© 2019 This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/

Laureano DP, Alves MB, Miguel PM, Machado TD, Reis AR, Mucellini AB, Cunha FS, Lampert C, Salvador APA, Dalle Molle R, Mosca PR, Pokhvisneva I, Desai M, Ross MG, Silveira PP. Intrauterine Growth Restriction Modifies the Accumbal Dopaminergic Response to Palatable Food Intake. Neuroscience. 2019 Feb 21;400:184-195. doi: 10.1016/j.neuroscience.2018.12.036. Epub 2018 Dec 30. PMID: 30599270./

Elsevier Editorial System(tm) for

Neuroscience

Manuscript Draft

Manuscript Number: NSC-18-1268R2

Title: Intrauterine growth restriction modifies the accumbal dopaminergic response to palatable food intake

Article Type: Research Paper

Section/Category: Behavioral and Cognitive Neuroscience

Keywords: Dopamine; Reward; Insulin

Corresponding Author: Mrs. Daniela Pereira Laureano, PhD

Corresponding Author's Institution: Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Brazil

First Author: Daniela Pereira Laureano, PhD

Order of Authors: Daniela Pereira Laureano, PhD; Márcio B Alves, Master; Patrícia M Miguel, Master; Tania D Machado, PhD; Adolfo R Reis, PhD; Amanda B Mucellini, PhD; Fábio da Silva Cunha, Phd; Ana Paula A Salvador; Carine Lampert, PhD; Roberta Dalle Molle, PhD; Paulo R MOSCA, PhD; Irina POKHVISNEVA, PhD; Mina Desai, PhD; Michael G Ross, PhD; Patricia P Silveira, PhD

Abstract: Intrauterine growth restriction (IUGR) associates with increased preference for palatable foods and altered insulin sensitivity. Insulin modulates the central dopaminergic response and changes behavioral responses to reward. We measured the release of dopamine in the accumbens during palatable food intake in IUGR rats both at baseline and in response to insulin. From pregnancy day 10 until birth, gestating Sprague-Dawley rats received either an ad libitum (Control), or a 50% food restricted (FR) diet. In adulthood, palatable food consumption and feeding behavior entropy was assessed using an electronic food intake monitor (BioDAQ®), and dopamine response to palatable food was measured by chronoamperometry recordings in the nucleus accumbens (NAcc). FR rats eat more palatable foods during the dark phase, and their eating pattern has a higher entropy compared to control rats. There was a delayed dopamine release in the FR group in response to palatable food and insulin administration reverted this delayed effect. Western blot showed a decrease in suppressor of cytokine signaling 3 protein (SOCS3) in the ventral tegmental area (VTA) and an increase in the ratio of phosphotyrosine hydroxylase to tyrosine hydroxylase (pTH/TH) in the NAcc of FR rats. Administration of insulin also abolished this latter effect in FR rats. FR rats showed metabolic alterations and a delay in the dopaminergic response to palatable foods. This could explain the increased palatable food intake and behavioral entropy found in FR rats. IUGR may lead to binge eating, obesity and its metabolic consequences by modifying the central dopaminergic response to sweet food.

Response to Reviewers: Reply to Reviewers

*Manuscript (Clear Copy)

© T2019 his manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/

Laureano DP, Alves MB, Miguel PM, Machado TD, Reis AR, Mucellini AB, Cunha FS, Lampert C, Salvador APA, Dalle Molle R, Mosca PR, Pokhvisneva I, Desai M, Ross MG, Silveira PP. Intrauterine Growth Restriction Modifies the Accumbal Dopaminergic Response to Palatable Food Intake. Neuroscience. 2019 Feb 21;400:184-195. doi: 10.1016/j.neuroscience.2018.12.036. Epub 2018 Dec 30. PMID: 30599270.

Intrauterine growth restriction modifies the accumbal dopaminergic response to palatable food intake

DP LAUREANO¹, MB ALVES², PM MIGUEL¹, TD MACHADO³, AR REIS³, AB MUCELLINI⁴, FDAS CUNHA³, C LAMPERT², APA SALVADOR⁵, R DALLE MOLLE³, PR MOSCA³, I POKHVISNEVA⁶, M DESAI⁷, MG ROSS⁷, PP SILVEIRA^{1,6,8}

¹ Programa de Pós Graduação em Neurociências, Instituto de Ciências Básicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ² Programa de Pós Graduação em Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ³ Programa de Pós Graduação em Saúde da Criança e do Adolescente, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁴Programa de Pós Graduação em Psiquiatria, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁵ Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁵ Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁶ Ludmer Centre for Neuroinformatics and Mental Health, Douglas Mental Health University Institute, McGill University, Montreal, QC, Canada;⁷ Dept of Ob/Gyn, Harbor-UCLA Med Ctr, Torrance, CA, United States;⁸ Department of Psychiatry, Faculty of Medicine, McGill University, Sackler Program for Epigenetics & Psychobiology at McGill University, Montreal, QC, Canada.

Corresponding author: Daniela Pereira Laureano, PhD. Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde. Universidade Federal do Rio Grande do Sul. Sarmento Leite, 500, Farroupilha, 90035-190, Porto Alegre, RS, Brazil. E-mail: dlaureano@hcpa.edu.br

Abstract

Intrauterine growth restriction (IUGR) associates with increased preference for palatable foods and altered insulin sensitivity. Insulin modulates the central dopaminergic response and changes behavioral responses to reward. We measured the release of dopamine in the accumbens during palatable food intake in IUGR rats both at baseline and in response to insulin. From pregnancy day 10 until birth, gestating Sprague–Dawley rats received either an ad libitum (Control), or a 50% food restricted (FR) diet. In adulthood, palatable food consumption and feeding behavior entropy was assessed using an electronic food intake monitor (BioDAQ®), and dopamine response to palatable food was measured by chronoamperometry recordings in the nucleus accumbens (NAcc). FR rats eat more palatable foods during the dark phase, and their eating pattern has a higher entropy compared to control rats. There was a delayed dopamine release in the FR group in response to palatable food and insulin administration reverted this delayed effect. Western blot showed a decrease in suppressor of cytokine signaling 3 protein (SOCS3) in the ventral tegmental area (VTA) and an increase in the ratio of phospho-tyrosine hydroxylase to tyrosine hydroxylase (pTH/TH) in the NAcc of FR rats. Administration of insulin also abolished this latter effect in FR rats. FR rats showed metabolic alterations and a delay in the dopaminergic response to palatable foods. This could explain the increased palatable food intake and behavioral entropy found in FR rats. IUGR may lead to binge eating, obesity and its metabolic consequences by modifying the central dopaminergic response to sweet food.

Introduction

Exposure to fetal adversity is associated with altered impulsivity-based behaviors such as, increased preference for sugary foods at different ages in humans (Barbieri, Portella et al. 2009, Ayres, Agranonik et al. 2012, Portella, Kajantie et al. 2012, Silveira, Agranonik et al. 2012, Portella and Silveira 2014, Silveira 2014, Dalle Molle and Silveira 2015, Laureano, Molle et al. 2015, Reis RS 2016) and animals (Alves, Molle et al. 2015, Cunha Fda, Dalle Molle et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016). Fetal adversity contributes to the development of prediabetes, type II diabetes, cardiovascular disease and mental health disorders. (Hales and Barker 1992, Eriksson, Wallander et al. 2004, Lahti, Eriksson et al. 2014, Li, Ley et al. 2015). Both humans and animals exposed to intrauterine growth restriction (IUGR) show altered behavior towards sweet rewards starting at birth (Ayres, Agranonik et al. 2012, Laureano, Dalle Molle et al. 2016). These findings suggest that fetal adversity programs neurobiological pathways involved in reward sensitivity (Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016). Insulin receptors are found in the ventral tegmental area (VTA), the nucleus accumbens (NAcc) and the prefrontal cortex (PFC). Insulin modulates the activity within these pathways (Sipols, Bayer et al. 2002, Figlewicz 2003). Fetal growth restriction impairs insulin secretion relative to insulin sensitivity in children and adults, and this phenomenon occurs before the onset of insulin resistance (Jensen, Storgaard et al. 2002, Veening, van Weissenbruch et al. 2003, Mericq, Ong et al. 2005). The behavioral phenotype of children and adults exposed to IUGR may be due to variations in insulin sensitivity within these neural pathways and these variations may in turn predispose these individuals to an enhanced risk for metabolic and psychiatric disorders in later life.

Insulin acts on the dopaminergic neurons of the ventral tegmental area (VTA), that project to the nucleus accumbens and PFC (Figlewicz, MacDonald Naleid et al. 2007), and insulin modulates behavioral responses including reward sensitivity and conditioning (Figlewicz, Bennett et al. 2008). Perinatal adversity affects insulin signaling within the mesocorticolimbic pathway and alters behavioral responses to sweet rewards suggesting changes in reward

sensitivity. (Portella, Silveira et al. 2015). Insulin acts on its receptors located on dopamine neurons in the midbrain including in the VTA and substantia nigra (Figlewicz, Evans et al. 2003). Insulin then triggers Mitogen-activated Protein Kinase (MAPK) and Phosphatidylinositol-3-Kinase (PI-3K) intracellular signaling cascades. The activation of PI-3K is especially linked to metabolic functions and induces the phosphorylation of protein kinase B (Akt). SOCS3 binds to distinct domains of the insulin receptor (Emanuelli, Peraldi et al. 2000), inhibiting the phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 in vivo and in vitro (Ueki, Kondo et al. 2004). This attenuation of insulin signaling by SOCS3 results in reduced activation of glycogen synthesis and glucose transport in cultured cells (Ueki, Kondo et al. 2004). SOCS3 activates an intracellular negative feedback loop within the insulin receptor that is regulated in both a phosphorylation and transcription-dependent manner. Changes in AKT phosphorylation and SOCS3 levels are indicative of modifications in insulin sensitivity within the tissues that are affected.

Maternal protein restriction during gestation alters brain systems involved in hedonic responses to sucrose. For example, these animals have increased levels of tyrosine-hydroxylase (TH) and dopamine in the VTA and PFC, respectively, and enhanced expression of genes responsible for TH and dopamine reuptake transporter (DAT) production in areas of the mesocorticolimbic system (Vucetic, Totoki et al. 2010). IUGR adult rats show robust reductions in the expression of enzymes involved in dopamine synthesis in the NAcc and orbitofrontal cortex (Alves, Molle et al. 2015, Dalle Molle, Laureano et al. 2015). In this current work we hypothesized that insulin would differentially modulate the behavioral and neurochemical DA responses to palatable foods in adult rats exposed to IUGR compared to controls.

Experimental Procedures

Animals

Primiparous Sprague Dawley dams were time-mated at approximately 80 days of age (CEMIB Laboratory Animal Reference Center, Campinas, SP,

Brazil) were time-mated within our institutional animal facility. Throughout pregnancy the dams were single-housed in Plexiglas home cages (49 x 34 x 16 cm) and maintained in a controlled environment. Lights were turned on between 7AM and 7PM, temperature was maintained at 22 \pm 2°C, and the cages were changed once per week. Food and water was provided ad libitum until gestational day 10. At 10 days of gestation, dams were randomly assigned to one of two groups: control (Adlib)(n=24), receiving an ad libitum (AdLib) diet of standard laboratory chow (Nuvilab®) or 50% food restriction (FR)(n=12), receiving 50% of the ad libitum-fed mothers intake. The FR group's ration of food was based on the daily consumption of normal chow in a cohort of gestating Sprague Dawley rats) (Desai, Gayle et al. 2005). These modified feeding conditions were maintained from gestational day 10 until the birth of the litters. Within 24 hours after birth, the pups litters were culled to 8 pups per litter (4 males and 4 females) and all pups were cross-fostered to other AdLib dams, forming the following groups (considering the biological/adoptive mother, i.e., gestation/lactation maternal diet): AdLib/AdLib (control - CTRL) and FR/AdLib (FR). Weaning occurred on postnatal day 21. Pups were separated by sex and housed four per cage. The excess male pups and the females were used in different studies (projects HCPA/GPPG 12-0353, 13-0544, 14-0289 and 140111). All rat pups were maintained on standard laboratory chow and water available ad libitum and were kept in a controlled environment as described above. Body weights were recorded weekly from weaning age using a digital scale with 0.01 g precision (Marte®, Canoas, Brazil). The males (n=64 animals) were assigned to one of two experiments, a) evaluation of feeding behavior (n=16 animals) and evaluation of insulin signaling in the mesocorticolimbic system (n=34 animals, Study 1) and b) chronoamperometric evaluation of the accumbal dopamine response to palatable food (n= 14 animals, Study 2). To avoid litter effects, a maximum of two rats per litter was used in each experiment.

The Research Ethics Committee of Hospital de Clínicas de Porto Alegre (GPPG/HCPA, project numbers 13-0420, 13-0264, 14-0111, 13-0544, 11–0053, 14-0289, 120353) approved all the animal procedures described here. All procedures were conducted in a climate-controlled environment within our

animal facility's behavioral testing rooms (Unidade de Experimentação Animal/HCPA).

Study 1: Evaluation of feeding behavior and insulin signaling in the mesocorticolimbic system

Analysis of feeding behavior

Rats were singly housed in cages equipped with a BioDAQ® food intake monitoring system (Research Diets) at 80 days of age. BioDAQ® is a computerbased software program that can provide detailed feeding behavior data. Total food intake and meal patterns were analyzed with the BioDAQ® system as previously described (Farley, Cook et al. 2003, Machado, Dalle Molle et al. 2013, Dalle Molle, Laureano et al. 2015). Briefly, the system uses a food hopper mounted on an electronic strain gauge-based load cell to measure food consumption. The food hopper is weighed 50 times per second (accurate to 0.01 g) and the mean and standard deviation (S.D.) of food consumption over approximately 1 sec is calculated by a peripheral computer. Food consumption is signaled by a change in the food hopper weight (defined as a S.D. > 2000 mg) caused by the animal ingesting food. Each feeding event including cage/animal number, start date and time, feeding duration, final hopper weight and amount of food eaten is recorded and exported to a central computer, where it is entered into a Microsoft Excel spreadsheet (Microsoft, Redmond, WA). There were two kinds of events recorded in BioDAQ®: feeding bouts and meals. The end of a feeding bout was signaled when the hopper was left undisturbed for 5s (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 >0.1g, separated from other feeding bouts within a range of 15 min (Surina-Baumgartner, Langhans et al. 1995, Eckel, Langhans et al. 1998). Rats feeding behavior was analyzed for 8 days. During the habituation phase, consisting of the first four days, the rats were given access to standard rat chow containing 2.95Kcal/g, 22% protein, 4,5% fat, 54% carbohydrate in each kg

(NUVILAB®) and water *ad libitum*. During the last four days, the rats were provided with access to both the previously described standard laboratory rat chow and a highly palatable diet containing 4.82 kcal/g, 8% mineral, 14% protein, 34% fat, 6% fiber, 30% carbohydrate in each kg (Prag Soluções Biociências®). This latter diet derived 20% of its carbohydrate content from sucrose. The rats were provided with a small quantity of the highly palatable diet in their home cages for 2 days prior to the start of the food choice experiment as a means to reduce possible effects of neophobia on the results. Each day, the diets were replenished at the same time that the cages and BioDAQ® system were cleaned and maintained (Kowalski, Farley et al. 2004).

Peripheral measurements – ELISA

At 100 days of age, the rats were fasted for 4h and injected with insulin (1 IU/kg) or saline through the intraperitoneal route (i.p.) and decapitated 15 minutes later for protein measurements or received no injection prior to decapitation for insulin measurements. Brain tissue and blood were collected. Blood was collected in tubes and centrifuged at +4°C at 4000 rpm for 10min. Plasma was separated in aliquots and frozen at -80°C until further analysis. Plasma insulin levels were measured using the Rat/Mouse Insulin ELISA Kit (Millipore, EZRMI-13K).

Central measurements – Western Blotting

Brains were flash frozen in isopentane and stored at -80 °C until further analyses. The brains were then warmed to -20 °C and cut into thick sections of 0.1 cm with the aid of an Atlas (Paxinos and Watson 2007). Bilateral 1 mm diameter punches were taken from the ventral tegmental area (VTA) and nucleus accumbens (NAcc) (Shahrokh, Zhang et al. 2010).

Tissue samples were homogenized in cytosol extraction buffer with the addition of a protease inhibitor cocktail, (Sigma- Aldrich, P8340) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets, Roche, 4906845001). The samples were then centrifuged at 6000 rpm for 2 min at +4 °C. Total protein was quantified using a Bicinchoninic Acid (BCA) kit with

bovine serum albumin as a standard (Pierce BCA Protein, Thermo Scientific, 23225). Aliquots of the supernatant containing 35 µg of VTA or 25 µg of NAcc protein were incubated with LDS (Invitrogen, NP0007) and DTT (Sigma-Aldrich, 43815) at 99°C for 3 min. The samples were subjected to electrophoresis using a 4 to 12% polyacrylamide gradient gel (Invitrogen, NP0323BOX) together with the addition of a standard molecular weight (Spectra[™] Multicolor Broad Range Protein Ladder, Thermo scientific, 26634), before being transferred to a nitrocellulose membrane (GE Healthcare, RPN303C). Blots were blocked in Tris buffered saline containing 5% non-fat milk concentrate and 1% Tween®20 (Sigma, P1379). We measured TH, pTH, in the NAcc, and SOCS3, AKT and pAKT in the VTA. The membranes were incubated overnight at +4°C with primary antibodies: TH 1:5000 (anti-tyrosine hydroxylase, Sigma-Aldrich, T2928), pTH 1:500 (anti-phospho tyrosine hydroxylase (Ser40), Invitrogen, 368600), SOCS3 1:500 (suppressor of cytokine signaling) (Cell Signaling, #2923), Akt (pan) (11E7) 1:1000 (Cell signaling, #4685), Phospho-Akt (Ser473) 1:1000 (Cell Signaling, #4060), followed by anti-mouse secondary antibodies (1:2500) (Anti-Mouse IgG, Cell Signaling, #7076s) or anti-rabbit (1:2500) (Anti-Rabbit IgG, Cell Signaling, #7074s) at room temperature for 1h. The membrane was then exposed on ImageQuant LAS 4000 GE Healthcare Life Sciences using ECL (ECL western blotting analysis system, GE healthcare, RNP2106). Results were calculated as a ratio of intensity of the protein of interest to that of actin 1:2000 (Sigma-Aldrich, A4700) in the same membrane, using a stripping protocol as needed (Laureano, Dalle Molle et al. 2016). The results were expressed as a percentage of control values (Colman, Laureano et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016).

Study 2: Chronoamperometric evaluation of the accumbal dopamine response to palatable food

Surgery

Between 80–120 days of age, rats (n=14) underwent stereotaxic surgery. They were anesthetized with ketamine 40 mg/kg (ip.) and xylazine 5mg/kg (ip.)

and maintained under isoflurane anesthesia during the surgery. Animals received tramadol 5 mg/kg (ip.) before surgery, and tramadol 2.5 mg/kg (ip.) immediately post-surgery, and sodium dipyrone 200 mg/kg (IM)was given to reduce pain post-surgery. Tramadol 5 mg/kg (ip.) was kept 12h/12 h thereafter, as well as and cetoprofen 5 mg/kg (ip.) 1x day for 2 consecutive days postoperative. Forty µL of bupivacaine with epinephrine (0.2 mg per animal) was injected subcutaneously at the surgical site. Electrochemical probes were lowered into the NAcc shell (coordinates: 1.2 mm anterior to bregma, 0.8 mm lateral to the midline, and 7.0 mm ventral to the surface of the cortex) (Shahrokh, Zhang et al. 2010). Animals were also implanted with a Ag/AgCI reference electrode in the contralateral parietal cortex. Miniature pin connectors soldered to the voltammetric and reference electrodes were inserted into a connector (9-pin ABS Plug, Ginger Scientific, GS09PLG-220). The assembly was secured with acrylic dental cement to four stainless steel screws threaded into the cranium (Paxinos and Watson 2007, Shahrokh, Zhang et al. 2010). Animals were returned to their home cages after a 2-5h recovery.

Electrochemical probes and measurements in freely moving rats

This experiment was performed during the early afternoon according to Shahrokh, Zhang et al. 2010). The voltammetric electrodes consisted of a bundle of three $30-\mu$ m-diameter carbon fibers (Textron Systems, Wilmington, MA) extending $50-100\mu$ m beyond the sealed tip of a pulled glass capillary (outer diameter 0.5 mm), and repeatedly coated with a 5% solution of Nafion® (Sigma-Aldrich, 274704), a perfluorinated ionomer that promotes the exchange of cations such as dopamine but impedes the exchange of interfering anionic species such as ascorbic acid (AA) and 3,4 dihydroxyphenylacetic acid. Each electrode was calibrated to determine dopamine sensitivity and selectivity compared with AA in 0.1 M PBS (pH 7.4) that contained 250 μ M AA to mimic brain extracellular conditions. The electrodes had a mean (±SEM) dopamine to AA selectivity ratio of 1619:1 ± 133:1 (Doherty and Gratton 1997, Shahrokh, Zhang et al. 2010, McCutcheon, Beeler et al. 2012).

Dopamine was detected as current through its oxidation and reduction, and measurements were recorded using a computer controlled, high-speed chronoamperometric apparatus (Quanteon, Lexington, KY). An oxidation potential of +0.55mV (with respect to the reference electrode) was applied to the electrode for 100 ms at a rate of 2 Hz. The oxidation current was digitally integrated during the last 80 ms of each pulse. The sum of every 10 digitized oxidative cycles from the chronoamperometric waveform was automatically converted into equivalent values of dopamine concentration using an in vitro calibration factor. The reduction current generated when the potential was returned to resting state (0.0 V for 100 ms) was digitized and summed in the same manner and served as an index to identify the main electroactive species contributing to the electrochemical signals. The magnitude of the increase in reduction current elicited by an elevation in dopamine concentration is typically 60-80% of the corresponding increase in oxidation current [reduction to oxidation ratio (red:ox)], 0.6–0.8 using nation-coated electrodes and a sampling rate of 2 Hz (Doherty and Gratton 1997, Gerhardt, Ksir et al. 1999, Shahrokh, Zhang et al. 2010, McCutcheon, Beeler et al. 2012). Supplementary information is available at <u>https://www.youtube.com/watch?v=Sf7L-34pqmc</u>.

In vivo electrochemical recordings

One day following surgery the rats received 6 Froot Loops® pellets in their home cages to reduce the influence of food neophobia during the experiment. The rats started to be habituated to the voltammetry apparatus two days following surgery. They were given 6 Froot Loops® pellets within a voltammetry cabinet ($60 \times 60 \times 60 \text{ cm}$) containing a single hole at the top of the box to connect the voltammetry cable and a glass front door. Rats were habituated to the box for 1 hr.

Voltammetry recordings took place on the 3rd and 5th days following surgery at afternoon. During the first two hours of recording, the rat was placed in the voltammetry cabinet to allow for habituation and electrochemical signal stabilization. The rat then received 30 grams of standard rat chow given in small pieces and the dopaminergic response to the presentation of standard rat chow

was recorded. The remaining food was removed and the rat remained in the box for 1 hour to allow for signal stabilization. Fifteen pellets of Froot Loops® were then offered for 30 minutes and the recordings continued for another hour to record the dopaminergic response to palatable food. Standard rat chow available at 80% of the free-feeding rate was available in the home cage between recording sessions. The next day, the rats underwent another 2 hours of acclimatization to the voltammetry cabinet. Rats were then injected with 100 microliters saline solution (ip.) and given15 Froot Loops® pellets for 30 minutes. The food was then removed and the recordings continued for another hour. On the third day of recordings, rats received an injection of 5 UI/kg insulin (i.p.) in a total volume of 100 microliters using saline for dilution.

Histological confirmation of electrode placement

On the sixth day, the electrode placement in the recording site was validated by histology. Rats were deeply anesthetized with isoflurane and a +4.5 DC current was applied to the carbon fiber electrode in order to produce a lesion at the exact recording site in the brain (Ledo, Lourenco et al. 2015). Rats were then decapitated, and the brains were fixed in 4% paraformaldehyde (PFA) solution in PBS for 24h. The brains were cryoprotected in 30% sucrose solution and frozen in isopentane and liquid nitrogen. Coronal sections (50 μ m) of the brain were made using a cryostat (Leica, Wetzlar, Germany) with the aid of an atlas (Paxinos and Watson 2007). The sections were mounted on gelatin-coated glass slides and stained with hematoxylin-eosin to verify the lesion site caused by the current applied to the microelectrodes. Rats with incorrect electrode placements in the recording site (n=5) were excluded from statistical analyses (Figure 1).

Statistical analyses

Data were expressed as means ± SEM and were analyzed using Student's t-tests when comparing two groups (FR vs. Adlib groups).

Generalized Estimating Equations (GEE) were used to analyze differences in birth weight and body weight at weaning since this method takes the interdependent effect of litter into account. We used group and sex as variables and adjusted by litter size. The models were adjusted for both litter number at birth and at weaning age.

BioDAQ® data was analyzed in three different ways: a) GEEs were used with group, experimental day, time of day (light vs. dark phase) as independent variables and total amount of food consumed was the dependent outcome variable. Food consumption was adjusted by body weight and analyzed as grams consumed per 100g of body weight; b) Using continuous recordings from the BioDAQ® system, we divided 24 hours in a day into 96 bins of 15 minutes and categorized the rat's behavior into one of three possible states: resting, eating rat chow or eating palatable food. We then measured unpredictability which is a measure of transitions from one type of state to another. We derived a measure of unpredictability by focusing on the conditional probabilities that describe transitions in behavior. This method is described in Molet et al. (2016). Briefly, we characterized the rats' behavior using an empirical transition matrix of conditional probabilities of changing from one behavioral state to another.

Entropy is a natural summary measure of randomness or the unpredictability of a distribution and can be defined in many ways. We focused on the Shannon entropy. We first calculated the probabilities to be in each of the three states for all rats together and used them as the stationary distribution of the Markov chain. The stationary probabilities serve as weights for the entropy values, which produce a measure known as the entropy rate (Molet, Heins et al. 2016), and these were compared between the groups using Student's t-test; c) Approximate entropy (ApEn) was calculated according to Richman and Moorman, 2000) using the continuous measure as described in b), we then calculated the tolerance, r, (0.3 times standard deviation of the consumption per 100g) for each subject. We defined that two sequences xi and xj will match if they are within a tolerance r, i.e. the distance between xi and xj is less than or equal to r. Then for each subject we calculated two probabilities: that two sequences of two consecutive periods will match and that two sequences of three consecutive periods will match. Further, for each subject we calculated approximate entropy, ApEn, as a negative logarithm of conditional probability that two sequences within a tolerance r for two consecutive periods will remain within tolerance r for the next period.

Voltammetry data was analyzed using GEEs, and group and type of food exposure (rat chow, Froot Loops®, IP Saline_Froot Loops®) IP Insulin_Froot Loops®) were independent factors within the model. Post hoc group comparisons were completed using the Bonferroni post hoc test. Three aspects of the dopaminergic response to food and injection presentation were analyzed based on the model developed by Joyce et al. (2007). The time it takes dopamine to reach its peak value (T rise), the amplitude of the dopaminergic response (in mMol) and the time it takes for 80% of the original dopamine content to be cleared (T80) were measured. The T80 value is a measure of dopamine reuptake.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) and R statistical software. Significance levels for all measures were set at p= 0.05.

RESULTS

Body weight

We found a significant effect of group [B=-0.834, p=0.004] on birth weight where FR rats weighed less than controls, and there was a significant effect of sex [B=-0.324, p<0.001] in which females weighed less than males (Figure 2A).

At weaning age, the body weight difference between FR and controls disappeared [B= -1.236, p=0.355], indicating catch up growth in the FR group. Females still weighed less than males at weaning age [B= -2.536, p<0.001], (Figure 2B). Sample size in this experiment reflects all the animals used in the different projects listed in the Methods since we decided not to trace the pups that were eventually assigned to each project at weaning age since we did not want to disturb the litters in the first 21 days of life.

The rats that were used in the analyses of BioDAQ® food consumption data and dopaminergic responses to food and injection presentation had no significant differences in body weights between the FR and control groups (body weights in BioDAQ® experiment, Student's T Test t(14)=0.530, p=0.605, (Figure 2C) and body weights in voltammetry experiment, Student's T Test t(7)=2.037, p=0.09, (Figure 2D).

Study 1

FR animals eat more palatable foods in the dark phase

Our analyses of food consumption during the last 4 days of study 1 showed that the rats ate very little or no regular rat chow. We focused our further analyses on the consumption of palatable food. We found no effect of the experimental day (1 to 4) (Wald= 3.17; df= 3; p= 0.37) and no effect of the group (Wald= 1.12; df= 1; p= 0.27) on palatable food consumption across the whole day. There was an effect of time of day (light vs. dark phase) (Wald= 9.63; df= 1; p= 0.002). There was also a significant interaction between group and time of day (Wald= 10.43; df= 1; p= 0.001), in which FR rats consumed more palatable food than control rats during the dark (active) phase of the light cycle (Figure 3A).

FR animals have differential behavioral entropy

There were no significant differences in behavioral entropy rates between FR and control groups over the course of the 96 hours food consumption experiment (Student's t-test, t(14)=1.79, p=0.09). However, the FR group showed significantly higher entropy during the dark phase (t(14)=2.72, p=0.02) but not during the light phase (t(14)=1.42, p=.18) (Figure 3 B, C, D) compared to the control group.

Approximate entropy, a measure that also takes into account the variability in the amount consumed (ApEn), was also calculated. The FR group had increased ApEn during the dark phase compared to the control group

 $(FR=0.282\pm0.020, \text{ control}=0.163\pm0.023, \text{ Student's t-test, t(14)}=3.76, p=0.002),$ but there were no group differences during the light phase (FR=0.091±0.010, control=0.115±0.012, Student's t-test, t(14)=1.51, p=0.153).

FR rats have higher peripheral insulin

Figure 4 shows that FR rats had significantly higher insulin levels than control rats (Student's t-test, t(11)=2.472 p=0.031) (Figure 4).

NAcc pTH/TH ratio is higher in the FR group but this is reverted by insulin

There were no statistically significant differences between groups in TH levels after saline [Student's t-test, t(10)=0.198, p=0.847] or insulin injection [Student's t-test, t(10)=0.633, p=0.541]. pTH levels were also similar between groups when exposed to saline [Student's t-test, t(10)=0.952, p=0.364] or insulin [Student's t-test, t(10)=0.800, p=0.442]. However, the ratio of pTH/TH was increased in the FR group compared to controls when exposed to saline [Student's t-test, t(10)=2.599, p=0.027], which was completely reverted by insulin injection [Student's t-test, t(10)=1.007, p=0.338] (Table 1).

VTA SOCS3 levels are diminished in FR animals

AKT levels in the VTA were similar between groups when exposed to saline [Student's t-test, t(7)=1.022, p=0.341] or insulin [Student's t-test, t(9)=1.009, p=0.340]. pAKT levels were also not significantly different between groups after saline [Student's t-test, t(7)=0.916, p=0.390] or insulin [Student's t-test, t(9)=1.501, p=0.168] injections. Similarly, the pAKT/AKT ratio was similar between groups after saline [Student's t-test, t(7)=0.621, p=0.554] and insulin injection [Student's T Test, t(9)=0.280, p=0.786]. SOCS3 levels were significantly diminished in the FR compared to control group after saline injection [Student's t-test, t(7)=3.584, p=0.009], but no significant differences were observed following insulin injection [Student's t-test, t(9)=0.428, p=0.678] (Table 2).

FR animals show a delayed dopaminergic response to palatable food and this effect is reversed by insulin

Food consumption of both regular rat chow and Froot Loops® presented in the voltammetry cabinet was measured. Rat chow consumption was minimal during the experiment, similar to the findings from Study 1 reported above. Rat chow intake frequency was similar between the FR and control groups during the 30 minutes of food presentation [Control = 7.8 ± 2.3 , FR= 10.7 ± 2.8 , Student's T Test, t(7)=1.007, p=0.35]. All the rats ate the entire portion of Froot Loops that was offered 1 hour later, as well as on the following days of the experiment.

The time it took for the dopaminergic response to reach peak amplitude (T rise) was not significantly different between the groups (Wald= 0.185; df= 1; p= 0.667), but there was an effect of food exposure (Wald= 8.972; df= 3; p= 0.030), and a significant interaction between the group and food exposure condition (Wald= 24.951; df= 3; p < 0.001). Posthoc analysis showed that the FR and control groups differed significantly after exposure to Froot Loops® (p = 0.006). The FR group took a significantly longer time to reach the dopaminergic peak. This finding is completely reversed when rats were injected with insulin (p = 0.006), Figure 5A.

The amplitude of the dopaminergic response was not significantly different between the FR and control groups (Wald= 1.134; df= 1; p= 0.287), but there was an effect of the food exposure condition (Wald= 8.215; df= 3; p= 0.042) and an interaction between group and the food exposure condition (Wald = 93.226; df= 3; p < 0.001). Bonferroni analyses showed that while there were no significant differences in amplitude in the different food exposure conditions in the control group, the FR rats responded with higher amplitude levels in the presence of Froot Loops® only in comparison to IP Saline_ Froot Loops® (p=0.038), Figure 5B.

The T80 value was not significantly different between the groups (Wald= 0.420; df= 1; p= 0.517), but there was an effect of the food exposure condition (Wald= 13.063; df= 3; p= 0.005) and an interaction between the group and food

exposure condition (Wald= 45.415; df= 3; p < 0.001). However, posthoc analysis was not able to detect a significant interaction. The only detectable difference was in the general comparison between the response after rat chow and IP Insulin_ Froot Loops®, in which the T80 value was longer after insulin injection (p= 0.024). Visual inspection of the graph suggests this finding is greater in the FR group (Figure 5C).

DISCUSSION

In this study, we confirmed previous findings of increased palatable food intake in FR (IUGR) animals, in agreement with several previous studies from our group (Alves, Molle et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016) and others (Vucetic, Totoki et al. 2010), and to the findings in humans that experienced IUGR (Lussana, Painter et al. 2008, Barbieri, Portella et al. 2009, Migraine, Nicklaus et al. 2013). We have previously shown that FR animals have increased preference for palatable food throughout the light-dark cycle(Dalle Molle, Laureano et al. 2015). In the current experiment, we focused exclusively on the consumption of palatable food, since the rats ate very little if any standard rat chow that was offered during the experiment. We showed that while FR and control rats ate a similar amount of palatable food over the course of the whole day, FR rats ate more palatable food during the dark phase of the light/dark cycle when rats are active. We also found a greater behavioral entropy or transitions between different behavioral states in the FR compared to the control rats. The FR group had a more unpredictable and disorganized behavior in the dark phase, as shown by the higher entropy rates. These refinements in measurements are important advances in our understanding of how gestational FR affects the behavioral response to palatable foods in adulthood.

In addition, we showed that FR animals have increased peripheral insulin, in agreement with the "thrifty phenotype" hypothesis proposed by Hales and Barker in 1992 (Hales and Barker 1992). We also found altered insulin sensitivity as reflected through lower levels of SOCS3 in the VTA of FR rats. FR rats also appear more sensitive to insulin's action at a molecular level by

showing normalization of the pTH/TH ratio after insulin injection. FR rats also showed a delayed dopaminergic peak response to palatable food in the accumbens and this finding was reversed by systemic insulin injection.

It is interesting that the well-known increased preference for palatable foods seen in FR rats is accompanied by higher behavioral unpredictability and higher approximate entropy, especially when considering that impulsivity seems to be a behavioral phenotype associated with the increased intake of palatable foods in humans exposed to IUGR (Ayres, Agranonik et al. 2012, Reis, Dalle Molle et al. 2016). It has been suggested that behavioral patterns are altered through the shifting of entropy to different regions across the brain, or that unpredictable behavioral patterns are coupled with more predictable brain activation patterns (Smotherman, Selz et al. 1996, Hong, Barton et al. 2012). This seems to be especially evident in models of altered striatal dopamine function (Hong, Barton et al. 2012). Therefore, our behavioral findings showing increased palatable food consumption and increased entropy rates in FR rats strongly suggests the involvement of altered dopaminergic responsivity to palatable foods, and this hypothesis was confirmed by the electrochemical experiment.

We found a delay in the peak dopaminergic response to sweet foods but not in response to regular chow in FR rats compared to controls. The increased intake of palatable foods observed in FR rats may be an attempt to reach the same dopaminergic peak in a similar amount of time compared to control rats. Activation of dopaminergic neurons facilitates the development of positive reinforcement during reward-seeking and behavioral flexibility (Adamantidis, Tsai et al. 2011). The delayed time it takes FR rats to reach a peak dopaminergic response to palatable food agrees with our previous finding of decreased place conditioning to palatable foods despite higher consumption in FR compared to control rats (Dalle Molle, Laureano et al. 2015). Deficits in striatal dopaminergic transmission are also related to impulsivity in humans and in animals (Trifilieff and Martinez 2014), a feature that characterizes individuals with IUGR..

Importantly, the time to reach the peak dopaminergic response to palatable foods is significantly lower in FR rats following insulin injection compared to control rats. These findings suggest that rats exposed to

gestational food restriction are more sensitive to the effects of systemic insulin in adulthood than control rats. The elimination of pTH/TH protein level differences in the nucleus accumbens of FR rats following insulin injection, the longer time to achieve dopamine reuptake (T80 value) and the lower levels of SOCS3 in the VTA are all aligned with the concept that gestational food restriction alters the response to palatable food possibly through alterations in insulin sensitivity in adulthood.

IUGR is associated with insulin resistance. In young rats, peripheral levels of insulin are increased in FR rats and this may facilitate insulin signaling in some brain regions before reaching exhaustion. Central insulin resistance first appears in the hypothalamus (Sears and Perry 2015). We have evidence that IUGR animals at this age already have increased hypothalamic SOCS3 levels (Mucellini et al., in prep). Therefore, the behavioral phenotype observed in IUGR individuals may be the result of different patterns of insulin resistance in different brain regions.

Our findings indicate that fetal adversity alters brain sensitivity to insulin and modulates behavioral responses to palatable foods. Our findings in rats corroborate with our findings in humans where individual differences in dopamine function are associated with increased palatable food consumption in IUGR individuals (Silveira, Pokhvisneva et al. 2018). These current findings have important implications as early life adversity during gestation associates with the development of chronic diseases later in life including increased risk for obesity, metabolic syndrome, cardiovascular disease and psychiatric disorders. Our further understanding of the molecular mechanisms involved in increasing the risk for these diseases in the face of early life gestational adversity allows us to develop preventative and therapeutic programs to target individuals at risk.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

Financial support was provided by the Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE/HCPA); Pró-Pesquisa/PROPESQ/UFRGS; Coordination for the Improvement of Higher Education Personnel (CAPES) and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

REFERENCES

Adamantidis, A. R., H. C. Tsai, B. Boutrel, F. Zhang, G. D. Stuber, E. A. Budygin, C. Tourino, A. Bonci, K. Deisseroth and L. de Lecea (2011). "Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior." <u>J Neurosci</u> 31(30): 10829-10835.

Alves, M. B., R. D. Molle, M. Desai, M. G. Ross and P. P. Silveira (2015). "Increased Palatable Food Intake and Response to Food Cues in Intrauterine Growth-Restricted Rats Are Related to Tyrosine Hydroxylase Content in the Orbitofrontal Cortex and Nucleus Accumbens." <u>Behav Brain Res</u>: 287:73-81.

Ayres, C., M. Agranonik, A. K. Portella, F. Filion, C. C. Johnston and P. P. Silveira (2012). "Intrauterine growth restriction and the fetal programming of the hedonic response to sweet taste in newborn infants." <u>Int J Pediatr</u> 2012: 657379.

Barbieri, M. A., A. K. Portella, P. P. Silveira, H. Bettiol, M. Agranonik, A. A. Silva and M. Z. Goldani (2009). "Severe intrauterine growth restriction is associated with higher spontaneous carbohydrate intake in young women." <u>Pediatr Res</u> 65(2): 215-220.

Colman, J. B., D. P. Laureano, T. M. Reis, R. Krolow, C. Dalmaz, S. Benetti Cda and P. P. Silveira (2015). "Variations in the neonatal environment modulate adult behavioral and brain responses to palatable food withdrawal in adult female rats." Int J Dev Neurosci 40: 70-75.

Cunha Fda, S., R. Dalle Molle, A. K. Portella, S. Benetti Cda, C. Noschang, M. Z. Goldani and P. P. Silveira (2015). "Both Food Restriction and High-Fat Diet during Gestation Induce Low Birth Weight and Altered Physical Activity in Adult

Rat Offspring: The "Similarities in the Inequalities" Model." <u>PLoS One</u> 10(3): e0118586.

Dalle Molle, R., D. P. Laureano, M. B. Alves, T. M. Reis, M. Desai, M. G. Ross and P. P. Silveira (2015). "Intrauterine growth restriction increases the preference for palatable foods and affects sensitivity to food rewards in male and female adult rats." <u>Brain Res</u> 1618: 41-49.

Dalle Molle, R. and P. P. Silveira (2015). "Small for gestational age children have specific food preferences." <u>J Pediatr</u> 166(6): 1547.

Desai, M., D. Gayle, J. Babu and M. G. Ross (2005). "Programmed obesity in intrauterine growth-restricted newborns: modulation by." <u>Am J Physiol Regul</u> <u>Integr Comp Physiol</u> 288(1): R91-96.

Doherty, M. D. and A. Gratton (1997). "NMDA receptors in nucleus accumbens modulate stress-induced dopamine release in nucleus accumbens and ventral tegmental area." <u>Synapse</u> 26(3): 225-234.

Eckel, L. A., W. Langhans, A. Kahler, L. A. Campfield, F. J. Smith and N. Geary (1998). "Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats." <u>Am J Physiol</u> 275(1 Pt 2): R186-193.

Emanuelli, B., P. Peraldi, C. Filloux, D. Sawka-Verhelle, D. Hilton and E. Van Obberghen (2000). "SOCS-3 is an insulin-induced negative regulator of insulin signaling." <u>J Biol Chem</u> 275(21): 15985-15991.

Eriksson, M., M. A. Wallander, I. Krakau, H. Wedel and K. Svardsudd (2004). "Birth weight and cardiovascular risk factors in a cohort followed until 80 years of age: the study of men born in 1913." <u>J Intern Med</u> 255(2): 236-246.

Farley, C., J. A. Cook, B. D. Spar, T. M. Austin and T. J. Kowalski (2003). "Meal pattern analysis of diet-induced obesity in susceptible and resistant rats." <u>Obes</u> <u>Res</u> 11(7): 845-851.

Figlewicz, D. P. (2003). "Insulin, food intake, and reward." <u>Semin Clin</u> <u>Neuropsychiatry</u> 8(2): 82-93.

Figlewicz, D. P., J. L. Bennett, S. Aliakbari, A. Zavosh and A. J. Sipols (2008). "Insulin acts at different CNS sites to decrease acute sucrose intake and sucrose self-administration in rats." <u>American Journal of Physiology-Regulatory</u> <u>Integrative and Comparative Physiology</u> 295(2): R388-R394.

Figlewicz, D. P., S. B. Evans, J. Murphy, M. Hoen and D. G. Baskin (2003). "Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat." <u>Brain Res</u> 964(1): 107-115.

Figlewicz, D. P., A. MacDonald Naleid and A. J. Sipols (2007). "Modulation of food reward by adiposity signals." <u>Physiol Behav</u> 91(5): 473-478.

Gerhardt, G. A., C. Ksir, C. Pivik, S. D. Dickinson, J. Sabeti and N. R. Zahniser (1999). "Methodology for coupling local application of dopamine and other chemicals with rapid in vivo electrochemical recordings in freely-moving rats." J <u>Neurosci Methods</u> 87(1): 67-76.

Hales, C. N. and D. J. Barker (1992). "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis." <u>Diabetologia</u> 35(7): 595-601.

Hong, S. L., S. J. Barton and G. V. Rebec (2012). "Neural correlates of unpredictability in behavioral patterns of wild-type and R6/2 mice." <u>Commun Integr Biol</u> 5(3): 259-261.

Jensen, C. B., H. Storgaard, F. Dela, J. J. Holst, S. Madsbad and A. A. Vaag (2002). "Early differential defects of insulin secretion and action in 19-year-old caucasian men who had low birth weight." <u>Diabetes</u> 51(4): 1271-1280.

Joyce, B. M., P. E. Glaser and G. A. Gerhardt (2007). "Adderall produces increased striatal dopamine release and a prolonged time course compared to amphetamine isomers." <u>Psychopharmacology (Berl)</u> 191(3): 669-677.

Kowalski, T. J., C. Farley, M. E. Cohen-Williams, G. Varty and B. D. Spar (2004). "Melanin-concentrating hormone-1 receptor antagonism decreases feeding by reducing meal size." <u>Eur J Pharmacol</u> 497(1): 41-47.

Lahti, M., J. G. Eriksson, K. Heinonen, E. Kajantie, J. Lahti, K. Wahlbeck, S. Tuovinen, A. K. Pesonen, M. Mikkonen, C. Osmond, D. J. Barker and K. Raikkonen (2014). "Late preterm birth, post-term birth, and abnormal fetal growth as risk factors for severe mental disorders from early to late adulthood." <u>Psychol Med</u>: 1-15.

Laureano, D. P., R. Dalle Molle, M. B. Alves, C. Luft, M. Desai, M. G. Ross and P. P. Silveira (2016). "Intrauterine growth restriction modifies the hedonic response to sweet taste in newborn pups - Role of the accumbal mu-opioid receptors." <u>Neuroscience</u> 322: 500-508.

Laureano, D. P., R. D. Molle, A. K. Portella and P. P. Silveira (2015). "Facial Expressions in Small for Gestational Age Newborns." <u>J Child Neurol</u>.

Ledo, A., C. F. Lourenco, M. Caetano, R. M. Barbosa and J. Laranjinha (2015). "Age-associated changes of nitric oxide concentration dynamics in the central nervous system of Fisher 344 rats." <u>Cell Mol Neurobiol</u> 35(1): 33-44.

Li, Y., S. H. Ley, D. K. Tobias, S. E. Chiuve, T. J. VanderWeele, J. W. Rich-Edwards, G. C. Curhan, W. C. Willett, J. E. Manson, F. B. Hu and L. Qi (2015). "Birth weight and later life adherence to unhealthy lifestyles in predicting type 2 diabetes: prospective cohort study." <u>BMJ</u> 351: h3672.

Lussana, F., R. C. Painter, M. C. Ocke, H. R. Buller, P. M. Bossuyt and T. J. Roseboom (2008). "Prenatal exposure to the Dutch famine is associated with a preference for fatty foods and a more atherogenic lipid profile." <u>American</u> <u>Journal of Clinical Nutrition</u> 88(6): 1648-1652.

Machado, T. D., R. Dalle Molle, D. P. Laureano, A. K. Portella, I. C. Werlang, S. Benetti Cda, C. Noschang and P. P. Silveira (2013). "Early life stress is associated with anxiety, increased stress responsivity and preference for "comfort foods" in adult female rats." <u>Stress</u> 16(5): 549-556.

McCutcheon, J. E., J. A. Beeler and M. F. Roitman (2012). "Sucrose-predictive cues evoke greater phasic dopamine release than saccharin-predictive cues." <u>Synapse</u> 66(4): 346-351.

Mericq, V., K. K. Ong, R. Bazaes, V. Pena, A. Avila, T. Salazar, N. Soto, G. Iniguez and D. B. Dunger (2005). "Longitudinal changes in insulin sensitivity and secretion from birth to age three years in small- and appropriate-for-gestational-age children." <u>Diabetologia</u> 48(12): 2609-2614.

Migraine, A., S. Nicklaus, P. Parnet, C. Lange, S. Monnery-Patris, C. Des Robert, D. Darmaun, C. Flamant, V. Amarger and J. C. Roze (2013). "Effect of preterm birth and birth weight on eating behavior at 2 y of age." <u>Am J Clin Nutr</u> 97(6): 1270-1277.

Molet, J., K. Heins, X. Zhuo, Y. T. Mei, L. Regev, T. Z. Baram and H. Stern (2016). "Fragmentation and high entropy of neonatal experience predict adolescent emotional outcome." <u>Transl Psychiatry</u> 6: e702.

Paxinos, G. and C. Watson (2007). <u>The rat brain in stereotaxic coordinates</u>. Amsterdam, Elsevier.

Portella, A. K., E. Kajantie, P. Hovi, M. Desai, M. G. Ross, M. Z. Goldani, T. J. Roseboom and P. P. Silveira (2012). "Effects of in utero conditions on adult feeding preferences." Journal of Developmental Origins of Health and Disease 3(3): 140-152.

Portella, A. K. and P. P. Silveira (2014). "Neurobehavioral determinants of nutritional security in fetal growth-restricted individuals." <u>Ann N Y Acad Sci</u>.

Portella, A. K., P. P. Silveira, D. P. Laureano, S. Cardoso, V. Bittencourt, C. Noschang, I. Werlang, F. U. Fontella, C. Dalmaz and M. Z. Goldani (2015). "Litter size reduction alters insulin signaling in the ventral tegmental area and influences dopamine-related behaviors in adult rats." <u>Behav Brain Res</u> 278: 66-73.

Reis RS, Dalle Molle R, Machado TD, Mucellini AB, Rodrigues DM, Bortoluzzi A, Bigonha SM, Toazza R, Salum GA, Minuzzi L, Buchweitz A, Franco AR, Pelúzio MCG, Manfro GG, Silveira PP. (2016). "Impulsivity-based thrifty eating phenotype and the protective role of n-3 PUFAs intake in adolescents." <u>Translational Psyhchiatry</u>. 15;6:e755.

Richman, J. S. and J. R. Moorman (2000). "Physiological time-series analysis using approximate entropy and sample entropy." <u>Am J Physiol Heart Circ</u> <u>Physiol</u> 278(6): H2039-2049.

Sears, B. and M. Perry (2015). "The role of fatty acids in insulin resistance." <u>Lipids Health Dis</u> 14: 121.

Shahrokh, D. K., T. Y. Zhang, J. Diorio, A. Gratton and M. J. Meaney (2010). "Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat." <u>Endocrinology</u> 151(5): 2276-2286. Silveira, P. P. (2014). "Playing with food: the fetal programming of food preferences." <u>Obesity (Silver Spring)</u> 22(5): 1210.

Silveira, P. P., M. Agranonik, H. Faras, A. K. Portella, M. J. Meaney, R. D. Levitan, V. Maternal Adversity and T. Neurodevelopment Study (2012). "Preliminary evidence for an impulsivity-based thrifty eating phenotype." <u>Pediatr</u> <u>Res</u> 71(3): 293-298.

Silveira, P. P., I. Pokhvisneva, H. Gaudreau, L. Atkinson, A. S. Fleming, M. B. Sokolowski, M. Steiner, J. L. Kennedy, L. Dube, R. D. Levitan, M. J. Meaney and M. r. team (2018). "Fetal growth interacts with multilocus genetic score reflecting dopamine signaling capacity to predict spontaneous sugar intake in children." <u>Appetite</u> 120: 596-601.

Sipols, A. J., J. Bayer, R. Bennett and D. P. Figlewicz (2002). "Intraventricular insulin decreases kappa opioid-mediated sucrose intake in rats." <u>Peptides</u> 23(12): 2181-2187.

Smotherman, W. P., K. A. Selz and A. J. Mandell (1996). "Dynamical entropy is conserved during cocaine-induced changes in fetal rat motor patterns." <u>Psychoneuroendocrinology</u> 21(2): 173-187.

Surina-Baumgartner, D. M., W. Langhans and N. Geary (1995). "Hepatic portal insulin antibody infusion increases, but insulin does not alter, spontaneous meal size in rats." <u>Am J Physiol</u> 269(5 Pt 2): R978-982.

Trifilieff, P. and D. Martinez (2014). "Imaging addiction: D2 receptors and dopamine signaling in the striatum as biomarkers for impulsivity." <u>Neuropharmacology</u> 76 Pt B: 498-509.

Ueki, K., T. Kondo and C. R. Kahn (2004). "Suppressor of Cytokine Signaling 1 (SOCS-1) and SOCS-3 Cause Insulin Resistance through Inhibition of Tyrosine Phosphorylation of Insulin Receptor Substrate Proteins by Discrete Mechanisms." <u>Molecular and Cellular Biology</u> 24(12): 5434-5446.

Veening, M. A., M. M. van Weissenbruch, R. J. Heine and H. A. Delemarre-van de Waal (2003). "Beta-cell capacity and insulin sensitivity in prepubertal children born small for gestational age: influence of body size during childhood." <u>Diabetes</u> 52(7): 1756-1760.

Vucetic, Z., K. Totoki, H. Schoch, K. W. Whitaker, T. Hill-Smith, I. Lucki and T. M. Reyes (2010). "Early Life Protein Restriction Alters Dopamine Circuitry." <u>Neuroscience</u> 168(2): 359-370.

FIGURE LEGENDS:

Figure 1: Schematic illustration of electrode placement in NAcc shell (A). Hematoxylin-eosin-stained coronal sections of rat brain were used to confirm the microelectrode recording site in the NAcc shell (B).

Figure 2: Body weight at birth and at weaning, in male and female rats from FR and control groups. Data is expressed as mean \pm SEM. A) Birth weight in grams. There is an effect of the group, in which FR animals weighed less than controls (GEE, n=114-176/group/sex, *p<0.05) and females weighed less than males (GEE, n=114-176/group/sex, *p<0.05). B) Body weight at weaning. Body weights were similar between the FR and control groups at weaning age. There was no effect of the group, demonstrating the catch-up growth in the FR animals. Females weighed less than males (GEE, n=51-82/group/sex, *p<0.05). C) There were no significant differences in body weight between FR and control rats used in Study 1 to measure food intake using the BioDAQ® system (Student's T Test, n=8/group, p>0.05). D) There were no significant differences in body weight between the FR and control rats used in Study 2 for chronoamperometric measurements (Student's T Test n=4-5/group, p>0.05).

Figure 3: Palatable and regular food consumption were measured using the BioDAQ® system. Data is expressed as mean \pm SEM. A) Average palatable food consumption/100g body weight measured during the light and dark phases of the 24 hr light cycle. GEE analysis showed a significant interaction between group and time of day, in which the FR group ate more palatable food during the dark phase (Generalized Estimating Equation, n=8/group,*p<0.05). B) Entropy rates across 96 hours and divided by the light (C) and dark phase (D).

In D, there was higher behavioral entropy in the FR group during the dark phase (Student's T test, n=8/group, *p<0.05).

Figure 4: Peripheral insulin levels. Data is expressed as mean \pm SEM. FR rats had significantly higher insulin levels than control rats (FR= 2,034 \pm 0,322, control= 1,086 \pm 0,244, Student's t-test, n=12-15/group, p=0.031).

Figure 5: In vivo electrochemical NAcc DA response to different conditions: exposure to rat chow and Froot loops at baseline and after IP saline or insulin. Data is expressed as mean ± SEM. Three variables summarize the DA response: A) T rise or time to reach DA peak after the stimulus. There was an interaction between group and food exposure condition in which FR rats took a longer time to reach the dopaminergic peak after Froot loops presentation and this finding is completely reversed by insulin injection (Generalized Estimating Equation, n=4-5/group,*p<0.05); B) Amplitude of the dopaminergic response. There was an interaction between food exposure condition and group, in which FR rats responded differently to Froot Loops® when comparing baseline vs. saline injection conditions (Generalized Estimating Equation, n=4-5/group, p= 0.038); C) T80 or time taken to reach 80% dopaminergic signal clearance following stimulus presentation. Although there was a significant interaction between group and food exposure condition, a post-hoc analysis showed only a longer T80 value after IP insulin and Froot Loops® compared to regular rat chow presentation chow. This pattern appears more evident in the FR group (Generalized Estimating Equation, n=4-5/group, p=0.024).

Intrauterine growth restriction modifies the accumbal dopaminergic response to palatable food intake

DP LAUREANO¹, MB ALVES², PM MIGUEL¹, TD MACHADO³, AR REIS³, AB MUCELLINI⁴, FDAS CUNHA³, C LAMPERT², APA SALVADOR⁵, R DALLE MOLLE³, PR MOSCA³, I POKHVISNEVA⁶, M DESAI⁷, MG ROSS⁷, PP SILVEIRA^{1,6,8}

¹ Programa de Pós Graduação em Neurociências, Instituto de Ciências Básicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ² Programa de Pós Graduação em Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ³ Programa de Pós Graduação em Saúde da Criança e do Adolescente, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁴Programa de Pós Graduação em Psiquiatria, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁵ Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁵ Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; ⁶ Ludmer Centre for Neuroinformatics and Mental Health, Douglas Mental Health University Institute, McGill University, Montreal, QC, Canada;⁷ Dept of Ob/Gyn, Harbor-UCLA Med Ctr, Torrance, CA, United States;⁸ Department of Psychiatry, Faculty of Medicine, McGill University, Sackler Program for Epigenetics & Psychobiology at McGill University, Montreal, QC, Canada.

Corresponding author: Daniela Pereira Laureano, PhD.
Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde.
Universidade Federal do Rio Grande do Sul.
Sarmento Leite, 500, Farroupilha, 90035-190, Porto Alegre, RS, Brazil.
E-mail: dlaureano@hcpa.edu.br

Abstract

Intrauterine growth restriction (IUGR) associates with increased preference for palatable foods and altered insulin sensitivity. Insulin modulates the central dopaminergic response and changes behavioral responses to reward. We measured the release of dopamine in the accumbens during palatable food intake in IUGR rats both at baseline and in response to insulin. From pregnancy day 10 until birth, gestating Sprague–Dawley rats received either an ad libitum (Control), or a 50% food restricted (FR) diet. In adulthood, palatable food consumption and feeding behavior entropy was assessed using an electronic food intake monitor (BioDAQ®), and dopamine response to palatable food was measured by chronoamperometry recordings in the nucleus accumbens (NAcc). FR rats eat more palatable foods during the dark phase, and their eating pattern has a higher entropy compared to control rats. There was a delayed dopamine release in the FR group in response to palatable food and insulin administration reverted this delayed effect. Western blot showed a decrease in suppressor of cytokine signaling 3 protein (SOCS3) in the ventral tegmental area (VTA) and an increase in the ratio of phospho-tyrosine hydroxylase to tyrosine hydroxylase (pTH/TH) in the NAcc of FR rats. Administration of insulin also abolished this latter effect in FR rats. FR rats showed metabolic alterations and a delay in the dopaminergic response to palatable foods. This could explain the increased palatable food intake and behavioral entropy found in FR rats. IUGR may lead to binge eating, obesity and its metabolic consequences by modifying the central dopaminergic response to sweet food.

Introduction

Exposure to fetal adversity is associated with altered impulsivity-based behaviors such as, increased preference for sugary foods at different ages in humans (Barbieri, Portella et al. 2009, Ayres, Agranonik et al. 2012, Portella, Kajantie et al. 2012, Silveira, Agranonik et al. 2012, Portella and Silveira 2014, Silveira 2014, Dalle Molle and Silveira 2015, Laureano, Molle et al. 2015, Reis RS 2016) and animals (Alves, Molle et al. 2015, Cunha Fda, Dalle Molle et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016). Fetal adversity contributes to the development of prediabetes, type II diabetes, cardiovascular disease and mental health disorders. (Hales and Barker 1992, Eriksson, Wallander et al. 2004, Lahti, Eriksson et al. 2014, Li, Ley et al. 2015). Both humans and animals exposed to intrauterine growth restriction (IUGR) show altered behavior towards sweet rewards starting at birth (Ayres, Agranonik et al. 2012, Laureano, Dalle Molle et al. 2016). These findings suggest that fetal adversity programs neurobiological pathways involved in reward sensitivity (Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016). Insulin receptors are found in the ventral tegmental area (VTA), the nucleus accumbens (NAcc) and the prefrontal cortex (PFC). Insulin modulates the activity within these pathways (Sipols, Bayer et al. 2002, Figlewicz 2003). Fetal growth restriction impairs insulin secretion relative to insulin sensitivity in children and adults, and this phenomenon occurs before the onset of insulin resistance (Jensen, Storgaard et al. 2002, Veening, van Weissenbruch et al. 2003, Mericq, Ong et al. 2005). The behavioral phenotype of children and adults exposed to IUGR may be due to variations in insulin sensitivity within these neural pathways and these variations may in turn predispose these individuals to an enhanced risk for metabolic and psychiatric disorders in later life.

Insulin acts on the dopaminergic neurons of the ventral tegmental area (VTA), that project to the nucleus accumbens and PFC (Figlewicz, MacDonald Naleid et al. 2007), and insulin modulates behavioral responses including reward sensitivity and conditioning (Figlewicz, Bennett et al. 2008). Perinatal adversity affects insulin signaling within the mesocorticolimbic pathway and alters behavioral responses to sweet rewards suggesting changes in reward

sensitivity. (Portella, Silveira et al. 2015). Insulin acts on its receptors located on dopamine neurons in the midbrain including in the VTA and substantia nigra (Figlewicz, Evans et al. 2003). Insulin then triggers Mitogen-activated Protein Kinase (MAPK) and Phosphatidylinositol-3-Kinase (PI-3K) intracellular signaling cascades. The activation of PI-3K is especially linked to metabolic functions and induces the phosphorylation of protein kinase B (Akt). SOCS3 binds to distinct domains of the insulin receptor (Emanuelli, Peraldi et al. 2000), inhibiting the phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 in vivo and in vitro (Ueki, Kondo et al. 2004). This attenuation of insulin signaling by SOCS3 results in reduced activation of glycogen synthesis and glucose transport in cultured cells (Ueki, Kondo et al. 2004). SOCS3 activates an intracellular negative feedback loop within the insulin receptor that is regulated in both a phosphorylation and transcription-dependent manner. Changes in AKT phosphorylation and SOCS3 levels are indicative of modifications in insulin sensitivity within the tissues that are affected.

Maternal protein restriction during gestation alters brain systems involved in hedonic responses to sucrose. For example, these animals have increased levels of tyrosine-hydroxylase (TH) and dopamine in the VTA and PFC, respectively, and enhanced expression of genes responsible for TH and dopamine reuptake transporter (DAT) production in areas of the mesocorticolimbic system (Vucetic, Totoki et al. 2010). IUGR adult rats show robust reductions in the expression of enzymes involved in dopamine synthesis in the NAcc and orbitofrontal cortex (Alves, Molle et al. 2015, Dalle Molle, Laureano et al. 2015). In this current work we hypothesized that insulin would differentially modulate the behavioral and neurochemical DA responses to palatable foods in adult rats exposed to IUGR compared to controls.

Experimental Procedures

Animals

Primiparous Sprague Dawley dams were time-mated at approximately 80 days of age (CEMIB Laboratory Animal Reference Center, Campinas, SP,

Brazil) were time-mated within our institutional animal facility. Throughout pregnancy the dams were single-housed in Plexiglas home cages (49 x 34 x 16 cm) and maintained in a controlled environment. Lights were turned on between 7AM and 7PM, temperature was maintained at 22 \pm 2°C, and the cages were changed once per week. Food and water was provided ad libitum until gestational day 10. At 10 days of gestation, dams were randomly assigned to one of two groups: control (Adlib)(n=24), receiving an ad libitum (AdLib) diet of standard laboratory chow (Nuvilab®) or 50% food restriction (FR)(n=12), receiving 50% of the ad libitum-fed mothers intake. The FR group's ration of food was based on the daily consumption of normal chow in a cohort of gestating Sprague Dawley rats) (Desai, Gayle et al. 2005). These modified feeding conditions were maintained from gestational day 10 until the birth of the litters. Within 24 hours after birth, the pups litters were culled to 8 pups per litter (4 males and 4 females) and all pups were cross-fostered to other AdLib dams, forming the following groups (considering the biological/adoptive mother, i.e., gestation/lactation maternal diet): AdLib/AdLib (control - CTRL) and FR/AdLib (FR). Weaning occurred on postnatal day 21. Pups were separated by sex and housed four per cage. The excess male pups and the females were used in different studies (projects HCPA/GPPG 12-0353, 13-0544, 14-0289 and 140111). All rat pups were maintained on standard laboratory chow and water available ad libitum and were kept in a controlled environment as described above. Body weights were recorded weekly from weaning age using a digital scale with 0.01 g precision (Marte®, Canoas, Brazil). The males (n=64 animals) were assigned to one of two experiments, a) evaluation of feeding behavior (n=16 animals) and evaluation of insulin signaling in the mesocorticolimbic system (n=34 animals, Study 1) and b) chronoamperometric evaluation of the accumbal dopamine response to palatable food (n= 14 animals, Study 2). To avoid litter effects, a maximum of two rats per litter was used in each experiment.

The Research Ethics Committee of Hospital de Clínicas de Porto Alegre (GPPG/HCPA, project numbers 13-0420, 13-0264, 14-0111, 13-0544, 11–0053, 14-0289, 120353) approved all the animal procedures described here. All procedures were conducted in a climate-controlled environment within our

animal facility's behavioral testing rooms (Unidade de Experimentação Animal/HCPA).

Study 1: Evaluation of feeding behavior and insulin signaling in the mesocorticolimbic system

Analysis of feeding behavior

Rats were singly housed in cages equipped with a BioDAQ® food intake monitoring system (Research Diets) at 80 days of age. BioDAQ® is a computerbased software program that can provide detailed feeding behavior data. Total food intake and meal patterns were analyzed with the BioDAQ® system as previously described (Farley, Cook et al. 2003, Machado, Dalle Molle et al. 2013, Dalle Molle, Laureano et al. 2015). Briefly, the system uses a food hopper mounted on an electronic strain gauge-based load cell to measure food consumption. The food hopper is weighed 50 times per second (accurate to 0.01 g) and the mean and standard deviation (S.D.) of food consumption over approximately 1 sec is calculated by a peripheral computer. Food consumption is signaled by a change in the food hopper weight (defined as a S.D. > 2000 mg) caused by the animal ingesting food. Each feeding event including cage/animal number, start date and time, feeding duration, final hopper weight and amount of food eaten is recorded and exported to a central computer, where it is entered into a Microsoft Excel spreadsheet (Microsoft, Redmond, WA). There were two kinds of events recorded in BioDAQ®: feeding bouts and meals. The end of a feeding bout was signaled when the hopper was left undisturbed for 5s (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 >0.1g, separated from other feeding bouts within a range of 15 min (Surina-Baumgartner, Langhans et al. 1995, Eckel, Langhans et al. 1998). Rats feeding behavior was analyzed for 8 days. During the habituation phase, consisting of the first four days, the rats were given access to standard rat chow containing 2.95Kcal/g, 22% protein, 4,5% fat, 54% carbohydrate in each kg

(NUVILAB®) and water *ad libitum*. During the last four days, the rats were provided with access to both the previously described standard laboratory rat chow and a highly palatable diet containing 4.82 kcal/g, 8% mineral, 14% protein, 34% fat, 6% fiber, 30% carbohydrate in each kg (Prag Soluções Biociências®). This latter diet derived 20% of its carbohydrate content from sucrose. The rats were provided with a small quantity of the highly palatable diet in their home cages for 2 days prior to the start of the food choice experiment as a means to reduce possible effects of neophobia on the results. Each day, the diets were replenished at the same time that the cages and BioDAQ® system were cleaned and maintained (Kowalski, Farley et al. 2004).

Peripheral measurements – ELISA

At 100 days of age, the rats were fasted for 4h and injected with insulin (1 IU/kg) or saline through the intraperitoneal route (i.p.) and decapitated 15 minutes later for protein measurements or received no injection prior to decapitation for insulin measurements. Brain tissue and blood were collected. Blood was collected in tubes and centrifuged at +4°C at 4000 rpm for 10min. Plasma was separated in aliquots and frozen at -80°C until further analysis. Plasma insulin levels were measured using the Rat/Mouse Insulin ELISA Kit (Millipore, EZRMI-13K).

Central measurements – Western Blotting

Brains were flash frozen in isopentane and stored at -80 °C until further analyses. The brains were then warmed to -20 °C and cut into thick sections of 0.1 cm with the aid of an Atlas (Paxinos and Watson 2007). Bilateral 1 mm diameter punches were taken from the ventral tegmental area (VTA) and nucleus accumbens (NAcc) (Shahrokh, Zhang et al. 2010).

Tissue samples were homogenized in cytosol extraction buffer with the addition of a protease inhibitor cocktail, (Sigma- Aldrich, P8340) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets, Roche, 4906845001). The samples were then centrifuged at 6000 rpm for 2 min at +4 °C. Total protein was quantified using a Bicinchoninic Acid (BCA) kit with

bovine serum albumin as a standard (Pierce BCA Protein, Thermo Scientific, 23225). Aliquots of the supernatant containing 35 µg of VTA or 25 µg of NAcc protein were incubated with LDS (Invitrogen, NP0007) and DTT (Sigma-Aldrich, 43815) at 99°C for 3 min. The samples were subjected to electrophoresis using a 4 to 12% polyacrylamide gradient gel (Invitrogen, NP0323BOX) together with the addition of a standard molecular weight (Spectra[™] Multicolor Broad Range Protein Ladder, Thermo scientific, 26634), before being transferred to a nitrocellulose membrane (GE Healthcare, RPN303C). Blots were blocked in Tris buffered saline containing 5% non-fat milk concentrate and 1% Tween®20 (Sigma, P1379). We measured TH, pTH, in the NAcc, and SOCS3, AKT and pAKT in the VTA. The membranes were incubated overnight at +4°C with primary antibodies: TH 1:5000 (anti-tyrosine hydroxylase, Sigma-Aldrich, T2928), pTH 1:500 (anti-phospho tyrosine hydroxylase (Ser40), Invitrogen, 368600), SOCS3 1:500 (suppressor of cytokine signaling) (Cell Signaling, #2923), Akt (pan) (11E7) 1:1000 (Cell signaling, #4685), Phospho-Akt (Ser473) 1:1000 (Cell Signaling, #4060), followed by anti-mouse secondary antibodies (1:2500) (Anti-Mouse IgG, Cell Signaling, #7076s) or anti-rabbit (1:2500) (Anti-Rabbit IgG, Cell Signaling, #7074s) at room temperature for 1h. The membrane was then exposed on ImageQuant LAS 4000 GE Healthcare Life Sciences using ECL (ECL western blotting analysis system, GE healthcare, RNP2106). Results were calculated as a ratio of intensity of the protein of interest to that of actin 1:2000 (Sigma-Aldrich, A4700) in the same membrane, using a stripping protocol as needed (Laureano, Dalle Molle et al. 2016). The results were expressed as a percentage of control values (Colman, Laureano et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016).

Study 2: Chronoamperometric evaluation of the accumbal dopamine response to palatable food

Surgery

Between 80–120 days of age, rats (n=14) underwent stereotaxic surgery. They were anesthetized with ketamine 40 mg/kg (ip.) and xylazine 5mg/kg (ip.)

and maintained under isoflurane anesthesia during the surgery. Animals received tramadol 5 mg/kg (ip.) before surgery, and tramadol 2.5 mg/kg (ip.) immediately post-surgery, and sodium dipyrone 200 mg/kg (IM)was given to reduce pain post-surgery. Tramadol 5 mg/kg (ip.) was kept 12h/12 h thereafter, as well as and cetoprofen 5 mg/kg (ip.) 1x day for 2 consecutive days postoperative. Forty µL of bupivacaine with epinephrine (0.2 mg per animal) was injected subcutaneously at the surgical site. Electrochemical probes were lowered into the NAcc shell (coordinates: 1.2 mm anterior to bregma, 0.8 mm lateral to the midline, and 7.0 mm ventral to the surface of the cortex) (Shahrokh, Zhang et al. 2010). Animals were also implanted with a Ag/AgCI reference electrode in the contralateral parietal cortex. Miniature pin connectors soldered to the voltammetric and reference electrodes were inserted into a connector (9-pin ABS Plug, Ginger Scientific, GS09PLG-220). The assembly was secured with acrylic dental cement to four stainless steel screws threaded into the cranium (Paxinos and Watson 2007, Shahrokh, Zhang et al. 2010). Animals were returned to their home cages after a 2-5h recovery.

Electrochemical probes and measurements in freely moving rats

This experiment was performed during the early afternoon according to Shahrokh, Zhang et al. 2010). The voltammetric electrodes consisted of a bundle of three $30-\mu$ m-diameter carbon fibers (Textron Systems, Wilmington, MA) extending $50-100\mu$ m beyond the sealed tip of a pulled glass capillary (outer diameter 0.5 mm), and repeatedly coated with a 5% solution of Nafion® (Sigma-Aldrich, 274704), a perfluorinated ionomer that promotes the exchange of cations such as dopamine but impedes the exchange of interfering anionic species such as ascorbic acid (AA) and 3,4 dihydroxyphenylacetic acid. Each electrode was calibrated to determine dopamine sensitivity and selectivity compared with AA in 0.1 M PBS (pH 7.4) that contained 250 μ M AA to mimic brain extracellular conditions. The electrodes had a mean (±SEM) dopamine to AA selectivity ratio of 1619:1 ± 133:1 (Doherty and Gratton 1997, Shahrokh, Zhang et al. 2010, McCutcheon, Beeler et al. 2012).

Dopamine was detected as current through its oxidation and reduction, and measurements were recorded using a computer controlled, high-speed chronoamperometric apparatus (Quanteon, Lexington, KY). An oxidation potential of +0.55mV (with respect to the reference electrode) was applied to the electrode for 100 ms at a rate of 2 Hz. The oxidation current was digitally integrated during the last 80 ms of each pulse. The sum of every 10 digitized oxidative cycles from the chronoamperometric waveform was automatically converted into equivalent values of dopamine concentration using an in vitro calibration factor. The reduction current generated when the potential was returned to resting state (0.0 V for 100 ms) was digitized and summed in the same manner and served as an index to identify the main electroactive species contributing to the electrochemical signals. The magnitude of the increase in reduction current elicited by an elevation in dopamine concentration is typically 60-80% of the corresponding increase in oxidation current [reduction to oxidation ratio (red:ox)], 0.6–0.8 using nation-coated electrodes and a sampling rate of 2 Hz (Doherty and Gratton 1997, Gerhardt, Ksir et al. 1999, Shahrokh, Zhang et al. 2010, McCutcheon, Beeler et al. 2012). Supplementary information is available at <u>https://www.youtube.com/watch?v=Sf7L-34pqmc</u>.

In vivo electrochemical recordings

One day following surgery the rats received 6 Froot Loops® pellets in their home cages to reduce the influence of food neophobia during the experiment. The rats started to be habituated to the voltammetry apparatus two days following surgery. They were given 6 Froot Loops® pellets within a voltammetry cabinet ($60 \times 60 \times 60 \text{ cm}$) containing a single hole at the top of the box to connect the voltammetry cable and a glass front door. Rats were habituated to the box for 1 hr.

Voltammetry recordings took place on the 3rd and 5th days following surgery at afternoon. During the first two hours of recording, the rat was placed in the voltammetry cabinet to allow for habituation and electrochemical signal stabilization. The rat then received 30 grams of standard rat chow given in small pieces and the dopaminergic response to the presentation of standard rat chow

was recorded. The remaining food was removed and the rat remained in the box for 1 hour to allow for signal stabilization. Fifteen pellets of Froot Loops® were then offered for 30 minutes and the recordings continued for another hour to record the dopaminergic response to palatable food. Standard rat chow available at 80% of the free-feeding rate was available in the home cage between recording sessions. The next day, the rats underwent another 2 hours of acclimatization to the voltammetry cabinet. Rats were then injected with 100 microliters saline solution (ip.) and given15 Froot Loops® pellets for 30 minutes. The food was then removed and the recordings continued for another hour. On the third day of recordings, rats received an injection of 5 UI/kg insulin (i.p.) in a total volume of 100 microliters using saline for dilution.

Histological confirmation of electrode placement

On the sixth day, the electrode placement in the recording site was validated by histology. Rats were deeply anesthetized with isoflurane and a +4.5 DC current was applied to the carbon fiber electrode in order to produce a lesion at the exact recording site in the brain (Ledo, Lourenco et al. 2015). Rats were then decapitated, and the brains were fixed in 4% paraformaldehyde (PFA) solution in PBS for 24h. The brains were cryoprotected in 30% sucrose solution and frozen in isopentane and liquid nitrogen. Coronal sections (50 μ m) of the brain were made using a cryostat (Leica, Wetzlar, Germany) with the aid of an atlas (Paxinos and Watson 2007). The sections were mounted on gelatin-coated glass slides and stained with hematoxylin-eosin to verify the lesion site caused by the current applied to the microelectrodes. Rats with incorrect electrode placements in the recording site (n=5) were excluded from statistical analyses (Figure 1).

Statistical analyses

Data were expressed as means ± SEM and were analyzed using Student's t-tests when comparing two groups (FR vs. Adlib groups).

Generalized Estimating Equations (GEE) were used to analyze differences in birth weight and body weight at weaning since this method takes the interdependent effect of litter into account. We used group and sex as variables and adjusted by litter size. The models were adjusted for both litter number at birth and at weaning age.

BioDAQ® data was analyzed in three different ways: a) GEEs were used with group, experimental day, time of day (light vs. dark phase) as independent variables and total amount of food consumed was the dependent outcome variable. Food consumption was adjusted by body weight and analyzed as grams consumed per 100g of body weight; b) Using continuous recordings from the BioDAQ® system, we divided 24 hours in a day into 96 bins of 15 minutes and categorized the rat's behavior into one of three possible states: resting, eating rat chow or eating palatable food. We then measured unpredictability which is a measure of transitions from one type of state to another. We derived a measure of unpredictability by focusing on the conditional probabilities that describe transitions in behavior. This method is described in Molet et al. (2016). Briefly, we characterized the rats' behavior using an empirical transition matrix of conditional probabilities of changing from one behavioral state to another.

Entropy is a natural summary measure of randomness or the unpredictability of a distribution and can be defined in many ways. We focused on the Shannon entropy. We first calculated the probabilities to be in each of the three states for all rats together and used them as the stationary distribution of the Markov chain. The stationary probabilities serve as weights for the entropy values, which produce a measure known as the entropy rate (Molet, Heins et al. 2016), and these were compared between the groups using Student's t-test; c) Approximate entropy (ApEn) was calculated according to Richman and Moorman, 2000) using the continuous measure as described in b), we then calculated the tolerance, r, (0.3 times standard deviation of the consumption per 100g) for each subject. We defined that two sequences xi and xj will match if they are within a tolerance r, i.e. the distance between xi and xj is less than or equal to r. Then for each subject we calculated two probabilities: that two sequences of two consecutive periods will match and that two sequences of three consecutive periods will match. Further, for each subject we calculated approximate entropy, ApEn, as a negative logarithm of conditional probability that two sequences within a tolerance r for two consecutive periods will remain within tolerance r for the next period.

Voltammetry data was analyzed using GEEs, and group and type of food exposure (rat chow, Froot Loops®, IP Saline_Froot Loops®) IP Insulin_Froot Loops®) were independent factors within the model. Post hoc group comparisons were completed using the Bonferroni post hoc test. Three aspects of the dopaminergic response to food and injection presentation were analyzed based on the model developed by Joyce et al. (2007). The time it takes dopamine to reach its peak value (T rise), the amplitude of the dopaminergic response (in mMol) and the time it takes for 80% of the original dopamine content to be cleared (T80) were measured. The T80 value is a measure of dopamine reuptake.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) and R statistical software. Significance levels for all measures were set at p= 0.05.

RESULTS

Body weight

We found a significant effect of group [B=-0.834, p=0.004] on birth weight where FR rats weighed less than controls, and there was a significant effect of sex [B=-0.324, p<0.001] in which females weighed less than males (Figure 2A).

At weaning age, the body weight difference between FR and controls disappeared [B= -1.236, p=0.355], indicating catch up growth in the FR group. Females still weighed less than males at weaning age [B= -2.536, p<0.001], (Figure 2B). Sample size in this experiment reflects all the animals used in the different projects listed in the Methods since we decided not to trace the pups that were eventually assigned to each project at weaning age since we did not want to disturb the litters in the first 21 days of life.

The rats that were used in the analyses of BioDAQ® food consumption data and dopaminergic responses to food and injection presentation had no significant differences in body weights between the FR and control groups (body weights in BioDAQ® experiment, Student's T Test t(14)=0.530, p=0.605, (Figure 2C) and body weights in voltammetry experiment, Student's T Test t(7)=2.037, p=0.09, (Figure 2D).

Study 1

FR animals eat more palatable foods in the dark phase

Our analyses of food consumption during the last 4 days of study 1 showed that the rats ate very little or no regular rat chow. We focused our further analyses on the consumption of palatable food. We found no effect of the experimental day (1 to 4) (Wald= 3.17; df= 3; p= 0.37) and no effect of the group (Wald= 1.12; df= 1; p= 0.27) on palatable food consumption across the whole day. There was an effect of time of day (light vs. dark phase) (Wald= 9.63; df= 1; p= 0.002). There was also a significant interaction between group and time of day (Wald= 10.43; df= 1; p= 0.001), in which FR rats consumed more palatable food than control rats during the dark (active) phase of the light cycle (Figure 3A).

FR animals have differential behavioral entropy

There were no significant differences in behavioral entropy rates between FR and control groups over the course of the 96 hours food consumption experiment (Student's t-test, t(14)=1.79, p=0.09). However, the FR group showed significantly higher entropy during the dark phase (t(14)=2.72, p=0.02) but not during the light phase (t(14)=1.42, p=.18) (Figure 3 B, C, D) compared to the control group.

Approximate entropy, a measure that also takes into account the variability in the amount consumed (ApEn), was also calculated. The FR group had increased ApEn during the dark phase compared to the control group

 $(FR=0.282\pm0.020, \text{ control}=0.163\pm0.023, \text{ Student's t-test, t(14)}=3.76, p=0.002),$ but there were no group differences during the light phase (FR=0.091±0.010, control=0.115±0.012, Student's t-test, t(14)=1.51, p=0.153).

FR rats have higher peripheral insulin

Figure 4 shows that FR rats had significantly higher insulin levels than control rats (Student's t-test, t(11)=2.472 p=0.031) (Figure 4).

NAcc pTH/TH ratio is higher in the FR group but this is reverted by insulin

There were no statistically significant differences between groups in TH levels after saline [Student's t-test, t(10)=0.198, p=0.847] or insulin injection [Student's t-test, t(10)=0.633, p=0.541]. pTH levels were also similar between groups when exposed to saline [Student's t-test, t(10)=0.952, p=0.364] or insulin [Student's t-test, t(10)=0.800, p=0.442]. However, the ratio of pTH/TH was increased in the FR group compared to controls when exposed to saline [Student's t-test, t(10)=2.599, p=0.027], which was completely reverted by insulin injection [Student's t-test, t(10)=1.007, p=0.338] (Table 1).

VTA SOCS3 levels are diminished in FR animals

AKT levels in the VTA were similar between groups when exposed to saline [Student's t-test, t(7)=1.022, p=0.341] or insulin [Student's t-test, t(9)=1.009, p=0.340]. pAKT levels were also not significantly different between groups after saline [Student's t-test, t(7)=0.916, p=0.390] or insulin [Student's t-test, t(9)=1.501, p=0.168] injections. Similarly, the pAKT/AKT ratio was similar between groups after saline [Student's t-test, t(7)=0.621, p=0.554] and insulin injection [Student's T Test, t(9)=0.280, p=0.786]. SOCS3 levels were significantly diminished in the FR compared to control group after saline injection [Student's t-test, t(7)=3.584, p=0.009], but no significant differences were observed following insulin injection [Student's t-test, t(9)=0.428, p=0.678] (Table 2).

FR animals show a delayed dopaminergic response to palatable food and this effect is reversed by insulin

Food consumption of both regular rat chow and Froot Loops® presented in the voltammetry cabinet was measured. Rat chow consumption was minimal during the experiment, similar to the findings from Study 1 reported above. Rat chow intake frequency was similar between the FR and control groups during the 30 minutes of food presentation [Control = 7.8 ± 2.3 , FR= 10.7 ± 2.8 , Student's T Test, t(7)=1.007, p=0.35]. All the rats ate the entire portion of Froot Loops that was offered 1 hour later, as well as on the following days of the experiment.

The time it took for the dopaminergic response to reach peak amplitude (T rise) was not significantly different between the groups (Wald= 0.185; df= 1; p= 0.667), but there was an effect of food exposure (Wald= 8.972; df= 3; p= 0.030), and a significant interaction between the group and food exposure condition (Wald= 24.951; df= 3; p < 0.001). Posthoc analysis showed that the FR and control groups differed significantly after exposure to Froot Loops® (p = 0.006). The FR group took a significantly longer time to reach the dopaminergic peak. This finding is completely reversed when rats were injected with insulin (p = 0.006), Figure 5A.

The amplitude of the dopaminergic response was not significantly different between the FR and control groups (Wald= 1.134; df= 1; p= 0.287), but there was an effect of the food exposure condition (Wald= 8.215; df= 3; p= 0.042) and an interaction between group and the food exposure condition (Wald = 93.226; df= 3; p < 0.001). Bonferroni analyses showed that while there were no significant differences in amplitude in the different food exposure conditions in the control group, the FR rats responded with higher amplitude levels in the presence of Froot Loops® only in comparison to IP Saline_ Froot Loops® (p=0.038), Figure 5B.

The T80 value was not significantly different between the groups (Wald= 0.420; df= 1; p= 0.517), but there was an effect of the food exposure condition (Wald= 13.063; df= 3; p= 0.005) and an interaction between the group and food

exposure condition (Wald= 45.415; df= 3; p < 0.001). However, posthoc analysis was not able to detect a significant interaction. The only detectable difference was in the general comparison between the response after rat chow and IP Insulin_ Froot Loops®, in which the T80 value was longer after insulin injection (p= 0.024). Visual inspection of the graph suggests this finding is greater in the FR group (Figure 5C).

DISCUSSION

In this study, we confirmed previous findings of increased palatable food intake in FR (IUGR) animals, in agreement with several previous studies from our group (Alves, Molle et al. 2015, Dalle Molle, Laureano et al. 2015, Laureano, Dalle Molle et al. 2016) and others (Vucetic, Totoki et al. 2010), and to the findings in humans that experienced IUGR (Lussana, Painter et al. 2008, Barbieri, Portella et al. 2009, Migraine, Nicklaus et al. 2013). We have previously shown that FR animals have increased preference for palatable food throughout the light-dark cycle(Dalle Molle, Laureano et al. 2015). In the current experiment, we focused exclusively on the consumption of palatable food, since the rats ate very little if any standard rat chow that was offered during the experiment. We showed that while FR and control rats ate a similar amount of palatable food over the course of the whole day, FR rats ate more palatable food during the dark phase of the light/dark cycle when rats are active. We also found a greater behavioral entropy or transitions between different behavioral states in the FR compared to the control rats. The FR group had a more unpredictable and disorganized behavior in the dark phase, as shown by the higher entropy rates. These refinements in measurements are important advances in our understanding of how gestational FR affects the behavioral response to palatable foods in adulthood.

In addition, we showed that FR animals have increased peripheral insulin, in agreement with the "thrifty phenotype" hypothesis proposed by Hales and Barker in 1992 (Hales and Barker 1992). We also found altered insulin sensitivity as reflected through lower levels of SOCS3 in the VTA of FR rats. FR rats also appear more sensitive to insulin's action at a molecular level by

showing normalization of the pTH/TH ratio after insulin injection. FR rats also showed a delayed dopaminergic peak response to palatable food in the accumbens and this finding was reversed by systemic insulin injection.

It is interesting that the well-known increased preference for palatable foods seen in FR rats is accompanied by higher behavioral unpredictability and higher approximate entropy, especially when considering that impulsivity seems to be a behavioral phenotype associated with the increased intake of palatable foods in humans exposed to IUGR (Ayres, Agranonik et al. 2012, Reis, Dalle Molle et al. 2016). It has been suggested that behavioral patterns are altered through the shifting of entropy to different regions across the brain, or that unpredictable behavioral patterns are coupled with more predictable brain activation patterns (Smotherman, Selz et al. 1996, Hong, Barton et al. 2012). This seems to be especially evident in models of altered striatal dopamine function (Hong, Barton et al. 2012). Therefore, our behavioral findings showing increased palatable food consumption and increased entropy rates in FR rats strongly suggests the involvement of altered dopaminergic responsivity to palatable foods, and this hypothesis was confirmed by the electrochemical experiment.

We found a delay in the peak dopaminergic response to sweet foods but not in response to regular chow in FR rats compared to controls. The increased intake of palatable foods observed in FR rats may be an attempt to reach the same dopaminergic peak in a similar amount of time compared to control rats. Activation of dopaminergic neurons facilitates the development of positive reinforcement during reward-seeking and behavioral flexibility (Adamantidis, Tsai et al. 2011). The delayed time it takes FR rats to reach a peak dopaminergic response to palatable food agrees with our previous finding of decreased place conditioning to palatable foods despite higher consumption in FR compared to control rats (Dalle Molle, Laureano et al. 2015). Deficits in striatal dopaminergic transmission are also related to impulsivity in humans and in animals (Trifilieff and Martinez 2014), a feature that characterizes individuals with IUGR..

Importantly, the time to reach the peak dopaminergic response to palatable foods is significantly lower in FR rats following insulin injection compared to control rats. These findings suggest that rats exposed to

gestational food restriction are more sensitive to the effects of systemic insulin in adulthood than control rats. The elimination of pTH/TH protein level differences in the nucleus accumbens of FR rats following insulin injection, the longer time to achieve dopamine reuptake (T80 value) and the lower levels of SOCS3 in the VTA are all aligned with the concept that gestational food restriction alters the response to palatable food possibly through alterations in insulin sensitivity in adulthood.

IUGR is associated with insulin resistance. In young rats, peripheral levels of insulin are increased in FR rats and this may facilitate insulin signaling in some brain regions before reaching exhaustion. Central insulin resistance first appears in the hypothalamus (Sears and Perry 2015). We have evidence that IUGR animals at this age already have increased hypothalamic SOCS3 levels (Mucellini et al., in prep). Therefore, the behavioral phenotype observed in IUGR individuals may be the result of different patterns of insulin resistance in different brain regions.

Our findings indicate that fetal adversity alters brain sensitivity to insulin and modulates behavioral responses to palatable foods. Our findings in rats corroborate with our findings in humans where individual differences in dopamine function are associated with increased palatable food consumption in IUGR individuals (Silveira, Pokhvisneva et al. 2018). These current findings have important implications as early life adversity during gestation associates with the development of chronic diseases later in life including increased risk for obesity, metabolic syndrome, cardiovascular disease and psychiatric disorders. Our further understanding of the molecular mechanisms involved in increasing the risk for these diseases in the face of early life gestational adversity allows us to develop preventative and therapeutic programs to target individuals at risk.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

Financial support was provided by the Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE/HCPA); Pró-Pesquisa/PROPESQ/UFRGS; Coordination for the Improvement of Higher Education Personnel (CAPES) and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

REFERENCES

Adamantidis, A. R., H. C. Tsai, B. Boutrel, F. Zhang, G. D. Stuber, E. A. Budygin, C. Tourino, A. Bonci, K. Deisseroth and L. de Lecea (2011). "Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior." <u>J Neurosci</u> 31(30): 10829-10835.

Alves, M. B., R. D. Molle, M. Desai, M. G. Ross and P. P. Silveira (2015). "Increased Palatable Food Intake and Response to Food Cues in Intrauterine Growth-Restricted Rats Are Related to Tyrosine Hydroxylase Content in the Orbitofrontal Cortex and Nucleus Accumbens." <u>Behav Brain Res</u>: 287:73-81.

Ayres, C., M. Agranonik, A. K. Portella, F. Filion, C. C. Johnston and P. P. Silveira (2012). "Intrauterine growth restriction and the fetal programming of the hedonic response to sweet taste in newborn infants." <u>Int J Pediatr</u> 2012: 657379.

Barbieri, M. A., A. K. Portella, P. P. Silveira, H. Bettiol, M. Agranonik, A. A. Silva and M. Z. Goldani (2009). "Severe intrauterine growth restriction is associated with higher spontaneous carbohydrate intake in young women." <u>Pediatr Res</u> 65(2): 215-220.

Colman, J. B., D. P. Laureano, T. M. Reis, R. Krolow, C. Dalmaz, S. Benetti Cda and P. P. Silveira (2015). "Variations in the neonatal environment modulate adult behavioral and brain responses to palatable food withdrawal in adult female rats." Int J Dev Neurosci 40: 70-75.

Cunha Fda, S., R. Dalle Molle, A. K. Portella, S. Benetti Cda, C. Noschang, M. Z. Goldani and P. P. Silveira (2015). "Both Food Restriction and High-Fat Diet during Gestation Induce Low Birth Weight and Altered Physical Activity in Adult

Rat Offspring: The "Similarities in the Inequalities" Model." <u>PLoS One</u> 10(3): e0118586.

Dalle Molle, R., D. P. Laureano, M. B. Alves, T. M. Reis, M. Desai, M. G. Ross and P. P. Silveira (2015). "Intrauterine growth restriction increases the preference for palatable foods and affects sensitivity to food rewards in male and female adult rats." <u>Brain Res</u> 1618: 41-49.

Dalle Molle, R. and P. P. Silveira (2015). "Small for gestational age children have specific food preferences." <u>J Pediatr</u> 166(6): 1547.

Desai, M., D. Gayle, J. Babu and M. G. Ross (2005). "Programmed obesity in intrauterine growth-restricted newborns: modulation by." <u>Am J Physiol Regul</u> <u>Integr Comp Physiol</u> 288(1): R91-96.

Doherty, M. D. and A. Gratton (1997). "NMDA receptors in nucleus accumbens modulate stress-induced dopamine release in nucleus accumbens and ventral tegmental area." <u>Synapse</u> 26(3): 225-234.

Eckel, L. A., W. Langhans, A. Kahler, L. A. Campfield, F. J. Smith and N. Geary (1998). "Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats." <u>Am J Physiol</u> 275(1 Pt 2): R186-193.

Emanuelli, B., P. Peraldi, C. Filloux, D. Sawka-Verhelle, D. Hilton and E. Van Obberghen (2000). "SOCS-3 is an insulin-induced negative regulator of insulin signaling." <u>J Biol Chem</u> 275(21): 15985-15991.

Eriksson, M., M. A. Wallander, I. Krakau, H. Wedel and K. Svardsudd (2004). "Birth weight and cardiovascular risk factors in a cohort followed until 80 years of age: the study of men born in 1913." <u>J Intern Med</u> 255(2): 236-246.

Farley, C., J. A. Cook, B. D. Spar, T. M. Austin and T. J. Kowalski (2003). "Meal pattern analysis of diet-induced obesity in susceptible and resistant rats." <u>Obes</u> <u>Res</u> 11(7): 845-851.

Figlewicz, D. P. (2003). "Insulin, food intake, and reward." <u>Semin Clin</u> <u>Neuropsychiatry</u> 8(2): 82-93.

Figlewicz, D. P., J. L. Bennett, S. Aliakbari, A. Zavosh and A. J. Sipols (2008). "Insulin acts at different CNS sites to decrease acute sucrose intake and sucrose self-administration in rats." <u>American Journal of Physiology-Regulatory</u> <u>Integrative and Comparative Physiology</u> 295(2): R388-R394.

Figlewicz, D. P., S. B. Evans, J. Murphy, M. Hoen and D. G. Baskin (2003). "Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat." <u>Brain Res</u> 964(1): 107-115.

Figlewicz, D. P., A. MacDonald Naleid and A. J. Sipols (2007). "Modulation of food reward by adiposity signals." <u>Physiol Behav</u> 91(5): 473-478.

Gerhardt, G. A., C. Ksir, C. Pivik, S. D. Dickinson, J. Sabeti and N. R. Zahniser (1999). "Methodology for coupling local application of dopamine and other chemicals with rapid in vivo electrochemical recordings in freely-moving rats." J <u>Neurosci Methods</u> 87(1): 67-76.

Hales, C. N. and D. J. Barker (1992). "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis." <u>Diabetologia</u> 35(7): 595-601.

Hong, S. L., S. J. Barton and G. V. Rebec (2012). "Neural correlates of unpredictability in behavioral patterns of wild-type and R6/2 mice." <u>Commun Integr Biol</u> 5(3): 259-261.

Jensen, C. B., H. Storgaard, F. Dela, J. J. Holst, S. Madsbad and A. A. Vaag (2002). "Early differential defects of insulin secretion and action in 19-year-old caucasian men who had low birth weight." <u>Diabetes</u> 51(4): 1271-1280.

Joyce, B. M., P. E. Glaser and G. A. Gerhardt (2007). "Adderall produces increased striatal dopamine release and a prolonged time course compared to amphetamine isomers." <u>Psychopharmacology (Berl)</u> 191(3): 669-677.

Kowalski, T. J., C. Farley, M. E. Cohen-Williams, G. Varty and B. D. Spar (2004). "Melanin-concentrating hormone-1 receptor antagonism decreases feeding by reducing meal size." <u>Eur J Pharmacol</u> 497(1): 41-47.

Lahti, M., J. G. Eriksson, K. Heinonen, E. Kajantie, J. Lahti, K. Wahlbeck, S. Tuovinen, A. K. Pesonen, M. Mikkonen, C. Osmond, D. J. Barker and K. Raikkonen (2014). "Late preterm birth, post-term birth, and abnormal fetal growth as risk factors for severe mental disorders from early to late adulthood." <u>Psychol Med</u>: 1-15.

Laureano, D. P., R. Dalle Molle, M. B. Alves, C. Luft, M. Desai, M. G. Ross and P. P. Silveira (2016). "Intrauterine growth restriction modifies the hedonic response to sweet taste in newborn pups - Role of the accumbal mu-opioid receptors." <u>Neuroscience</u> 322: 500-508.

Laureano, D. P., R. D. Molle, A. K. Portella and P. P. Silveira (2015). "Facial Expressions in Small for Gestational Age Newborns." <u>J Child Neurol</u>.

Ledo, A., C. F. Lourenco, M. Caetano, R. M. Barbosa and J. Laranjinha (2015). "Age-associated changes of nitric oxide concentration dynamics in the central nervous system of Fisher 344 rats." <u>Cell Mol Neurobiol</u> 35(1): 33-44.

Li, Y., S. H. Ley, D. K. Tobias, S. E. Chiuve, T. J. VanderWeele, J. W. Rich-Edwards, G. C. Curhan, W. C. Willett, J. E. Manson, F. B. Hu and L. Qi (2015). "Birth weight and later life adherence to unhealthy lifestyles in predicting type 2 diabetes: prospective cohort study." <u>BMJ</u> 351: h3672.

Lussana, F., R. C. Painter, M. C. Ocke, H. R. Buller, P. M. Bossuyt and T. J. Roseboom (2008). "Prenatal exposure to the Dutch famine is associated with a preference for fatty foods and a more atherogenic lipid profile." <u>American</u> <u>Journal of Clinical Nutrition</u> 88(6): 1648-1652.

Machado, T. D., R. Dalle Molle, D. P. Laureano, A. K. Portella, I. C. Werlang, S. Benetti Cda, C. Noschang and P. P. Silveira (2013). "Early life stress is associated with anxiety, increased stress responsivity and preference for "comfort foods" in adult female rats." <u>Stress</u> 16(5): 549-556.

McCutcheon, J. E., J. A. Beeler and M. F. Roitman (2012). "Sucrose-predictive cues evoke greater phasic dopamine release than saccharin-predictive cues." <u>Synapse</u> 66(4): 346-351.

Mericq, V., K. K. Ong, R. Bazaes, V. Pena, A. Avila, T. Salazar, N. Soto, G. Iniguez and D. B. Dunger (2005). "Longitudinal changes in insulin sensitivity and secretion from birth to age three years in small- and appropriate-for-gestational-age children." <u>Diabetologia</u> 48(12): 2609-2614.

Migraine, A., S. Nicklaus, P. Parnet, C. Lange, S. Monnery-Patris, C. Des Robert, D. Darmaun, C. Flamant, V. Amarger and J. C. Roze (2013). "Effect of preterm birth and birth weight on eating behavior at 2 y of age." <u>Am J Clin Nutr</u> 97(6): 1270-1277.

Molet, J., K. Heins, X. Zhuo, Y. T. Mei, L. Regev, T. Z. Baram and H. Stern (2016). "Fragmentation and high entropy of neonatal experience predict adolescent emotional outcome." <u>Transl Psychiatry</u> 6: e702.

Paxinos, G. and C. Watson (2007). <u>The rat brain in stereotaxic coordinates</u>. Amsterdam, Elsevier.

Portella, A. K., E. Kajantie, P. Hovi, M. Desai, M. G. Ross, M. Z. Goldani, T. J. Roseboom and P. P. Silveira (2012). "Effects of in utero conditions on adult feeding preferences." Journal of Developmental Origins of Health and Disease 3(3): 140-152.

Portella, A. K. and P. P. Silveira (2014). "Neurobehavioral determinants of nutritional security in fetal growth-restricted individuals." <u>Ann N Y Acad Sci</u>.

Portella, A. K., P. P. Silveira, D. P. Laureano, S. Cardoso, V. Bittencourt, C. Noschang, I. Werlang, F. U. Fontella, C. Dalmaz and M. Z. Goldani (2015). "Litter size reduction alters insulin signaling in the ventral tegmental area and influences dopamine-related behaviors in adult rats." <u>Behav Brain Res</u> 278: 66-73.

Reis RS, Dalle Molle R, Machado TD, Mucellini AB, Rodrigues DM, Bortoluzzi A, Bigonha SM, Toazza R, Salum GA, Minuzzi L, Buchweitz A, Franco AR, Pelúzio MCG, Manfro GG, Silveira PP. (2016). "Impulsivity-based thrifty eating phenotype and the protective role of n-3 PUFAs intake in adolescents." <u>Translational Psyhchiatry</u>. 15;6:e755.

Richman, J. S. and J. R. Moorman (2000). "Physiological time-series analysis using approximate entropy and sample entropy." <u>Am J Physiol Heart Circ</u> <u>Physiol</u> 278(6): H2039-2049.

Sears, B. and M. Perry (2015). "The role of fatty acids in insulin resistance." <u>Lipids Health Dis</u> 14: 121.

Shahrokh, D. K., T. Y. Zhang, J. Diorio, A. Gratton and M. J. Meaney (2010). "Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat." <u>Endocrinology</u> 151(5): 2276-2286. Silveira, P. P. (2014). "Playing with food: the fetal programming of food preferences." <u>Obesity (Silver Spring)</u> 22(5): 1210.

Silveira, P. P., M. Agranonik, H. Faras, A. K. Portella, M. J. Meaney, R. D. Levitan, V. Maternal Adversity and T. Neurodevelopment Study (2012). "Preliminary evidence for an impulsivity-based thrifty eating phenotype." <u>Pediatr</u> <u>Res</u> 71(3): 293-298.

Silveira, P. P., I. Pokhvisneva, H. Gaudreau, L. Atkinson, A. S. Fleming, M. B. Sokolowski, M. Steiner, J. L. Kennedy, L. Dube, R. D. Levitan, M. J. Meaney and M. r. team (2018). "Fetal growth interacts with multilocus genetic score reflecting dopamine signaling capacity to predict spontaneous sugar intake in children." <u>Appetite</u> 120: 596-601.

Sipols, A. J., J. Bayer, R. Bennett and D. P. Figlewicz (2002). "Intraventricular insulin decreases kappa opioid-mediated sucrose intake in rats." <u>Peptides</u> 23(12): 2181-2187.

Smotherman, W. P., K. A. Selz and A. J. Mandell (1996). "Dynamical entropy is conserved during cocaine-induced changes in fetal rat motor patterns." <u>Psychoneuroendocrinology</u> 21(2): 173-187.

Surina-Baumgartner, D. M., W. Langhans and N. Geary (1995). "Hepatic portal insulin antibody infusion increases, but insulin does not alter, spontaneous meal size in rats." <u>Am J Physiol</u> 269(5 Pt 2): R978-982.

Trifilieff, P. and D. Martinez (2014). "Imaging addiction: D2 receptors and dopamine signaling in the striatum as biomarkers for impulsivity." <u>Neuropharmacology</u> 76 Pt B: 498-509.

Ueki, K., T. Kondo and C. R. Kahn (2004). "Suppressor of Cytokine Signaling 1 (SOCS-1) and SOCS-3 Cause Insulin Resistance through Inhibition of Tyrosine Phosphorylation of Insulin Receptor Substrate Proteins by Discrete Mechanisms." <u>Molecular and Cellular Biology</u> 24(12): 5434-5446.

Veening, M. A., M. M. van Weissenbruch, R. J. Heine and H. A. Delemarre-van de Waal (2003). "Beta-cell capacity and insulin sensitivity in prepubertal children born small for gestational age: influence of body size during childhood." <u>Diabetes</u> 52(7): 1756-1760.

Vucetic, Z., K. Totoki, H. Schoch, K. W. Whitaker, T. Hill-Smith, I. Lucki and T. M. Reyes (2010). "Early Life Protein Restriction Alters Dopamine Circuitry." <u>Neuroscience</u> 168(2): 359-370.

FIGURE LEGENDS:

Figure 1: Schematic illustration of electrode placement in NAcc shell (A). Hematoxylin-eosin-stained coronal sections of rat brain were used to confirm the microelectrode recording site in the NAcc shell (B).

Figure 2: Body weight at birth and at weaning, in male and female rats from FR and control groups. Data is expressed as mean \pm SEM. A) Birth weight in grams. There is an effect of the group, in which FR animals weighed less than controls (GEE, n=114-176/group/sex, *p<0.05) and females weighed less than males (GEE, n=114-176/group/sex, *p<0.05). B) Body weight at weaning. Body weights were similar between the FR and control groups at weaning age. There was no effect of the group, demonstrating the catch-up growth in the FR animals. Females weighed less than males (GEE, n=51-82/group/sex, *p<0.05). C) There were no significant differences in body weight between FR and control rats used in Study 1 to measure food intake using the BioDAQ® system (Student's T Test, n=8/group, p>0.05). D) There were no significant differences in body weight between the FR and control rats used in Study 2 for chronoamperometric measurements (Student's T Test n=4-5/group, p>0.05).

Figure 3: Palatable and regular food consumption were measured using the BioDAQ® system. Data is expressed as mean \pm SEM. A) Average palatable food consumption/100g body weight measured during the light and dark phases of the 24 hr light cycle. GEE analysis showed a significant interaction between group and time of day, in which the FR group ate more palatable food during the dark phase (Generalized Estimating Equation, n=8/group,*p<0.05). B) Entropy rates across 96 hours and divided by the light (C) and dark phase (D).

In D, there was higher behavioral entropy in the FR group during the dark phase (Student's T test, n=8/group, *p<0.05).

Figure 4: Peripheral insulin levels. Data is expressed as mean \pm SEM. FR rats had significantly higher insulin levels than control rats (FR= 2,034 \pm 0,322, control= 1,086 \pm 0,244, Student's t-test, n=12-15/group, p=0.031).

Figure 5: In vivo electrochemical NAcc DA response to different conditions: exposure to rat chow and Froot loops at baseline and after IP saline or insulin. Data is expressed as mean ± SEM. Three variables summarize the DA response: A) T rise or time to reach DA peak after the stimulus. There was an interaction between group and food exposure condition in which FR rats took a longer time to reach the dopaminergic peak after Froot loops presentation and this finding is completely reversed by insulin injection (Generalized Estimating Equation, n=4-5/group,*p<0.05); B) Amplitude of the dopaminergic response. There was an interaction between food exposure condition and group, in which FR rats responded differently to Froot Loops® when comparing baseline vs. saline injection conditions (Generalized Estimating Equation, n=4-5/group, p= 0.038); C) T80 or time taken to reach 80% dopaminergic signal clearance following stimulus presentation. Although there was a significant interaction between group and food exposure condition, a post-hoc analysis showed only a longer T80 value after IP insulin and Froot Loops® compared to regular rat chow presentation chow. This pattern appears more evident in the FR group (Generalized Estimating Equation, n=4-5/group, p=0.024).

Figure 1 Click here to download Figure: Figure 1 (1)_review_december.xlsx



Figure 2 Click here to download Figure: Figure 2(1)_review_december.xlsx



Figure 3 Click here to download Figure: Figure 3(1)_review_december.xlsx



Figure 4 Click here to download Figure: Figure 4(1)_review_december.xlsx





Protein	Controls		FR	
	Saline	Insulin	Saline	Insulin
TH	100 <u>+</u> 33.92	100 <u>+</u> 24.84	109.59 <u>+</u> 34.70	83.66 <u>+</u> 6.99
		1		ļ
рТН	100 <u>+</u> 27.59	100 <u>+</u> 24.96	149.92 <u>+</u> 44.62	79.28 <u>+</u> 6.90
pTH/TH	1.11 <u>+</u> 0.10	1.07 <u>+</u> 0.10	1.40 <u>+</u> 0.06*	0.96 <u>+</u> 0.06
Actin	ĺ	1	ļ	ĺ

Table 1 – Ratio protein OD/Actin OD expressed as percentage of controls in NAcc

Student's t-test; data are expressed as mean \pm S.E.M.; * significant difference from Control Saline (p<0.05).

Protein	Con	Controls		FR	
	Saline	Insulin	Saline	Insulin	
AKT	100 <u>+</u> 11.41	100 <u>+</u> 21.24	77.56 <u>+</u> 17.23	128.41 <u>+</u> 18.66	
pAKT	100 <u>+</u> 11.25	100 <u>+</u> 17.62	80.77 <u>+</u> 16.33	136.97 <u>+</u> 17.02	
		1		-	
pAKT/AKT	1.01 <u>+</u> 0.09	1.06+0.09	1.06 <u>+</u> 0.03	1.09 <u>+</u> 0.07	
SOCS3	100 <u>+</u> 7.42	100 <u>+</u> 22.99	46,72 <u>+</u> 11,78*	111.93 <u>+</u> 16.81	
Actin		3	1	-	

Table 2 – Ratio protein OD/Actin OD expressed as percentage of controls in VTA

Student's t-test; data are expressed as mean \pm S.E.M.; * significant difference from Control Saline (p<0.05).