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ORIGINAL INVESTIGATION

Changes in gene expression and sensitivity of cocaine reward produced by a continuous fat diet

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Abstract
Rationale Preclinical studies report that free access to a high-fat diet (HFD) alters the response to psychostimulants.
Objectives The aim of the present study was to examine how HFD exposure during adolescence modifies cocaine effects. Gene expression of CB1 and mu-opioid receptors (MOr) in the nucleus accumbens (N Acc) and prefrontal cortex (PFC) and ghrelin receptor (GHSR) in the ventral tegmental area (VTA) were assessed.
Methods Mice were allowed continuous access to fat from PND 29, and the locomotor (10 mg/kg) and reinforcing effects of cocaine (1 and 6 mg/kg) on conditioned place preference (CPP) were evaluated on PND 69. Another group of mice was exposed to a standard diet until the day of post-conditioning, on which free access to the HFD began.
Results HFD induced an increase of MOr gene expression in the N Acc, but decreased CB1 receptor in the N Acc and PFC. After fat withdrawal, the reduction of CB1 receptor in the N Acc was maintained. Gene expression of GHSR in the VTA decreased during the HFD and increased after withdrawal. Following fat discontinuation, mice exhibited increased anxiety, augmented locomotor response to cocaine, and developed CPP for 1 mg/kg cocaine. HFD reduced the number of sessions required to extinguish the preference and decreased sensitivity to drug priming-induced reinstatement.
Conclusion Our results suggest that consumption of a HFD during adolescence induces neurobiochemical changes that increased sensitivity to cocaine when fat is withdrawn, acting as an alternative reward.

Keywords Cocaine · High-fat diet · Conditioned place preference · CB1 · Mu-opioid receptor

Introduction

Among the factors that contribute to increased vulnerability to drug use, dietary conditions might play a greater role than previously thought (Baladi et al. 2012; Daws et al. 2011; Spear 2000). Currently, there is an increasingly high-fat, "fast-food" culture and a rising prevalence of obesity in developed countries, particularly among adolescents (Baladi et al. 2012; Herpertz-Dahlmann 2015; Volkow et al. 2013). Drug addiction and overeating cause high comorbidity (Swanson et al. 2011), and several studies have highlighted that palatable food increases vulnerability to psychostimulant use. The acute locomotor response to cocaine is enhanced in mice that consume a continuous diet high in fat and/or sucrose (Collins et al. 2015), and two recent reports described the development of locomotor sensitization to cocaine in adolescent mice exposed to a restricted or continuous high-fat diet (Baladi et al. 2015; Serafine et al. 2015). In contrast, several reports, most of them performed in adult animals, suggest that continuous access to fat diminishes the reinforcing efficacy of cocaine (Davis et al. 2008; Morales et al. 2012; Thanos et al. 2010; Wellman et al. 2007).

Like drugs of abuse, food presents intense reinforcing properties, and both share common mechanisms in the brain reward system (DiLeone et al. 2012). Preclinical studies provided robust evidence confirming that free access to a high-fat

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diet (HFD) has considerable effects on the brain reward system, producing changes in the dopaminergic system. Ingestion of palatable foods activates dopaminergic neurons within the nucleus accumbens (N Acc) and other reward centers (Kelley et al. 2005; Rada et al. 2005; Narayanaswami et al. 2013), thereby decreasing DAT density (Huang et al. 2006). Over time, striatal DA D2 receptors become downregulated in obese rats (Davis et al. 2008; Johnson and Kenny 2010). Cone et al. (2010) observed that cocaine caused a dramatic increase in evoked DA in low-fat diet rats, but a much smaller increase in HFD animals. Besides DA, the opioid and the endocannabinoid systems also play important roles in the reward process (de Macedo et al. 2016).

DA release in the N Acc is generally associated with the reinforcing effects of food, whereas 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). Several studies show that palatable food increases DA release in the N Acc via activation of the mu-opioid receptor pathway in the VTA (Kawahara et al. 2013; Pitman and Borgland 2015).

On the other hand, the endocannabinoid system (ECS) plays a pivotal role in reward/reinforcement circuits of the mesolimbic system (Cristino et al. 2014). The CB1 receptor agonist raises extracellular DA, leading to an increase in the frequency and amplitude of rapid dopamine transients in the N Acc (Cheer et al. 2004). 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 N Acc mediated by a novel intake of highly palatable food (Mellis et al. 2007).

In light of the aforementioned neuroadaptations, the concept of food addiction has been suggested in recent years (Volkow et al. 2013). Moreover, similarly to drugs of abuse, withdrawal from and craving for specific kinds of foods have also been observed and measured in humans (Rogers and Smit 2000). Several studies showed that sugar consumption leads to a withdrawal syndrome similar to that which occurs under opiate withdrawal (Avena et al. 2009). While the same has not been confirmed with respect to high-fat food (Bocarsly et al. 2011), Teegarden and Bale (2007) did confirm that discontinuation of a high-fat diet led to an increased stress response and the drive to seek palatable food.

To summarize, the literature shows that ingestion of a HFD induces neuroadaptations that alter the reward system, affecting dopaminergic, opioidergic, and endocannabinoid pathways, which modifies the response of animals to the effects of drugs of abuse. The aim of the present study was to evaluate how exposure to a HFD during adolescence interacts with the motor and conditioned rewarding effects of cocaine. As no studies have previously tested if sensitivity to the rewarding effects of cocaine is altered after cessation of a HFD, we also evaluated whether withdrawal of fat intake modifies these effects. In a first experiment, we assessed the biochemical effects of HFD exposure during adolescence, confirming if, as expected (Ahrén and Scheurink 1998; Lin et al. 2000), fat induced increases in serum leptin and decreases in ghrelin levels, with a return to normal levels after 2 weeks of fat abstinence. In addition, we determined CB1 receptor gene expression in the N Acc and prefrontal cortex (PFC), μ receptor gene expression in the N Acc, and ghrelin receptor gene expression in the VTA. As expected, fat exposure during adolescence increased leptin and ghrelin plasmatic levels, while withdrawal from fat normalized them. Equally, withdrawal of the HFD normalized several of the fat-induced changes in CB1r and MOr gene expression. These results confirm that HFD induces biochemical changes in brain reward structures that can modify cocaine-induced motor and rewarding responses. Based on the literature, our first behavioral hypothesis was that continuous exposure to a HFD would reduce the conditioned rewarding effects of cocaine. To test this hypothesis, we induced CPP with an effective dose of cocaine (6 mg/kg). The lack of effect during both HFD ingestion and withdrawal suggested that HFD exposure during adolescence did not undermine the rewarding effects of cocaine. Subsequently, we induced CPP with a subthreshold, noneffective dose of cocaine (1 mg/kg) to test an increase in CPP sensitivity. Although no effect was detected while the HFD was maintained, during fat withdrawal, an increased sensitivity to the conditioned rewarding and motor effects of cocaine was observed. These results suggest that continuous exposure to fat during adolescence induces neuroadaptations that will be expressed after cessation of fat consumption and which will increase anxiety levels. Therefore, our results support the hypothesis that high-fat food presents addictive properties. Our last experiment, based on this endorsement of our original hypothesis, aimed to test if a HFD acts as an alternative reinforcer that competes with cocaine to decrease drug priming-induced reinstatement of CPP.

Several human studies report that cessation of drug abuse following a period of chronic intake is related to hyperphagia and weight gain (Edge and Gold 2011). However, there are no preclinical studies which confirm that food helps people to quit drugs. Only Orsini et al. (2014) recently reported that rats with a history of chronic amphetamine exposure increased their consumption of palatable food. Our results confirm that fat can act as an alternative reinforcer, as reinstatement of cocaine-induced CPP was decreased in mice exposed to HFD.

Materials and methods

Subjects

A total of 179 male mice of the OF1 outbred strain were acquired commercially from Charles River (Barcelona, Spain). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28 × 28 × 14.5 cm) for 8 days prior to initiating the experimental feeding condition, at a constant temperature (21 ± 2 °C), with a reversed light schedule (white lights on 19:30–7:30 hours) and food and water available ad libitum (except during the behavioral tests). 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 approved the study.

Feeding conditions

Two different types of diet were used in this study. The control group was fed with a standard diet (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates, and 20% kcal protein; 2.9 kcal/g) and the high-fat diet group with a high-fat diet (TD.06415, 45 kcal % fat, 36 kcal % carbohydrates, and 19% kcal protein; 4.6 kcal/g). Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to as the standard diet (control) and the HFD from this point forward.

Mice were acclimated for 8 days before initiating experiments. They were then randomly divided into groups with similar average bodyweight (25–26 g) and assigned either the control (C) or HFD. Water was freely available at all times.

Drug treatment

For CPP, animals were injected i.p. with 1, 6, or 25 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S. A., Madrid, Spain) diluted in physiological saline. The dose of 1 mg/kg 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 subthreshold dose. The dose of 6 mg/kg cocaine has been demonstrated to be effective for inducing CPP but not reinstatement (Maldonado et al. 2006). For the acute response to the motor effects of cocaine, naive animals were injected with 10 mg/kg cocaine. The highest dose of cocaine employed (25 m/kg) induced strong CPP and reinstatement of the preference with progressively lower priming doses (Ribeiro Do Couto et al. 2009).

Apparatus and procedure

Experimental design

An overall and more detailed description of the experimental procedure of each experiment is provided in Table 1. In the first experiment, animals were divided into three groups: control, fed the standard diet; continuous HFD, with access to fat throughout the whole study; and HFD, *15-day withdrawal* (HFD 15W) which had access to fat until 15 days before the initiation of behavioral tests. Both HFD groups were fed fat for 40 days (from PND 29 until PND 69), while the HFD group continued to consume the diet until the end of the behavioral studies. Mice in the HFD 15W group arrived at the laboratory 15 days before control and HFD groups. They were exposed to the same experimental procedures but were switched on PND 69 to a standard diet and remained undisturbed in their home cages until PND 84, when the CPP procedure was initiated.

One set of animals (n = 10/condition) was employed to extract blood samples and brains on PND 69 to carry out gene expression studies with real-time PCR analyses and to determine circulating leptin and ghrelin levels. In another set of mice, behavioral tests started on PND 69 for control and HFD groups or on PND 84 for the HFD 15W group. A first set of animals was conditioned with 6 mg/kg cocaine in the CPP (control n = 12; HFD n = 14; HFD 15W n = 9). A second set of animals performed the elevated plus maze (EPM) and then underwent 1 mg/kg cocaine-induced CPP (control n = 11; HFD n = 13; HFD 15W n = 15). Finally, a third set of mice (n = 15/condition) was challenged with an effective dose of cocaine (10 mg/kg) and locomotor activity was measured in the open field.

In the second experiment, only two groups of mice—control and HFD condition (n = 15 in both groups)—were exposed to a standard diet until the day of post-conditioning on which HFD animals began to have free access to high-fat food in order to evaluate its effects on the extinction of the preference.

Determination of plasma leptin and ghrelin concentrations

Plasma leptin concentrations were measured with an ELISA kit from B-Bridge International (Cupertino, CA, USA) and from Sigma-Aldrich (San Louis, EEUU) for ghrelin following the manufacturer's instructions. The sensitivity of the test is 0.2. All samples were run in duplicate.

Gene expression analyses: real-time PCR

For gene expression analyses, the protocol described previously (Rodriguez-Arias et al. 2016) was followed. Brain sections were cut (500 μm) in a cryostat (−10 °C) at levels

Table 1 Experimental design

PND	29–68	69			70–77	78	
					84–91 (HFD 15W)	92 (HFD 15W)	
Experiment 1, n = 149	Standard diet						Control
	High-fat diet						HFD
	High-fat diet						HFD 15W
	n = 30			Blood and brain samples			
	n = 35				CPP (6 mg/kg)	Extinction and reinstatement tests	
	n = 39	Elevated plus maze			CPP (1 mg/kg)		
	n = 45		Motor activity				
PND	29–69	70–77	78–141				
Experiment 2, n = 30	Standard diet				Control		
	Standard diet	CPP (25 mg/kg)	High-fat diet Extinction and reinstatement tests		HFD		

containing the regions of interest according to Paxinos and Franklin (2001), mounted onto slides, and stored at −80 °C. 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, mu-opioid receptor and GHSR gene expressions was performed with the Step One Real Time PCR System (Life Technologies, Madrid, Spain). All reagents were obtained from Applied Biosystems, and manufacturer's protocols were followed. The reference gene used was 18S rRNA, detected using Taqman® ribosomal RNA control reagents. 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).

Conditioned place preference

For place conditioning, we employed 12 identical Plexiglas boxes with two equally 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).

The place conditioning procedure, unbiased in terms of initial spontaneous preference, was performed as described previously (Maldonado et al. 2006) and consisted of three phases. To summarize, in the first phase, known as preconditioning (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 15 min (900 s) per day on 3 days. On day 3, the time spent in each compartment during 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 experiment (the total number of animals excluded in the three CPP studies was 16). 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 vs vehicle-paired compartment 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 30 min. 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 postconditioning (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 preference for the drug-paired compartment had been established underwent a weekly extinction session that consisted of placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 min. The extinction condition was fulfilled when there was a lack of significant differences between CPP scores in the extinction sessions and Pre-C test values in two consecutive sessions.

Reinstatement of CPP Twenty-four 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 min), except that animals were tested 15 min after administration of the respective dose of cocaine. When reinstatement of the preference was achieved, and 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 noncontingent place to that of the previous conditioning procedure.

Acute locomotor response to cocaine

Acute locomotor response to 10 mg/kg of cocaine was assessed in an open field for a period of 30 min. The open field test was performed in an opaque plastic box (30 × 30 × 15 cm) opened at the top. The animal was placed in the box 30 min before the injection to become habituated and was subsequently injected i.p. with 10 mg/kg of cocaine. Locomotor activity was then recorded for 30 min by an automated tracking control (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The parameter studied was total distance traveled (cm).

Elevated plus maze

The EPM consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5 cm). 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 additional grip for the animals. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1 h 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).

Statistics

Data related to body weight in the first experiment were analyzed by a one-way analysis of variance (ANOVA) with a within variable PND with nine levels—PND 29, 36, 43, 50, 57, 64, 69, 76, and 78. In experiment 2, bodyweight and food intake were analyzed by a one-way ANOVA with a within variable PND with 14 levels—PND 29, 36, 43, 50, 57, 64, 69, 76, 78, 82, 89, 96, 101, and 107. The EPM data were analyzed by a one-way ANOVA with a between variable—"Diet"—with three levels: control, HFD, and HFD 15W.

For CPP, the time spent in the drug-paired compartment was analyzed by means of a mixed ANOVA with one between variable—Diet, with three levels (control, HFD, HFD 15W)—and a within variable—days, with two 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. Leptin and ghrelin levels were analyzed by one-way ANOVA with a between variable—Diet—with three levels (control, HFD, HFD 15W). Gene expression values were analyzed by a one-way ANOVA.

Results

Experiment 1: effects of HFD during adolescence on the motor and the conditioned rewarding effects of cocaine

Body weight and food intake

As seen in Fig. 1a, the ANOVA for body weight revealed a significant difference of the variable Days [$F(8.336) = 655.873$; $p < 0.001$], as all animals exhibited an increase in body weight throughout the duration of the experiment. There was also an effect of the variable Diet [$F(2.42) = 13.461$; $p < 0.001$] and the interaction Days × Diet [$F(16.336) = 9.856$; $p < 0.001$). Both groups of mice exposed to fat (HFD and HFD 15W) displayed significantly higher weight with respect to the control group on days 36, 43, 50, 57, 64, 69, 76, and 78 ($p < 0.01$).

With respect to daily food intake (see Fig. 1b, c), the ANOVA of the grams and kcal of food intake revealed an

effect of the variable Diet [$F(2.9) = 17.388$; $p < 0.001$] and [$F(2.9) = 8.567$; $p < 0.01$] and the interaction Days × Diet [$F(16.72) = 9.394$; $p < 0.001$] and [$F(16.72) = 3.713$; $p < 0.001$]. Animals in both HFD groups showed a decrease in grams of food intake from PND 29 to PND 69 and also on PND 78 with respect to the control group. On PND 76, after 7 days without access to fat, animals in the HFD 15W group increased their intake in grams of standard food with respect to the HFD group ($p < 0.01$). In terms of intake of kcal, animals in both HFD groups showed an increase with respect to the control group on PND 29 ($p < 0.001$), 36 and 43 ($p < 0.01$), and PND 78 (only HFD group, $p < 0.05$).

Effects of a HFD on circulating leptin and ghrelin levels and MOr, CB1r, and GHSR gene expression

With respect to circulating leptin levels (Table 2), the ANOVA revealed [$F(2.27) = 4.59$; $p < 0.05$] that animals in the HFD group showed an increase with respect to those in the standard diet group ($p < 0.05$). With respect to circulating ghrelin levels (Table 2), the ANOVA [$F(2.27) = 4.294$; $p < 0.05$] revealed a decrease in the HFD group ($p < 0.05$).

On the other hand, real-time PCR analyses (Fig. 2.) showed an effect of the variable Diet in the CB1r expression in N Acc [$F(2.27) = 7234$; $p < 0.01$] and PFC [$F(2.27) = 6364$; $p < 0.01$], MOr gene expression [$F(2.27) = 4641$; $p < 0.01$] and GHSR expression [$F(2.27) = 16{,}019$; $p < 0.001$]. Bonferroni post hoc analyses indicated that exposure to a HFD during adolescence decreased CB1 receptor gene expression in the N Acc ($p < 0.05$) and PFC ($p < 0.01$) (Fig. 2a, b). Although animals in the HFD 15W group also exhibited decreased expression of the CB1 receptor in the N Acc with respect to the control ($p < 0.001$) group, expression levels in the PFC became normalized, showing a decrease when compared to the HFD group ($p < 0.05$). In relation to MOr gene expression, values in the HFD group were increased in the N Acc with respect to the control group ($p < 0.01$) (Fig. 2c), but became normalized in the HFD 15W ($p < 0.05$ with respect HFD group). Finally, regarding GHSR gene expression (Fig. 2d), animals in the HFD group presented decreased gene expression with respect to controls ($p < 0.01$). However, the HFD 15W group showed a significant increase with respect to the control and HFD groups ($p < 0.001$).

Conditioned place preference induced by 1 and 6 mg/kg of cocaine

The ANOVA for the 6 mg/kg of cocaine-induced CPP (Fig. 3a) revealed a significant effect of the variable Days

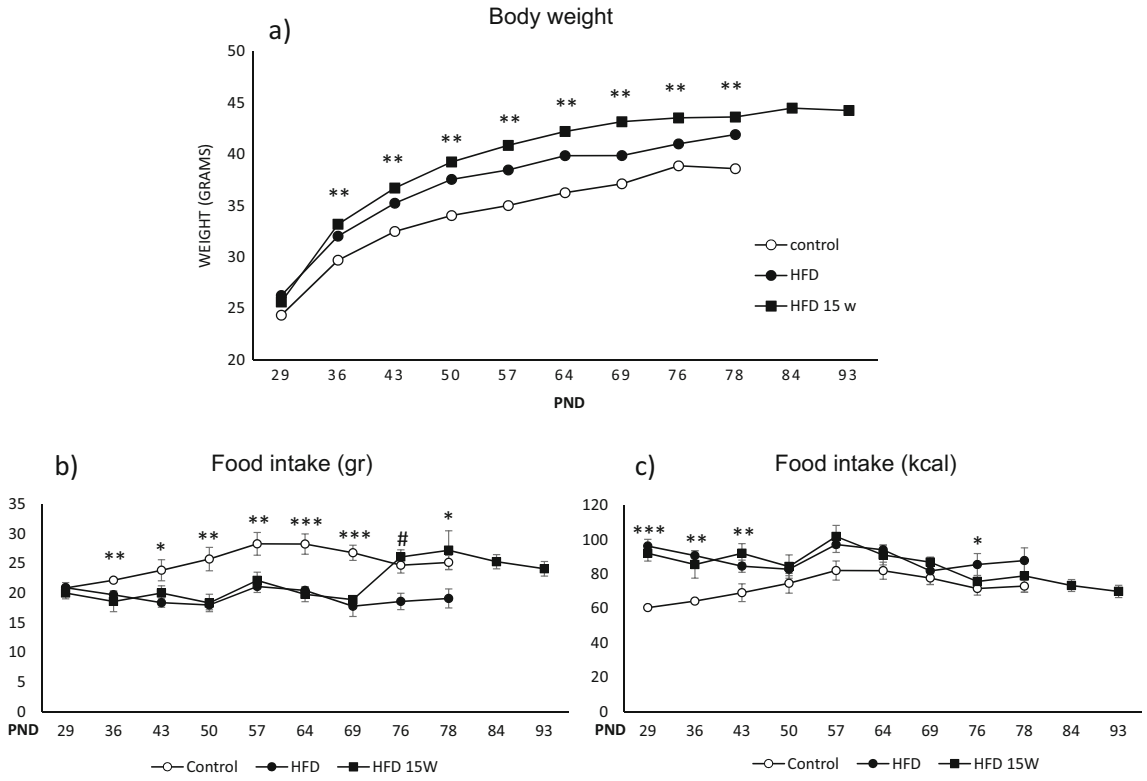

Fig. 1 **a** Body weight (measured weekly) of animals in the control group, the HFD group, and the HFD 15W group. **b** Food intake in grams during the whole procedure. **c** Food intake in kcal during the whole procedure. Data are represented as the mean (± SEM) amount of body weight measured weekly. HFD and HFD 15W groups showed a significant difference with respect to the control group, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 2 Effects of a continuous HFD during adolescence on circulating leptin (ng/ml) and ghrelin (pg/ml) levels in controls and the HFD group on PND 69, and in the HFD 15W group on PND 84

	Plasma leptin (ng/ml)	Ghrelin (pg/ml)
Control	2,3 ± 0,4	560 ± 65
HFD	5,5 ± 1,2 *	401 ± 18 *
HFD 15W	2,9 ± 1,3	488 ± 29

Data are presented as mean values ± SEM (ng/ml)

**$p < 0.01$; *$p < 0.05$ with respect to the control group

[$F(1.32) = 34.148$; $p < 0.001$], 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 test showed no differences between groups in the time required to achieve extinction (control required 4 sessions, HFD required 5.7 sessions, and HFD 15W required 6.25 sessions). No reinstatement of the preference was achieved with a priming dose of 3 mg/kg of cocaine.

Results obtained for 1 mg/kg cocaine-induced CPP are presented in Fig. 3b. The ANOVA revealed an effect of the interaction of Days × Diet [$F(2.36) = 4.204$; $p < 0.05$]. CPP developed only in the HFD 15W group, which spent more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.01$).

Acute response to 10 mg/kg cocaine

The ANOVA (see Fig. 3c) of the locomotor response to 10 mg/kg cocaine presented an effect of the variable Diet [$F(2.42) = 3.622$; $p < 0.05$], showing that, after a single injection of 10 mg/kg cocaine, animals of the HFD 15W exhibited an increased locomotor response to cocaine when compared to control mice ($p < 0.05$).

Effects of exposure to a continuous HFD during adolescence on performance in the elevated plus maze in adulthood

In order to evaluate if cessation of fat administration produces withdrawal symptoms, the behavior in the EPM was tested on PND 68. Mice undergoing fat withdrawal (HFD 15W) showed a higher anxiogenic profile than control and HFD groups (see Table 3), spending less time [$F(2.42) = 11.901$; $p < 0.001$] and percentage of time [$F(2.42) = 12.957$; $p < 0.001$] in the open arms of the maze ($p < 0.001$ in all cases); performing a lower number [$F(2.42) = 5.456$; $p < 0.01$] ($p < 0.01$ with respect to control), and percentage [$F(2.42) = 7.938$ $p < 0.001$) of open arm entries ($p < 0.01$ in all cases); and spending more time in the closed arms of the maze [$F(2.42) = 6.929$; $p < 0.01$] ($p < 0.01$ with respect to control).

Experiment 2: effects of a high-fat diet during the extinction and reinstatement of a 25 mg/kg cocaine-induced CPP

Body weight and food intake

As seen in Fig. 4a, the ANOVA for body weight revealed no significant differences between groups. There was an effect of the variable Days [$F(12.336) = 403.640$; $p < 0.001$], as mice in both groups showed an increase in body weight throughout the study.

With respect to daily food intake (see Fig. 4b, c), the ANOVA of the grams and kcal of food intake revealed an effect of the variable Days [$F(12.72) = 23.208$; $p < 0,001$] and [$F(12.72) = 41.445$; $p < 0.001$], and the interaction Days × Diet [$F(12.72) = 15.584$; $p < 0.001$] and [$F(12.72) = 38.155$; $p < 0.001$]. Animals of the HFD group showed a decrease of food intake in grams (Fig. 4b) on PND 96, 101, and 107 with respect to the control group ($p < 0.05$ and $p < 0.01$). The intake in kcal (Fig. 4c) showed that animals in the HFD group exhibited an increase in their kcal intake on PND 82 ($p < 0.001$), PND 89 ($p < 0.01$), and PND 96 ($p < 0.05$) with respect to the control group.

Extinction and reinstatement of 25 mg/kg cocaine-induced CPP

The ANOVA revealed a significant effect of the variable Days [$F(1.26) = 21.527$; $p < 0.001$]. Both groups spent more time in the drug-paired compartment in the Post-C than in the Pre-C test ($p < 0.01$) (see Fig. 5), and required five (control) and two (HFD) sessions, respectively, to achieve extinction after Post-C. The Kaplan-Meier test confirmed that the HFD group required significantly fewer sessions to achieve extinction ($\chi^2 = 20.648$; $p < 0.001$). A Student's *t*-test showed that a priming dose of 12.5 mg/kg of cocaine reinstated the preference in both control ($p < 0.01$) and HFD ($p < 0.05$) groups. After this, animals in both groups required one session to achieve extinction. No further reinstatement with 6.25 mg/kg was obtained in the HFD group. However, once extinction was achieved in the control group, the preference was reinstated with 6.25 mg/kg ($p < 0.05$) and 3.125 mg/kg ($p < 0.01$). No further reinstatement was achieved.

Discussion

Our results confirm that continuous exposure to a HFD during adolescence induces neurobiological alterations that only partially return to normal after fat withdrawal. We show that prolonged consumption of a HFD during adolescence deeply alters endogenous cannabinoid and opioid systems, leading to a decreased CB1 receptors gene expression in the N Acc and

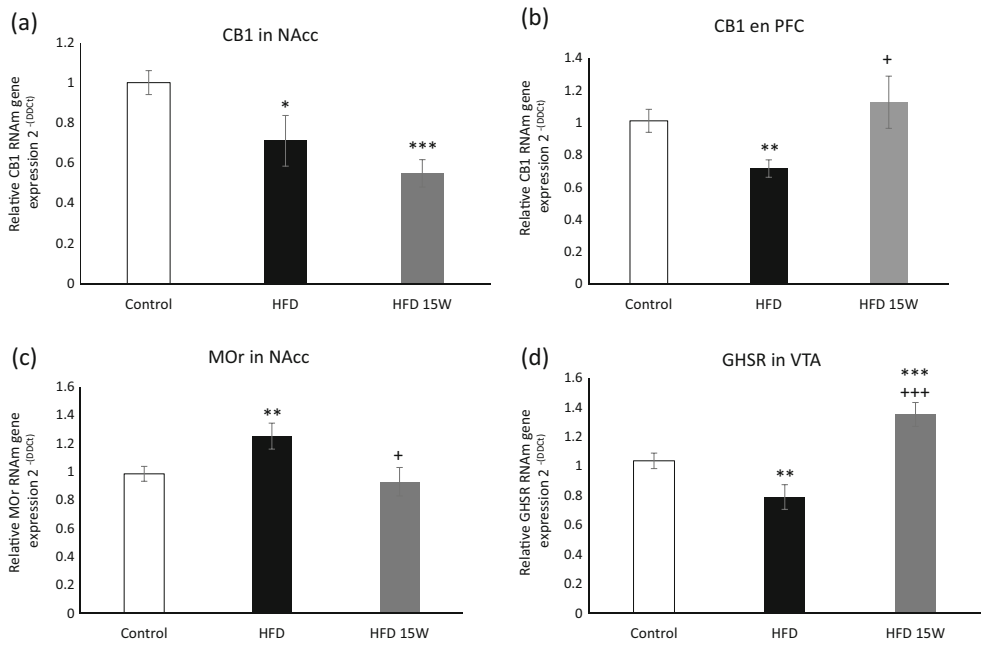

Fig. 2 Real-time PCR CB1 receptor relative gene expression evaluation in the N Acc (**a**) and PFC (**b**) brain regions of control, HFD, and HFD 15W animals on PND 69 (control and HFD) and PND 84 (15W animals) (n = 10 per group). **c** MOr relative gene expression evaluation in the N Acc brain region of control, HFD, and HFD 15W animals on PND 69 (control and HFD) and PND 84 (15W animals) (n = 10 per group). **d** GHSR relative gene expression evaluation in the VTA brain region of control, HFD, and HFD 15W animals on PND 69 (control and HFD) and PND 84 (15W animals). The *columns* represent means and the vertical lines ± SEM of relative (2−ΔΔCt method) gene expression in the PFC, N Acc, and VTA of OF1 mice. *, **, *** represent the values that differ significantly ($p < 0.05$, $p < 0.01$, and $p < 0.001$) from those of their corresponding control mice. +, +++ represent the values that differ from the HFD group ($p < 0.05$ and $p < 0.001$)

PFC and increased mu-opioid receptor gene expression in the N Acc. After withdrawal from fat, these changes return to control levels, with the exception of CB1 receptor gene expression in N Acc, which continues to be decreased. Equally, plasmatic concentrations of leptin and ghrelin are altered during fat ingestion, normalizing after cessation of fat ingestion. However, GHSR gene expression in the VTA, which decreases during fat diet, increases during withdrawal. These neuroadaptations are accompanied by alterations in the conditioned rewarding effects of cocaine. Although no changes in cocaine-induced CPP were observed when our animals continued to consume the HFD, there was an increase in the rewarding and motor effects of cocaine after cessation of said diet. Finally, we demonstrate that continuous exposure to a HFD during the extinction period of cocaine-induced CPP reduces the time required to achieve extinction and diminishes reinstatement of the preference induced by a priming dose of cocaine, which confirms the ability of fat ingestion to act as a reinforcer.

In agreement with previous reports, our model of continuous access to fat induced significant differences in body weight between the standard diet and the HFD groups (Wellman et al. 2007; Morales et al. 2012). Hence, our data suggest that a HFD during adolescence induces a more marked progressive weight gain than that observed in control mice, which eventually leads to obesity in adulthood. As expected, animals in the HFD group showed increased leptin plasma concentrations with respect to the standard diet group (Ahrén and Scheurink 1998; Lin et al. 2000). As we have previously reported in mice exposed to a high-fat binge, plasmatic ghrelin concentrations were significantly lower in mice on the HFD (Blanco-Gandia et al. 2017). Ghrelin plays an important role in nutritional homeostasis (Schellekens et al. 2013), and most reports show that ghrelin secretion is downregulated by a HFD (Beck et al. 2002; Lindqvist et al. 2005; Bello et al. 2009), suggesting a deficit in satiety signals as a result of exposure to such diets. Cessation of HFD ingestion tended to normalize leptin and ghrelin in the HFD 15W group, which did not differ from controls. These results suggest that after 2 weeks of withdrawal of HFD intake, there is an ongoing normalization process of the hormonal disturbances.

Reward-driven overeating is characterized by repeated cycles of abstinence and craving, turning obesity into a chronic condition (Alsiö et al. 2012), and its dopaminergic phenotype is comparable to that of drug addicts. Obese subjects display significantly less D2 binding than healthy normal weight subjects (Wang et al. 2001) and numerous studies in animal models confirm these data. Chronic intake of fat induces lower basal DA levels in the N Acc and VTA (Geiger et al. 2007, 2009; Cone et al. 2010; Rada et al. 2010), lower DA turnover (Davis et al. 2008), lower DA release (York et al. 2010), and reduced DA clearance in the N Acc (Speed et al. 2011).

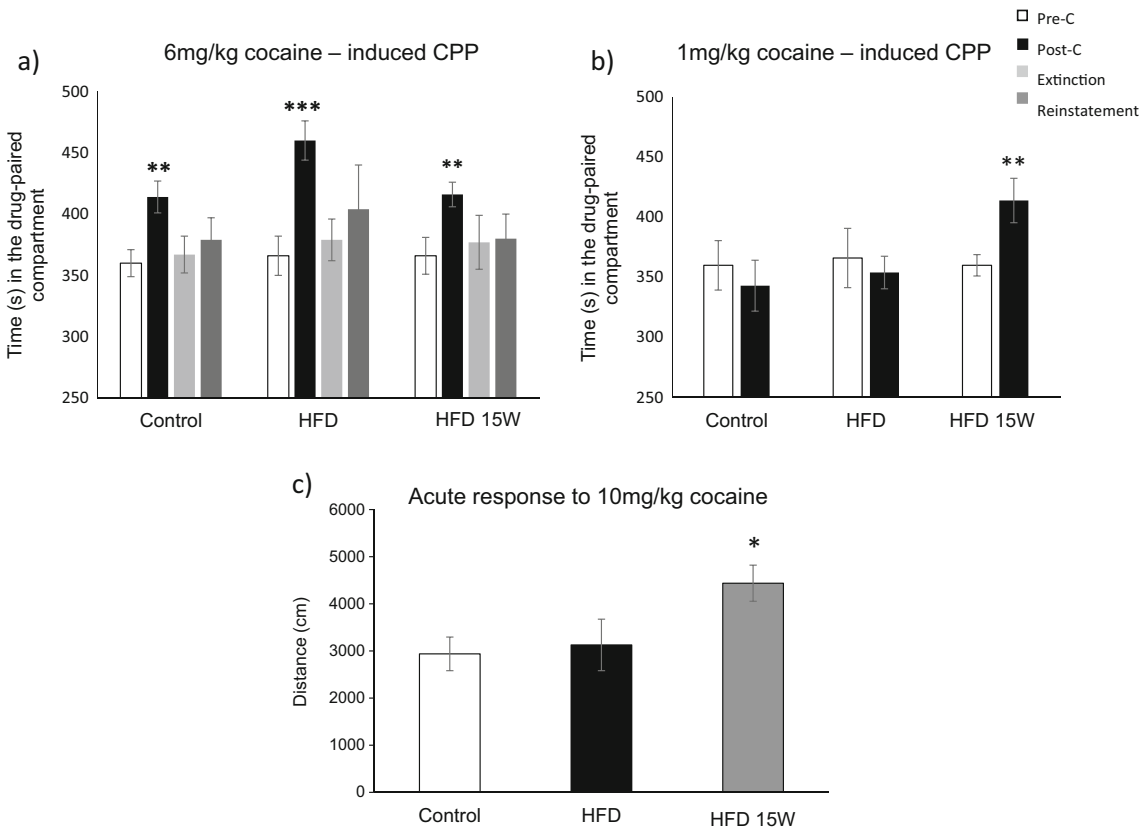

Fig. 3 **a** CPP induced by 6 mg/kg of cocaine in mice exposed to a continuous HFD. *Bars* represent the time (± SEM) in seconds spent in the drug-paired compartment before conditioning sessions in the preconditioning test (*white bars*), after conditioning sessions in the postconditioning test (*black bars*), in the last extinction session (*light gray bars*) and during the reinstatement test (*dark gray bars*). The reinstatement test was evaluated 15 min after a priming dose of 3 mg/kg cocaine. $^{**}p < 0.01$, $^{***}p < 0.001$ significant difference in the time spent in Post-C vs Pre-C sessions. **b** CPP induced by 1 mg/kg of cocaine in mice exposed to a continuous HFD. *Bars* represent the mean (± SEM) time in seconds spent in the drug-paired compartment before conditioning sessions in the preconditioning test (*white bars*), after conditioning sessions in the postconditioning test (*black bars*). $^{**}p < 0.01$ with respect to the Pre-C day. **c** Acute locomotor response to cocaine. The *bars* represent the mean value (± SEM) of the total distance (cm) in a period of 10 min after the cocaine injection (10 mg/kg). $^{*}p < 0.05$ with respect to control

Endogenous opioid and endocannabinoid systems interact very closely with DA, modulating the reward system. The endogenous opioid system is strongly implicated in the regulation of appetite, and specifically in fat consumption (Sakamoto et al. 2015). We have observed that exposure to a HFD during adolescence increases MOr gene expression in the N Acc of animals on a HFD. In line with our results, MOr binding has been reported to be increased in reward-related

Table 3 Effects of a HFD on the performance of adolescent mice in the elevated plus maze

	Control	HFD	HFD 15w
Time in open arms	124.6 ± 12.9	129.8 ± 11.3	$54.9 \pm 13.4^{***+++}$
% Time in open arms	53.5 ± 4.7	59.3 ± 3.9	$27.4 \pm 5.9^{***+++}$
Time in central platform	59.5 ± 5.7	75.6 ± 6.2	90.2 ± 11.7
Time in closed arms	104.5 ± 10	83.8 ± 5.5	$140.1 \pm 15.7^{++}$
Entries in open arms	26.8 ± 2.6	35.3 ± 2.7	$20.5 \pm 4.2^{++}$
% Open entries	63.2 ± 4.7	64.8 ± 3.7	$43.6 \pm 4.5^{**++}$
Entries in closed arms	15.3 ± 2	21.7 ± 4.9	23.7 ± 3.2
Total entries	42.1 ± 2.4	57 ± 6.3	44,3 ± 6,8

. Data are presented as mean values ± SEM

Differences with respect to the control group $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; differences with respect to the HFD group $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$

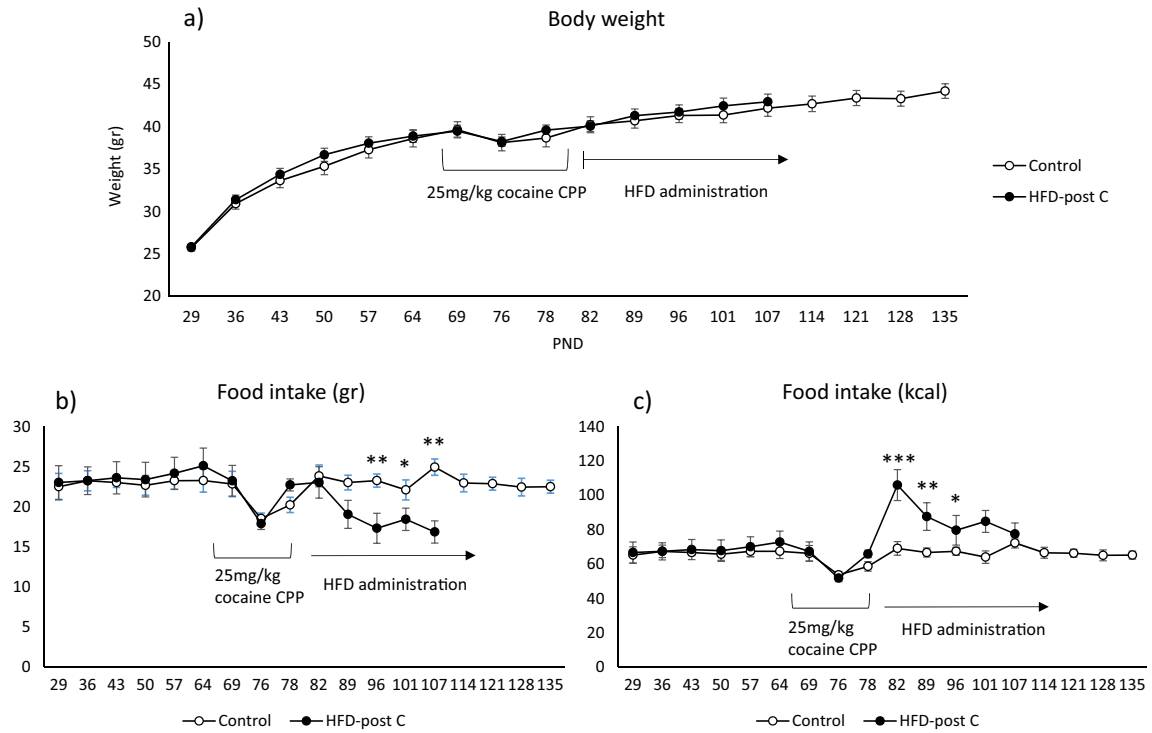

Fig. 4 **a** Body weight (measured weekly) of animals in the control group and the HFD Post-C group. **b** Food intake in grams during the whole procedure. **c** Food intake in kcal during the whole procedure. Data are represented as the mean (± SEM) amount of body weight measured weekly. HFD and HFD 15W groups showed a significant difference with respect to the control group, $*p < 0.05$; $**p < 0.01$, $***p < 0.001$

sites in HFD-obese rats, such as the basomedial or basolateral amygdala, or the hypothalamus (Smith et al. 2002; Barnes et al. 2003). This increase in MOr expression may reflect a decreased release of endogenous opioid peptides. The mu-opioid system in reward-related areas may be inhibited in dietary obesity, probably by increased plasma leptin and/or insulin. In support of this hypothesis, we have previously reported contrary results after binge exposure to a fat diet during

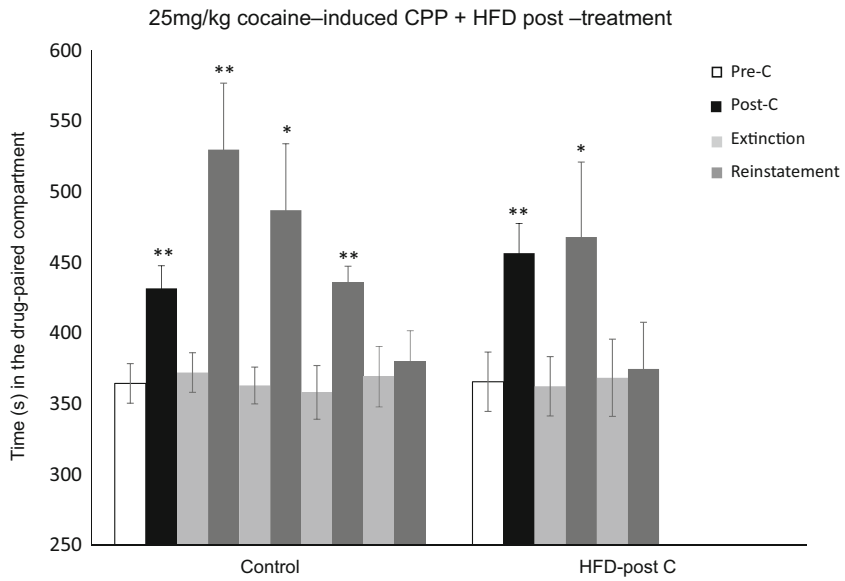

Fig. 5 CPP induced by 25 mg/kg of cocaine in mice receiving a standard diet. After Post-C, animals in the HFD Post-C group were fed a continuous high-fat diet to explore its effect on extinction and reinstatement. *Bars* represent the time (± SEM) in seconds spent in the drug-paired compartment before conditioning sessions in the pre-conditioning test (*white bars*), after conditioning sessions in the post-conditioning test (*black bars*), in the last extinction session (*light gray bars*) and during the reinstatement test (*dark gray bars* correspond to reinstatement of the following doses *from left to right*: 12.5, 6.25, 3.125, and 1.56 mg/kg). $*p < 0.05$; $**p < 0.01$ significant difference in the time spent in Post-C vs Pre-C sessions and reinstatement vs extinction

adolescence, showing a decreased MOr gene expression without altering leptin levels (Blanco-Gandia et al. 2017).

Endocannabinoids affect appetite for specific dietary components through CB1 receptors, with N Acc constituting a critically involved area of the brain (South and Huang 2008; Higuchi et al. 2011; Deshmukh and Sharma 2012). In the present study, we have shown how animals on a HFD exhibit decreased CB1 receptor gene expression in the PFC and N Acc. In the same line, certain studies report that CB1 receptor density in the N Acc or in the hypothalamus is reduced by 20% in HFD-fed animals (Di Marzo et al. 2001; Bello et al. 2012; Martire et al. 2014; Blanco-Gandia et al. 2017). Several reports have pointed out that leptin regulates not only DA activity but also opioidergic and endocannabinoid systems. Leptin injections reduce endocannabinoid levels in the hypothalamus (Di Marzo et al. 2001) or reverse mu-opioid-stimulated sucrose feeding in the VTA (Figlewicz et al. 2007). Our results confirm these interactions, since a HFD during adolescence increased levels of leptin, which would interact with the opioid and endocannabinoid neurotransmission systems, among others.

Two weeks after cessation of the HFD, the hormonal disturbances and most of the changes in MOr and CB1 receptor gene expressions induced by the diet were normalized. However, the decrease in CB1 receptor gene expression in the N Acc was maintained. Few studies have evaluated the effects of fat withdrawal, but Martire et al. (2014) showed that a 15-week cafeteria diet induced a reduction of mRNA expression of MOr and CB1 receptors in the VTA that was maintained 48 h after cessation of said diet. The longer withdrawal period (2 weeks) in our study was probably responsible for the different results obtained. In the same line, Ong et al. (2013) showed that after 72 h of withdrawal of a cafeteria diet, μ-opioid receptor expression was reduced in CD and CD-W males but not females.

In addition, although GHSR in the VTA was reduced during fat consumption, a significant increase was observed after 2 weeks of abstinence. In agreement with our findings, 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), but no reports have evaluated GHSR gene expression after a period of withdrawal. Although their study was not focused on fat deprivation, Wellman and Abizaid (2015) also reported increases in hypothalamic GHSR1a mRNA in response to food restriction. Ghrelin signaling in the VTA is implicated in natural and drug-induced reward (Wellman et al. 2013), suggesting that ghrelin receptors facilitate the activation of DA circuits by psychostimulant drugs. In this context, numerous studies have pointed out that ghrelin increases the rewarding and locomotor effects of cocaine (Wellman et al. 2005; Davis et al. 2007; Abizaid et al. 2011). GHSR are expressed in DA neurons (Naleid et al. 2005; Skibicka et al. 2011a; King et al. 2011) and ghrelin induces food-motivated behavior via interaction with MOr (Kawahara et al. 2009; Skibicka et al. 2011b). Similarly to our results obtained during fat withdrawal, GHSR mRNA was reported to be upregulated in the hypothalamus of hamsters after food deprivation and accompanied by an elevation of circulating ghrelin concentration (Tups et al. 2004). Therefore, the increase in GHSR expression could be a compensatory response to the previous decrease in circulating plasma ghrelin levels during a HFD.

Human studies show that obese individuals are less prone to use recreational drugs and show less prevalence of substance abuse disorders (Simon et al. 2006; Warren et al. 2005; Mather et al. 2009). Preclinical data also suggest that obesity alters the neural processing of rewarding stimuli, since both food and drugs of abuse activate the reward system (Gambarana et al. 2003; Salamone et al. 2005; Pontieri et al. 1995). Although Lockie et al. (2015) observed a normal development of cocaine-induced CPP in adult mice exposed to a HFD, continuous exposure diminished cocaine- or food-induced CPP in adolescent rats (Morales et al. 2012), which suggests that adolescence is a period of higher vulnerability. However, after exposure to a HFD during the entire period of adolescence, our mice did not exhibit such an attenuation of cocaine-induced CPP. Given the range of doses studied (1 and 6 mg/kg), our results are in accordance with those of Morales et al. (2012), who observed a decreased sensitivity of cocaine-induced CPP with 2 mg/kg of the drug but not with the other doses administered (1, 4, and 8 mg/kg). Although the CPP procedure of that study was different (biased CPP), like them, we also observed that HFD mice did not develop CPP when conditioned with a low dose of cocaine (1 mg/kg), as they behaved in the same way as mice fed standard chow. Likewise, no differences were observed in the CPP induced by 6 mg/kg of cocaine, as both groups developed CPP and required the same number of sessions for the preference to be extinguished, while, in agreement with previous results, preference was reinstated in neither group (Maldonado et al. 2006). A recent report shows that leptin attenuates cocaine-induced increases in DA levels in the N Acc and reduces the ability of cocaine-predictive stimuli to establish CPP and to prolong the response of cocaine-seeking during extinction (You et al. 2016). A similar response to the acute locomotor effects of cocaine was also seen in controls and in HFD-feed animals in our study, as it has been previously reported (Baladi et al. 2012; Fordahl et al. 2016). However, Collins et al. (2015) observed enhanced motor response in mice consuming a HFD in comparison to mice consuming standard chow. The lack of differences in the present study between cocaine-induced CPP or locomotor activation in fat-fed mice vs controls does not seem to be due to the lack of a leptin response, since there was a significant increase of this hormone in the HFD group.

Prolonged exposure to sugar-rich diets leads to physical dependence, inducing physical symptoms of withdrawal

when the food is removed (Avena 2007). In order to evaluate if continuous exposure to a HFD induced similar alterations to those seen in diets rich in sugar, we included an additional group of mice that was exposed to fat during the whole of the adolescent period, but which was changed to a standard chow diet 15 days before initiation of the CPP (HFD 15W). Data provided by the EPM confirmed that the HFD 15W group showed an anxiogenic profile when compared with the rest of groups, as shown by a reduction in the time spent in the open arms. In line with our results, several studies have reported an increase in anxiety levels up to 24 h after cessation of continuous access to a HFD (Teegarden and Bale 2007; Cottone et al. 2009; Sharma et al. 2013). Moreover, our data show that withdrawal of continuous access to a HFD induced a long-lasting increase in anxiety that was noticeable for up to 2 weeks. In addition, appetitive behavior to palatable foods increases during cessation of such diets and can induce cross-sensitization behavior with drugs of abuse. In agreement with these results, we have observed that mice under withdrawal from a fatty diet developed CPP after conditioning with a subthreshold dose of cocaine (1 mg/kg), suggesting increased sensitivity to the conditioned rewarding effects of cocaine, which did not occur when HFD was consumed during the CPP procedure. Equally, animals in the HFD 15W group exhibited an increased acute locomotor response to 10 mg/kg cocaine. Overall, our behavioral data are in line with those of previous studies reporting an enhanced response to alcohol, methamphetamine, and cocaine in animals forced to abstain from sucrose (Avena et al. 2004; Avena and Hoebel 2003; Gosnell 2005). There are practically no studies evaluating the effect of abrupt cessation of a HFD on the response to drugs of abuse. It is known that food restriction increases the locomotor response of DA agonists such as quinpirole (Carr et al. 2003), amphetamine (Deroche et al. 1993), and cocaine (Stamp et al. 2008). Only one study has evaluated the effect of withdrawal from chronic exposure to HFD on the locomotor response of rats to cocaine, with no changes observed after cessation of the HFD (Baladi et al. 2012). Differences in the time exposed to HFD and the withdrawal period—shorter and longer, respectively, in the Baladis' study—and the use of different rodent species (rats) could explain the divergent results. In short, we did not observe changes in CPP or in the locomotor response to cocaine when our mice continued consuming a HFD, but an increased response to conditioned rewarding and stronger motor effects of cocaine were apparent when HFD was discontinued.

We have previously reported comparable results in mice exposed to a high-fat binge during adolescence, which showed CPP with a subthreshold dose of cocaine (Blanco-Gandía et al. 2017). Therefore, mice consuming a high-fat binge diet and mice under withdrawal from a HFD show an increased sensitivity to the conditioned rewarding effects of cocaine. In both cases, mice present a similar hormonal and neurobiochemical profile and plasmatic levels of leptin and ghrelin are within normal values. More remarkable, CB1 receptor gene expression in the N Acc is decreased and GHSR in VTA is increased in both cases. Recent studies suggest that leptin represents an endogenous antagonist of responses to cocaine (You et al. 2016). A subset of VTA dopamine neurons was shown to express leptin receptors (Hommel et al. 2006; Leshan et al. 2010), which hyperpolarized DA neurons when stimulated, thus decreasing their action potential firing frequency (Hommel et al. 2006) and reducing extracellular DA in the NAc (Krügel et al. 2003). These data suggest that leptin directly inhibits DA neurons in the VTA. The possibility of leptin resistance in our HFD obese mice cannot be ruled out (Munzberg et al. 2005). However, several studies report dopamine inhibition in obese leptin-resistant animals (Davis et al. 2009; Thanos et al. 2008). Therefore, we can hypothesize that, while animals are feeding on fat, the elevation of leptin levels will decrease the response of the dopaminergic mesolimbic system to cocaine. Our results suggest that this decrease is not enough to block the conditioned rewarding effect of an effective dose of cocaine (6 mg/kg). However, after abrupt cessation of fat ingestion, DA neurons would uncover the neuroadaptation due to their chronic inhibition for higher leptin levels. When able to function without that negative influence, a temporary increase in their responsiveness to drug stimuli would be observed.

An undermining of the endocannabinoid system can modulate the dopaminergic system and contribute to the sensitization of 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 have contributed to the increase in the rewarding effects of cocaine observed in these animals. This hypothesis would explain why HFD 15W mice developed preference for a noneffective dose of cocaine.

Our results suggest that continuous exposure to fat during adolescence induces neuroadaptations that continue to be expressed after cessation of fat ingestion. Therefore, our results give support to the hypothesis that high fat food has addictive properties. Clinical practice often reports that subjects under treatment for cocaine dependence experience significant weight gain during recovery, developing a pronounced appetite, especially for high-fat food (VanBuskirk and Potenza 2010; Billing and Ersche 2015; Balopole et al. 1979), and similar results have been obtained in animal models (Bane et al. 1993; Avena and Hoebel 2003; Orsini et al. 2014). Based on these studies, a second experiment was performed to evaluate if a HFD, acting as an alternative reinforcer, reduced cocaine-seeking during extinction of the CPP and/or reinstatement. Our results confirmed that animals with free access to high-fat food after conditioning with 25 mg/kg cocaine in the CPP showed an attenuated cocaine-induced reinstatement and needed less time than control

animals for the preference to be extinguished. A few studies have evaluated these effects in animals. Kearns and Weiss (2007) reported that pairing cocaine-related stimuli with alternative reinforcers, such as food, prevented reinstatement of self-administration. Our results support some human studies that point to the concept of "addiction transfer," whereby one addiction is replaced by another (Chechlacz et al. 2009). In our case, the rewarding effects of cocaine conditioning would seem to be replaced by food reward.

Conclusion

To sum up, our results provide biochemical and behavioral evidence that nutritional manipulations can modify the response and sensitivity to the rewarding effects of cocaine in mice. Continuous exposure to fat alters the endocannabinoid and endogenous opioid systems, perhaps through leptin increase, and some of these alterations are maintained after fat withdrawal. While abrupt discontinuation of fat induces increased sensitivity to the rewarding and motor effects of cocaine, chronic fat intake during cocaine withdrawal accelerates extinction of cocaine memories and undermined reinstatement, therefore acting as an alternative reward. Our results highlight the close relationship between chronic intake of palatable food and the rewarding effects of cocaine.

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