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Intrauterine growth restriction increases the preference for palatable foods and affects sensitivity to food rewards in male and female adult rats

Roberta Dalle Molle[a,*], *Daniela Pereira Laureano*[b], *Márcio Bonesso Alves*[b], *Tatiane Madeira Reis*[a], *Mina Desai*[c], *Michael G. Ross*[c], *Patrícia Pelufo Silveira*[a,b]

[a]*Programa de Pós-Graduação em Saúde da Criança e do Adolescente. Faculdade de Medicina, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2350, Largo Eduardo Zaccaro Faraco, CEP 90035-903 Porto Alegre, RS, Brazil*
[b]*Programa de Pós-Graduação em Neurociências. Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, Sala 107 (Campus Centro), CEP 90046-900 Porto Alegre, RS, Brazil*
[c]*Department of Obstetrics and Gynecology, Harbor-UCLA Medical Center, 1124 West Carson Street, Box 446, RB-1 Bldg., Torrance, CA 90502, USA*

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ABSTRACT

Clinical evidence suggests that intrauterine growth restriction (IUGR) can cause persistent changes in the preference for palatable foods. In this study, we compared food preferences, the response to food rewards, and the role of the mesolimbic dopaminergic system in feeding behavior, between IUGR and control rats. Time-mated pregnant Sprague–Dawley rats were randomly allocated to a control group (standard chow *ad libitum*) or a 50% food restriction (FR) group, which received 50% of the control dams' habitual intake. These diets were provided from gestation day 10 to the 21st day of lactation. Within 24 h of birth, pups were cross-fostered and divided into four groups: *Adlib/Adlib, FR/Adlib, FR/FR, Adlib/FR*. Standard chow consumption was compared between all groups. Food preferences, conditioned place preference to a palatable diet, and the levels of tyrosine hydroxylase (TH) phosphorylation and D2 receptors in the nucleus accumbens were analyzed and compared between the two groups of interest: *Adlib/Adlib* (control) and *FR/Adlib* (exposed to growth restriction during the fetal period only). IUGR adult rats had a stronger preference for palatable foods, but showed less conditioned place preference to a palatable diet than controls. D2 receptors levels were lower in IUGR rats. At baseline, TH and pTH levels were higher in FR/Adlib than control males. Measurements taken after exposure to sweet foods revealed higher levels of TH and pTH in FR/Adlib than control females. These data showed

Abbreviations: Adlib, *Ad libitum*; CPP, conditioned place preference; FR, food restriction; GEE, Generalized Estimating Equations; IUGR, intrauterine growth restriction; NAcc, nucleus accumbens; OD, optic density; PFC, prefrontal cortex; pTH, phospho-tyrosine hydroxylase; SGA, small for gestational age; TH, tyrosine hydroxylase; VTA, ventral tegmental area

*Corresponding author.
E-mail address: betinha_dm@yahoo.com (R. Dalle Molle).

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that IUGR rats exhibited a preference for palatable foods, potentially due to alterations in their mesolimbic reward pathway. Additionally, the changes observed in the mesolimbic dopaminergic system of IUGR rats proved to be sex-specific.

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1. Introduction

Environmental events in early life, especially those related to nutrition, can lead to adaptations in growth and metabolism which may increase the risk of chronic conditions in adulthood (Barker, 2004), including cardiovascular disease (Barker et al., 2005), hypertension (Law et al., 2002), insulin resistance (Barker, 1999; Eriksson et al., 2002) and obesity (Pilgaard et al., 2011; Ravelli et al., 1999). According to Barker (2006), this developmental programming is more strongly associated with low birth weight for gestational age (*i.e.*, intrauterine growth restriction; IUGR) than with premature birth. The interaction between fetal life events and subsequent environmental factors may also result in an increased or decreased risk for the aforementioned conditions (Barker, 2004). As such, exposure to certain foods over the life course (*e.g.* palatable foods, rich in sugar and/or fat) could modulate the development of chronic non-communicable diseases in adult individuals with IUGR.

Several recent clinical studies have found an association between IUGR and offspring preference for highly palatable foods (rich in sugar and/or fat) throughout the life course. These findings point to a persistent programming effect with behavioral repercussions ranging from impulsiveness for sweet rewards in childhood (Silveira et al., 2012), to a preference for carbohydrates and low fruit and vegetable intake in young adulthood (Barbieri et al., 2009; Kaseva et al., 2013), and higher fat intake in older age (Lussana et al., 2008; Perala et al., 2012; Stein et al., 2009).

IUGR seems to program feeding behavior before metabolic changes (*e.g.* insulin and leptin resistance, obesity) have taken place, as evidenced by the distinct responses to sweet stimuli observed in individuals with IUGR from the very first day of life (Ayres et al., 2012). The preference for palatable foods may lead to subtle but persistent nutritional imbalance, and contribute to the development of obesity and chronic diseases in adulthood (Portella and Silveira, 2014).

The apparent specificity of the effects of IUGR on responses to palatable foods suggests that the brain mechanisms involved in food rewards are likely to be implicated in this phenomenon. Vucetic et al. (2010) found that the offspring of pregnant females exposed to protein restriction during gestation show an increased number of tyrosine hydroxylase (TH) immunoreactive neurons in the ventral tegmental area (VTA), increased expression of DAT in the VTA and nucleus accumbens (NAcc), and higher levels of dopamine in the prefrontal cortex (PFC). Not surprisingly, these changes were associated with dysfunctions in dopamine-dependent behaviors, including preference for sweet solutions and hyperactivity in response to cocaine or a high fat diet. However, as far as we know, the cerebral dopamine mechanisms linked to altered feeding behavior and food choices in animal models of IUGR induced by maternal food restriction have not yet been explored. Therefore, the aim of this study was to evaluate food preferences and food reward, as well as the putative role of the mesolimbic dopaminergic system in these behaviors in IUGR rats.

2. Results

2.1. *Feeding behavior: standard rat chow consumption and food preference*

Rats were habituated to cages equipped with BioDAQ® monitoring systems. During this period, no interactions between group and time were observed (GEE, Wald=12.26; gl=6; $p=0.056$). During these first three days of the study, when only standard chow was available, FR/Adlib males ate more than Adlib/Adlib (Bonferroni $p=0.024$) and Adlib/FR males (Bonferroni $p=0.005$) (interaction group*sex, Wald=16.26; gl=3;$p=0.001$). In females, standard chow consumption was found to be higher in the Adlib/Adlib group than in the FR/Adlib (Bonferroni $p=0.042$) and Adlib/FR groups (Bonferroni $p=0.009$). Habituation was faster in males (Bonferroni $p<0.001$) than females (Bonferroni $p=0.015$) (interaction sex*time, Wald=19.34; gl=2; $p<0.001$) (Fig. 1). In both sexes, food consumption increased over time.

Standard rat chow consumption in the 24 h following habituation was analyzed by two-way ANOVA with group and sex as independent factors and body weight as a covariate. No main effects or interactions were observed on any of the variables except for meal size, which was influenced by a group by sex interaction [F(3,49)=3.64; $p=0.019$],

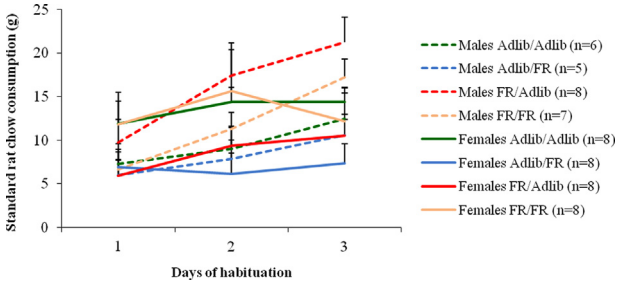

Fig. 1 – Standard rat chow consumption during the habituation period. Data expressed as mean ± SEM. Means adjusted for body weight.

whereby FR/Adlib males and FR/FR males ate larger meals than females in the same groups (Tables 1 and 2).

A comparison of the two groups of interest (Adlib/Adlib and FR/Adlib) in the food preference test revealed a higher preference index for the highly palatable diet in the FR/Adlib group (1.00±0.20) as compared to the Adlib/Adlib group (0.94±0.20) ($p=0.05$). This group effect was more consistent when the dark cycle was evaluated separately [$F(1,27)=4.64$; $p=0.04$] (Fig. 2). No main effects of sex [$F(1,27)=2.66$;$p=0.115$] or interactions between variables [$F(1,27)=1.44$; $p=0.24$] were identified.

2.2. *Conditioned place preference (CPP)*

Rats have a natural preference for dark environments (Matsuo and Tsuji, 1989), and as such, all animals spent more time in the dark side of the cage on day 1 (mean±SD of the percentage of time spent on the dark side at baseline (day 1)=66.54±10.58). The FR/Adlib group showed less conditioning to the lit side (which was paired with the palatable food) than the Adlib/Adlib group, as evidenced by their significantly lower delta values (time spent in the light side on test day - time spent in the light side at baseline), [Two-Way ANOVA, $F(1,22)=5.80$; $p=0.025$] (Fig. 3). No effects of sex [$F(1,22)=3.76$; $p=0.065$] or interaction between variables were observed [$F(1,22)=0.16$; $p=0.69$].

2.3. *Tyrosine hydroxylase (TH) and phospho-tyrosine hydroxylase (pTH) in the NAcc*

Figs. 4 and 5 show the levels of TH and pTH in the NAcc of rats in each group normalized to the control condition (Adlib/Adlib baseline or Adlib/Adlib sweet). In males (Fig. 4A–B), a two-way ANOVA revealed an interaction between group and metabolic status on TH [$F(1,16)=4.716$; $p=0.045$] and pTH levels [$F(1,17)=7.155$; $p=0.016$], with the FR/Adlib group showing increased TH and pTH levels at baseline as compared to control animals (Absolute OD TH/β actin values of controls: baseline=1.23; sweet=1.31. Absolute OD pTH/β actin values of controls: baseline=0.06; sweet=0.63). In females (Fig. 5A and B), an interaction between group and metabolic status was also found to affect TH [$F(1,18)=5.699$; $p=0.028$] and pTH levels [$F(1,18)=4.958$; $p=0.039$], with the FR/Adlib group showing higher TH and pTH levels than control rats after sweet food intake (Absolute OD TH/β actin values of controls: baseline=1.29; sweet=1.35. Absolute OD pTH/β actin values of controls: baseline=0.59; sweet=0.32).

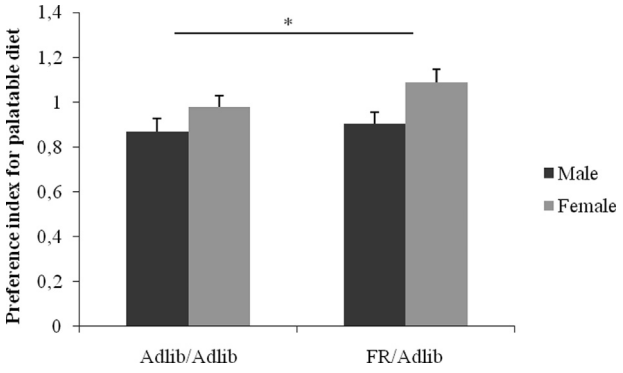

Fig. 2 – Preference index for palatable diet during dark cycles in Adlib/Adlib (males: $n=8$, females: $n=8$) and FR/Adlib (males: $n=8$, females: $n=8$) animals. Data expressed as mean±SEM. A two-way ANOVA revealed a main effect of group (*$p=0.04$). Means adjusted for body weight.

Table 1 – Characterization of males' standard rat chow consumption (24 h period).

Measures	Adlib/Adlib ($n=6$)	FR/Adlib ($n=8$)	Adlib/FR ($n=5$)	FR/FR ($n=7$)
24 h consumption (g)	17.26±4.25	23.83±3.49	21.88±3.25	21.05±2.52
24 h consumption (kcal)	50.92±12.54	70.30±10.29	64.55±9.59	62.10±7.43
Number of bouts	67.11±14.34	79.10±11.79	76.66±10.96	72.30±8.49
Bout size (g/bout)	0.18±0.069	0.32±0.057	0.29±0.053	0.35±0.041
Number of meals	9.26±2.05	9.12±1.68	9.07±1.56	8.24±1.21
Meal size (g/meal)*	1.89±0.69	3.22±0.57[a]	2.81±0.53	2.81±0.41[a]

Two-way ANOVA; data are expressed as mean±S.E.M.
Analyzes adjusted for body weight.
[a,b] different letters show significant difference ($p<0.05$).
* Compare to Table 2.

Table 2 – Characterization of females' standard rat chow consumption (24 h period).

Measures	Adlib/Adlib ($n=8$)	FR/Adlib ($n=8$)	Adlib/FR ($n=8$)	FR/FR ($n=8$)
24 h consumption (g)	16.04±2.37	13.34±2.76	10.99±3.02	12.13±3.29
24 h consumption (kcal)	47.32±6.99	39.53±8.14	32.42±8.91	35.78±9.70
Number of bouts	57.48±7.99	46.09±9.33	37.30±10.19	37.91±11.10
Bout size (g/bout)	0.30±0.039	0.32±0.045	0.33±0.049	0.34±0.054
Number of meals	7.69±1.14	9.43±1.33	8.28±1.45	11.27±1.58
Meal size (g/meal)*	2.27±0.39	1.42±0.45[b]	1.36±0.49	1.21±0.54[b]

Two-way ANOVA; data are expressed as mean±S.E.M.
Analyzes adjusted for body weight.
[a,b] different letters show significant difference ($p<0.05$).
* Compare to Table 1.

2.4. D2 dopamine receptors in the NAcc

D2 receptor protein levels were found to be lower in the NAcc of FR/Adlib males (Absolute D2/ β actin values: Adlib/Adlib=1.40±0.15; FR/Adlib=0.82±0.05) (Student's *t*-test, t=3.027, $p=0.016$) and females (Absolute D2/β actin values: Adlib/Adlib=2.14±0.16; FR/Adlib=1.12±0.11) (Student's *t*-test, t=5.107, $p<0.001$) than in control animals.

3. Discussion

This study demonstrated that IUGR adult rats show a preference for highly palatable foods, are less prone to conditioned place preference for this type of food, and have significant alterations in the mesolimbic reward pathway, as evidenced by altered levels of TH, pTH and D2 receptor proteins in the NAcc.

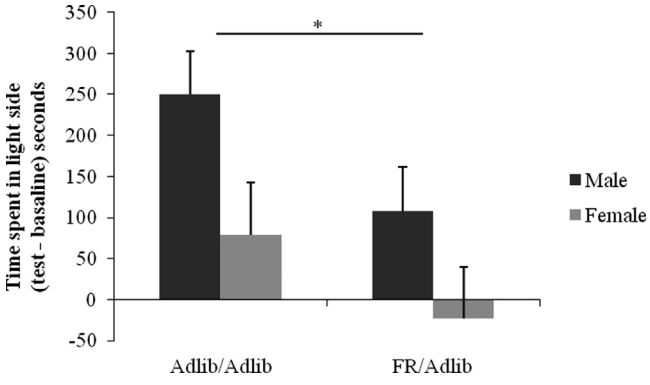

Fig. 3 – Time spent by Adlib/Adlib (males: $n=8$, females: $n=6$) and FR/Adlib (males: $n=8$, females: $n=6$) rats in the lit side of the chamber (test - baseline) during the conditioned place preference test. Data expressed as mean±SEM. A two-way ANOVA showed a main effect of group (*$p=0.025$). Means adjusted for mean palatable food intake during training, and number of chamber crossings on test day.

Standard rat chow consumption after habituation to cages equipped with BioDAQ® did not differ between groups. This finding is in agreement with that of Desai et al. (2005), who found that the increased consumption of standard chow observed in IUGR rats only lasted until the 8th week of life (after adjusting the analysis for body weight). However, an interesting result was obtained during the habituation period: although all groups habituated to the new environment over the three-day interval, IUGR rats (FR/Adlib), but not controls (Adlib/Adlib), displayed a sex-specific pattern of adaptation.

The interaction between group and sex during the habituation period may reflect sex-specific differences in the adaptation to new environments in IUGR offspring, since these differences were observed only during, but not following, habituation. Grissom and Reyes (2013) observed that small for gestational age (SGA) rats are hyperactive in light–dark boxes, indicating hypersensitivity to the stress of the novel chamber, and providing indirect support for increased anxiety in SGA animals. In our study, the sex differences in the chow consumption of IUGR rats during the habituation period could be related to variations in the interpretation of new environmental cues or to a differential response to the stress of the new apparatus in males *vs.* females, resulting in higher chow consumption in IUGR males and lower consumption in IUGR females during this period. Interestingly, other studies from our research group had also found that FR/Adlib males and females behave in opposite ways when placed in an environment containing a running wheel, with IUGR males showing decreased spontaneous physical activity and IUGR females showing increased activity as compared to controls (Cunha Fda et al., 2015). Thus, it appears that IUGR males and females exhibit a peculiar pattern of energy intake/expenditure when facing a novel environment.

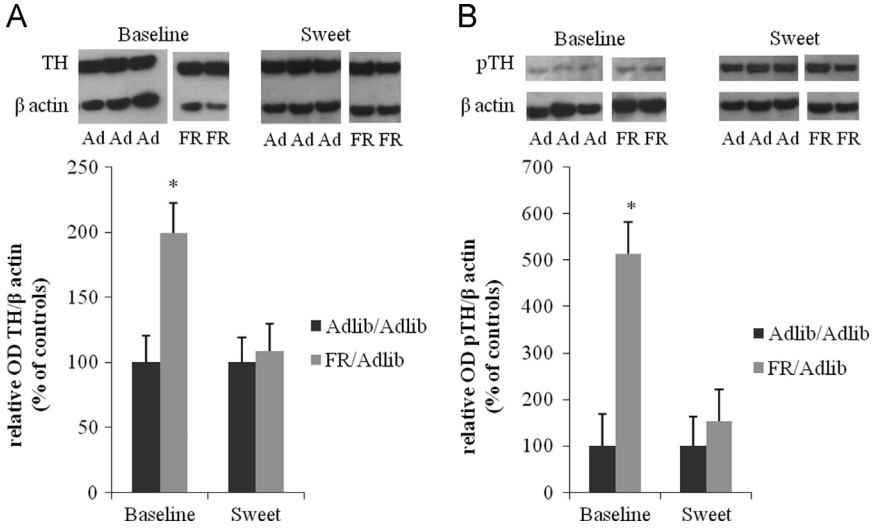

Fig. 4 – (A) TH and (B) pTH levels in the nucleus accumbens of male rats at baseline ($n=4-5$) and after exposure to sweet food ($n=5-6$). Data expressed as percent of control levels (Adlib/Adlib group). A two-way ANOVA showed a significant group*metabolic status interaction on TH (*$p=0.045$) and pTH levels (*$p=0.016$). Absolute OD TH/β actin values in control rats: baseline=1.23; sweet food=1.31. Absolute OD pTH/β actin values in control rats: baseline=0.06; sweet food=0.63.

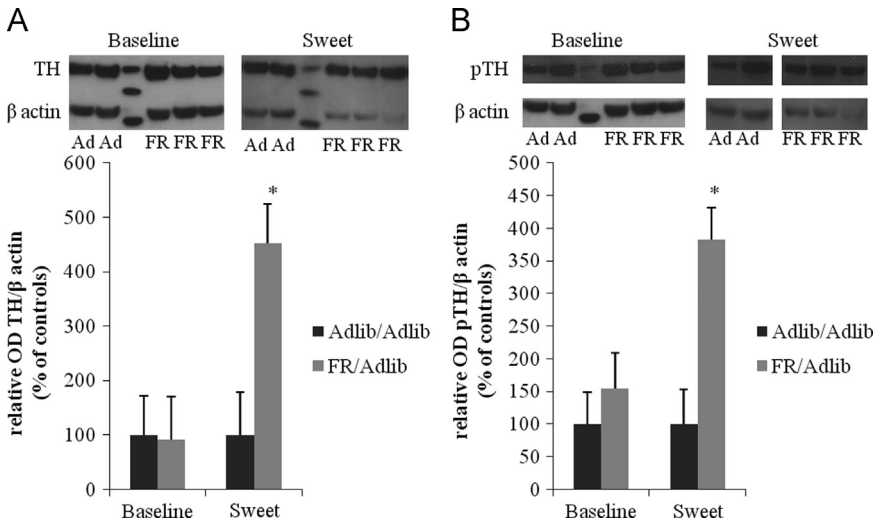

Fig. 5 – (A) TH and (B) pTH levels in the nucleus accumbens of female rats at baseline ($n=5-6$) and after exposure to sweet food ($n=5–6$). Data expressed as percent of control levels (Adlib/Adlib group). A two-way ANOVA showed a significant group*metabolic status interaction on TH (*$p=0.028$) and pTH levels (*$p=0.039$). Absolute OD TH/β actin values of control rats: baseline=1.29; sweet food=1.35. Absolute OD pTH/β actin values of control rats: baseline=0.59; sweet food=0.32.

When exposed simultaneously to a highly palatable diet and the standard rat chow, IUGR rats showed a stronger preference for the former. Vickers et al. (2000) also observed hyperphagia in adult IUGR animals (30% of *ad libiutm* diet throughout gestation) when exposed to a high calorie diet. Similarly to the present study, Bellinger et al. (2004) offered the choice between three types of diet (high-fat, high-protein and high carbohydrate) to adult rats exposed to protein restriction prenatally and have noticed a preference for the high-fat diet. However, our study is the first, to our knowledge, to assess the feeding preference of adult IUGR rats exposed to 50% food restriction, resulting in maternal undernutrition from gestation day 10. The association found between IUGR and preference for highly palatable food corroborates the results of some studies performed in humans (Barbieri et al., 2009; Lussana et al., 2008).

This study also assessed NAcc dopamine release, albeit indirectly, by evaluating TH and pTH levels in the NAcc of animals exposed to sweet food *vs.* those unexposed to such stimuli (baseline). TH is the rate-limiting enzyme in dopamine synthesis and therefore the principal regulator of cytosolic dopamine levels (Dunkley et al., 2004). Phosphorylated TH serine 40 is one of the two major active forms of TH, and its levels are positively related to the speed of dopamine synthesis (Dunkley et al., 2004). We found increased TH and pTH levels in FR/Adlib males at baseline and in FR/Adlib females after sweet food intake. The latter results are in accordance with studies showing increased NAcc dopamine in response to sucrose licking (Hajnal and Norgren, 2001; Hajnal et al., 2004). These findings are also corroborated by the fact that FR/Adlib females showed a stronger response to sweet food than Adlib/Adlib females. The TH and pTH levels displayed by IUGR males are indicative of increased dopamine synthesis even in the absence of a stimulus (sweet food). This finding agrees with previous studies reporting higher TH activity (Marichich et al., 1979) and an increased number of TH immunoreactive neurons in the VTA (Vucetic et al., 2010) of animals exposed to perinatal and prenatal protein restriction, respectively. The increased TH levels observed at baseline also agree with another study which evaluated early adverse life events using a model of postnatal overfeeding (Portella et al., 2014). In our study, the absence of differences between male IUGR and control rats following exposure to sweet foods could be explained by a ceiling effect. The sex differences observed in pTH and TH levels following sweet food intake once again indicate sex specificity in the fetal programming of food preferences in IUGR rats.

Intriguingly, IUGR rats—known to prefer highly palatable foods and to have higher baseline TH phosphorylation—showed less conditioned place preference in response to palatable foods than control animals. Studies have demonstrated this type of conditioning to be dopamine dependent, since the blockage of certain dopamine receptors decreases conditioning to some types of drug in this same paradigm (Cervo and Samanin, 1995; Cunningham et al., 2000; Hachimine et al., 2014; Maldonado et al., 1997). In line with this reasoning, we found decreased levels of D2 receptors in the NAcc of IUGR rats compared to controls. We recognize that there is little consensus in the literature regarding the importance of D2 receptors for CPP conditioning to drugs (Nazarian et al., 2004; Smith et al., 2002; Welter et al., 2007). However, our results demonstrate an association between decreased D2 receptors in the NAcc and less CPP in IUGR rats using palatable food as a reward. Other researchers have obtained similar results in animal models of adverse early life events, though the mechanisms underlying this relationship may differ from those involved in the present study. For instance, Portella et al. (2014) found that animals exposed to postnatal overfeeding did not develop a CPP to sweet foods and had decreased levels of dopamine D2 receptors in the NAcc. Silveira et al. (2010) also obtained the puzzling findings of increased palatable food intake accompanied by reduced

CPP in a model of early life stress (neonatal handling). Although these data suggest that adverse early life events may be linked to alterations in the functioning of the mesolimbic dopamine pathway, we do not yet know whether the lack of CPP in these animals represents a "pure" measure of the reward value of palatable foods or if it reflects a difference in the metabolic value of food between experimental and control animals. Lastly, it is important to note that other research groups have also described apparently discrepant findings concerning reward-seeking behavior and the actual pleasure elicited by the reward (Tindell et al., 2009).

These apparently contradicting results (fewer D2 receptors and a greater preference for highly palatable foods) are consistent with the reward deficiency syndrome theory, developed based on the lower density of striatal D2 receptors observed in obese individuals (Morton et al., 2006; Stice et al., 2008; Wang et al., 2001). According to this theory, the low density of striatal D2 receptors could lead to the overconsumption of energy-dense foods in an attempt to increase dopamine levels (Stice et al., 2010; Wang et al., 2004). Researchers speculate that the association between obesity and lower D2 receptor density could be explained by an insensitive reward system (Wang et al., 2004). Therefore, the diminished levels of D2 receptors found in IUGR rats may help explain the decreased reward sensitivity observed in these animals during CPP, as well as their increased palatable food intake during the preference test.

3.1. *Conclusions*

IUGR rats have a uniquely programmed mesolimbic reward pathway which favors a preference for highly palatable foods (rich in fat and/or sugar). According to our results, the mesolimbic dopaminergic system is likely to play an important role in the food preferences of IUGR rats. We propose that the excess food intake and preference for highly palatable foods displayed by IUGR individuals may place them at greater risk for obesity and related comorbidities. Further knowledge regarding these behaviors and the mechanisms involved in their development may help design programs to prevent the development of chronic diseases.

4. Experimental procedures

Primiparous Sprague Dawley rats (CEMIB, Campinas, SP, Brazil) of approximately 70 days of age were single-housed in Plexiglas home cages (49 cm × 34 cm × 16 cm) and maintained in a controlled environment: standard dark/light cycle (lights on between 7:00 a.m. and 7:00 p.m.), temperature of 22±2 °C, cage cleaning once a week, food and water provided *ad libitum*. Estrous cycle was determined daily by vaginal smearing, and females were placed with males only when receptive (proestrous). Sixteen males were used for mating. Gestation was confirmed at day 1 by sperm-positive vaginal smears. On gestation day 10, dams were randomly allocated to a control group (*Adlib*), which received an *ad libitum* diet of standard laboratory chow (NUVILAB®) or a 50% food restriction group (FR), which was given 50% of the intake of the *ad libitum*-fed dams (determined by the quantification of normal intake in a cohort of pregnant Sprague Dawley rats) (Cunha Fda et al., 2015; Desai et al., 2005). These diets were provided from gestation day 10 through to the 21st day of lactation. Within 24 h of birth, all pups were weighed and litters were culled to eight (4 males and 4 females) before being cross-fostered to other dams forming the following groups based on maternal diet during gestation/lactation: *Adlib/Adlib, FR/Adlib, Adlib/FR, FR/FR*. Since our objective was to investigate the fetal programming effects of IUGR, data analysis focused mainly on the two groups of interest: *Adlib/Adlib* (control) and *FR/Adlib* (growth restriction during the fetal period, but not during lactation). Evidence has already identified important metabolic changes in adult FR/Adlib animals, such as increased body weight and percent body fat, as well as higher plasma leptin levels, insulin resistance and hypertriglyceridemia. However, FR/FR and Adlib/FR pups do not present these same metabolic alterations in adulthood (Desai et al., 2005, 2007). Thus, the analysis of food preference in groups with worse metabolic outcomes might contribute significantly to our understanding of the relationship between changes in feeding behavior and the development of chronic diseases in adulthood. Data from the other experimental groups were utilized in a separate study.

On postnatal day (PND) 21, pups were weaned, separated into groups of two or three same-sex individuals per cage, and kept in a controlled environment as previously described. Sixty rats (Adlib/Adlib males=8; Adlib/Adlib females=8; FR/Adlib males=8; FR/Adlib females=8; Adlib/FR males=5; Adlib/FR females=8; FR/FR males=7; FR/FR females=8), derived from 24 litters, were subjected to behavioral tasks starting at approximately PND80. Animals were weighed before the tasks and prior to decapitation using a scale accurate to 0.01 g (Marte®, Canoas, Brazil). No more than two same-sex pups per litter were randomly allocated to each experimental condition.

All procedures were approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (GPPG/HCPA, project number 12-0353), and performed according to National (Brazilian Law No. 11,794, 2008) and International (Directive 86/609/EEC) guidelines for animal research. All efforts were made to minimize animal suffering and reduce the number of animals used in the study. Tasks were performed in climate-controlled rooms within our animal research facility (Unidade de Experimentação Animal/HCPA).

4.1. *Feeding behavior: standard rat chow consumption and food preference*

At ~80 days of life, rats were transferred into cages equipped with BioDAQ® monitoring systems (Research Diets) consisting of two food hoppers mounted on electronic strain gauge-based load cells to measure food intake. Food hoppers were weighed 50 times/second (accurate to 0.01 g) and the mean and standard deviation (SD) of food intake over the course of each second were calculated by a peripheral computer. Feeding was signaled by a fluctuation in food hopper weight (defined as a S.D > 2000 mg) caused by the animal eating. Each feeding event (cage/animal number, start date and time, feeding duration, final hopper weight and amount eaten)

was recorded and exported to a central computer, where it was entered into a Microsoft Excel spreadsheet (Microsoft, Redmond, WA) for the calculation of desired parameters (see below) and data summarization.

The system recorded two types of feeding event: bouts and meals. The end of a feeding bout was signaled when the hopper was left undisturbed for 5 s (defined as a S.D < 2000 mg), at which point the duration of the feeding event and the amount eaten (initial hopper weight minus final hopper weight) was calculated. A meal was defined as a difference in hopper weight of > 0.1 g, separated from other feeding bouts by > 15 min (Eckel et al., 1998; Surina-Baumgartner et al., 1995).

During the experiment, rats were individually housed for 8 days in cages equipped with a BioDAQ® system. In the first 4 days, rats were given access to standard rat chow (2.95 Kcal/g, 15% protein, 12% fat, 73% carbohydrate; NUVILAB®) and water *ad libitum*. The first 3 days were considered a habituation period, so that standard chow consumption was only calculated on the fourth day of the experiment. Food preference was evaluated in the following 4 days, during which the animals were allowed to choose between standard rat chow (2.95 Kcal/g, 15% protein, 12% fat, 73% carbohydrate; no sucrose; NUVILAB®) and a highly palatable diet (4.59 Kcal/g, 25% protein, 23% fat, 47% carbohydrate—20% from sucrose; Prag Soluções®, SP, Brazil). A preference index for the palatable diet during the 4 days on which both diets were available was calculated by dividing the caloric intake from the palatable diet by total calorie intake. To avoid possible effects of neophobia, the animals received a small amount of the palatable diet inside their cages for two days before the beginning of the food choice experiment. Both diets were replenished daily, simultaneously with the cleaning and maintenance of the BioDAQ® system.

4.2. Conditioned place preference (CPP)

After the evaluation of feeding behavior using the BioDAQ® system, the animals were housed in individual cages for the CPP procedure. The experiment lasted 8 days, and was performed in an acrylic chamber with two compartments, a dark/black side (21 cm × 35 cm × 41 cm) and a lit/white side (21 cm × 45 cm × 41 cm), separated by a removable door. The lights were turned off in the experimental room, and the lit/white compartment was illuminated with a 60 W lamp. The animals were habituated daily to the experimental room for approximately 20 min before the start of each session. All sessions were performed at 9:00 a.m.

On the first day (baseline), rats were placed in the apparatus for 15 min with free access to both compartments, so that their natural preference for each side could be evaluated. The palatable diet was not available on the first day. At the start of the session, rats were placed in the middle of the apparatus, facing the wall, and spontaneously chose one of the two sides. The time spent in each compartment and the number of crossings were scored.

Over the next six days, rats were placed on alternating sides of the chamber every other day for a 30-min training session, and given access to 50 g of the palatable diet (see composition above) on the non-preferred side (lit compartment). No stimulus was present in the other compartment.

On test day (day-8), rats were placed in the apparatus for 15 min and given free access to both sides, again without the palatable diet. The animals' movements in the apparatus were filmed in the first (baseline) and eighth day (test day) of the experiment. The time spent in each compartment and the number of times the animals crossed between compartments were recorded by a trained researcher blinded to treatment condition. Rats were kept under food restriction (receiving about 80% of habitual chow intake) during both training and test days. The difference between the times spent in the lit side in the last session (test) and the first session (baseline) was taken as an indicator of a CPP to the presence of a food reward.

4.3. Tissue collection and neurochemical analysis

One week after the last behavioral test, animals were decapitated for brain dissection. Half the rats were decapitated immediately after a 4 h fast. The remaining animals were habituated to a sweet food (Froot Loops®) for 4 days by receiving a few pellets inside the home cages, and exposed to 4 h of fasting then 1 h of free access to Froot Loops® before being decapitated. This cereal was chosen due to its high sugar content (~50% of carbohydrate derived from sugar). Studies have found that sucrose increases dopamine levels in the NAcc and, similarly, increased dopamine in the NAcc is associated with a higher intake of sweet foods, in a positive feedback fashion (Hajnal and Norgren, 2001, 2002, 2004). Therefore, we planned to examine whether the cerebral response to sucrose would differ between groups by evaluating TH and pTH in the NAcc at both time points (baseline and after exposure to sweet food). D2 receptors were evaluated only at baseline, since we assumed that one hour of exposure to a sweet food in adulthood (an acute intervention) would not influence receptor density.

The brain was quickly removed, flash frozen in isopentane and stored at −80 °C. To dissect the NAcc, the brains were warmed to −20 °C and 0.15 cm-thick coronal slices were obtained (bregma +2.7 to +0.7), before bilateral punches (1.0 mm diameter) were collected at $L = 1.5$ and $V = -7.0$ mm, frozen and stored at −80 °C until use (Paxinos and Watson, 2007). Punches were subjected to Western blot analysis, as described below.

Tissue samples were homogenized in a cytosolic extraction buffer with protease (Protease Inhibitor Cocktail, Sigma-Aldrich, P8340) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets, Roche, 4906845001). The samples used for TH and pTH quantification were centrifuged at 5,500 rpm for 1 min at 4 °C for cytosolic protein extraction. The samples used to quantify D2 receptors underwent additional centrifugation at 13,000 rpm for 30 min at 4 °C to further purify the cytosolic fraction. Part of the supernatant (2 μl) was used to quantify total protein using a BCA protein assay with bovine serum albumin as standard (Pierce BCA Protein Thermo Scientific).

Aliquots of the supernatant containing 30 μg (TH and pTH) or 40 μg of protein (D2 receptor) were incubated with LDS (Invitrogen, NP0007) and DTT (Sigma-Aldrich, 43815) at 99°C

for 3 min. These samples were loaded on 4% to 12% polyacrylamide gradient gels (Invitrogen, NP0323BOX) together with a standard molecular weight (Magic Marker, Invitrogen, LC5602), and submitted to electrophoresis before being transferred to a nitrocellulose membrane (GE Healthcare, RPN303C). Blots were blocked in a Tris-buffered saline containing 5% non-fat dried milk and 1% Tween®20 (Sigma, P1379). Membranes were incubated overnight with the primary antibody, and for 1 h with the secondary antibody on the following day (anti-rabbit 1:2000, Cell Signaling, 7074s or anti-mouse 1:2000, Cell Signaling, 7076s). Membranes were then exposed on film (GE healthcare, 28906836) using ECL (ECL western blotting analysis system, GE healthcare, RNP2106). The primary antibodies and concentrations used were as follows: TH 1:5000 (anti-tyrosine hydroxylase, Sigma-Aldrich, T2928), pTH 1:1000 (anti-phospho tyrosine hydroxylase (Ser40), Invitrogen, 368600) and D2 1:1000 (anti-dopamine D2 receptor, Millipore, AB5084P). The intensity of Western blot bands was quantified by densitometry using the ImageJ® software (Research Services Branch, National Institute of Mental Health, Maryland, USA). Results were expressed as the ratio between the protein of interest and β-actin (1:1000, Sigma-Aldrich, A4700).

4.4. *Statistical analysis*

Data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 18.0 (SPSS Inc., Chicago, IL, USA). Variables were described using mean ± SEM or median and interquartile range.

Standard rat chow consumption (during habituation to the BioDAQ® system as well as usual consumption) was analyzed for all experimental groups (*Adlib/Adlib, FR/Adlib, Adlib/FR, FR/FR*). Since our goal was to investigate the fetal programming effects of IUGR, all subsequent analyses focused only on the two groups of interest: *Adlib/Adlib* (control) and *FR/Adlib* (exposed to growth restriction during the fetal period, but not during lactation).

Generalized Estimating Equations (GEE) with group, sex and time as independent variables were used to evaluate longitudinal data (BioDAQ® habituation period). A two-way ANOVA with group and sex as independent variables was employed to analyze other behavioral outcomes (usual chow consumption, food preference index and conditioned place preference). All analyses of feeding behavior were adjusted for body weight. CPP results were adjusted for mean palatable food intake during training and the number of crossings on test day. Bonferroni *post hoc* tests were used where appropriate.

Western blot analyses were performed separately by sex. TH and pTH levels were analyzed by Two-Way ANOVA, with group and metabolic status (baseline or exposure to sweet food) as independent variables. Results were expressed as percentages of control group values (Control groups: Adlib/Adlib baseline for nitrocellulose membrane with baseline samples; Adlib/Adlib sweet for nitrocellulose membrane with samples collected following exposure to sweet foods). Student's *t*-tests were used to analyze dopamine D2 receptor levels. Significance for all measures was set at $p<0.05$.

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