Changes in reward-induced neural activity upon Cafeteria Diet consumption

Heijkoop, R.[1], Lalanza, J.F.[1], Solanas, M.[2,3], Álvarez-Monell, A.[2,3], Subias-Gusils, A.[2,4], Escorihuela, R.M.[2,4], Snoeren, E.M.S.[1*].

[1]*Department of Psychology, UiT The Arctic University of Norway, Norway*

[2]*Institut de Neurociències, Universitat Autònoma de Barcelona, Spain*

[3]*Medical Physiology Unit, Department of Cell Biology, Physiology and Immunology, Faculty of Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.*

[4]*Unitat de Psicologia Mèdica, Departament de Psiquiatria i Medicina Legal, Universitat Autònoma de Barcelona, Spain.*

* Corresponding author. Current address: Department of Psychology, UiT The Arctic University of Norway, 9037, Tromsø, Norway.

E-mail address: eelke.snoeren@uit.no (E.M.S. Snoeren).

Abstract

Excessive consumption of highly palatable foods rich in sugar and fat, often referred to as “junk” or “fast” foods, plays a central role in the development of obesity. The highly palatable characteristics of these foods activate hedonic and motivational mechanisms to promote food-seeking behavior and overeating, which is largely regulated by the brain reward system. Excessive junk food consumption can alter the functioning of this reward system, but exact mechanisms of these changes are still largely unknown. This study investigated whether long-term junk food consumption, in the form of Cafeteria (CAF) diet, can alter the reward system in female rats, and whether different regimes of CAF diet influence the extent of these changes. To this end, rats were exposed to a 6-week diet with either standard chow, or *ad libitum* daily access to CAF diet, 30% restricted but daily access to CAF diet, or one-day-a-week (intermittent) *ad libitum* access to CAF diet, after which c-Fos expression in the Nucleus Accumbens (NAc), Prefrontal Cortex (PFC), and Ventral Tegmental Area (VTA) following consumption of a CAF reward was examined. We found that all CAF diet regimes decreased c-Fos expression in the NAc-shell when presented with a CAF reward. While no changes in c-Fos expression upon the different diet regimes were found in the PFC, future research should reveal potential decreases in neural activity in the VTA. Our data suggests that long-term junk food exposure can affect the brain reward system, resulting in an attenuated activity of the NAc-shell.

Keywords: Obesity, cafeteria diet, reward system, rats, junk food, c-Fos

1. Introduction

As the prevalence of obesity continues to rise worldwide, its importance as a significant health concern grows. The condition can for example contribute to the development of cardiovascular disease, type 2 diabetes, hypertension and some cancers [1]. One of the key factors contributing to overweight and obesity is a lack of control over food intake [2], and large portion sizes and snacking patterns have been found to be risk factors in the development of obesity [3]. However, also excessive consumption of highly palatable foods rich in sugar and fat, often referred to as “junk” or “fast” foods, plays a central role in the development of obesity in humans [4]. The highly palatable characteristics of these foods activate hedonic and motivational mechanisms to promote food-seeking behavior and overeating [5], and it has been shown (also in rodents) that modern energy-rich diets are consumed not only because of their nutrient value but also because they are hedonically pleasant [6, 7].

The brain reward system has a central role in regulating motivation and reward-related responses. In normal circumstances, naturally rewarding behaviors like feeding, but also evaluation of palatability of the food, are regulated in part by dopamine transmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) [8, 9]. While dopamine synthesis and release increase following acute exposure to palatable foods [10, 11], dopamine release in the NAc appears to play a central role in differentiating and encoding rewarding stimuli [12], resulting in an increase in the drive to engage in food seeking [13].

Excessive junk food consumption, however, can alter the functioning of this reward system [14-17]. The exact mechanisms of these changes are still largely unknown, but some studies have shown that rats that are obesity-prone show higher levels of motivation to work for food rewards than obesity-resistant or control animals [18-21]. Others, however, found that rats on a high-fat diet show reduced interest in cues for reward and show attenuated operant responses for sucrose [22, 23] or cocaine rewards [24].

Despite the large body of evidence indicating a relationship between junk food consumption and the reward system, it remains unclear what factors contribute to changes in the reward system and subsequent overeating. Besides the palatability of food, also the element of choice plays an important role in overeating. It has been shown that the body weight of rats on a high-fat, high-sugar diet of free choice increases more than that of rats on a forced high-fat, high-sugar diet, probably caused by the consumption of more meals per day without compensating meal size [25]. More variety in a meal and the greater dietary variation is an important element as well. The presentation of a wide range of foods evokes over-eating, known as the “buffet effect” [26]. The experimental

Cafeteria (CAF) diet consists of several unhealthy, often ultraprocessed, but tasty products that humans consume too (e.g. bacon, muffins and cookies), and has because of that multiple advantages over other high-calory diets as obesogenic diet[27, 28]. It combines the orosensory properties (such as smell and texture) and palatability of the foodstuffs that promote overconsumption, but it also contains the element of free choice when the different products are provided simultaneously [28]. This way CAF diet mimics a certain pattern of problematic human consumption, which makes it very suitable as experimental diet model to study the effects of junk food consumption on the reward system.

Interestingly, when access to CAF diet is limited to a few days per week (often referred to as intermittent access), increases in amount of CAF consumption have been found [29, 30]. However, rats on an intermittent access scheme show intermediate to no responses to sucrose consumption compared to rats on a full-time CAF diet [29]. When rats had access to a restricted amount of CAF diet (30% of calorie restriction) besides standard chow, they showed decreased levels of sucrose preference similar to rats with *ad libitum* access to CAF diet compared with control rats, but had an intermediate phenotype on total sucrose intake in the two-bottle preference test and on the number of trials initiated in the brief access licking test, and no difference in hedonic responses to sucrose in the taste reactivity test [31, 32]. Altogether, this suggests that different exposures to CAF diet could have different effects on the reward system.

Therefore, this study investigates whether long-term CAF diet consumption can alter the reward system in female rats, and whether different regimes of CAF diet influence the extent of these changes. To this end, rats received a 6-week diet with either standard chow, or *ad libitum* daily access to CAF diet, 30% restricted but daily access to CAF diet, or one-day-a-week (intermittent) *ad libitum* access to CAF diet. Then all rats were exposed to either a standard chow or CAF reward in order to investigate c-Fos expression as a marker for neural activity in the NAc, PFC and VTA. Changes in calory intake and biometric variables were also compared. We hypothesized that chronic, daily *ad libitum* access to CAF diet would reduce the neural activation upon a food reward in the NAc, VTA and PFC, and that restricted or intermittent access to the CAF diet would result in intermediate effects compared to control diet.

2. Methods

All animals were cared for according to an institutionally approved experimental animal protocol, following Spanish legislation. The experimental protocol was approved by the Generalitat de

Catalunya (approval number DAAM 9978) and was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.1 Animals and design

A total of 40 female Long-Evans rats (average body weight circa 230g upon arrival at an age of 13 weeks) were obtained from Janvier (Le Genest-Saint-Isle, France). The rats were pair-housed in standard polycarbonate cages at a temperature of 21±1°C and a humidity of 50±10% under a light/dark period of 12 h (lights on at 08:00) during the whole experiment.

After one week of habituation to the animal facility, the rats were weighed and randomly assigned to five treatment groups (n=8), which at baseline did not differ in average body weight. Each group underwent a different 6-week dietary regimen (Table 1). At the beginning of the seventh week rats were exposed to a food reward before perfusion for c-Fos expression analysis in the brain.

Table 1. Experimental groups exposed to standard chow or cafeteria (CAF) diet and reward

Group	6 week diet	Reward
CTR-Chow	*ad libitum* access to standard chow and water	Chow
CTR-CAF	*ad libitum* access to standard chow and water	CAF
CAFchr	*ad libitum* access to standard chow and water and daily *ad libitum* access to CAF diet	CAF
CAFint	*ad libitum* access to standard chow and water + 1 day per week *ad libitum* access to CAF diet	CAF
CAFres	*ad libitum* access to standard chow and water + daily access to 30% CAF diet (relative to CAFchr)	CAF

2.2 Diet procedure

The rats received their assigned diets daily for six weeks until reward exposure and subsequent transcardiac perfusion. All groups of rats had *ad libitum* access to standard chow (Teklad Global 18% Protein Rodent Diet 2018" (Harlan, Barcelona, Spain)) and tap water. The rats in the CAFchr and CAFint diet groups received a daily-fresh CAF meal in addition, which consisted of a combination of the following products: pork jowl, biscuit with pâté, biscuit with cheese, muffins, carrots, and "flam" (jellied sugared milk). These meals were prepared fresh daily. CAFres diet consisted of a 30% calorie-restricted CAF diet (relative to the energy intake consumed by the CAFchr group). Rats in the CAFint group had ad libitum access to a CAF meal only once per week. The mean daily food intake (g) during

the 6-week diet exposure per cage, and the nutritional composition of the standard chow and CAF diet products are shown in tables S1-S3. Some food product labels reported salt as NaCl. These values were recalculated to Na to enable uniform analyses of all food products.

Food consumption was assessed once per week, except for the CAFint group which was assessed twice per week; once for CAF diet and standard chow, and once for standard chow only. Food consumption was calculated as the difference between the received amount of food product and the unconsumed amount of food product that was remaining 24 hours later.

2.3 Biometric variables

At the beginning of each week (Monday or Tuesday) during the 6-week diet exposure, body weight, abdominal perimeter, and length (nose-anus distance) were measured. From the combination of body weight and length we calculated the body mass index (BMI: body weight (g) / ano-nasal length $(cm)^2$) and the Lee index (cube root of body weight (g) / length (cm)), which is an adaptation of the BMI for rodents [33].

2.4 Reward exposure

After six weeks of their respective diets, the rats were exposed to a food reward (chow for CTR-Chow, CAF for the other groups). On the day of the reward exposure, the food was removed from the home cage at 07:00 to motivate rats to eat the reward. Approximately two hours before transcardiac perfusion, the rats were single-housed and exposed to 15% of the CAF diet or STD chow quantity (depending on the experimental group). The rats had access to the reward for 30 minutes (counted from the start of eating), before the remaining food was removed from their cages. Perfusion took place 90 minutes after removal of the food. No significant differences in latency to start eating were found between groups. Only one cage took more than 30 min to start eating and was therefore re-tested (and perfused) at the end of the day. Perfusions took place on three consecutive days and cages were randomly assigned to each day balancing the experiment group.

2.5 Transcardiac perfusion

The rats were deeply anesthetized with sodium pentobarbital (Doletal ®, 200 mg/kg i.p.) and then transcardially perfused with 0.1M phosphate-buffered saline (PBS; pH 7.4) followed by formaldehyde (3.7-4% buffered to pH=7 and stabilized with methanol <=3%, Casa Alvarez, Madrid, Spain). The brains were removed and post-fixed in individual vials with 4% formaldehyde for 48 hours. Subsequently, brains were transferred to 10% sucrose in 0.1M PBS solution, followed by a 20% and then a 30% sucrose in 0.1M PBS solution until they had sunken. A preservative (ProClin 150, Sigma-

Aldrich, cat. 49377, 1:1000) was added to the final sucrose solution in which the brains were shipped to UiT The Arctic University of Norway for further analysis.

2.6 Brain sectioning and regions of interest

The brains were snap frozen in isopentane and kept at -80°C until sectioning. Brains were sectioned on a vibratome (Leica VT1200, Leica Biosystems, Nussloch, Germany) into 40 μm thick sections and stored in cryoprotectant solution (30% sucrose w/v, 30% ethylene glycol in 0.1 M PBS, pH 7.4) until further use. The brain regions of interest, of which the coordinates were determined using a rat brain atlas[34], were the nucleus accumbens shell and core (NAc-sh, NAc-c: AP between +2.28mm and +0.98mm), the anterior (aVTA) and posterior ventral (pVTA) tegmental area (-AP between -4.68mm and -6.12mm), and the prelimbic and infralimbic parts of the prefrontal cortex (PrL, IL: AP between +3.72mm and +2.52mm).

2.7 Immunohistochemistry

Sections were washed in Tris-buffered saline (TBS, 0.1M) before and after incubations. First, sections were blocked in a TBS solution with 0.5% BSA and 0.1% Triton-X-100 for 30 min at room temperature. Then, sections of each brain area were incubated in primary antibody for c-Fos (monoclonal rabbit anti-c-Fos, Cell Signaling, cat mAb-9F6, 1:4000) for 24h at room temperature, followed by 24h at 4°C. The samples were then incubated in secondary antibody solution (polyclonal biotinylated goat anti-rabbit antibody, Abcam, cat. Ab6720, 1:400) for 30min at room temperature, and subsequently with avidin-biotin-complex solution (Vectastain Elite ABC-HRP kit, Vector Laboratories, cat. PK-6100) in TBS for 30min at room temperature. Finally, the brain sections were incubated with 3,3'-diaminobenzidine (DAB substrate kit (HRP), Vector Laboratories, cat. SK-4100) for 5 min. After final washes, they were mounted on glass slides in 0.01M PB, dehydrated in ethanol, cleared with Xylene (Sigma-Aldrich, cat. 534056) and coverslipped using mounting medium (Entellan, Sigma-Aldrich, cat. 1.07961).

2.8 c-Fos immune-responsive cell quantification

After coverslipping, the slides were loaded into an Olympus VS120 slide scanner microscope. Image scans were obtained for each section using a 10x objective (NA 0.40), automatic focus settings in single plane, and fixed exposure of 1.13 ms. Using Olyvia online database software, high resolution cropped images (5x digital zoom) of the brain regions of interest were downloaded and opened in ImageJ, and bilateral counting boxes of always the same size and location were drawn to fit into the regions of interest. The following sizes of the boxes were used: NAc-shell and NAc-core: 0.81 mm x 0.51 mm; aVTA and pVTA: 0.6 mm x 0.5 mm; PrL: 1.0 mm x 0.4 mm; IL: 0.5 mm x 0.4 mm. Because

the shape and size of the VTA vary substantially along the anteroposterior axis, placement templates for each AP location were created in ImageJ, in accordance with VTA locations in the Paxinos & Watson brain atlas. Subsequently, AP coordinates of each VTA section were determined using the brain atlas and the corresponding templates were used to ensure correct placement of the ROI boxes. Examples of the placement of ROI boxes can be found in Figure S1.

On these photomicrographs, all stained cells inside the zones were manually counted using Cell Counter plugin in ImageJ. Stained cells that overlapped with the counting frame border were included only if they overlapped with the dorsal or the lateral border. Cells that overlapped the ventral or medial border were not counted. This was performed for 2-3 sections per brain area per rat. Each section was counted twice when less than 10% difference in cell count was obtained, and 3 times when more than 10% difference was observed. Then the average of the two counts with less than 10% count difference was taken as data point. Per rat and brain region, the average of the 2-4 sections was used for further analysis. The number of counted cells was divided by the surface area of the zone to obtain the outcome measure of number of c-Fos-IR cells per mm^2.

2.9 Statistical analysis

Since the rats were pair-housed, food intake and subsequent nutritional data was calculated per cage and converted in a mean daily intake of the 6 weeks diet. The other parameters were calculated for each individual animal and per week.

First, the CTR-Chow and CTR-CAF groups were compared, after which the different diet regime groups were compared only to CTR-CAF. For the food intake and nutritional data, an independent t-test was used to compare CTR-Chow and CTR-CAF, while the experimental groups were compared to CTR-CAF with a one-way ANOVA (taking the results of the Levene's test for homogeneity of variances into account), followed by a Bonferroni post-hoc analysis.

A repeated measures ANOVA was employed for comparison of the biometric variables between the groups. When Mauchly's test of sphericity was below 0.05, Greenhouse-Geisser was used for further analysis. In addition, a one-way ANOVA was performed to compare the differences between groups for each week, followed by a Bonferroni post-hoc analysis.

For the c-Fos data, t-test analysis was employed to compare CTR-Chow with CTR-CAF, and one-way ANOVA analyses were used for the different diet regime comparisons, followed by a Bonferroni post-hoc analysis. A p-value <0.05 was considered significant.

3. Results

3.1 Food intake and nutritional data

An overview of the intake of diet components and correlating nutritional values per experimental group is shown in tables S1-S3. As expected, rats in the CTR-CAF and CTR-Chow groups did consume the same amount of water ($t_{(6)}$=--0.775, NS, table S1) and chow ($t_{(6)}$=-0.043, NS), and thus took a same amount of mean daily energy during the 6-week exposure to the diets per cage. In contrast, all three CAF groups (CAFchr, CAFint, CAFres) consumed significantly less standard chow and water during six weeks than the CTR-CAF control group (chow: $F_{(4,19)=}$424.052, p<0.001; water: $F_{(4,19)}$=59.229, p<0.001, table S1), except when the rats exposed to an intermittent access to CAF diet (CAFint) received the standard chow and they ate and drank the same amount as the control group. The intake of energy and nutrients differed significantly over the groups (energy: F(4,19)=216.672, p<0.001; protein: F(4,19)=20.743, p<0.001; sugar: F(4,19)=58.098, p<0.001; F(4,19)=184.426, p<0.001; carbohydrate: F(4,19)=10.871, p<0.001; and salt: F(4,19)=159.350, p<0.001). Of note, a post hoc analysis showed that CAFres had consumed more sugars, lipids and salt than the CTR-CAF control group, but less than CAFchr and CAFint (detailed comparisons can be found in table S2). As expected, no differences were found between CTR-Chow and CTR-CAF group ($t_{(6)}$=-0.043, NS for energy, protein, carbohydrate and salt; $t_{(6)}$=-0.047, NS for lipid, table S2).

3.2 Biometric data:

We confirmed that no differences in body weight ($F_{(1,14)}$=0.031, NS, Fig. 1A), abdominal perimeter ($F_{(1,14)}$=0.231, NS, Fig. 1B), body mass index ($F_{(1,14)}$=0.227, NS, Fig. 1C) and Lee index ($F_{(1,14)}$=0.455, NS, Fig. 1D) were found between the two control diet groups before they were exposed to either a standard chow or CAF reward. In these control groups, there was also an expected increase in body weight ($F_{(2.794,\ 39.121)}$=42.667, p<0.001), abdominal perimeter ($F_{(5,70)}$=2.871, p=0.020), body mass index ($F_{(2.757,38.603)}$=30.792, p<0.001) and Lee index ($F_{(2.786,39.010)}$=51.458, p<0.001) over the course of the 6 weeks of diet exposure, but no interaction effect of diet*weeks was found (body weight: $F_{(2.794,39.121)}$=0.438, NS; abdominal perimeter: $F_{(5,70)}$=2.224, p=0.061, NS; body mass index $F_{(2.757,38.603)}$=1.380, NS; Lee index: $F_{(2.786,39.010)}$=1.431, NS).

When we compared the different CAF regimes to the control diet exposed to CAF-reward (CTR-CAF), we found an effect of diet and weeks on body weight (diet: $F_{(3,28)}$=10.918, p<0.001; weeks: $F_{(2.984,83.562)}$=225.375, p<0.001, Fig. 1A), abdominal perimeter (diet: $F_{(3,28)}$=16.745, p<0.001; weeks: $F_{(3.584,100.352)}$=63.631, p<0.001, Fig. 1B), body mass index (diet: $F_{(3,28)}$=12.351, p<0.001; weeks: $F_{(5,140)}$=3.953, p<0.001, Fig. 1C), and Lee index (diet: $F_{(3,28)}$=10.462, p<0.001; weeks: $F_{(5,140)}$=117.559, p<0.001, Fig. 1D). An interaction effect of diet*weeks was also found for body weight.

($F_{(8.953,83.562)}$=14.417, p<0.001), abdominal perimeter ($F_{(10.752,100.352)}$=7.464, p<0.001), body mass index ($F_{(3,140)}$=6.644, p<0.001) and Lee index ($F_{(15,140)}$=4.353, p<0.001). Post-hoc analysis revealed that

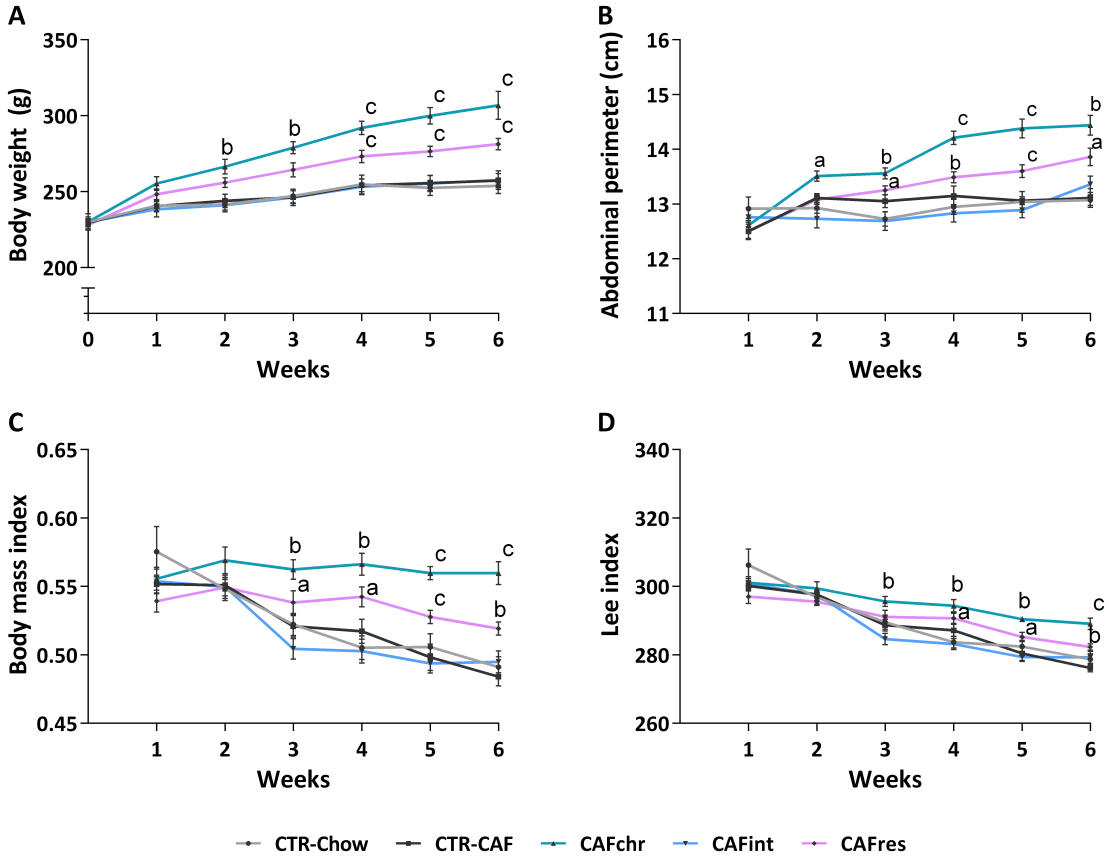

Figure 1. *Biometric data of CTR-Chow (light grey line, n=8), CTR-CAF (dark grey line, n=8), CAFchr (green line, n=8), CAFint (blue line, n=8), and CAFres (purple line, n=8) female Long-Evans rats. (A) Body weight over the course of the 6 week diet exposure in grams. (B) Abdominal perimeter in centimeters. (C) Body mass index (body weight (g)/ ano-nasal length (cm)^2). (D) Lee index (cube root of body weight (g) / ano-nasal length (cm)). a = p<0.05 significantly different from either CTR-CAF or CAFint (see results for details), b = p<0.05 significantly different from both CTR-CAF and CAFint, c= p<0.05 significantly different from all other groups.*

chronic *ad libitum* access to CAF (CAFchr) diet resulted in significantly higher body weights compared to control diet (CTR-CAF, p<0.01) or intermittent access (CAFint, p>0.001) to CAF diet, starting in the second week of exposure (Fig. 1A). From the second week of diet exposure, CAFchr also developed an increased abdominal perimeter compared to all other diet regimes (CTR-CAF: p<0.01, CAFint: p=0.010, CAFres: p<0.01). Restricted, but daily access to CAF diet (CARres) resulted in a significantly different abdominal perimeter than intermittent access to CAF diet (CAFint, p=0.032) from the third week as well (Fig. 1B). The body mass index and Lee index were also significantly higher in CAFchr compared to CTR-CAF (body mass index: p<0.001, Lee index: p>0.001), CAFint (body mass index: p<0.001, Lee index: p>0.001), and CAFres (body mass index: p=0.221, Lee index: p=0.21) from the third week of exposure (Fig. 1C-D). Interestingly, intermittent access to CAF diet (CAFint) did not result in any different biometric outcomes compared to the control group (CTR-CAF).

3.3 c-Fos expression in the NAc, VTA and PFC

First, we tested whether acute exposure to a CAF reward induced c-Fos expression in the NAc, VTA and PFC in rats on control diet (standard chow) for 6 weeks. When we did not distinguish between NAc shell and NAc core, we did not find a significant increase in c-Fos expression upon an acute exposure to a CAF reward compared to a standard chow reward ($t_{(14)}$=-1.930, p=0.074, Fig. 2A). However, when we looked at the NAc shell separately, the number of c-Fos IR cells was significantly higher in control animals that had received CAF (CTR-CAF) as a reward vs animals that had received chow (CTR-chow) ($t_{(14)}$=-3.080, p=0.008, Fig. 2B/D). We did not find these differences in the NAc core ($t_{(14)}$=-0.828, NS, Fig. 2C/E). No differences in number of c-Fos-IR (immunoreactive) cells were found between CAF and standard chow reward in the VTA (VTA anterior: $t_{(10)}$=-1.429, NS; VTA posterior: $t_{(10)}$=-0.276, NS; average between aVTA and pVTA: $t_{(7)}$=-1.346, NS, Fig. 3) or PFC (PFC: $t_{(12)}$=-0.696, NS; of which PrL: $t_{(12)}$=-0.611, NS; IL: $t_{(12)}$=-0.213, NS, Fig. 4).

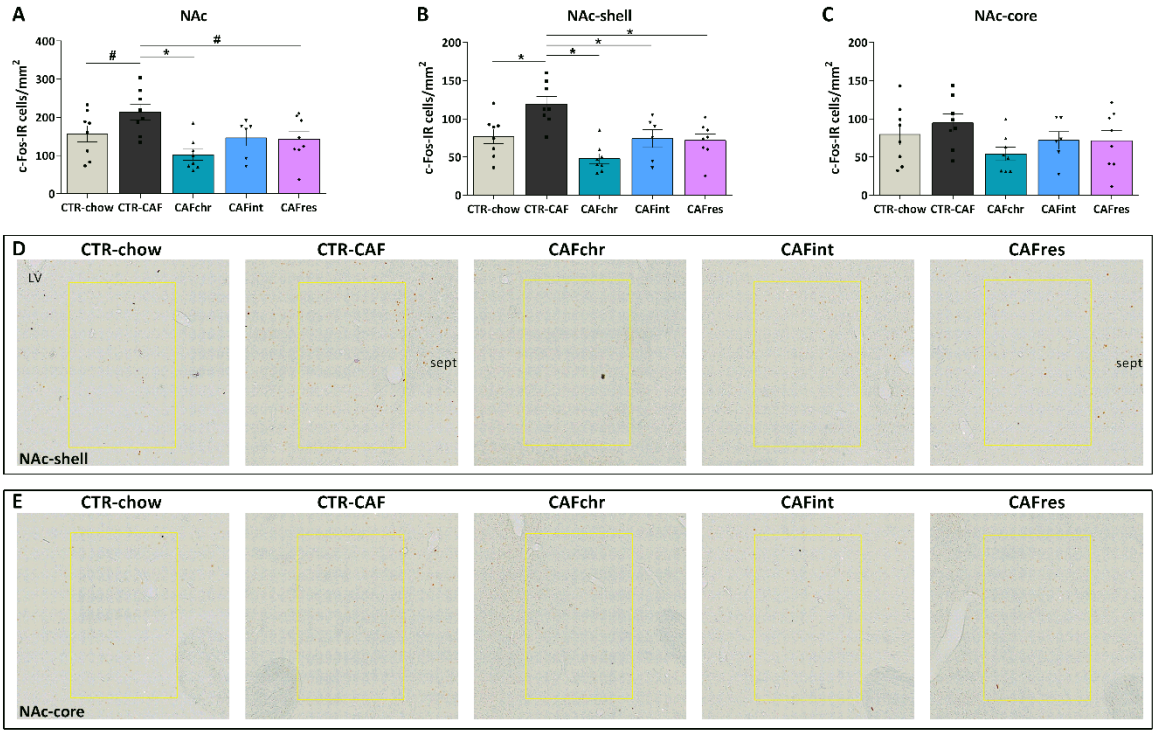

Figure 2. *A) The total count of c-Fos-IR cells per mm2 in the nucleus accumbens (NAc), B) The total count of c-Fos- IR cells per mm² in the NAc-shell, C) The total count of c-Fos-IR cells per mm2 in the NAc-core. D) Examples of microphotographs with the counted c-Fos-IR cells of the different diet groups in the nucleus accumbens shell (NAc-shell) and E) the nucleus accumbens core (NAc-core). The yellow outlines represent the zone that was used for counting. The data are shown with individual data points, with the bars representing the mean ± standard error of the mean for CTR-Chow (light grey bar, n=8), CTR-CAF (dark grey bar, n=8), CAFchr (green bar, n=8), CAFint (blue bar, n=6), and CAFres (purple bar, n=8) female Long-Evans rats. LV = lateral ventrical, Sept = Septum. * p<0.05, # p<0.08.*

Second, we studied the effects of different regimes of CAF diet on c-Fos expression upon exposure to a CAF reward. We found that long-term CAF diet (CAFchr) decreases the number of c-Fos-IR cells in the NAc compared to a control diet of standard chow after exposure to a CAF reward ($F_{(3,29)}$=6.164,

p=0.003, Fig. 2A), which is caused by a decrease in the NAc-shell ($F_{(3,29)}$=11.839, p<0.001, Fig. 2B/D), but not the NAc-core ($F_{(3,29)}$=2.182, NS, Fig. 2C/E). A post-hoc analysis revealed that rats on a chronic *ad libitum* showed reduced levels of c-Fos expression in the NAc as a whole (p=0.001) and in the NAc-shell (p<0.01), but not the NAc-core compared to CTR-CAF. With regard to the rats on a restricted CAF diet, no significant changes were found in c-Fos expression in the whole NAc (p=0.076) but reduced c-Fos expression was found in the NAc-shell (p=0.004 compared to CTR-CAF) Rats on an intermittent CAF diet only showed reduced levels of c-Fos in the NAc-shell (p=0.013), compared to control rats that received CAF as reward.

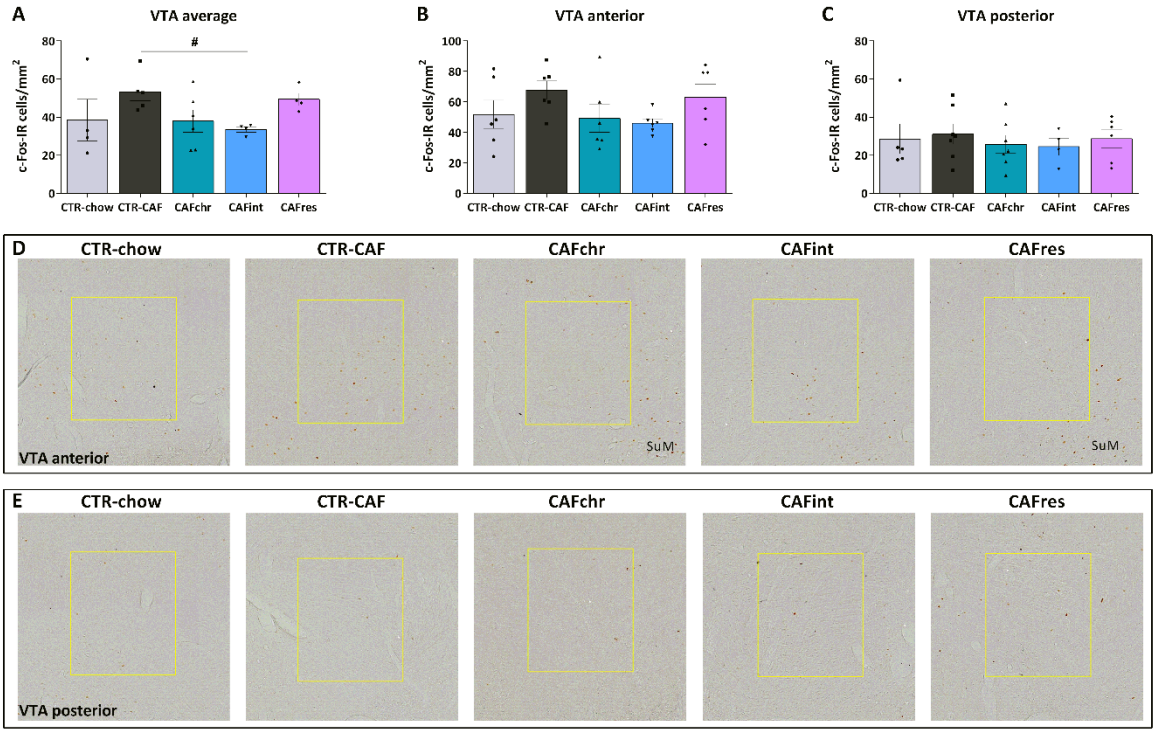

Figure 3. *A) The total count of c-Fos-IR cells per mm² the average of the anterior and posterior ventral tegmental area (VTA), B) The total count of c-Fos-IR cells per mm² in the anterior VTA, C) The total count of c-Fos-IR cells per mm² in the posterior VTA. D) Examples of microphotographs with the counted c-Fos-IR cells of the different diet groups in the anterior VTA and E) the posterior VTA. The yellow outlines represent the zone that was used for counting. The data are shown with individual data points, with the bars representing the mean ± standard error of the mean for CTR-Chow (light grey bar, n=4-6), CTR-CAF (dark grey bar, n=5-7), CAFchr (green bar, n=6-7), CAFint (blue bar, n=4-6), and CAFres (purple bar, n=4-6) female Long-Evans rats. SuM=supramammillary nucleus. # p<0.08.*

No differences in c-Fos expression between the diet regimes were found in the anterior ($F_{(3,23)}$=2.179, NS, Fig. 3B/D) or posterior ($F_{(3,23)}$=0.339, NS, Fig. 3C/E) VTA. However, when the average of the anterior and posterior VTA was calculated, it was found that CAF diet results in reduced levels c-Fos expression ($F_{(3,18)}$=3.771, p=0.034, Fig. 3A). But this effect was not confirmed in post-hoc analysis.

At last, no differences in c-Fos expression between diet regimes were found in the PFC as a whole ($F_{(3,30)}$=1.334, NS, Fig. 4A), nor in the PrL ($F_{(3,30)}$=1.486, NS, Fig. 4B/D) or IL ($F_{(3,30)}$=1.201, NS, Fig. 4C/E) separately.

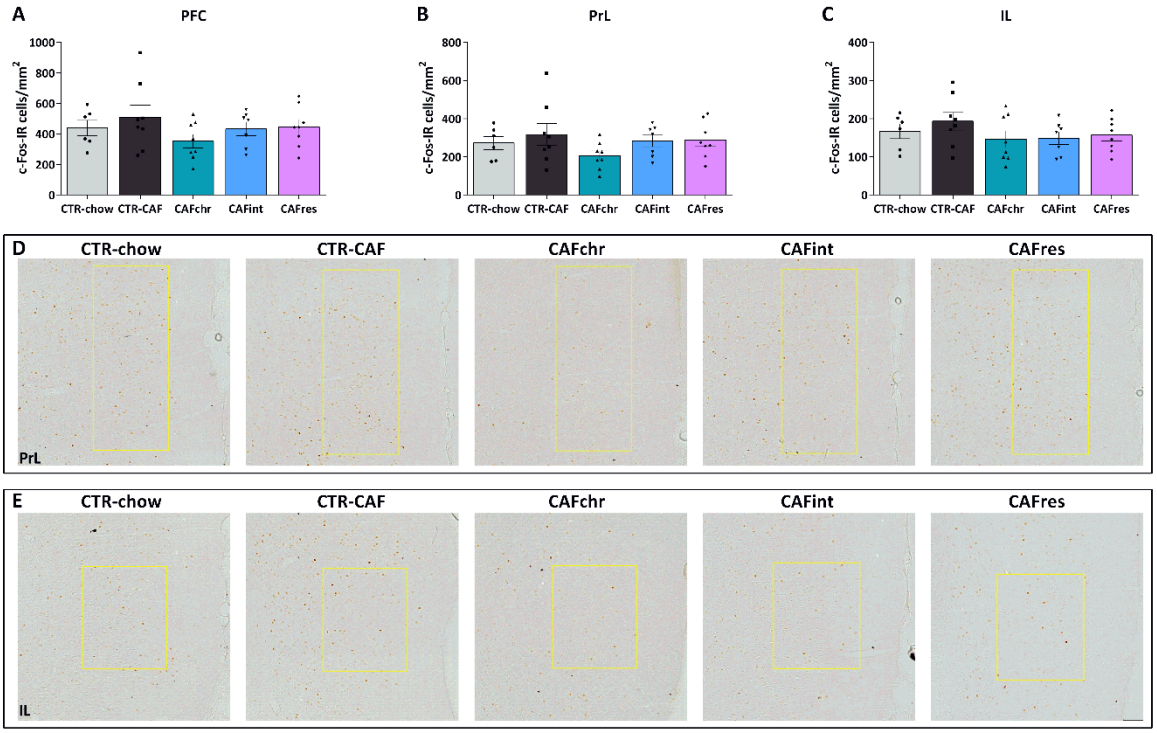

Figure 4. *A) The total count of c-Fos-IR cells per mm² in the prefrontal cortex (PFC, (PrL+IL)), B) The total count of c-Fos-IR cells per mm² in the prelimbic prefrontal cortex (PrL), C) The total count of c-Fos-IR cells per mm² in the infralimbic prefrontal cortex (IL). D) Examples of microphotographs with the counted c-Fos-IR cells of the different diet groups in the PrL and E) IL. The yellow outlines represent the zone that was used for counting. The data are shown with individual data points, with the bars representing the mean ± standard error of the mean for CTR-Chow (light grey bar, n=6), CTR-CAF (dark grey bar, n=8), CAFchr (green bar, n=8), CAFint (blue bar, n=7), and CAFres (purple bar, n=8) female Long-Evans rats.*

4. Discussion

In this study we investigated the effects of different long-term cafeteria diet regimes on the brain reward system in response to a food reward. First of all, we found that an acute first-time exposure to a CAF reward caused an increase in c-Fos expression in the NAc-shell, but not in the VTA and PFC. In addition, we found that only chronic access to CAF diet (both restricted and *ad libitum*) resulted in higher biometric outcomes (body weight, abdominal perimeter, body mass index and Lee index) as other studies similarly reported [29, 35, 36]. However, chronic but also intermittent exposure to CAF diet caused a decrease in c-Fos expression in the NAc-shell upon a CAF reward compared to rats on a control diet. No differences in c-Fos expression levels were found in the PrL and IL of the PFC, nor in the aVTA and pVTA Interestingly though not significant, intermittent access to CAF diet (and not chronic access (*ad libitum* or restricted)) did seem to trend towards a decrease in average of c-Fos-IR cells of the aVTA and pVTA. These results suggest that regular junk food consumption, even if consumed only 1 day per week, can change the brain reward system, mainly by affecting the NAc-shell.

The increase in c-Fos expression in the NAc-shell upon an acute first-time CAF reward is in line with other studies that found increases in c-Fos expression in the NAc after drinking palatable glucose or sucrose [37, 38]. Although surprising at first sight, the lack of differences in c-Fos expression between a control and palatable food reward in the VTA and PFC has also been shown before [37]. However, in that study an increased neural activity has been found in other regions of the PFC (the ventrolateral and lateral parts) after drinking glucose [37]. Since dopamine synthesis and release increases following acute exposure to palatable foods [10, 39], and dopamine signaling plays a central role in differentiating and encoding rewarding stimuli [12], we expected that dopamine-rich brain regions like the VTA and dopamine-receiving areas like the PFC would have increased levels of neural activity, indicated by higher levels of c-Fos in those regions, in response to a CAF reward as well. As a tendency of increased levels of c-Fos-IR cells in the anterior VTA is seen, the lack of significant increases might in part have been caused by the anatomical sparseness of (dopamine) cells in the VTA and the limited number of brain sections that were available for this study.

All long-term CAF diet regimes did result in attenuated activity of the NAc-shell neurons in response to a CAF reward compared to rats on a control diet. That chronic *ad libitum* access to junk food can decrease neural activity in the NAc-shell is in line with another study that showed reduced levels of pERK after a chronic CAF diet [40]. In addition, many studies have shown that rats fed high in calorie food show lower dopamine turnover in the NAc [22, 41-45]. This reduction in dopamine levels in the nucleus accumbens is suggested to be due to reduced stimulated dopamine release and vesicle size [43, 44]. In addition, it was found previously that rats on CAF or high-fat, high-sugar diets show reduced levels of ethanol or sucrose intake [46-48]. The diets seem to attenuate the rewarding properties of these substances, which could explain the lower levels of neural activation in the NAc-shell. It should be mentioned, though, that others have not been able to find changes in c-Fos expression in the NAc in rats on a high-fat diet, but this could be explained by differences in the duration of the palatable food exposure [37].

The interesting aspect of our study can mainly be found in the fact that also chronic restricted access to a CAF diet, and even 1 day a week exposure, already reduces c-Fos expression in the NAc-shell. One could argue that familiarity with the CAF reward may cause this decreased neural activation. The CTR-CAF rats have never been exposed to CAF before and could therefore experience the CAF reward as more rewarding. However, limited access to CAF has been shown to increase the amount of CAF consumption compared to chronic access [29, 30]. In addition, when exposed to a sucrose solution, rats exhibit more licks per cluster than rats on a chronic CAF diet [29]. If novelty plays a role in our finding, it would then be expected that also the CAFint rats would show increased, instead of

decreased c-Fos expression in the NAc-shell. Either way, more research is needed to unravel the reasons behind these changes in NAc-shell neural activation.

As mentioned above, our findings surprisingly did not show an effect of exposure to an acute CAF reward on c-Fos-IR cell levels in the VTA in control rats. Due to unforeseen circumstances during sectioning, there were fewer sections of the VTA with sufficient quality available, which ultimately led to lower numbers of animals in the VTA comparison. The visual representation in Fig. 3 and the similarity of the directionality of the results with those of the NAc, however, do suggest that differences in the VTA may be found in a higher-powered study. Previously, it has been found that a short 3 day high-fat diet does also not change c-Fos expression in the VTA in response to a palatable glucose solution [37], but several reward-related receptors in the VTA are found to be down-regulated after long-term high-calories food diets [49-51]. Unpublished data from our laboratory, gathered with fiber photometry, also suggests that neurons in the VTA become activated upon exposure to a CAF reward in control animals, while these levels of VTA activation are reduced in the context of chronic *ad libitum* CAF diet treatment. Future research is needed to reveal whether or not different regimes of CAF diet exposure can cause desensitization of VTA neurons.

In addition, we found that different regimes of CAF diet exposure did not change the neural activation of the PFC in response to a food reward. Since the c-Fos-IR cell levels of CTR-CAF rats were also not significantly different from CTR-chow rats in the PFC, we conclude that the PrL and IL of the PFC are not involved in this aspect of reward processing. This is in line with another study that also failed to detect changes in monoamine neurotransmitters in the PFC after CAF exposure [52]. However, this does not mean that the PFC is not affected by junk food at all. It has been shown before, for example, that inbred obesity-prone rats have lower dopamine turn-over in the PFC in response to acute feeding [43]. It is then logic to assume that changes in neural activation within the PFC could be found, if different reward-related aspects were explored.

At last, we would like to stress that our study was performed in female Long-Evans rats. To the best of our knowledge, this is the first study exploring the effects of chronic junk food exposure on the brain reward system in females. It is important to consider that sex differences can occur and that male and female rats can respond differently to CAF diet. As such, sex differences in changes in reward relevant parameters such the TH mRNA levels and I-opioid receptors expression have already been reported [53]. This highlights the importance of investigating sex differences in the neurobiological response to palatable food intake and the need for more studies in this area being conducted with female rats.

5. Conclusion

In conclusion, our study revealed that 6 weeks exposure to CAF diet can decrease c-Fos expression in the NAc-shell when presented with a CAF reward. This decrease was found in all CAF diet regimes: with chronic *ad libitum* and restricted access, and with intermittent (1 day a week) exposure to CAF diet. While no changes in c-Fos expression upon the different diet regimes were found in the PFC, future research should reveal potential decreases in neural activity in the VTA. Our data suggests that long-term junk food exposure can affect the brain reward system, resulting in an attenuated activity of the NAc-shell.

Acknowledgements

Financial support was received from Helse Nord (HNF1443-19). We thank Carles Baldellou, Laia Oliva and the technical staff of the Unitat d'Experimentació Animal in the Campus Mundet of Universitat de Barcelona (UB) for the excellent care of the animals. Finally, we thank the Advanced Microscopy Core Facility of UiT The Arctic University of Tromsø for access to their equipment for the brain imaging analyses.

Author contributions

RH: Experimental design, methodology, data gathering, data curation, analysis, supervision, writing – original draft, funding acquisition

JFL: Experimental design, data gathering, writing – review and editing

MS: Methodology, writing - review and editing

AMA: Data gathering

SGA: Data gathering

RME: Methodology, writing - review and editing

EMSS: Experimental design, methodology, data gathering, data curation, analysis, supervision, writing – original draft, funding acquisition

References

[1] Guh, D. P., Zhang, W., Bansback, N., Amarsi, Z., Birmingham, C. L., Anis, A. H. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC public health. 2009,9:88.

[2] Yu, Y. H., Vasselli, J. R., Zhang, Y., Mechanick, J. I., Korner, J., Peterli, R. Metabolic vs. hedonic obesity: a conceptual distinction and its clinical implications. Obes Rev. 2015,16:234-47.

[3] Bellisle, F. Meals and snacking, diet quality and energy balance. Physiol Behav. 2014,134:38-43.

[4] Malik, V. S., Willett, W. C., Hu, F. B. Global obesity: trends, risk factors and policy implications. Nat Rev Endocrinol. 2013,9:13-27.
[5] Morris, M. J., Beilharz, J. E., Maniam, J., Reichelt, A. C., Westbrook, R. F. Why is obesity such a problem in the 21st century? The intersection of palatable food, cues and reward pathways, stress, and cognition. Neurosci Biobehav Rev. 2015,58:36-45.
[6] Berridge, K. C., Ho, C. Y., Richard, J. M., DiFeliceantonio, A. G. The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. Brain Res. 2010,1350:43-64.
[7] Zheng, H., Berthoud, H. R. Eating for pleasure or calories. Curr Opin Pharmacol. 2007,7:607-12.
[8] Berridge, K. C., Robinson, T. E. Parsing reward. Trends in neurosciences. 2003,26:507-13.
[9] Yoshida, M., Yokoo, H., Mizoguchi, K., Kawahara, H., Tsuda, A., Nishikawa, T., et al. Eating and drinking cause increased dopamine release in the nucleus accumbens and ventral tegmental area in the rat: measurement by in vivo microdialysis. Neurosci Lett. 1992,139:73-6.
[10] Martel, P., Fantino, M. Influence of the amount of food ingested on mesolimbic dopaminergic system activity: a microdialysis study. Pharmacol Biochem Behav. 1996,55:297-302.
[11] Hajnal, A., Smith, G. P., Norgren, R. Oral sucrose stimulation increases accumbens dopamine in the rat. American journal of physiology. Regulatory, integrative and comparative physiology. 2004,286:R31-7.
[12] Roitman, M. F., Wheeler, R. A., Wightman, R. M., Carelli, R. M. Real-time chemical responses in the nucleus accumbens differentiate rewarding and aversive stimuli. Nat Neurosci. 2008,11:1376-7.
[13] Pecina, S., Cagniard, B., Berridge, K. C., Aldridge, J. W., Zhuang, X. Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. J Neurosci. 2003,23:9395-402.
[14] de Wit, L., Luppino, F., van Straten, A., Penninx, B., Zitman, F., Cuijpers, P. Depression and obesity: a meta-analysis of community-based studies. Psychiatry Res. 2010,178:230-5.
[15] Tomasi, D., Volkow, N. D. Striatocortical pathway dysfunction in addiction and obesity: differences and similarities. Crit Rev Biochem Mol Biol. 2013,48:1-19.
[16] Rothemund, Y., Preuschhof, C., Bohner, G., Bauknecht, H. C., Klingebiel, R., Flor, H., et al. Differential activation of the dorsal striatum by high-calorie visual food stimuli in obese individuals. Neuroimage. 2007,37:410-21.
[17] Tellez, L. A., Medina, S., Han, W., Ferreira, J. G., Licona-Limon, P., Ren, X., et al. A gut lipid messenger links excess dietary fat to dopamine deficiency. Science. 2013,341:800-2.
[18] la Fleur, S. E., Vanderschuren, L. J. M. J., Luijendijk, M. C., Kloeze, B. M., Tiesjema, B., Adan, R. A. H. A reciprocal interaction between food-motivated behavior and diet-induced obesity. International journal of obesity. 2007,31:1286-94.
[19] Vasselli, J. R., Cleary, M. P., Jen, K. L., Greenwood, M. R. Development of food motivated behavior in free feeding and food restricted Zucker fatty (fa/fa) rats. Physiol Behav. 1980,25:565-73.

[20] Greenwood, M. R., Quartermain, D., Johnson, P. R., Cruce, J. A., Hirsch, J. Food motivated behavior in genetically obese and hypothalamic-hyperphagic rats and mice. Physiol Behav. 1974,13:687-92.
[21] Narayanaswami, V., Thompson, A. C., Cassis, L. A., Bardo, M. T., Dwoskin, L. P. Diet-induced obesity: dopamine transporter function, impulsivity and motivation. International journal of obesity. 2013,37:1095-103.
[22] Davis, J. F., Tracy, A. L., Schurdak, J. D., Tschop, M. H., Lipton, J. W., Clegg, D. J., et al. Exposure to elevated levels of dietary fat attenuates psychostimulant reward and mesolimbic dopamine turnover in the rat. Behav Neurosci. 2008,122:1257-63.
[23] Steele, C. C., Pirkle, J. R. A., Davis, I. R., Kirkpatrick, K. Dietary effects on the determinants of food choice: Impulsive choice, discrimination, incentive motivation, preference, and liking in male rats. Appetite. 2019,136:160-72.
[24] Wellman, P. J., Nation, J. R., Davis, K. W. Impairment of acquisition of cocaine self-administration in rats maintained on a high-fat diet. Pharmacol Biochem Behav. 2007,88:89-93.
[25] la Fleur, S. E., Luijendijk, M. C., van der Zwaal, E. M., Brans, M. A., Adan, R. A. The snacking rat as model of human obesity: effects of a free-choice high-fat high-sugar diet on meal patterns. International journal of obesity. 2014,38:643-9.
[26] Rolls, B. J., Rowe, E. A., Rolls, E. T., Kingston, B., Megson, A., Gunary, R. Variety in a meal enhances food intake in man. Physiol Behav. 1981,26:215-21.
[27] Corwin, R. L., Avena, N. M., Boggiano, M. M. Feeding and reward: perspectives from three rat models of binge eating. Physiol Behav. 2011,104:87-97.
[28] Lalanza, J. F., Snoeren, E. M. S. The cafeteria diet: A standardized protocol and its effects on behavior. Neurosci Biobehav Rev. 2021,122:92-119.
[29] Martire, S. I., Westbrook, R. F., Morris, M. J. Effects of long-term cycling between palatable cafeteria diet and regular chow on intake, eating patterns, and response to saccharin and sucrose. Physiol Behav. 2015,139:80-8.
[30] Tenk, C. M., Felfeli, T. Sucrose and fat content significantly affects palatable food consumption in adolescent male and female rats. Appetite. 2017,118:49-59.
[31] Subias-Gusils, A., Alvarez-Monell, A., Boque, N., Caimari, A., Del Bas, J. M., Marine-Casado, R., et al. Behavioral and Metabolic Effects of a Calorie-Restricted Cafeteria Diet and Oleuropein Supplementation in Obese Male Rats. Nutrients. 2021,13.
[32] Alvarez-Monell, A., Subias-Gusils, A., Mariné-Casadó, R., Boqué , N., Caimari, A., Solanas, M., Escorihuela, R.E. Impact of Calorie-Restricted Cafeteria Diet and Treadmill Exercise on Sweet Taste in Diet-Induced Obese Female and Male Rats. Nutrients 2023, 15(1), 144.

[33] Lee, M. O. Determination of the surface area of the white rat with its application to the expression of metabolic results. American Journal of Physiology. 1929,89:24-33.
[34] Paxinos, G., Watson, C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press; 1998.
[35] Thompson, J. L., Drysdale, M., Baimel, C., Kaur, M., MacGowan, T., Pitman, K. A., et al. Obesity-Induced Structural and Neuronal Plasticity in the Lateral Orbitofrontal Cortex. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2017,42:1480-90.
[36] Johnson, P. M., Kenny, P. J. Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats. Nature neuroscience. 2010,13:635-41.
[37] Delaere, F., Akaoka, H., De Vadder, F., Duchampt, A., Mithieux, G. Portal glucose influences the sensory, cortical and reward systems in rats. Eur J Neurosci. 2013,38:3476-86.
[38] Koekkoek, L. L., Masis-Vargas, A., Kool, T., Eggels, L., van der Gun, L. L., Lamuadni, K., et al. Sucrose drinking mimics effects of nucleus accumbens micro-opioid receptor stimulation on fat intake and brain c-Fos-expression. Nutr Neurosci. 2021:1-13.
[39] South, T., Westbrook, F., Morris, M. J. Neurological and stress related effects of shifting obese rats from a palatable diet to chow and lean rats from chow to a palatable diet. Physiol Behav. 2012,105:1052-7.
[40] Fam, J., Clemens, K. J., Westbrook, R. F., Morris, M. J., Kendig, M. D. Chronic exposure to cafeteria-style diet in rats alters sweet taste preference and reduces motivation for, but not 'liking' of sucrose. Appetite. 2022,168:105742.
[41] Geiger, B. M., Haburcak, M., Avena, N. M., Moyer, M. C., Hoebel, B. G., Pothos, E. N. Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity. Neuroscience. 2009,159:1193-9.
[42] Nguyen, J. C., Ali, S. F., Kosari, S., Woodman, O. L., Spencer, S. J., Killcross, A. S., et al. Western Diet Chow Consumption in Rats Induces Striatal Neuronal Activation While Reducing Dopamine Levels without Affecting Spatial Memory in the Radial Arm Maze. Frontiers in behavioral neuroscience. 2017,11:22.
[43] Geiger, B. M., Behr, G. G., Frank, L. E., Caldera-Siu, A. D., Beinfeld, M. C., Kokkotou, E. G., et al. Evidence for defective mesolimbic dopamine exocytosis in obesity-prone rats. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2008,22:2740-6.
[44] Pothos, E. N., Sulzer, D., Hoebel, B. G. Plasticity of quantal size in ventral midbrain dopamine neurons: possible implications for the neurochemistry of feeding and reward. Appetite. 1998,31:405.
[45] Rada, P., Bocarsly, M. E., Barson, J. R., Hoebel, B. G., Leibowitz, S. F. Reduced accumbens dopamine in Sprague-Dawley rats prone to overeating a fat-rich diet. Physiol Behav. 2010,101:394-400.

[46] Cook, J. B., Hendrickson, L. M., Garwood, G. M., Toungate, K. M., Nania, C. V., Morikawa, H. Junk food diet-induced obesity increases D2 receptor autoinhibition in the ventral tegmental area and reduces ethanol drinking. PLoS One. 2017,12:e0183685.
[47] Takase, K., Tsuneoka, Y., Oda, S., Kuroda, M., Funato, H. High-fat diet feeding alters olfactory-, social-, and reward-related behaviors of mice independent of obesity. Obesity. 2016,24:886-94.
[48] Gac, L., Kanaly, V., Ramirez, V., Teske, J. A., Pinto, M. P., Perez-Leighton, C. E. Behavioral characterization of a model of differential susceptibility to obesity induced by standard and personalized cafeteria diet feeding. Physiol Behav. 2015,152:315-22.
[49] Martire, S. I., Maniam, J., South, T., Holmes, N., Westbrook, R. F., Morris, M. J. Extended exposure to a palatable cafeteria diet alters gene expression in brain regions implicated in reward, and withdrawal from this diet alters gene expression in brain regions associated with stress. Behav Brain Res. 2014,265:132-41.
[50] Bourdy, R., Hertz, A., Filliol, D., Andry, V., Goumon, Y., Mendoza, J., et al. The endocannabinoid system is modulated in reward and homeostatic brain regions following diet-induced obesity in rats: a cluster analysis approach. Eur J Nutr. 2021,60:4621-33.
[51] Carlin, J., Hill-Smith, T. E., Lucki, I., Reyes, T. M. Reversal of dopamine system dysfunction in response to high-fat diet. Obesity. 2013,21:2513-21.
[52] Virtuoso, A., Tveden-Nyborg, P., Schou-Pedersen, A. M. V., Lykkesfeldt, J., Muller, H. K., Elfving, B., et al. A Long-Term Energy-Rich Diet Increases Prefrontal BDNF in Sprague-Dawley Rats. Nutrients. 2021,14.
[53] Ong, Z. Y., Wanasuria, A. F., Lin, M. Z., Hiscock, J., Muhlhausler, B. S. Chronic intake of a cafeteria diet and subsequent abstinence. Sex-specific effects on gene expression in the mesolimbic reward system. Appetite. 2013,65:189-99.