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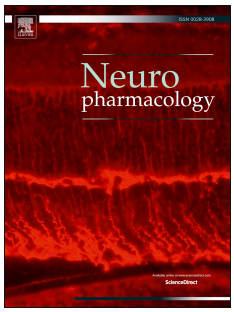

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Effects of bingeing on fat during adolescence on the reinforcing effects of cocaine in adult male mice

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Abstract

Binge eating is a specific form of overeating characterized by intermittent excessive eating. In addition to altering the neurobiological reward system, several studies have highlighted that consumption of palatable food increases vulnerability to drug use. The aim of the present study was to evaluate the effects of a high-fat diet consumed in a binge pattern during adolescence on the reinforcing effects of cocaine.

After 40 days of binge-eating for 2h, three days a week (PND 29-69), the reinforcing effects of cocaine on conditioning place preference and intravenous self-administration paradigm were evaluated in adolescent male mice. Circulating leptin and ghrelin levels and the effects of bingeing on fat on CB1 mu opioid receptor (MOr) and ghrelin receptor (GHSR) gene expression in the Nucleus Accumbens (NAcc) and ventral tegmental area (VTA) were also assessed.

Our results showed a significant escalation in the consumption of a high-fat diet between the first and last week. High-fat binge (HFB) animals were more sensitive to the reinforcing effects of a subthreshold dose of cocaine in the paradigms assayed, and animals under fat withdrawal were more vulnerable to the reinstatement of conditioned place preference. HFB mice also showed enhanced cocaine self-administration. After fat withdrawal, exposure to a new fat binge reinstated cocaine seeking. Although HFB did not modify leptin levels, a decrease in plasmatic ghrelin was observed. Moreover, this pattern of fatty diet resulted in a reduction of MOr and CB1 gene expression in the NAcc and an increase in GHSR expression in the VTA.

We propose that bingeing on fat during adolescence induces long-lasting changes in the brain through the sensitization of brain reward circuits, which predisposes individuals to seek cocaine during adulthood.

Highlights

- High-fat binging increases sensitivity to cocaine in the conditioned place preference
- High-fat binging enhances acquisition and reinstatement of cocaine self-administration
- High-fat binging reduces MOr and CB1r gene expression in the Nucleus Accumbens
- High-fat binging increases ghrelin receptor expression in the ventral tegmental area
- Fat withdrawal prolongs extinction and facilitates reinstatement of the preference

Keywords: binge eating, fat, cocaine, conditioned place preference, self-administration, gene expression

1. Introduction

Adolescent development is associated with major changes in emotional and cognitive functions. It is also a period of brain maturation marked by structural alterations in many limbic and cortical regions. Drug use during this critical period of development often predicts an increased likelihood of continued use into adulthood (Arteaga et al., 2010; Merline et al., 2004; Young et al., 2002). For example, the adolescent brain is especially sensitive to some effects of ethanol, such as memory impairment (White & Swartzwelder, 2005), ethanol binge drinking-induced brain damage (Crews et al., 2000) or epigenetic alterations (Pascual et al., 2012). Therefore, exposure to ethanol binge drinking during the juvenile/adolescent stage can sensitize some of the brain regions and/or developmental processes involved in drug addiction-like behavior (Pascual et al., 2009).

Factors contributing to increased vulnerability to drug use during adolescence also include social, economic, hormonal, neurochemical and dietary conditions that influence individual responses to drugs (Baladi et al., 2012; Daws et al., 2011; Spear, 2000). Indeed, with the growing high-fat “fast-food” culture and prevalence of obesity, particularly among adolescents, diet might play a greater role than previously thought in determining the sensitivity of an individual to drugs, as well as his/her predisposition to drug abuse (Baladi et al., 2012; Herpertz-Dahlmann, 2015; Volkow et al., 2013). Statistics suggest that binge eating is more common than other eating disorders (Hudson et al., 2007). The DSM-5 defines binge eating as recurring episodes of rapid and excessive food

consumption in a short period of time, marked by feelings of lack of control (5th ed., DSM-5; American Psychiatric Association, 2013). Moreover, it is not necessarily driven by hunger or metabolic need (Brownley et al., 2007; Davis et al., 2007). Foods that are consumed during a binge episode are typically high in calories, fat and/or sugar (Guertin and Conger, 1999; Hadigan et al., 1989; Kales, 1990). Although binge eating is related to obesity, many people who binge are not obese, and most obese people do not present binge eating disorders (Hudson et al., 2007). Binge eating in animals is characterized by behavior patterns similar to those seen in humans. To be classified as a binge, animals must consume large quantities of food in a brief, defined period of time, and this quantity should exceed that which would be consumed by control animals under similar circumstances, and must be stable and maintained over long periods of time (Corwin & Buda-Levin, 2004).

Similarly to drugs of abuse, ingestion of palatable foods activates dopaminergic neurons within the Nucleus Accumbens (NAcc) and other reward centers (Kelley et al., 2005; Rada et al., 2005). Brain regions such as the lateral hypothalamus, ventral tegmental area (VTA), prefrontal cortex and amygdala are activated in response to palatable food (de Macedo et al., 2016). An acute high-fat diet activates c-Fos expression in the VTA, NAcc, central amygdala and lateral hypothalamic area (Valdivia et al., 2015). Dietary-induced binge eating of fat results in sustained dopamine (DA) stimulation within the NAcc (Bello and Hajnal, 2010). Similarly, it was observed by Liang and co-workers (2006) that DA release in the NAcc is significantly increased during licking of corn oil compared with baseline.

Other neurotransmitter systems, such as the opioid and the cannabinoid systems are also important to the reward process (Wang et al., 2004). DA release in the NAcc is generally associated with the reinforcing effects of food, while opioid signaling in this area regulates its palatability and hedonic properties (Cota et al., 2006; Esch and Stefano, 2004). The MOr pathway plays a major role in the stimulatory effect of high reward food on the mesolimbic DA system (Tanda and Di Chiara, 1998), and MOr agonists in the VTA stimulate feeding behavior (Figlewicz and Sipols, 2010). In an elegantly executed study, Kawahara and co-workers (2013) showed that palatable food without food deprivation increased DA release in the NAcc via activation of the mu opioid receptor pathway in the VTA. Activation of MOr located in GABAergic interneurons inhibits GABA release in the VTA, resulting in disinhibition of DA neurons (Chefer et al., 2009; Johnson and North, 1992), therefore increasing DA release in the NAcc (Spanagel et al., 1990; Chefer et al., 2009). The authors hypothesized that beta – endorphin could be released in the VTA in response to food reward. In addition, changes in endogenous opioid systems have also been identified in individuals with binge eating disorders. Naloxone decreases the intake of palatable foods only in individuals fitting the criteria for bulimia nervosa and binge eating disorders, but does not alter food intake in non-bingeing obese or normal weight individuals (Drewnowski et al., 1995). Similarly, opioid receptor binding within the insular cortex in individuals with bulimia nervosa is decreased compared to individuals with no symptoms or who binge eat (Bencherif et al., 2005). The endocannabinoid system, besides playing a pivotal role in reward/reinforcement circuits of the mesolimbic system,

regulates a wide variety of processes, including pain, mood, memory and appetite and energy metabolism (Cristino et al., 2014). In the NAcc and VTA, CB1 activation modulates both dopaminergic and opioidergic pathways, thereby helping to reinforce both the 'liking' for and 'wanting' of highly palatable food (Mellis et al., 2007). Although addictive drugs initially produce strong feelings of pleasure (liking), with the transition to addiction the role of the pleasure produced by the drug becomes less important. According to the incentive sensitization theory, repeated drug use sensitizes only the neural systems that mediate the motivational process of incentive salience (wanting), but not those that mediate the pleasurable effects of drugs (liking). Thus, continued use makes drugs more wanted than liked, and this dissociation progressively increases with the development of addiction (Robinson and Berridge, 2008). High-fat diets upregulate hippocampal endocannabinoid system levels and hypothalamic 2-Arachidonylglycerol (2-AG), indicating that highly palatable foods may be more satisfying under these conditions (Massa et al., 2010; Higuchi et al., 2012). Accordingly, CB1r antagonists reduce binge-like intake (Parylak et al., 2012) and the increase in extracellular DA release in the NAcc mediated by a novel intake of high palatable food (Mellis et al., 2007).

Consequently, several studies have pointed out that, due to the common neurobiological pathways that stimulate fat intake and drugs of abuse, palatable food increases vulnerability to drug use. Acute locomotor response to cocaine is enhanced in mice consuming a continuous diet high in fat and/or sucrose (Collins et al., 2015). Two recent reports have described the development of locomotor sensitization to cocaine in adolescent mice exposed to a restricted or

continuous high-fat diet, while no response was observed in adult animals (Baladi et al., 2015; Serafine et al., 2015). Although a continuous high-fat diet attenuates cocaine and food reward in the Conditioned Place Preference (CPP) protocol (Morales et al., 2012), adult rats exposed to a binge-type intake of fat exhibit more robust "addiction-like" behaviors toward a substance of abuse. Although no significant differences have been observed, these mice tend to consume more cocaine in fixed ratio training, while they persist in their efforts to obtain cocaine in the face of signaled non-availability, work harder for cocaine in a progressive ratio schedule of reinforcement, and exhibit more goal-directed behavior toward the cocaine (Puhl et al., 2011).

To date, no studies have evaluated how bingeing on fat during adolescence modulates drug consumption. The aim of this study was to evaluate the effect of adolescent exposure to a binge pattern of a high-fat diet on the rewarding effects of cocaine. For this purpose, we employed the limited access model of a fatty diet based on that proposed by Corwin and co-workers (1998). This model provides limited access to palatable food for 2 h, three times a week (on Monday, Wednesday and Friday), which produces an escalation of intake, while animals have constant access to standard chow (Corwin et al., 2011). We assessed the effects of this high-fat diet binging during adolescence on the reinforcing properties of cocaine using the CPP and the intravenous self-administration paradigm (SA). There is evidence of a clinical overlap between binge-eating disorders and drug addiction (Davis and Carter, 2009), with typical addictive behaviors such as tolerance, withdrawal and compulsive food-seeking having been demonstrated in animal models of high-fat and sugar-rich

overeating (Avena et al., 2008). For this reason, we also studied whether the reinforcing and reinstating effects of cocaine were affected by the withdrawal of an intermittent HFB. Finally, we performed real-time polymerase chain reaction (PCR) experiments in mice exposed during adolescence to a HFB and their corresponding controls in order to study gene expression changes in opioid (MOr) and endocannabinoid (CB1) receptors in the NAcc and ghrelin receptors (GHSR) in the VTA, which are involved in the brain's responses to dietary nutrients and drug reward processes. Given their known modulatory effect on the activity of mesolimbic DA neurons, circulating levels of leptin, ghrelin and corticosterone were also determined (Figlewicz and Benoit, 2009; Murray et al., 2014).

2. Material and Methods

2.1. Animals

A total of 180 male mice of the OF1 and CD1 strains were acquired commercially from Charles River (Barcelona, Spain). Animals were 21 days old on arrival at the laboratory and were all housed in groups of 4, under standard conditions (cage size 28x28x14.5cm), for 8 days prior to initiating the experimental feeding schedule, at a constant temperature (21±2℃), with a reverse light cycle (white lights on 19:30 -7:30h).

For CPP, gene expression analysis and leptin analyses, a total 140 male mice of the OF1 outbred strain were employed, while for self-administration studies, 40 CD1 male mice were employed. CD1 mice were used for self-administration studies due to their greater sensitivity to this technique (Rodriguez-Arias et al., 2016). Mice were housed under standard conditions in groups of 4 (as above),

and were exposed to a reverse light-dark cycle (12:12), with lights turned off at 08.00 hours and on at 20.00 hours. Animal rooms were controlled for temperature (21±1ºC) and humidity (55±10%). Food (standard diet) and water were available *ad libitum* in all the experiments (except during the behavioral tests). Mice were manipulated at the same time on each test day to minimize inter-day variability. All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia and the PRBB approved the present study.

2.2. Drugs

For CPP, animals were injected i.p. with 1 or 6 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S. A. Madrid, Spain) diluted in physiological saline. The 1 mg/kg dose of cocaine used to induce CPP was based on previous studies (Vidal-Infer et al., 2012; Maldonado et al., 2006) in which it was shown to be a sub-threshold dose. The 6 mg/kg dose of cocaine has been demonstrated to be an effective dose that does not induce reinstatement (Maldonado et al., 2006). For cocaine self-administration studies the dose of cocaine selected was 0.5 mg/kg/infusion diluted in sterile 0.9% physiological saline in a volume of 20 µl, in accordance with previous studies by our team (Soria et al., 2005).

2.3. Procedure

2.3.1. Feeding Conditions

Our feeding procedure is based on the limited access model described by Corwin et al. (1998), in which non-food-deprived animals with sporadic and limited access to a high-fat food develop binge-type behaviors. Two different types of diet were administered in the study. A standard diet (Teklad Global Diet 2014, 13 Kcal % fat, 67 Kcal % carbohydrates and 20% Kcal protein; 2,9 Kcal/g) was given to the control group and a high-fat diet (TD.06415, 45 Kcal % fat, 36 Kcal % carbohydrates and 19% Kcal protein; 4,6 Kcal/g) was administered in a limited way to the high-fat diet binge group. Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to from now on as the standard diet and the high-fat diet, while the sporadic limited access to the high-fat food will be referred to as the high-fat diet binge (HFB).

On PND 29, mice were randomly divided into groups (n=15/condition) with similar average body weight (25-26g) and assigned either a Control (C) diet or HFB (2h access on Monday, Wednesday and Friday). All groups were fed with the standard diet in their own cages 3 days a week and were exposed to a 2-h binge session in a different plastic cage (standard diet for the control group and high-fat diet for the HFB groups). Water was freely available at all times. Binge sessions took place 2-3h after the beginning of the dark phase. Animals were weighed every Monday, Wednesday and Friday throughout the study, at which point their intake of standard diet in their home cage was also measured.

2.3.2. Experimental Design

An overall and more detailed description of the sets of animals and experimental procedure related to 1mg/kg or 6mg/kg cocaine CPP, SA and brain extraction is provided in Table 1.

Behavioral Tests began on PND 69, after 18 binge-eating sessions (for control, HFB, and HFB 1-day withdrawal groups (HFB 1w) and PND 84 (for HFB 15-day withdrawal groups (HFB 15w).

In experiment 1, OF1 mice (n=120) performed the Elevated Plus Maze (EPM) prior to the first Pre-Conditioning session of CPP, in which mice were conditioned with 1mg/kg or 6mg/kg cocaine. Four groups were employed in this experiment: Control, HFB, HFB 1w and HFB 15w.

In experiment 2, a different set of CD1 mouse strain (n=40) was exposed to 18 binge sessions (from PND29 to PND69), following the same procedure as in Experiment 1. Two groups were employed in this experiment: Control and HFB. Subsequently, mice underwent catheter implantation surgery and then performed the operant SA procedure with 0.5mg/kg/infusion from PND 77 to 88. During the surgery recovery period and SA procedure, mice were exposed to food binge sessions every Monday, Wednesday and Friday. After completing the SA procedure, mice were left undisturbed for a period of 20 days. On PND 108, after a further 4 2-hour sessions of food bingeing, the animals underwent a single session in the SA operant chamber without receiving cocaine.

Finally, a further set of OF1 mice (n=20) was employed to extract blood samples and brains on PND 69 for the assessment of circulating leptin, ghrelin and corticosterone levels and to carry out gene expression studies with real-

time PCR in the NAcc and VTA. Again, two groups were employed in this experiment: Control and HFB.

2.4. Apparatus

2.4.1. Elevated Plus Maze

The Elevated Plus Maze (EPM) consisted of two open arms (30x5x0.25cm) and two enclosed arms (30x5x15cm). The junction of the four arms formed a central platform (5x5cm). The floor of the maze was made of black Plexiglas and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1h prior to testing. At the beginning of each trial, subjects were placed on the central platform so that they were facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was recorded automatically by an automated tracking control (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The measurements recorded during the test period were frequency of entries and time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open arm entries, time spent in open arms and percentage of open arm entries are generally used to characterize the anxiolytic effects of drugs (Pellow and File, 1986; Rodgers et al., 1997).

2.4.2. Conditioning Place Preference

For Place Conditioning we employed twelve identical Plexiglas boxes with two equal sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a gray central area (13.8 cm, length × 31.5 cm, width × 34.5 cm height). The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously (Maldonado et al., 2006) and consisted of three phases. To summarize the main aspects, in the first phase, known as Pre-Conditioning (Pre-C), mice of 69 PND (and 84 PND in the case of the withdrawal groups) were allowed access to both compartments of the apparatus for 15min (900 s) per day on 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment. Half the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the preconditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined

to the vehicle-paired compartment for 30min. After an interval of 4 h, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. During the third phase, known as post-conditioning (Post-C), the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has developed.

Extinction of CPP

All groups in which a preference for the drug-paired compartment was established

underwent a weekly extinction session that consisted of placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 minutes. The extinction condition was fulfilled when there was a lack of significant differences between CPP scores and Pre-C test values in two consecutive sessions.

Reinstatement of CPP

24 hours after extinction had been confirmed, the effects of a priming dose of cocaine were evaluated. Reinstatement tests were the same as those carried out in Post-C (free ambulation for 15 minutes), except that animals were tested

15 minutes after administration of the respective dose of cocaine. When reinstatement of the preference was achieved, after a subsequent weekly extinction process, a new reinstatement test was conducted with progressively lower doses of the drug until the CPP was completely extinguished. This procedure of extinction-reinstatement was repeated with decreasing doses (half the previous dose) until a priming dose was confirmed to be ineffective. Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure.

2.4.3 Self – administration procedure

Mice were anesthetized with ketamine/xylacine solution and implanted with an indwelling i.v. silastic catheter in the right jugular vein, as previously described (Soria et al., 2005; Touriño et al., 2012). For the SA experiments, surgical implantation of the catheter into the jugular vein was performed following anesthetization with a mixture of ketamine hydrochloride (100 mg/ml; Imalgène®1000, Rhône Mérieux, Lyon, France) and Xylazine hydrochloride (20 mg/kg; Sigma Chemical Co., Madrid, Spain). Both compounds were dissolved in distilled water to obtain a final concentration of 5 mg/mL of ketamine and 1 mg/mL of xylazine. The anaesthetic solution was injected i.p. in a volume of 0.15 mL per 10 g body weight. We used meloxicam (Metacam®, 5mg/mL, Boehringer Ingelheim, Barcelona, Spain) dissolved in 0.9% physiological saline to 0.5 mg/kg as an analgesic during surgery. The analgesic solution was injected subcutaneously (s.c.) in a volume of 0.1 mL per 10 g body weight. Enrofloxacin (Baytril® 2.5%; Bayer, Barcelona, Spain) dissolved in 0.9%

physiological saline and was injected (i.p.) at a dose of 7.5 mg/kg just before surgery, as a preventive antibiotic.

After surgery, mice were housed individually and allowed to recover for at least 4 days prior to the first SA session. Mice were trained to receive cocaine infusions for 1h per day on 10 consecutive days under a fixed ratio 1 (FR1) schedule of reinforcement. Cocaine dose (0.5 mg/kg/infusion) was selected as in previous studies performed in our laboratory (Soria et al., 2005; Touriño et al., 2012).

Mice were considered to have acquired stable SA when the following criteria were met on three consecutive days: i) 80% stability in reinforcements (the number of reinforcements on each day deviated by <20% from the mean number of reinforcements over the three consecutive days); ii) ≥70% of responses were received at the active nose-poke; and iii) ≥5 responses were received at the active nose-poke (excluding priming reinforcement).

After the SA procedure, mice spent 20 days without receiving any treatment and with a standard diet. After this period, they were once again exposed to bingeing on a high fat diet for one week and were then assayed in the SA chamber during a one-hour session.

For SA procedures, we used 8 operant chambers with two nose-pokes (Model ENV-307A-CT, Med Associates, Inc. Cibertec. Madrid. Spain). Active and inactive nose-pokes were selected randomly. Cocaine was delivered in a 2 s 20 µl injection via a syringe mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) connected via Tygon tubing (0.96 mm outer

diameter, Portex Fine Bore Polythene Tubing, Portex Limited, Kent, England) to a single-channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and the mouse's intravenous catheter. All sessions began with a priming injection of cocaine. When mice responded at the reinforcing hole, the stimulus lights (one located inside the nose-poke and the other above it) lit up for 4s and a cocaine infusion was delivered automatically over 2s. The number of reinforcements was limited to 50 infusions per session. Each infusion was followed by a 30s time-out period in which an active nose-poke had no consequences. After each session, mice were returned to their home cages. The patency of the catheter was evaluated by passing a 0.1 mL infusion of thiopental through it (5 mg/mL; B. Braun Medical S.A., Barcelona, Spain). If clear signs of anesthesia were not apparent within 3s of the infusion, the animal was removed from the experiment.

2.4.4. Determination of plasma leptin, ghrelin and corticosterone

Plasma levels were measured with an ELISA kit from B-Bridge International (Cupertino, CA, USA) for leptin; Sigma Aldrich (San Louis, EEUU) for ghrelin; and Enzo® Life Sciences (Catalog No. ADI-900-097) for corticosterone, following the manufacturer's instructions. The sensitivity of the test is 0.2. All samples were run in duplicate.

2.4.5. Gene expression analyses. Real time PCR

Brain sections were cut (500 µm) in a cryostat (-10ºC) at levels containing the regions of interest according to Paxinos and Franklin (2001), and were then mounted onto slides and stored at −80 ℃. Sections were dissected following the method described by Palkovits (1983). Total RNA was isolated from brain

tissue micropunches using TRI Reagent® (Ambion) and subsequently retrotranscribed to cDNA. Quantitative analysis of the relative abundance of CB1, MOr and ghrelin receptor mRNA was measured by means of Taqman® Gene Expression assays (Mm00432621_s1 Cnr1, Mm01188089_m1 Oprm and Mm00616415_m1 Ghsr, respectively) (Thermo Fisher Scientific, Madrid, Spain), which is a double-stranded DNA-specific fluorescent dye, using the Step One Real Time PCR System (Life Technologies, Madrid, Spain). The reference gene was 18S rRNA, detected using Taqman® ribosomal RNA control reagents (Mm03928990_g1 Rn18s). The data for each target gene were normalized to the endogenous reference gene, and the fold-change in target gene mRNA abundance was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

2.5. Statistics

Data relating to body weight and binge intake were analyzed by a mixed ANOVA with one between-subjects variable – Diet, with 4 levels (Control, HFB, HFB 1w, HFB 15w) - and a within variable – Days, with 7 levels (PND 29, 36, 43, 50, 57, 64 and 69). The EPM, leptin, corticosterone and ghrelin data were analyzed by one – way ANOVA with a between variable: Diet, with 3 levels (Control, HFB, HFB 15w) or 5 levels (Control PND 69, Control PND 81, HFB PND 69, HFB PND 81 and, HFB 15d W PND 96). For CPP, the time spent in the drug-paired compartment was analyzed by means of a mixed analysis of variance (ANOVA) with one between variable – Diet, with 4 levels (Control, HFB, HFB 1w, HFB 15w) - and a within variable - Days, with 2 levels (Pre-C, and Post-C). Data related to extinction and reinstatement values in the groups showing CPP were analyzed by means of Student's t-tests. The time required

for the preference to be extinguished in each animal was analyzed by means of the Kaplan–Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. To analyze acquisition of cocaine SA during the 10-day training phase, a three-way ANOVA was calculated with Nose-poke (active or inactive) and Diet (standard or HFB) as the *between* factors, and Days (1-10) as the *within* factor. Subsequent Bonferroni post – hoc tests were calculated when required. Data related to gene expression values were analyzed by means of Student's t-tests. Data are presented as mean ± SEM. A p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS v22.

3. Results

3.1 Bingeing on fat and body weight (Experiment 1)

Results obtained in the statistical analysis of body weight revealed no differences between the groups during the course of the procedure (Figure 1a).

An escalation in the intake of the high-fat diet (see Fig. 1b and c) was confirmed by ANOVA, which revealed a significant difference of the variable Diet; from PND 36 onwards, mice in the HFB groups exhibited a significant increase in the intake (Kcal and g) [F (3,101) = 26.067; p<0,001] and [F (3,101) = 15.224; p<0,001] of high-fat diet with respect to controls (p<0.001). There was also an effect of the interaction Days*Diet (Kcal and g) [F(18,606)= 5,428; p<0.001] and [F(18,606)= 5,982; p<0.001], with significant differences observed between days 29 and 36 and days 43, 50, 57, 64 and 69 (p<0.001 in all cases) in the groups bingeing on fat, thus confirming an escalation in the intake of high-fat diet. No differences were detected over time in the control group.

With respect to daily standard food intake, the ANOVA did not reveal significant differences in intake between groups (Figure 1d).

3.2. Effects of exposure to a HFB during adolescence on anxiety (Experiment 1)

For the time and the percentage of time spent in open arms, the ANOVA (see Table 2) showed an effect of the variable Diet [F (2,42)=13,489; p<0,001] and [F (2,42)=15,479 p<0,001]. HFB 15w animals spent less time and percentage of time in the open arms than control and HFB mice (p<0.001), which represented an increase in anxiety levels after withdrawal.

For the number of entries into open arms and percentage of open entries, the ANOVA also showed an effect of the variable Diet [F(2,42) = 6,769; p<0.01] and [F (2,42) = 8,277 p<0,001). HFB 15w mice made fewer entries into the open arms and a smaller percentage of open entries with respect to the HFB group (p<0.01) and the control group (p<0.01).

The time spent in the closed arms and the number of entries into closed arms also revealed an effect of Diet [F(2,42) = 12; p<0,001] and [F(2,42) = 4,637; p<0,05]. AgaiN, HFB 15w mice spent more time in the closed arms with respect to the control and HFB groups (p<0.01) and performed a higher number of entries into closed arms with respect to the control group (p<0.05).

3.3. *Effects of exposure to a HFB during adolescence on cocaine-induced CPP in adulthood (Experiment 1)*

Results of the cocaine-induced CPP in animals receiving a dose of 1mg/kg cocaine are presented in Fig. 2a. The ANOVA for the time spent in the drug-

paired compartment revealed an effect of the interaction Days x Diet [F(3,52) = 3,747; p< 0,05]. The HFB group spent more time in the drug-paired compartment in Post-C than in Pre-C (p<0,001). HFB mice required 2 sessions for the preference to be extinguished. A priming dose of 0.5 mg/kg cocaine reinstated the preference [F(1,11)=17,211; p<0,01], and reinstatement with a priming dose of 0.25mg/kg cocaine [F(1,11)=7,990; p<0,05] was also achieved after a single extinction session. The other groups did not develop preference for the drug-paired compartment, as it was a subthreshold dose of cocaine.

The results regarding the effects of a HFB on a 6 mg/kg cocaine-induced CPP are presented in Figure 2b. The ANOVA revealed a significant effect of the variable Days [F(1,50) =32.846; p<0.001], as all the groups spent more time in the drug-paired compartment in the Post-C test than in the Pre-C test (p<0.001). The Kaplan-Meier analysis (see Figure 2c) revealed that the HFB 1w and HFB 15w groups required more time to achieve extinction (10 and 9 sessions, respectively) than the Control (5 sessions; χ^2 = 5,828; p<0.05 and χ^2 = 4,423; p<0.05) and the HFB (3 sessions; χ^2 = 6,114; p<0.05 and χ^2 = 4,857; p<0.05) groups. The student's t-test showed that a priming dose of 3 mg/kg of cocaine only reinstated the preference in the HFB 1w and HFB 15w groups (p<0.05). The Kaplan-Meier analysis revealed that the HFB 1w group required more time to achieve extinction (4 sessions) than the HFB 15w group (1 session) (χ^2 = 11,345; p<0.001). The student's t-test showed that a priming dose of 1.5 mg/kg of cocaine reinstated the preference only in the HFB 15w group (p<0.05). No further reinstatement was achieved.

3.4. Effects of exposure to HFB during adolescence on cocaine self-administration in adulthood (Experiment 2)

The results of the cocaine SA procedure with a dose of 0.5 mg/kg/infusion under a FR1 schedule of reinforcement are presented in Figure 3. A three-way ANOVA revealed an effect of diet and nose-poke (active vs. inactive hole) [$F(3,44)=13.69$; $p<0.001$], while the variable Day did not have an effect, and an interaction between day, nose-poke and diet was detected [$F(27,396)=2.971$; $p<0.001$]. Subsequent Bonferroni's post-hoc analyses showed significant differences in the number of active nose-pokes between HFB and control groups on the following training days: 2, 3, 8, 9 ($p<0.05$), 4 ($p<0.01$), 5 and 7 ($p<0.001$). Bonferroni's post-hoc comparisons also indicated that mice exposed to HFB during adolescence could significantly discriminate between active and inactive nose-pokes from day 2 to 10 ($p<0.001$) (Figure 3a). This suggests that mice that undergo HFB during adolescence show increased drug-seeking and drug-taking behavior in the cocaine SA paradigm in adulthood.

As a subthreshold dose of cocaine was employed, the percentage of mice that met the acquisition criteria was 40% in the control group and 71.4% in the HFB group. However, we observed no significant difference in the number of sessions required to achieve the acquisition criteria between the control group (6.75 ± 1.49 days) and the HFB group (6.7 ± 0.63 days).

To evaluate reinstatement, 20 days after completing the SA procedure, in which mice did not have access to high-fat food, they were re-exposed to 3 HFB sessions. On PND 117 mice were exposed to the SA chamber without receiving any dose of cocaine. The number of responses to the active or inactive holes

was recorded (Figure 3b). Two-way ANOVA indicated an effect of Nose-poke (Active vs. Inactive) [$F(1,34)=5.322$; $p=0.027$]. A subsequent Bonferroni's post-hoc test revealed a significant difference between active and inactive nose-pokes in the HFB group ($p<0.05$), but not in the control group. These results suggest that mice exposed to HFB during adolescence and later undergoing a 10-day SA training continue to exhibit drug-seeking behavior in the operant SA chamber, even after a period of cocaine withdrawal.

3.5. *Effects of a HFB on circulating leptin, corticosterone, and ghrelin levels and MOr, CB1 and Ghelin receptors gene expression*

There was no significant effect of the variable Diet on circulating leptin levels (see Table 3). However, those mice feed a HFB [$F(4,35)=6.449$; $p=0.001$] showed significantly lower ghrelin levels than controls on PND 69 ($p< 0.01$) and no difference after developing cocaine CPP (see Table 4). Although no differences in corticosterone levels were observed among mice feed a HFB vs. standard diet before cocaine CPP (PND 69), animals undergoing withdrawal (HFB 15d w PND 96) displayed significantly higher corticosterone levels [$F(4,34)=3.907$; $p=0.01$] than their corresponding control or HFB groups ($p< 0.02$, in both cases) (see Table 5).

Real-time PCR analyses indicated that exposure during adolescence to a HFB decreased CB1 and MOr gene expression in the NAcc with respect to the control group (Student's t-test, $t=3.160$, 16 d.f. $p<0.01$ and $t=3.539$, 16 d.f., $p<0.01$ respectively) (see Fig. 4a and b). Conversely, GHSR gene expression values in the VTA were significantly higher in mice exposed to a HFB during adolescence (Student's t – test, $t= -3.653$, 14 d.f. $p<0.01$) (see Figure 4c).

4. Discussion

Nowadays, a compulsive and intermittent intake of high-fat meals represents an increasing problem among teenagers, who are at a vulnerable age with respect to initiating substance abuse. The present study demonstrates for the first time that high-fat binge-eating during adolescence enhances sensitivity to the rewarding and reinforcing effects of cocaine. Mice exposed to a binge-type intake of fat developed CPP with a non-effective dose of cocaine (1 mg/kg) and displayed a reinstated preference following extinction. Moreover, when an effective dose of cocaine was administered, mice undergoing withdrawal from fat showed a more persistent memory of the conditioned reward and an increased sensitivity to the reinstatement of CPP. Similar results were obtained with cocaine SA; HFB mice consumed more cocaine and learned the SA task faster than their counterparts. Moreover, after a period of withdrawal, re-exposure to fat bingeing induced relapse into cocaine seeking.

Rodent models of binge eating are based on clinical criteria that characterize binge episodes in human. Therefore, animal models of bingeing need to demonstrate large intakes within defined brief periods, with similar environmental conditions being provided for control animals (Corwin and Babbs, 2012). The model used in the present study did not include energy restriction to induce bingeing, but instead relied on intermittent limited access to palatable food to drive escalations in intake. The fact that the animals are never food-deprived renders the model more relevant, as they eat in the absence of hunger. This model results in significantly higher palatable food intake and

progressive-ratio response in comparison to animals allowed daily access to fat (Wojnicki et al. 2010). In agreement with previous reports, we have observed that the limited access protocol led to the development of fat-bingeing behaviors (Corwin et al., 1998; Corwin, 2004; Wojnicki et al., 2008). There was a significant increase in the amount of food and Kcal ingested by HFB mice in every binge session from the second week of exposure to fat.

HFB mice that continued to binge throughout the whole procedure were more sensitive to 1 mg/kg of cocaine, a subthreshold dose that had no effect on the standard diet control group. Moreover, this preference was reinstated with very low priming doses (up to 0.25 mg/kg of cocaine), thus suggesting greater sensitivity to reinstatement of the extinguished preference by the cocaine-priming dose. On the other hand, 6 mg/kg of cocaine has been shown to be an effective dose that induces preference but not reinstatement (Maldonado et al., 2006). However, cocaine priming-induced reinstatement was observed in the HFB group. To date, only two studies have related a high-fat diet and obesity to cocaine-induced CPP. Morales and coworkers (2012) reported that mice exposed to a continuous fat diet that induced obesity showed a decrease in cocaine reward. In the same line, obesity-resistant rats show greater cocaine CPP than obesity-prone rats (Thanos et al., 2010). Human studies have produced controversial results, but animal studies have consistently shown that diet – induced obesity decreases striatal DA concentrations (Davis et al., 2007; Zhang et al., 2013) and thyroxin hydroxylase levels (Li et a., 2009; Ong et al., 2013). On the other hand, human studies have shown that obesity induces a

decrease in dopamine D2 receptor concentration in the striatum (de Weijer et al., 2011), though there is a lack of consensus among animal studies (Johnson and Kenny, 2010; Sharma and Fulton, 2013). All these results feed into the reward deficiency hypothesis of obesity, which holds that reduced DA tone leads to overeating as an attempt to restore striatal DA concentrations (Naef et al., 2015). It is possible that signals sent out by adipose tissue - leptin being the most likely candidate - control this response (Fulton et al., 2006). Our intermittent access-to-fat model induced the opposite effects in the CPP, increasing sensitivity to the conditioned rewarding effects of cocaine. Binge eating disorders seem to have a different effect on DA metabolism, with several studies reporting an elevation of striatal DA (Rada et al., 2005; Hajnal and Norgren, 2002), although all used food restriction, which is known to increase DA tone. Intermittent fat access was not accompanied by increased body weight or leptin levels in the HFB groups, in line with previous reports (Corwin et al., 1998). However, ghrelin levels were significantly lower in mice exposed to HFB. Ghrelin plays an important role in nutritional homeostasis (Schellekens et al., 2013), and recent data based on GHSR KO mice specifically suggest that HFB behavior requires ghrelin signaling (Valdivia et al., 2015; King et al., 2016). Although some studies have reported no such effect (Bake et al., 2014), most reports show that ghrelin secretion is downregulated by high-fat diets (Beck et al., 2002; Lindqvist et al., 2005; Bello et al., 2009), suggesting a deficit in satiety signals after exposure to a high-fat diet. In accordance with these results, our HFB group showed significantly lower plasmatic ghrelin levels than controls. Although the latter mice continued to display higher levels of plasmatic ghrelin after cocaine CPP, this difference did not reach statistical significance.

Data obtained in the SA paradigm confirm the abovementioned results. Active seeking of active nose-pokes, total cocaine infused and infusions during the last three days of training increased in HFB mice, revealing higher cocaine values in animals exposed to the high-fat diet. It is noteworthy that the cocaine dose selected (0.5 mg/kg/infusion) is a threshold dose in the SA paradigm and that, consequently, a low proportion of animals acquired the task (40% of the mice fed a standard diet). However, exposure to the HFB facilitated the effect of cocaine significantly, with increased consumption of the drug and a higher percentage of animals acquiring the SA task (70%). Therefore, HFB mice seem to learn the SA task faster than mice fed a standard diet. Early studies suggested that reinforcers could change behaviors by promoting learning and storage of information processes (White and Milner, 1992). Cocaine and other drugs of abuse can serve as reinforcers, and it has been demonstrated that cocaine can facilitate learning in different learning tasks when received in moderate doses (Rkieh et al., 2014). Hence, we can argue that HFB enhances the value of cocaine as a reinforcer, leading to a faster learning process during the operant task. Interestingly, 20-day abstinence of fat bingeing and cocaine produced an extinction of operant behavior in mice exposed to the standard diet, whereas animals exposed to the HFB persisted in their cocaine-seeking behavior, suggesting the development of a long-lasting neuroadaptive state related to exposure to a HFB, in accordance with the aforementioned more robust learning process (White and Milner, 1992; Rkieh et al., 2014). Only one previous study has evaluated fat bingeing in adult rats, and provided similar results (Puhl et al., 2011). However, in said study, although rats exposed to fat

bingeing worked harder to achieve cocaine, no significant increment in intake was detected. The fact that rats abstained from fat during the whole SA procedure could explain this discrepancy.

After sustained *ad libitum* exposure to a high-fat diet, blocking access to said diet potentiates anxiety, elevation of the stress state and a reduction in the reward response (Teegarden and Bale, 2007; Sharma et a., 2013). Regarding fat bingeing, no signs of withdrawal have previously been described (Bocarsly et al., 2011). However, in our study, the data provided by the EPM confirmed that the groups in which fat bingeing was discontinued at the beginning of the CPP or 15 days earlier had higher levels of anxiety, since they spent less time and percentage of time in the open arms of the maze. Measure of plasma corticosterone levels also confirmed an increase in this group, suggesting that a state of withdrawal (dysphoria) arises when binge sessions terminate. These results, which contradict those of the Bocarsly report, can be explained by two main factors. Firstly, adult animals were employed in Bocarsly's work (instead of adolescent mice), and secondly, animals were allowed access to fat for only 25 days, a much shorter period than in our study (40 days).

Stress – related pathways are involved in the withdrawal state, and corticotropin-releasing factor creates an aversive state after cessation of palatable food, which provokes further compulsive intake when palatable food becomes available once again (Cottone et al., 2009; Koob and Zorrilla, 2010). Craving of fat and cross-sensitization between sugar intake and drugs of abuse have been documented in laboratory animals; for example, rats that binge on

sugar display decreased DA release in the NAcc after 36h of deprivation (Avena et al., 2008), although no results have been obtained to date with fat diets. Neither one of our withdrawal groups responded to a subthreshold cocaine dose in the CPP, proving that the increased sensitivity of the HFB group to this dose of cocaine was temporary and no longer present after cessation of bingeing. However, HFB withdrawal groups were more resistant to the extinction of memories associated with reward when conditioned with an effective cocaine dose. In addition, these groups were more vulnerable to reinstatement of the preference. These results are in line with those of the SA study. Re-exposure to fat after a period of abstinence significantly increased the number of active nose-pokes. The CPP and SA results obtained in our study show, for the first time, that withdrawal from fat bingeing increases vulnerability to reinstatement of cocaine-seeking.

The neurobiological alterations that occur after intermittent opportunities to consume palatable foods are only now starting to be revealed. Parallel neural systems to the hypothalamus have been shown to control feeding. Motivation disorders such as anorexia may involve disturbances in the NAcc (Jean et al., 2007). Basic research suggests that stimulation of serotonin 4 receptors activates an addictive molecular facet of anorexia involving production in the NAcc of the same transcripts stimulated in response to cocaine and amphetamine (CART) (Jean et al., 2007). In fact, the NAcc/serotonin 4/CART molecular pathway triggers not only anorexia but also motor hyperactivity (Jean et al., 2012).

Fat consumption-induced changes in the DA system mimic that which occurs after exposure to substances of abuse after either continuous (Narayanaswami et al., 2013) or limited access (Liang et al., 2006). Other changes in the DA system after continuous fat access involve changes in striatal D2-receptor density and DAT expression and function (South and Huang, 2008; Narayanaswami et al., 2013). In a recent work, Valdivia and co-workers (2015) showed that a subset of dopaminergic neurons in the VTA is activated by an escalation of fat intake, in a response that can be considered sensitization. This response may lead to neuroadaptations that activate the dopaminergic system persistently, as occurs with drugs of abuse (Valdivia et al., 2015). However, mechanisms other than DA might also play a role. Baladi and co-workers (2015) have recently described that, though sensitization to the locomotor effects of cocaine were enhanced in adolescent rats fed a high-fat chow, the DA clearance rate in the striatum was decreased in rats fed a fatty diet.

Bingeing on a fat-rich diet is known to affect the opioid system in the NAcc by decreasing enkephalin mRNA, an effect that is not observed with acute access (Kelley et al., 2003). Continuous access to a high fat diet induces a significant reduction in mu-opioid receptor mRNA in the VTA (Blendy et al., 2005; Vucetic et al., 2011). A recent report suggests that long-term access to a cafeteria (high sugar + fat) diet suppresses the transcription mechanisms necessary for MOr receptor mRNA synthesis, since it decreases MOr receptor levels in the VTA, which increase 48 h after withdrawal of the diet (Martire et al., 2014). Equally, continuous access to a high-fat (Vucetic et al., 2011) or cafeteria (Ong et al., 2013) diet was shown to reduce MOr receptor mRNA in the NAcc of male mice

and rats, although other authors reported no changes (Smith et al., 2002). In agreement with these results, we observed that intermittent access to a high-fat diet also decreased mRNA expression of the MOr receptor in the NAcc of HFB mice on PND 69.

Both endogenous opioids and ghrelin act on the mesolimbic dopamine system and may interact to regulate food reward. Ghrelin induces food-motivated behavior via interaction with mu opioid receptors (Kawahara et al., 2009; Skibicka et al., 2012) and GHSR expressed in dopamine neurons (Naleid et al., 2005; Skibicka et al., 2011; King et al., 2011). Ghrelin/GHSR signaling in the VTA has been identified as a crucial component of food reward, other natural rewards, and drugs of abuse (for review see Wellman et al., 2013). Several studies using ghrelin-KO mice or systemic ghrelin administration have shown that this hormone enhances locomotor and rewarding effects of cocaine (Wellman et al., 2005; Davis et al., 2007; Abizaid et al., 2011). On the other hand, pharmacological inactivation of GHSR has been reported to attenuate locomotive and CPP properties of cocaine (Jerlhag et al., 2010). These findings indicate that ghrelin receptors exert a permissive function for the activation of DA circuits by psychostimulant drugs. We observed an increased expression of GHSR in VTA in mice exposed to HFB during adolescence compared to those fed a standard diet. Previous reports have associated a reduction of GHSR expression with continuous exposure to a fatty diet or adiposity (Kurose et al., 2005; Zhang et al., 2013). These discrepant results can be explained by several methodological differences, including the species used (mice vs rats and sheep), the anatomical structure in which the gene expression was evaluated

(VTA vs hypothalamus), the age at which animals were exposed to fat (adolescent vs adult), or the schedule of fat administration (intermittent vs continuous). The literature shows that changes in ghrelin signaling is complex, as GHSR mRNA is also up-regulated in the hypothalamus in hamsters after food deprivation and is accompanied by an elevation of circulating ghrelin concentration (Tups et al., 2004). In this context, the increase in GHSR expression in our study could be a compensatory response to the significant decrease in circulating plasma ghrelin levels.

There are data to suggest an important modulatory role for cannabinoid receptors in the expression of feeding behaviors and that the NAcc is a critical site of such activity. Intra – accumbens administration of 2-AG enhanced fat consumption, an effect that was attenuated by a CB1r antagonist (Deshmukh and Sharma, 2012). Levels of 2-AG and binding to the cannabinoid CB1r in the hypothalamus are also increased by a high-fat compared to low-fat diet (Higuchi et al., 2011; South and Huang, 2008), while CB1r expression is upregulated by sucrose intake (Lindqvist et al., 2008), suggesting that sucrose decreases endocannabinoid levels in this brain region. Giving further support for a role of CB1r, mice lacking the central CB1r exhibit a delayed onset of preference for high-fat vs. standard chow when compared with WT mice (Ravinet-Trillou et al., 2004). All these results suggest that endocannabinoids affect appetite for specific dietary components through CB1r. In line with this, our study shows that CB1 receptor gene expression in the NAcc is decreased after exposure to fat bingeing during adolescence. We found that a binge pattern of excessive and intermittent consumption of fat made animals more sensitive to cocaine reward.

We hypothesize that this decrease in the opioid and endocannabinoid systems can induce modifications of the dopaminergic system that sensitize cocaine reward. Since an increased ghrelin signal in the VTA has been associated with more potent effects of cocaine, the enhanced expression of GHSR in the VTA of mice exposed to a HFB may modulate the increase in the rewarding effects of cocaine observed in these animals, perhaps through the endogenous opioid system.

5. Conclusions

The animal model we have used in the present study is a useful paradigm to study the consequences of binge eating in humans, which is not driven by metabolic needs. The escalation of high-fat food consumption observed in this model mimics that which occurs with drug abuse, since there is a transition from controlled to compulsive intake and a subsequent loss of control (Goeders et al., 2009). For many authors, it is the manner in which the substance is consumed, rather than the substance itself, which alters the reward system (Avena et al., 2008; Corwin et al., 2011). Our results provide behavioral evidence that bingeing on palatable (high-fat) food during adolescence can modify the vulnerability of mice to the effects of cocaine. Our data highlight a relationship between eating disorders and substance abuse disorders, which have a high comorbidity in humans. To date, many studies have been designed to explore the role of food as an addictive disorder, but few have investigated the interaction between drugs of abuse and eating disorders such as binge eating. We show that a limited and intermittent schedule of high-fat bingeing modulates orexigenic circuits through alterations of ghrelin and its receptor, thus

producing the phenomenon of sensitization, affecting not only the dopaminergic system, but also the opioid and endocannabinoid systems. This sensitizes animals to cocaine and predisposes them to seek and consume the drug. The present study highlights nutritional patterns as an important variable to take into consideration when treating psychostimulant disorders, and provides new pharmacological targets for pharmacological intervention.

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Conflicts of interest

None

6. References

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Table 1. **Experimental Design.** Control mice received a standard diet during the binge sessions (n=15) and animals in the HFB condition underwent three additional temporary conditions: the HFB group (n=15) had 2h access every Monday, Wednesday and Friday throughout the study; the HFB 1w group (n=15) binged throughout the study until the beginning of behavioral tests; the HFB 15w group (n=15) binged throughout the study until 15 days before the beginning of the behavioral tests.

PND	**29-68**	**69** **83 (HFD 15w)**	**70-77** **84 - 91 (HFD 15w)**	**79 -110** **93 - 237**
1st set of mice (Exp 1) (n=120)	**Control (Standard diet)** **HFB** **HFB 1w** **HFB 15w**	Elevated Plus Maze	1mg/kg CPP 6mg/kg CPP	Reinst. 0.5mg/kg 3 and 1.5mg/kg

PND	**29-68**	**68-72**	**77-88**	**89-107**	**108 - 114**	**117**
2nd set of mice (Exp. 2) (n=40)	**Control (Standard diet)** **HFB**	Catheter implantation surgery	Cocaine self-administration	Free Period	Control (Standard diet) HFB	SA chamber re-exposure

PND	**29-68**	**69**	**81 and 96**
3rd set of mice (n=20)	**Control (Standard diet)** **HFB**	Brain extraction and Blood samples	Blood samples

Table 2. Effects of a HFB on adolescent mice in the EPM on PND 69. Control mice received a standard diet during the binge sessions (n=15); HFB and HFB 1w groups had 2h access every Monday, Wednesday and Friday throughout the study until PND 69; HFB 15w group binged throughout the study until 15 days before PND 83. Data are presented as mean values ± S.E.M. Differences with respect the control group *p<0.05; **p<0.01; ***p<0.001; Differences with respect the HFB group ++p<0.01; +++ <0.001

	Control	HFB/HFB 1w	HFB 15w
Time in open arms	125 ± 13	118 ± 8	58 ± 9***+++
% Time in open arms	53 ± 5	53 ± 3	27 ± 4 ***+++
Time in central platform	59 ± 6	69 ± 4	78 ± 6 *
Time in closed arms	104 ± 10	104 ± 4	1515 ± 9 ***+++
Entries in open arms	27 ± 3	31 ± 2	19 ± 2 ++
% Open entries	54 ± 5	60 ± 2	43 ± 4 **++
Entries in closed arms	15± 2	22 ± 3	25 ± 2 *
Total entries	42 ± 2	53 ± 4 *	45 ± 3

Table 3. Leptin. Effects of exposure of adolescent mice to a HFB on circulating leptin levels on PND 69 (controls and HFB group), and PND 83 (HFB 15w group). Control mice received a standard diet during the binge sessions; HFB group had 2h access every Monday, Wednesday and Friday throughout the study until PND 69; HFB 15w group binged throughout the study until 15 days before PND 83. Data are presented as mean values ± S.E.M. (ng/ml).

	Plasma Leptin (ng/ml) ± S.E.M.
Control	2.01 ± 0,7
HFB	3.1 ± 0,6
HFB 15w	3 ± 0,6

Table 4. Ghrelin. Effects of exposure of adolescent mice to a HFB on circulating ghrelin levels on PND 69 (controls and HFB group), PND 81 (controls and HFB group) and PND 96 (HFB 15w group). Levels were measured on PND 69 for Control mice which received standard diet during the binge sessions; and HFB group which had 2h access every Monday, Wednesday and Friday. Levels were also measured in three more groups after 1 mg/kg of cocaine-induced CPP: Controls and HFB group (PND 81) and HFB 15w group (PND 96) which binged throughout the study until 15 days before of initiating the CPP. Data are presented as mean values ± S.E.M. (pg/ml).

	Plasma Ghrelin (pg/ml) ± S.E.M.
Control PND 69	636 ± 78
HFB PND 69	373 ± 23 **
Control PND 81	441 ± 26
HFB PND 81	268 ± 15
HFB 15 w PND 96	382 ± 33

Table 5. Corticosterone. Effects of exposure of adolescent mice to a HFB on circulating corticosterone levels on PND 69 (controls and HFB group), PND 81 (controls and HFB group) and PND 96 (HFB 15w group). Levels were measured on PND 69 for Control mice which received standard diet during the binge sessions; and HFB group which had 2h access every Monday, Wednesday and Friday. Levels were also measured in three more groups after 1 mg/kg of cocaine-induced CPP: Controls and HFB group (PND 81) and HFB 15w group (PND 96) which binged throughout the study until 15 days before of initiating the CPP. Data are presented as mean values ± S.E.M. (pg/ml).

	Plasma Corticosterone (pg/ml) ± S.E.M.
Control PND 69	3180 ± 55
HFB PND 69	3123 ± 101
Control PND 81	2786 ± 226
HFB PND 81	2817 ± 116
HFB 15 w PND 96	3523 ± 149 **

Figure 1. (a) Bodyweight of mice over the procedure. Control mice received a standard diet during the binge sessions and animals in the HFB condition underwent three additional temporary conditions: the HFB group had 2h access every Monday, Wednesday and Friday throughout the study; the HFB 1w group binged throughout the study until the beginning of behavioral tests; the HFB 15w group binged throughout the study until 15 days before the beginning of the behavioral tests. Mean (± SEM) amount of body weight measured weekly of animals in the control group and those exposed to HFB, the HFB 1w and HFB 15w groups (n=15 per condition). **(b and c) Binge sessions.** Intake (kcal and g) in the 2-h High-fat binge-eating sessions that took place on Monday, Wednesday and Friday. The mean (± SEM) amount of kcal consumed in 2 hours of limited access to high fat food (control group had access to standard food) and stated here weekly to confirm the escalation of intake. **(d) Standard food intake.** Daily intake (g) of standard food per cage of 4 mice (mean ± SEM). The data correspond with the same days evaluated for the binge sessions. ***p<0.001 significant difference with respect the control group. + p<0.001 significant difference with respect to PND 29.

Figure 2 (a) Effects of a HFB on adolescent mice in the Conditioned Place Preference. CPP induced by 1 mg/kg of cocaine in mice exposed to standard diet during the binge sessions (n=15) (controls) or high fat binge (HFB) with three additional temporary conditions: the HFB group (n=15) had 2h access every Monday, Wednesday and Friday throughout the study; the HFB 1w group (n=15) binged throughout the study until the beginning of behavioral tests; the HFB 15w group (n=15) binged throughout the study until 15 days before the

beginning of the behavioral tests. Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white), post-conditioning (black), the last extinction session (light grey) and reinstatement (dark grey). The reinstatement test was evaluated 15 mins after a priming dose of 0.5 mg/kg and 0.25mg/kg cocaine. *p<0.05; **p<0.01; ***p<0.001 significant difference vs Pre-C or the last extinction sessions. **(b)** CPP induced by 6 mg/kg of cocaine in mice exposed to a High-Fat Binge. The reinstatement test was evaluated 15 mins after a priming dose of 3 and 1.5 /kg cocaine. *p<0.05; **p<0.01; ***p<0.001 significant difference vs Pre-C or the last extinction sessions. **(c)** The bars represent the mean value (± SEM) of the number of daily sessions required for the preference to be extinguished after the Post-C test. Preference was considered to be extinguished when an animal spent 370 seconds or less in the drug-paired compartment on two consecutive days. When the preference was not extinguished in a mouse, the number of days needed to achieve extinction in the whole group was assigned to that animal. * p<0.05 with respect to Control and HFB.

Figure 3. (a) Effects of a HFB on adolescent mice in the cocaine self-administration. Acquisition of cocaine (0.5mg/kg/infusion) self-administration in mice exposed to a standard diet (control) or high fat binge (HFB) during adolescence. Number of active (NA) and inactive (NI) nose-pokes in 1-hour sessions over 10 consecutive days. Data are presented as mean ± SEM (n=10 or 14 per group). Two-way ANOVA (repeated measures) and Bonferroni's post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 Control (active hole) vs. HFB (active hole); ###p<0.001 HFB (active hole) vs. HFB (inactive hole). **(b)** Number

of active or inactive nose-pokes in the operant SA chamber during a single reinstatement 1-hour session. Two-way ANOVA and Bonferroni's post-hoc analysis. *p<0.05 Active vs. Inactive nose-poke.

Figure 4. Effects of a high fat binge (HFB) on adolescent mice on gene expression in the Nucleus Accumbens (NAcc)**. (a)** Real-time PCR CB1 relative gene expression evaluation in the NAcc brain region of control and HFB groups (n = 9). **(b)** MOr relative gene expression evaluation in the NAcc brain region of these groups (n = 9). The columns represent means and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the NAcc of OF1 mice. **Represents the values of HFB mice that are significantly different (P<0.01) from those of their corresponding controls.

PND	**29-68**	**69** **83 (HFD 15w)**	**70-77** **84 - 91 (HFD 15w)**	**79 -110** **93 - 237**
1st set of mice (Exp 1) (n=120)	**Control (Standard diet)** **HFB** **HFB 1w** **HFB 15w**	Elevated Plus Maze	1mg/kg CPP 6mg/kg CPP	Reinst. 0.5mg/kg 3 and 1.5mg/kg

PND	**29-68**	**68-72**	**77-88**	**89-107**	**108 - 114**	**117**
2nd set of mice (Exp. 2) (n=40)	**Control (Standard diet)** **HFB**	Catheter implantation surgery	Cocaine self-administration	Free Period	Control (Standard diet HFB	SA chamber re-exposure

PND	**29-68**	**69**		**81 and 96**
3rd set of mice (n=20)	**Control (Standard diet)** **HFB**	Brain extraction and Blood samples		Blood samples

	Control	HFB/HFB-1W	HFB -*15 W*
Time in open arms	125 ± 13	118 ± 8	58 ± 9***+++
% Time in open arms	53 ± 5	53 ± 3	27 ± 4 ***+++
Time in central platform	59 ± 6	69 ± 4	78 ± 6 *
Time in closed arms	104 ± 10	104 ± 4	1515 ± 9 ***+++
Entries in open arms	27 ± 3	31 ± 2	19 ± 2 ++
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	Plasma Corticosterone (pg/ml) ± S.E.M.
Control PND 69	3180 ± 55
HFB PND 69	3123 ± 101
Control PND 81	2786 ± 226
HFB PND 81	2817 ± 116
HFB 15 w PND 96	3523 ± 149 **

Fig. 1

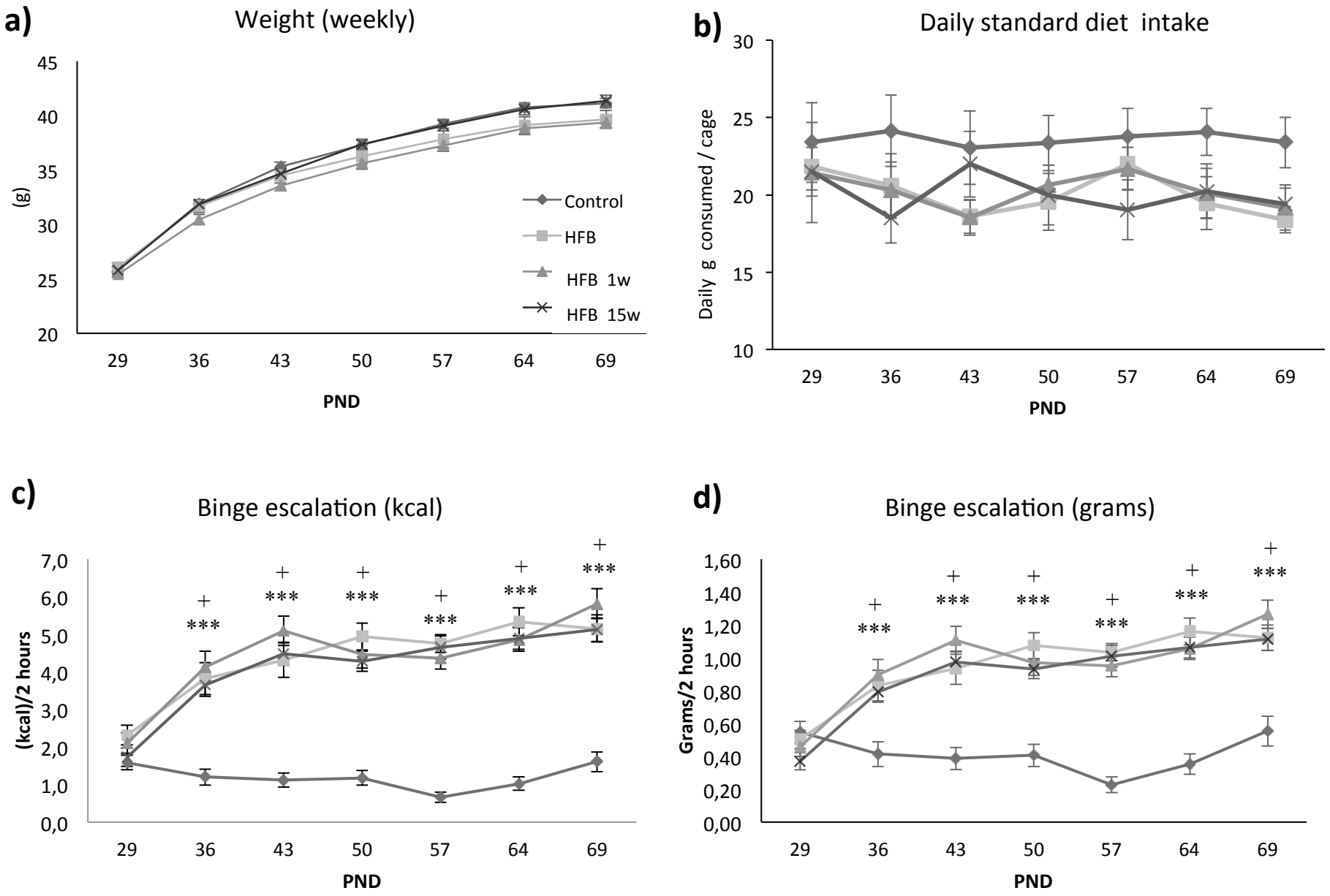

a)
Weight (weekly)
45
40
35
30
25
20
(g)
29
36
43
50
57
64
69
PND
Control
HFB
HFB 1w
HFB 15w
b)
Daily standard diet intake
30
25
20
15
10
Daily g consumed / cage
29
36
43
50
57
64
69
PND
c)
Binge escalation (kcal)
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
(kcal)/2 hours
***
+
29
36
43
50
57
64
69
PND
d)
Binge escalation (grams)
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Grams/2 hours
***
+
29
36
43
50
57
64
69
PND

Fig. 2

CPP

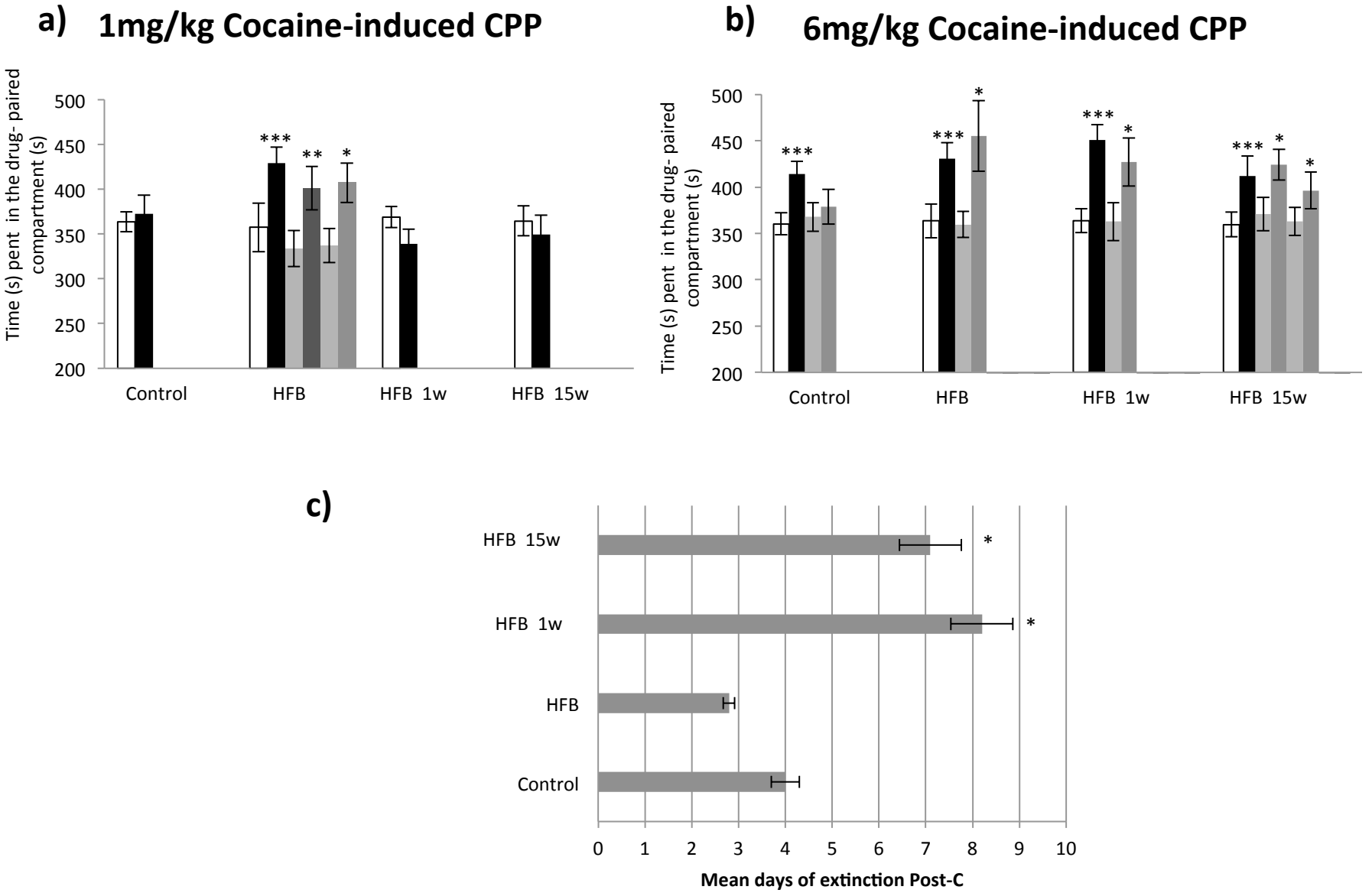
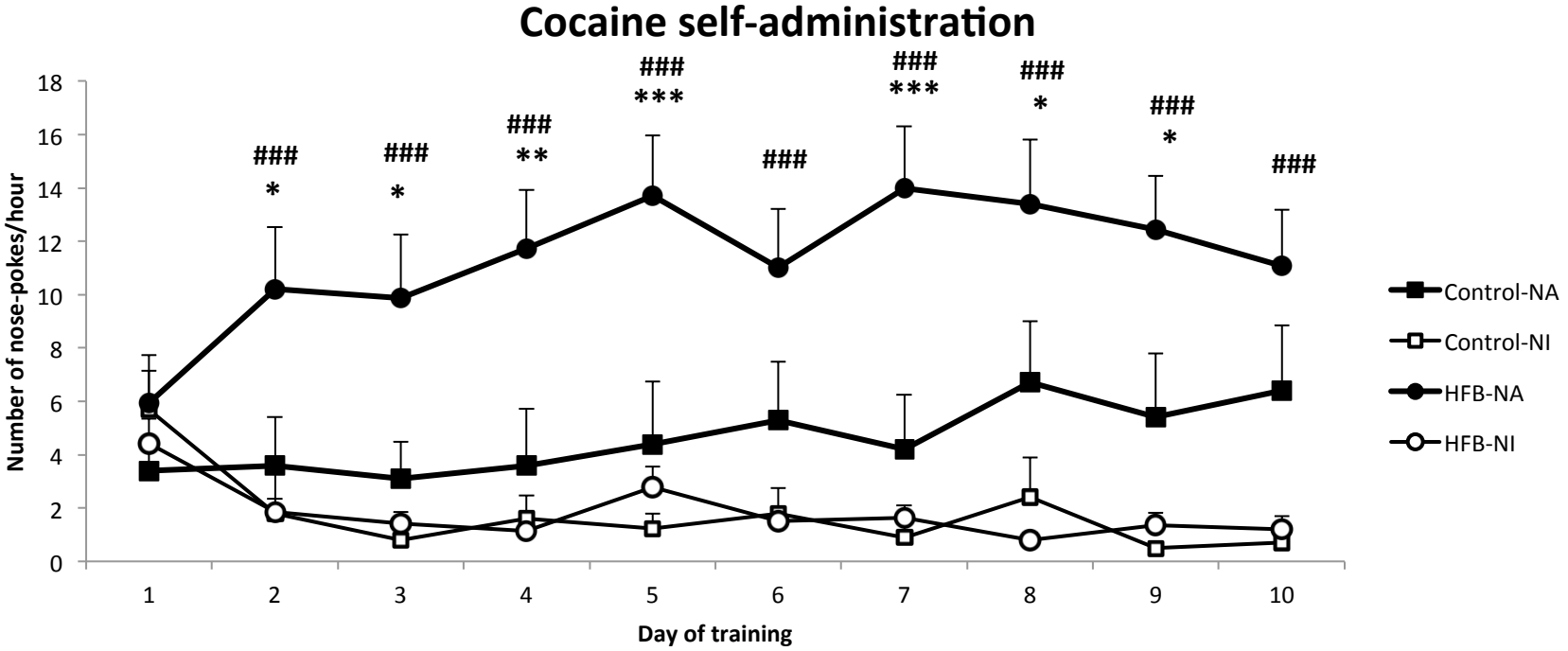

a)
1mg/kg Cocaine-induced CPP
Time (s) pent in the drug- paired compartment (s)
500
450
400
350
300
250
200
***
**
*
Control
HFB
HFB 1w
HFB 15w
b)
6mg/kg Cocaine-induced CPP
Time (s) pent in the drug- paired compartment (s)
500
450
400
350
300
250
200
***
***
*
***
*
***
*
*
Control
HFB
HFB 1w
HFB 15w
c)
HFB 15w
HFB 1w
HFB
Control
*
*
0
1
2
3
4
5
6
7
8
9
10
Mean days of extinction Post-C

Fig. 3

a)


Cocaine self-administration
Number of nose-pokes/hour
Day of training
Control-NA
Control-NI
HFB-NA
HFB-NI
*
*
**
***
###
***
*
*
###

b)

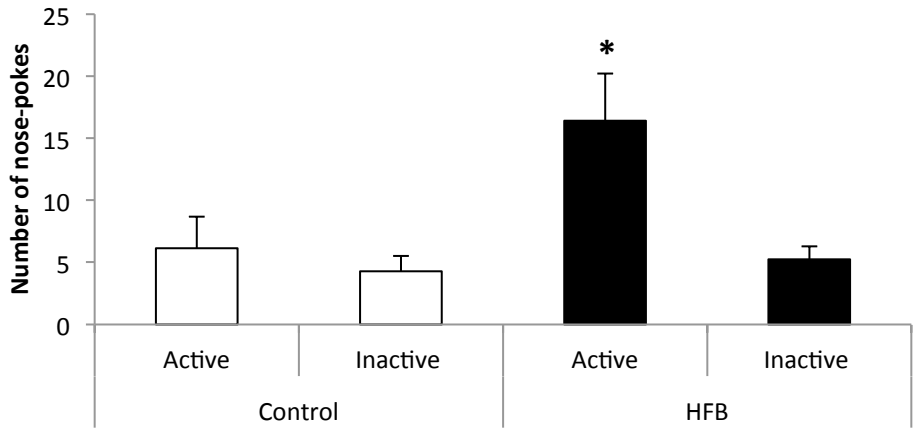
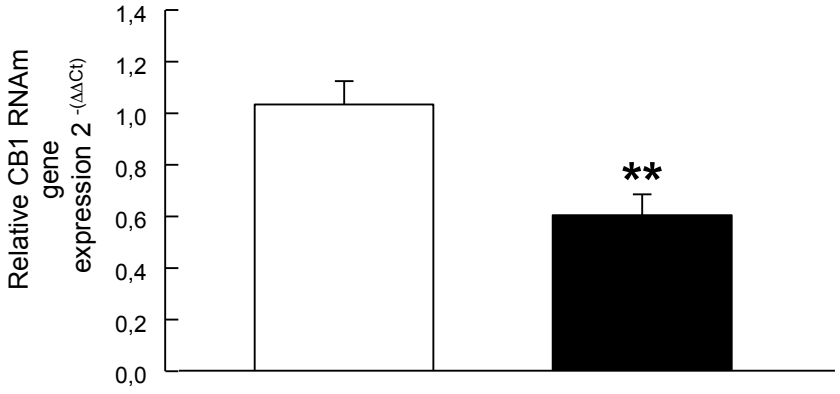

SA Reinstatement session
Number of nose-pokes
Active
Inactive
Active
Inactive
Control
HFB
*

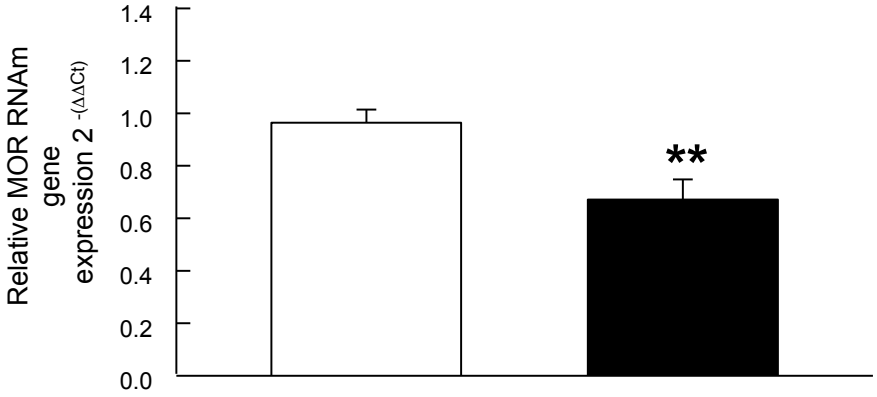
Fig. 4 **rt-PCR**

a)



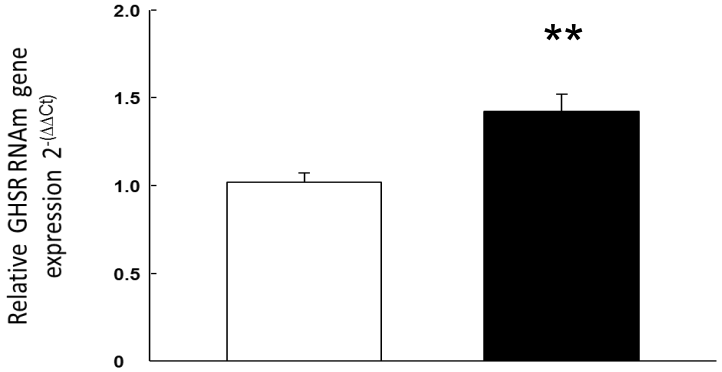
CB1 in NAcc

b)



MOR in NAcc

c)



GHSR in VTA

Control

HFB