAA	NM_012765.3
5ht2c	ATTTGTGCCCCGTCTGGATT	CGCGAATTGAACCGGCTATG	NM_012765.3
Sert	AGCATCTGGAAAGGCGGTCAA	ACACCCCTGTCTCCAAGAGT	NM_013034.4
Drd1	GTTTGTGTGGTTTGGGTGGG	GCTCATGGTGGCTGGAAAAC	NM_012546
Drd2	GAGCCAACCTGAAGACACCA	GCATCCATTCTCCGCCTGTT	NM_012547
β2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG	NM_012512.2

*Reference number on PUBMED.

2.8 Statistical Analysis

Regular T-tests were performed to evaluate the effects of early weaning, comparing EW *vs*. RW, by the following traits; (a) the 24-hour food intake, (b) food intake after food deprivation, (c) body mass, and (d) each mRNA expression. Two-way ANOVA with Bonferroni post-hoc was used to evaluate the effects of the early weaning, and comparing EW *vs*. RW, by the following traits; (a) food intake per phase of the circadian cycle. Further, the data obtained were presented in the form of Mean ± SEM, with the results being considered significant when $p < 0.05$. Moreover, the software GraphPad Prism v6 was utilized, for carrying out the analysis.

3. RESULTS

3.1 Early weaning increases food intake after food deprivation during later adult age in male rats

Regular t-test showed that EW increased food intake in male adult rats when compared to RW animals (t=2.283, df=12,99, p=0.0399) (Fig 1A).

3.2 Early weaning changed the feeding patterns in male adult rats

Regular t-test demonstrated that EW increased food intake in a 24h – period (t=3.188, df=15.99, p=0.0057) (Fig. 1B). ANOVA two-way showed interactions between early weaning and phases ($F_{1, 30} = 4{,}933$, $p = 0.0340$), and isolated efffect of

phases ($F_{1, 30}$ = 186,3, p < 0.0001), but no effects of early weaning ($F_{1, 30}$ = 2,989, p = 0.0941). Bonferroni post-hoc test demonstrated that early weaning increased food intake only in the dark phase (p = 0.0180) (Fig. 1C).

3.3 Early weaning incrases palatable food intake

Regular t-tests showed that EW promotes increase in palatable food intake (t=2,506 df=9,405, p = 0.0325) (Fig 1D).

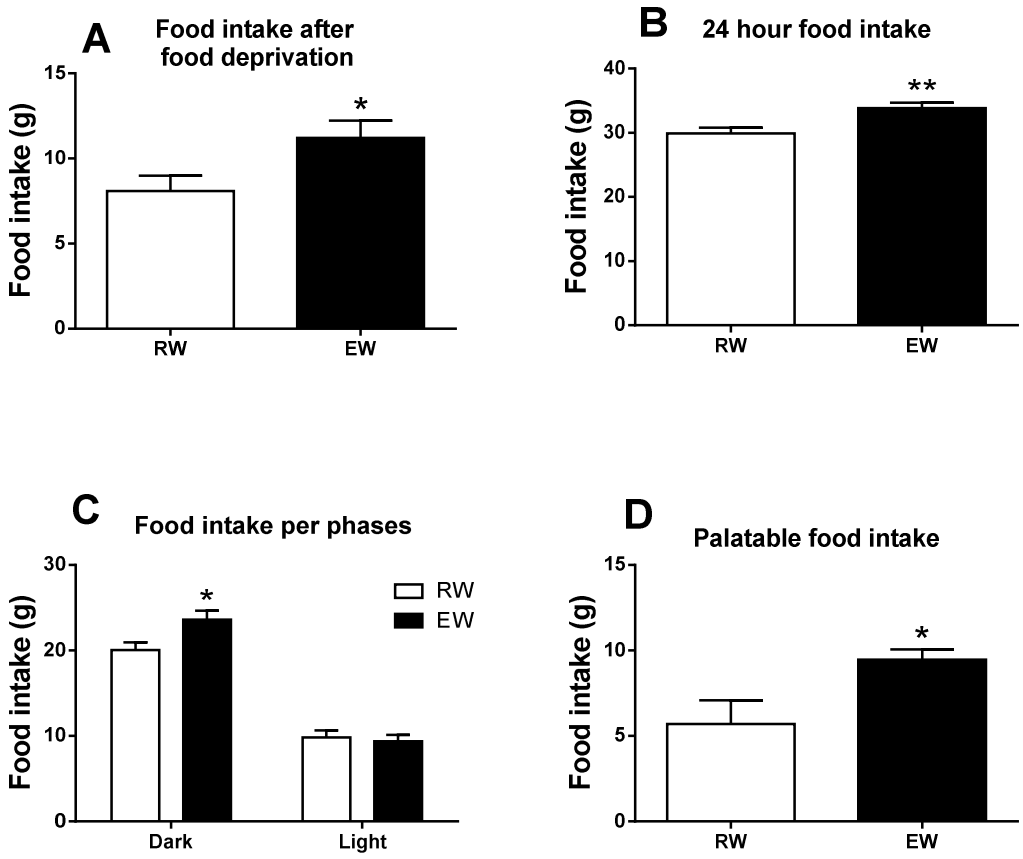

Figure 1. *The figure reveals the effects of the early weaning on the feeding patterns of male adult rats. Between PND250 and PND300, early weaned male adult rats had their food intake assessed after a 4-hour food deprivation (n = 07 – 08) (A) in a 24-hour cycle (n = 08 – 09) (B), in each phase of the circadian cycle (n = 08 – 09) (C), and also palatable food intake was measured (n = 08) (D). Data are expressed as the mean ± SEM, *p < 0.05, **p < 0.01. (□) Regular weaning, (■) Early weaning.*

3.4 Early weaning dos not modify the body mass of male adult rats

No differences on body mass were found between early weaning and regular weaning (t=1.117, df=16.85, p=0.2799) (Fig. 2).

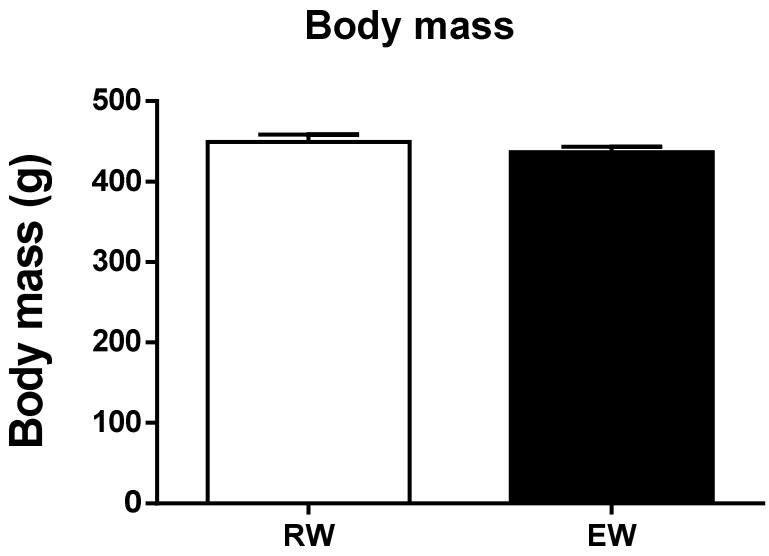

Figure 2. *The figure demonstrates the effects of early weaning on body mass of male adult rats. The body mass of early weaned male adult rats aged between PND250 and PND300 was measured (n = 10). Data are expressed as the mean ± SEM. (□) Regular weaning, (■) Early weaning.*

3.5 Early weaning modulated the mRNA expression of SERT and 5HT and DA receptors associated to food intake on brainstem and hypothalamus

On hypothalamus, EW increased the mRNA expression of SERT (t=4.185, df=6, p=0.0058), and DRD1 (t=5,560, df=10, p = 0.0002), but decreased the mRNA expression of 5HT-1b (t=2.862, df=10, p=0.0169) (Fig. 3A). Additionally, on brainstem the EW increased the mRNA expression of 5HT-1b (t=5.389, df=6, p=0.0017), 5HT-2c (t=2.793, df=6, p=0.0014) and SERT (t=3.652, df=5, p=0.0147), DRD1 (t=4,681, df=6, p = 0.0034), and DRD2 (t=6,520, df=6, p = 0.0006) (Fig. 3B).

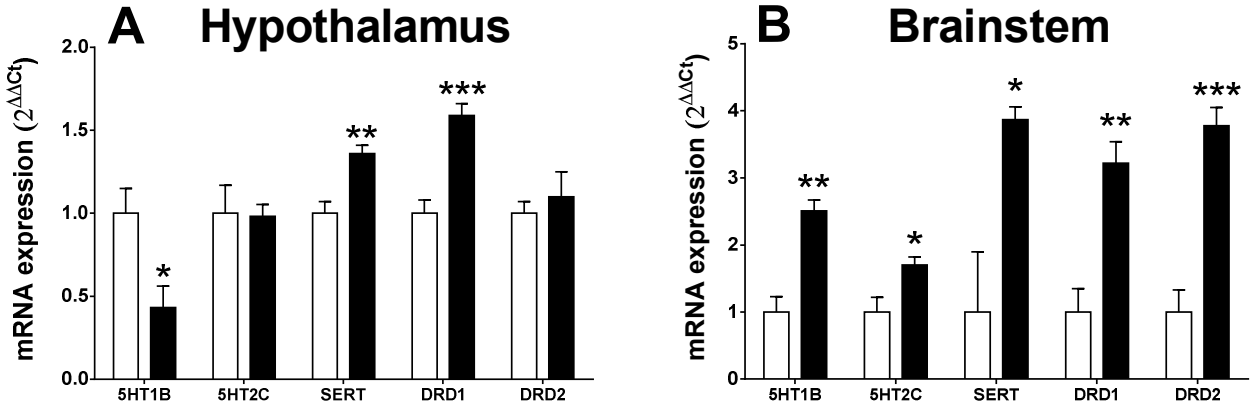

Figure 3. *The figure unveils the effects of early weaning on gene expression of 5HT and DA systems components on hypothalamus and brainstem. Rats were decapitated between 04:00 pm and 05:00 pm, in the dark phase of circadian cycle, and samples of hypothalamus and brainstem were collected (n = 03 – 06). Gene expression of 5HT-1b, 5HT-2c, SERT, DRD1 and DRD2 was performed in the hypothalamus (A) and brainstem (B). Data is shown as the mean ± SEM, *p<0.05, **p < 0.01, ***p < 0.001. (□) Regular weaning, (■) Early weaning.*

4. DISCUSSION

To the best of our knowledge, this is the first study that seeks to evaluate the effects of early weaning on long-term eating behavior and neurotransmitter systems. Also, no study has sought to evaluate the effects of early weaning on hedonic eating behavior at the molecular level. Here, we observed that early weaning promoted hyperphagia with a standard diet and increased palatable food intake in adult male rats, associated with changes in mRNA expression of 5HT and DA systems components. Thus, this study provides new insights into the effects of early life stressors such as early weaning on neurobehavioral development.

When subjected to homeostatic food intake tests, the rats showed increased food intake in response to 4h food deprivation, during the 24h cycle, and the dark phase of the circadian cycle. Other studies using the same weaning model also point out that this stress can promote hyperphagia, a delay in the satiety point when submitted to the behavioral satiety sequence test, with a consequent increase in meal size [25]. In other study, also with the same weaning model, hyperphagia and feeding rhythm modulation were observed in the 24h cycle in young female rats submitted to early weaning [26]. Using an early weaning model with teat bandages of dams in the last 3 days of lactation (PND17-PND20) it was also observed hyperphagia in adult male rats [37]. In another early weaning model that uses bromocriptine to inhibit lactation, no changes in food intake are observed, but several metabolic disorders are observed, such as increased body mass, increased fat mass and leptin resistance, suggesting susceptibility to the development of obesity in these animals [15,38]. Despite the differences observed in the literature, the evidence that early weaning changes the eating behavior of rats are strong.

In addition to homeostatic modifications, changes in rats' hedonic eating behavior were also observed. When submitted to the palatable food intake test, the early weaned rats presented increased intake. Similar data were observed by Oliveira, who demonstrated that adult male rats show increased palatable food intake and preference for high-fat food [24]. Thus, we observed that early weaning alters both homeostatic and hedonic food intake, suggesting susceptibility to physiological disorders such as obesity and metabolic syndrome.

Interestingly, when body mass was assessed, no changes were observed. In the literature, no changes were observed in body mass of early weaned male rats up to

PND150[25]. However, in a study conducted with young females, it was demonstrated that the same model of early weaning used in this study is capable of promoting body mass increase [26]. These inconsistences may be due to sexual differences, once males and females differently respond to challenges in their growth periods [39,40]. Hence, further studies are necessary to elucidate those sex differences.

Regarding the 5HT system, the data show that early weaning promoted the hypothalamic increase of SERT mRNA expression, but decreased 5HT1B mRNA expression. The 5HT system is well known to signals satiety trough the action of 5HT on receptors 5HT1B, which inhibits hungry and trough receptors 5HT2C, which signals satiety [28]. In the hypothalamus, SERT acts by promoting serotonin reuptake into presynaptic terminals, whereas the 5HT1B receptor acts by stimulating satiety by inhibiting orexigenic neurons in the arcuate nucleus [28,29,41,42]. The increase in SERT along with the 5HT1B decrease seems to be related to the hyperphagia presented by the animals, since increased SERT may lead to lower serotonin availability in the synaptic clefts and the decrease of 5HT1B may impair satiety signaling. Thus, both modifications appear to act synergistically in the hypothalamus to decrease satiety signaling. In the brainstem, increased gene expression of SERT, 5HT1B and 5HT2C was observed. Tavares et al., (2019) found the same result in young female rats and suggested a mechanism for inhibiting the activity of 5HT neurons in the brainstem, where serotonin is mainly produced, specifically in the raphe nuclei [43], as SERT, 5HT1B and 5HT2C have inhibitory activity on brainstem serotonergic neurons [42,44–47]. Thus, the hypothalamus and brainstem appear to act together to decrease serotonergic activity and satiety, suggesting that early weaning programs the rats to be hyperphagic.

On the other hand, the DA system has been more associated with the control of palatable food intake, although evidence also indicates its participation in the homeostatic regulation of food intake [31,33,48,49]. Here we find increased DRD1 mRNA expression in the hypothalamus and increased DRD1 and DRD2 mRNA expression in the brainstem. DRD1 receptors present postsynaptic action, thus being responsible for the DA signaling response [50,51]. In the hypothalamus, increased levels of this receptor have been associated with increased both standard food intake and palatable food intake in case of obesity [52,53]. The DRD2 receptor acts as a self-receptor, inhibiting DA neuron activity and consequent DA release [51]. In the midbrain, the rostral region of the

brainstem, where the main bodies of the DA neurons are found, specifically in the ventral tegmental area (VTA) and substantia nigra (SN), only DRD2 expression is described [54]. Increasing this receptor in this brainstem region suggests decreased activity of DA neurons. Still in the brainstem, DRD1 receptors present localization in several areas, among them the raphe nuclei, and seem to be more related to food reward signaling through circuits between the raphe nuclei and VTA and SN [33]. Studies show that abuse of reward-promoting substances is usually accompanied by a deficiency in neural signaling of this reward, which causes the individual to seek this reward incessantly [55]. Thus, the possibility of inhibition of DA neurons may be generating a compensatory mechanism, with increased expression of DRD1, in an attempt to signal reward. And it is this flaw that may be associated with the increased palatable food intake noted here, since the early weaned rats seem to need to eat more palatable food for the food reward to be recognized. This type of effect caused by perinatal insults is well described in the literature. Rodents submitted to neonatal maternal separation, perinatal malnutrition or even obesogenic diets in the perinatal period have increased palatable food intake and dopaminergic system dysfunction [8,53,56,57].

Of course, we cannot dissociate homeostatic and hedonic systems and behaviors. Today we know that both act in an integrated manner for the expression of behavior. We already know that the 5HT system, which has long been rated as a homeostasis regulator, also acts on hedonic signaling, just as the DA system that has long been rated as a food reward regulator, also acts as a regulator of homeostasis. However, despite this difficulty in associating these two systems, we note here that early weaning causes a central disorder on eating behavior that involves both 5HT and DA systems. In fact, we cannot assume these data for all individuals as a whole because the study was conducted only with males, and we know there are various sexual differences, especially regarding hormonal regulation, that could affect neurotransmission systems. In addition, some limitations of this study were the evaluation of brain regions as a whole and the lack of protein expression evaluation, since we know that changes in mRNA do not always cause changes in protein expression. Thus, further studies are needed to fill this gap. However, relevant data are presented which is consistent with behavioral data and other data already presented in the literature about the effects of early weaning on the long-term feeding behavior of rats.

5. CONCLUSION

Neonatal stress caused by early weaning, which consists of early and abrupt interruption of breastfeeding and maternal care, promoted changes in the eating behavior of male rats in adulthood. According to the data presented here, both homeostatic and hedonic eating behavior were sensitive to early weaning, and the 5HT and DA systems were modulated, which seems to be associated with behavioral changes. Further studies are needed to fill some gaps present in this study. However, the present study provides important data about the association of disorders during the critical periods of development with the onset of neurobehavioral disorders that can lead to problems such as obesity.

Author contributions

SS proposed the idea; GT and SS designed the study; GT, LA, FS, and VV conducted the behavioral assessments; GT, CL, and SCS conducted the molecular assays and the partial (i.e. molecular assays) statistical analyses; GT, JS, BK and SS analyzed the data, conducted the statistical analyses, and wrote the manuscript; GT and BK edited the manuscript. All the authors agreed with the final article and declare that there is no conflict of interests.

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