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# Influence of a high-fat diet during adolescence on the reinforcing effects of cocaine and alcohol.

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**TESIS DOCTORAL**

Presentada por:

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CERTIFICA:

Que la Tesis Doctoral presentada por Doña María del Carmen Blanco Gandía, con el título "Influence of a high fat diet during adolescence on the reinforcing effects of cocaine and alcohol" ha sido realizada bajo su dirección. Tras haberla examinado hace constar su autorización para que se realicen los trámites conducentes a su defensa.

Y para que conste a los efectos oportunos, firma el presente certificado en Valencia a 18 de Mayo de 2017.

Fdo.: Dra. Marta Rodríguez Arias

*A mis padres, Rafael y M. Carmen*

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RESUMEN

La adolescencia es un período de especial vulnerabilidad en el desarrollo del ser humano, en el que se dan grandes cambios a nivel estructural y funcional (Spear, 2000; Chambers et al., 2003). En esta etapa de gran vulnerabilidad, los jóvenes muestran trastornos de la alimentación, consumo de sustancias, búsqueda de sensaciones y conductas de riesgo (Bava and Tapert, 2010). Actualmente, el consumo excesivo de comida especialmente rica en azúcares y grasas, se ha convertido en un serio problema para nuestra sociedad, habiéndose producido un incremento en las tasas de obesidad a nivel mundial que afecta particularmente a la población adolescente.

La alimentación está regulada tanto por mecanismos homeostáticos como hedónicos. Un malfuncionamiento de cualquiera de estos sistemas puede provocar una alteración en la alimentación y producir obesidad (Kenny et al., 2011). El sistema de refuerzo, constituido esencialmente por el sistema dopaminérgico mesocorticolímbico, regula la motivación para buscar o consumir estímulos reforzantes como las drogas o la comida altamente palatable. Por lo tanto, el consumo de drogas o la alimentación por razones hedónicas activan las mismas vías del refuerzo. Inicialmente nuestro organismo se desarrolló en un contexto caracterizado por la escasez nutricional y conseguir alimentos era el principal objetivo a alcanzar en el día a día. Ya que nuestro cuerpo procesa los alimentos de forma rápida, mostramos una preferencia innata por las comidas con alto contenido calórico y energético. Este tipo de alimentos, al igual que la cocaína o el alcohol, producen liberación de dopamina en el sistema de recompensa cerebral. Hoy en día, en la sociedad de la abundancia y la variedad de alimentos, comemos no sólo por hambre, sino también por placer (Gold, 2011). Por lo tanto, comer por placer afecta a los mecanismos neurales relacionados con el refuerzo y promueve el mantenimiento de esta conducta (ver revisión Avena et al., 2008; Volkow et al., 2013). En resumen, tanto los reforzadores naturales como las drogas estimulan el circuito cerebral de recompensa, produciendo placer y euforia.

El estado nutricional es un importante factor modulador en el desarrollo de la adicción, y algunos estudios han mostrado similitudes psicológicas y biológicas entre la ingesta de comida rápida y la adicción a las drogas, (Garber and Lustig, 2011; Avena et al., 2012). Por ejemplo, tanto la adicción a las drogas como la obesidad pueden ser definidas como trastornos en los que el valor de determinado refuerzo (droga o comida) está anormalmente incrementado en relación y a expensas de otros refuerzos. La adicción a drogas y los atracones de comida se caracterizan por una pérdida de control sobre la conducta consumatoria, la escalada, la dependencia y el ansia por consumir (*craving*), mostrando ambos trastornos una elevada comorbilidad (Swanson et al., 2011).

Numerosos estudios han puesto de manifiesto que el consumo de drogas durante la adolescencia incrementa la probabilidad de desarrollar dependencia y problemas relacionados con estas sustancias en la edad adulta (Arteaga et al., 2010; Merline et al., 2004). Se ha propuesto igualmente una teoría de puerta de entrada también para la comida y el abuso de sustancias (Degenhardt y cols., 2008). Esta teoría postula que la ingesta compulsiva de comida puede facilitar el desarrollo de otros comportamientos desadaptativos, como es el consumo de drogas. Por el momento, los estudios preclínicos indican que el atracón de comida rica en azúcar provoca a una sensibilización conductual a la anfetamina (Avena y Hoebel, 2003) y aumenta la autoadministración de alcohol (Avena y cols., 2004).

Se han realizado estudios utilizando comida rica en azúcar, sin embargo, los estudios con comida rica en grasa son escasos. Hasta la fecha todavía no se han evaluado las consecuencias de realizar atracones de grasa durante la adolescencia sobre el consumo de drogas como la cocaína y el alcohol. Tampoco conocemos los efectos de suspender el consumo de una dieta alta en grasas, sobre los efectos gratificantes de la cocaína o el alcohol.

Así pues, el objetivo de la presente tesis doctoral ha sido evaluar cómo una dieta alta en grasa consumida durante la adolescencia, puede modificar los efectos reforzantes de la cocaína y el alcohol. Estudiamos dos tipos

diferentes de consumo de la dieta alta en grasa, el acceso continuado a la dieta rica en grasa (los animales consumen la dieta durante todo el periodo de tiempo correspondiente, sin limitar su acceso) y el atracón, que es un acceso limitado e intermitente. Este último patrón se ha demostrado que induce un consumo de grasa en forma de atracones (Puhl y cols., 2011; Bocarsly y cols., 2011). En el protocolo de administración intermitente los ratones tienen acceso a la dieta rica en grasa solo durante 2 horas los lunes, miércoles y viernes con un acceso ilimitado a la dieta estándar.

Como metodología principal para evaluar la respuesta a la cocaína y el alcohol utilizamos los paradigmas de Condicionamiento de la Preferencia de Lugar y el procedimiento de Autoadministración operante. El Condicionamiento de la Preferencia de lugar es el paradigma más utilizado para medir el efecto de las claves ambientadas asociadas con los efectos reforzantes de las drogas (Aguilar et al., 2013). Por otro lado, el modelo de autoadministración operante evalúa directamente la motivación del animal por conseguir la sustancia. Ambos modelos proporcionan una evaluación completa de los efectos reforzantes de las drogas, ya que nos permiten medir tanto las claves individuales como las externas.

También hemos estudiado los efectos metabólicos que estos tipos de dieta grasa producían en los niveles plasmáticos de leptina, grelina y corticosterona. Igualmente, hemos estudiado mediante la reacción en cadena de la polimerasa (PCR) en tiempo real, la expresión de genes pertenecientes a los sistemas dopaminérgico, cannabinoide y opioide, debido a su implicación tanto en el refuerzo inducido por las drogas como por la comida.

En cuanto a los resultados obtenidos, en el primer estudio hemos observado que, aunque no hay cambios en ninguna de las conductas estudiadas mientras se mantiene el consumo de grasa, el cese de esta dieta produce un incremento en el nivel de ansiedad, aumenta la respuesta aguda a la cocaína y produce condicionamiento de la preferencia de lugar con dosis subumbrales de cocaína. Todo esto se acompaña por cambios a nivel de expresión del gen para el receptor opioide mu, el cannabinoide CB1 y GHSR.

Por otro lado, observamos el poder de la dieta rica en grasa como refuerzo alternativo, ya que cuando la administramos tras condicionar a los animales con una dosis efectiva, éstos tardan menos en extinguir la preferencia y no presentan reinstauración con dosis con las que el grupo control sí reinstaura la preferencia de lugar.

En el segundo estudio hemos evaluado el efecto de realizar atracones de una dieta rica en grasa y hemos observado que provocan un aumento en la sensibilidad a dosis subumbrales de cocaína en el condicionamiento de la preferencia de lugar y se autoadministran más cocaína que el grupo con dieta estándar. Por otro lado, observamos también cambios en la expresión de los genes responsables de los receptores opioide mu, cannabinoide CB1 y de la grelina GHSR.

En el tercer estudio realizamos el mismo procedimiento que en el estudio anterior, pero con el fin de evaluar si este tipo de administración de dieta rica en grasa también incrementaba la sensibilidad al alcohol. De nuevo encontramos que los animales que realizan atracones son más sensibles a dosis subumbrales de alcohol y se autoadministran más que el grupo con dieta estándar. Además, presentan cambios a nivel de expresión de genes tras la autoadministración, diferentes a los obtenidos con la simple administración de la grasa en estudios anteriores.

En el cuarto estudio evaluamos si estos efectos sobre la ingesta de alcohol tenían un efecto a largo plazo, incluso habiendo cesado el consumo de grasa. Observamos cómo los animales que realizaron atracones durante la adolescencia y llevaban 15 días sin acceso a la grasa seguían presentando un incremento en la autoadministración de alcohol, pero ya no eran más sensibles a los efectos reforzantes de una dosis subumbral de alcohol en el condicionamiento de la preferencia de lugar.

En el quinto estudio evaluamos el papel que tiene un estrés crónico, como es el aislamiento, en la modulación que produce la dieta rica en grasa en atracón sobre los efectos reforzantes de la cocaína. Observamos resultados opuestos a los obtenidos con animales agrupados, ya que los animales aislados con

una dieta estándar fueron más sensibles a dosis subumbrales de cocaína que los que realizaron atracones de dieta rica en grasa. Además, los grupos que desarrollaron la preferencia (aislados dieta estándar y agrupados dieta atracón grasa) también presentaron niveles incrementados de leptina.

En el sexto y último estudio trazamos un perfil conductual con todos los grupos que hemos utilizado en la presente tesis doctoral, observando que los animales que realizan atracones de grasa presentan pocas alteraciones conductuales. Únicamente exhiben un incremento en la actividad locomotora y conductas agresivas en la interacción social con un oponente. Sí que observamos déficits a nivel de aprendizaje espacial con los animales que comen comida rica en grasa de forma continuada y además de un comportamiento agresivo. En ambos tipos de dieta, el cese del consumo produce un incremento en el nivel de ansiedad.

Los resultados obtenidos nos indican el consumo de una dieta alta en grasa de forma continuada produce efectos muy diferentes a los producidos por el consumo intermitente en atracón. Mientras que el consumo en forma de atracón no produce grandes efectos metabólicos, incrementa la sensibilidad a los efectos reforzantes del alcohol y la cocaína. Sin embargo, el consumo de grasa de forma continuada produce grandes cambios metabólicos, como hiperleptinemia y aumento de peso, pero no afecta la respuesta a la cocaína. Sin embargo, tras el cese en el consumo continuado de una dieta alta en grasa, aparece un incremento en la respuesta a los efectos condicionados reforzantes de la cocaína, confirmando así que el sistema de refuerzo ha quedado sensibilizado tras el consumo de grasa. Ambos tipos de dieta alta en grasa produce cambios en la expresión génica de los receptores opioide mu, cannabinoide CB1 y del receptor de grelina GHSR, indicando que ambos patrones de consumo de grasa modifican de forma diferente la función del sistema de refuerzo cerebral, produciendo consecuencias a largo plazo, incluso cuando la comida rica en grasa ya no está disponible.

Nuestro trabajo demuestra que los hábitos nutricionales no solo producen alteraciones metabólicas en nuestro organismo o modifican nuestro peso corporal. También modifican nuestro SNC y alteran la forma en que

respondemos a las drogas de abuso. Una de las principales aportaciones de este trabajo es la demostración de que la dieta grasa consumida de forma continuada, que induce grandes cambios metabólicos y obesidad, así como alteraciones conductuales, no parece modular la respuesta a las drogas hasta que esta deja de consumirse, momento en el que observamos una respuesta incrementada. Sin embargo, los atracones de grasa, que no alteran metabólicamente al sujeto, ni modifican su peso corporal o su conducta, sí que incrementan la respuesta a drogas como la cocaína o el etanol, incluso cuando ya ha dejado de consumirse. Por lo tanto, esperamos que los artículos que componen la presente Tesis Doctoral ayuden a incrementar el conocimiento y la conciencia sobre la importancia de los hábitos nutricionales en el abordaje de un problema multifactorial como es la adicción a las drogas.

## ABSTRACT

Adolescence is a highly vulnerable developmental period in which numerous structural and functional maturational changes occur (Spear, 2000; Chambers et al., 2003). In this early stage of vulnerability, adolescents exhibit eating disorders, substance abuse, novelty-seeking and risk-taking behaviors (Bava and Tapert, 2010). Regarding these environmental hazards, the rise in obesity rates worldwide has encouraged extensive research to improve understanding of this problem, in which the excessive intake of food, especially sugar-rich and high-fat food has become a serious problem for society.

As we know, addiction is a chronic and multifactorial relapsing disorder resulting from an interaction of biological and environmental aspects, characterized by a loss of control in the use of the substance and relapse (Koob and Volkow, 2010). Developmental factors are important components of vulnerability, with strong evidence supporting that exposure to drugs of abuse during adolescence leads to a significantly higher likelihood of drug dependence and drug-related problems in adulthood.

Feeding is regulated by homeostatic and hedonic mechanisms. Abnormalities in these functions can cause several feeding alterations and produce obesity (Kenny et al., 2011). The reward system, which is essentially constituted by the dopamine mesocorticolimbic system, regulates motivation to seek and take rewarding stimuli, such as drugs or highly palatable food. Therefore, drug abuse or hedonic eating activate the same reward pathways. Our body was initially developed in a context characterized by nutritional deficiencies and getting food was the daily main objective to achieve. It processes foods quickly and we have innate preference for high energy meals, especially those high in fat and sugar, because in a situation of scarcity those would be the energy stores that would help us to survive. This type of food, like drugs of abuse, produces a dopamine release in the brain reward system, which explains its pleasurable effects. Nowadays, there is such abundance

and variety of food, that this survival-oriented evolutionary adaptation has lost its sense, since we eat not only for hunger, but also for pleasure (Gold, 2011). Thus, hedonic eating affects neural mechanisms connected with reward and maintains this behavior (For reviews: Avena et al., 2008; Volkow et al., 2013). In summary, both natural reinforcers and drugs stimulate the brain reward circuit, producing pleasure and euphoria.

The nutritional status is an important factor in the development of addiction, since some studies indicate psychological and biological similarities between fast food intake and addiction to drugs, sharing common reward mechanisms (Garber and Lustig, 2011; Avena et al., 2012). For example, both drug addiction and obesity can be defined as disorders in which the value of the type of reinforcement (drug or food, respectively) is abnormally increased in relationship to other reinforcements (Avena et al., 2012; Volkow et al., 2013). Drug addiction and binge eating are characterized by a loss of control over consummatory behaviors, exhibiting escalation, dependence and craving when the reward is not available, and both present a high comorbidity (Swanson et al., 2011).

Several studies report that drug use during adolescence often predicts an increased likelihood of continued use into adulthood (Arteaga et al., 2010; Merline et al., 2004). Based on these relationships, a *Theory of Gateway* has also been proposed for eating disorders and substance abuse (Degenhardt et al., 2008), in which it is postulated that eating disorders, such as binge eating, can lead to the development of another desadaptive behavior, such as drug abuse. At the moment, preclinical studies indicate, for example, that intake of certain types of sugar leads to a sensitization to amphetamine (Avena and Hoebel, 2003) and to an increase in alcohol self-administration (Avena et al., 2004).

Studies with sugar are abundant, unlike studies with high-fat food and drug vulnerability. For example, there are no studies reporting the effects of bingeing on fat during adolescence and vulnerability to drug abuse, like cocaine or alcohol, nor are there studies examining the effects of interrupting

fat consumption on the subsequent vulnerability to drugs like cocaine or alcohol.

Thus, the objective of the present work was to evaluate how a high-fat diet exposure during adolescence modulates the reinforcing effects of cocaine and alcohol. We studied two different types of fat diet consumption: continuous access (animals have *ad libitum* access to high-fat food without a limit) and the intermittent and limited access. The latter has been shown to induce a binge eating pattern (Puhl and cols., 2011; Bocarsly et al., 2011), and our protocol is based on that used by Corwin et al. (1998). Here, mice had access to the high-fat diet only for 2 hours every Monday, Wednesday and Friday, with ad libitum access to the standard diet.

The main methodology employed to assess the vulnerability to cocaine and alcohol rewarding effects was the Conditioned Place Preference paradigm and the operant Self-Administration procedure. The conditioned place preference is the most commonly used test to evaluate the environmental cues conditioned to the rewarding effects of the drug (Aguilar et al., 2013), while the self-administration procedure evaluates directly the animals' motivation to obtain the substance of abuse. Both models provide a complete scenario to evaluate vulnerability to drug abuse, since they allow us to measure both the external and the individual cues.

We have also studied some metabolic effects on circulating leptin, ghrelin and corticosterone levels and examined changes in gene expression with real time polymerase chain reaction (rt-PCR) related to dopaminergic, opioid and endocannabinoid systems, because of their involvement in the rewarding properties of drugs and food.

Regarding our results, in the first study we observed no effects on the behavioral response to drugs while fat is available. However, following fat discontinuation, mice exhibited increased anxiety, augmented locomotor response to cocaine, and developed preference for subthreshold doses of cocaine. In addition, there were changes in MOr, CB1 and GHSR gene expression. On the other hand, we observed how high-fat diets act as

an alternative reinforcer, as its administration after conditioned place preference reduces the number of sessions required to extinguish the preference and decreased sensitivity to drug priming-induced reinstatement.

In the second study, we observed that animals that binged on fat were more sensitive to the reinforcing effects of a subthreshold dose of cocaine in the conditioned place preference and enhanced self-administration. We also observed changes in MOr, CB1 and GHSR gene expression.

In the third study, we employed the same procedure as in the former study, but with the aim of evaluating if the increased sensitivity also arises with alcohol. Animals in the high-fat binge group presented more sensitivity to the rewarding effects of subthreshold doses of ethanol and greater ethanol consumption with a higher motivation to obtain the drug. In addition, they presented changes in gene expression after the self-administration procedure which are different from those found with the fat administration alone.

In the fourth study, we evaluated if these effects of bingeing on fat and alcohol intake would have a long term consequences, even when fat consumption is interrupted. Our results showed how after 15 days from the last binge session, animals still presented a greater ethanol consumption, but they were not sensitive to subthreshold doses in the conditioned place preference anymore.

The fifth study aimed to evaluate the role of chronic stress, such as isolation, on the modulatory effects of fat on the rewarding properties of cocaine. We observed opposite effects as those found in grouped animals, as isolated mice with a standard diet access were more sensitive to subthreshold doses of cocaine than those fed with a fat binge eating pattern. In addition, the groups that developed preference (isolated with standard diet and grouped with high-fat binge) were those who presented increased circulating leptin levels.

In the sixth and last study, we drew a behavioral profile with the groups

used in the present doctoral thesis, and found a few alterations in animals that binge on fat, such as hyperlocomotion and aggressive behaviors. On the other hand, we observed marked spatial learning deficits in animals eating fat continuously as well as increased attack behaviors with conspecifics. In both diet patterns, discontinuation of fat led to an increase in anxiety levels.

Our results show that bingeing on fat is quite different from eating fat continuously. While the former does not produce great metabolic effects, it produces significant changes in the sensitivity to drugs such as alcohol and cocaine. On the other hand, continuously eating fat produces big metabolic changes like hyperleptinemia and increased bodyweight and but has no effect regarding vulnerability to drug use. However, when access to fat is interrupted (withdrawal period), the increased response to the rewarding effects of drugs arises, confirming that the reward system has become sensitized. Both fat consumption patterns produce several changes in gene expression of mu opioid receptor, of cannabinoid receptor CB1 and of ghrelin receptor GHSR, indicating that both patterns of fat consumption changed the reward system function in different manners, having long-lasting effects, even when fat is no longer available.

Our work shows that the nutritional habits not only produce metabolic alterations in our organism or modify our body weight, they also modify our CNS and change the way we respond to drug abuse. One of the main contributions of this work is the demonstration that consuming a high-fat diet on a continuous form, which induces several metabolic changes and obesity, as well as behavioral alterations, does not seem to modulate the drug sensitivity until fat consumption is interrupted. At this moment, we observe an increased response to cocaine. However, bingeing on fat does not modify metabolism or bodyweight, but it increases the sensitivity to drugs like cocaine or ethanol, even when fat is no longer available. Therefore, we hope that the studies that constitute this Doctoral Thesis help to increase knowledge and awareness about the relevance of nutritional habits on the intervention in the multifactorial disorder that is drug addiction.

PREFACE

This Doctoral Thesis is based on the following six studies:

- Blanco-Gandía MC, Aracil-Fernández A, Montagud-Romero S, Aguilar MA, Manzanares J, Miñarro J, Rodríguez-Arias M, (2017). Changes in gene expression and sensitivity of cocaine reward produced by a continuous fat diet. *Psychopharmacology, 2017* doi: 10.1007/s00213-017-4630-9 **(Study 1)**
- Blanco-Gandía MC, Cantacorps L, Aracil-Fernández A, Montagud-Romero S, Aguilar MA, Manzanares J, Valverde O, Miñarro J, Rodríguez-Arias M. (2017) Effects of bingeing on fat during adolescence on the reinforcing effects of cocaine in adult male mice. *Neuropharmacology* 113:31-44. doi: 10.1016/j.neuropharm.2016.09.020 **(Study 2)**
- Blanco-Gandía MC, Rodríguez-Arias M (2017). Bingeing on fat increases cocaine reward. *Oncotarget*, 8 (10), pp 16105-16106 doi: 10.18632/oncotarget.15260 **(Study 2)**
- Blanco-Gandía MC, Ledesma JC, Aracil – Fernández A, Navarrete F, Montagud-Romero S, Aguilar MA, Manzanares J, Miñarro J, Rodríguez-Arias M (2017). The rewarding effects of ethanol are modulated by binge eating of a high-fat diet during adolescence. *Neuropharmacology, 2017* doi: 10.1016/j.neuropharm.2017.04.040 **(Study 3)**
- Blanco-Gandía MC, Aguilar MA, Miñarro J, Rodríguez-Arias M. Ethanol consumption is enhanced 15 days after fat binge eating interruption. *In preparation* **(Study 4)**
- Blanco-Gandía MC, Montagud-Romero S, Aguilar MA, Miñarro J, Rodríguez-Arias M. Housing conditions modulate the reinforcing properties of cocaine in adolescent mice that binge on fat.

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## ABBREVIATIONS

| | |
|---|---|
| 2-AG | 2-arachidonoylglycerol |
| AgRP | Agouti-related protein |
| Anandamide | N-arachidonoylethanolamine |
| CART | Cocaine-amphetamine regulated transcript |
| CB1 and CB2 | Cannabinoid receptor 1 and 2 |
| CCK | Cholecystokinin |
| CPP | Conditioned Place Preference |
| D1, D2, D3 | Dopamine receptors 1, 2 and 3 |
| DA | Dopamine |
| DAT | Dopamine transporter |
| DSM-V | Diagnostic and Statistical Manual of Mental Disorders 5$^{th}$ ed. |
| ECS | Endocannabinoid system |
| EtOH | ethanol |
| GABA | gamma-Aminobutyric acid |
| GHSR | Growth hormone secretagogue receptor |
| HFD | High-fat diet |
| HFB | High-fat binge |
| KO | Knockout |
| L-DOPA | Levodopa |
| MCH | Melanin concentrating hormone |
| mRNA | Messenger Ribonucleic acid |
| NAcc | Nucleus Accumbens |
| NPY | Neuropeptide Y |
| PFC | Prefrontal Cortex |
| PND | Postnatal Day |
| POMC | Proopiomelanocortin |
| PYY | Peptide YY |
| rt-PCR | Real time Polymerase Chain Reaction |
| SA | Self-Administration |
| TH | Tirosine hidroxilase |
| THC | delta-9-tetrahydrocannabinol |
| VTA | Ventral Tegmental Area |
| WHO | World Health Organization |

# 1. INTRODUCTION

# 1. General Introduction

# 1. General 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. Moreover, factors contributing to increasing 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).

Among the factors that contribute to an 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 prevalent high-fat, "fast-food" culture, rising rates of obesity in developed countries, particularly among adolescents (Baladi et al., 2012; Herpertz-Dahlmann, 2015; Volkow et al., 2013). Feeding behavior is regulated by the homeostatic and the hedonic systems. Malfunction of any one of these systems can lead to overeating and obesity (Kenny et al., 2011). The hedonic or reward circuit constituted by the mesocorticolimbic dopaminergic pathway regulates the motivation to seek or consume rewarding stimuli such as drugs or palatable foods. Therefore, consumption of drugs of abuse and hedonic eating, besides sharing a high comorbidity, activate common reward pathways.

Obesity is a serious problem worldwide and the second cause of death, despite being preventable, with overeating of palatable and energy-dense foods contributing to the epidemic (WHO, 2015). Preclinical studies have provided robust evidence confirming that free access to a high-fat 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 (NAcc) and other reward centers (Kelley et al., 2005; Rada et al., 2005; Narayanaswami et al., 2013), thereby decreasing the dopamine transporter (DAT) density (Huang et al., 2006). Over time, striatal dopamine (DA) D2 receptors

become downregulated in obese rats (Davis et al., 2008; Johnson and Kenny, 2010). Several reports suggest that continuous access to fat diminishes the reinforcing efficacy of cocaine, attenuating the conditioned place preference (CPP) induced by cocaine, food, and amphetamine (Morales et al., 2012; Davis et al., 2008). Likewise, Osborne-Mendel rats, genetically prone to obesity, do not develop a preference for cocaine in the CPP (Thanos et al., 2010). Diminished acquisition of cocaine self-administration (SA) and an attenuation of operant response to sucrose were also described after continuous access to a fatty diet (Wellman et al., 2007).

Among other disorders, binge eating is highly common (Hudson et al., 2007). The DSM-V 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-V; American Psychiatric Association, 2013), and 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).

Similarly to drugs of abuse, withdrawal from and craving for specific kinds of food have also been observed in humans (Rogers and Smit, 2000). Pickering and coworkers (2009) reported that obesity-prone animals, chronically fed a palatable diet that was drastically interrupted, exhibited signs of craving, increased anxiety and symptoms of withdrawal. Several studies showed that sugar consumption leads to a withdrawal syndrome similar to that which occurs under opiate removal (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 HFD led to an increased stress response and the drive to seek palatable food.

To date, there is strong evidence of sugar dependence in animal models of sugar bingeing, as there are changes in limbic system neurochemistry that are similar for both drugs and sugar. In clinical literature, when one drug

leads to the consumption of another, it is referred to as a "gateway effect", for example when a legal drug (e.g. nicotine) acts as a gateway to an illegal drug (e.g. cocaine) (Lai et al., 2000). Sugar acts as a gateway to drugs like alcohol or cocaine (Avena et al., 2004; Carroll et al., 2007). However, there are few studies that explore the role of fatty foods and specifically the role of bingeing on fat as a gateway to drugs of abuse. Thus, the aim of the present work was to evaluate the effects of a HFD consumed during adolescence either in a binge pattern, or in a continuous form, on the rewarding properties of cocaine and alcohol. In addition, we explored the metabolic consequences derived from this by measuring the circulating leptin and ghrelin levels and the role of the dopaminergic, endocannabinoid and opioid systems.

## Risk factors in addiction

As with other disorders, humans do not have the same risk of developing an addiction. There are many different factors, characteristics and variables involved in the addictive process which can determine the individuals' vulnerability to develop this disease. In this section, we will discuss the two main variables that can lead to the development of cocaine or alcohol addiction, such as adolescence or eating patterns.

# 2. Adolescence

## 2. Adolescence

Adolescence is the gradual transition from childhood to adulthood, a critical period of brain maturation marked by numerous cognitive, behavioral and biological changes that are related to major changes in emotional and cognitive functions (Pickles et al., 1998, Spear, 2000; Bava and Tapert, 2010).

In both humans and animals, adolescence is a period generally co-occurring with puberty (i.e. with the development of reproductive functions), which marks the beginnings of sexual maturation (Graber and Brooks-Gunn, 1998; Laviola et al., 2003). Evolutionarily, this is the period in which the individual acquires the independence skills to increase success upon separation from the family (Kelley et al., 2004). Independence seeking behaviors are prevalent across species, exhibiting certain common behaviors, such as peer-directed social interactions, novelty-seeking and risk-taking behaviors (Spear, 2000). According to Steinberg (2004), risky behaviors are the product of a biologically driven imbalance between increased novelty and sensation-seeking in conjunction with immature self-regulatory competence.

In rodents, the term 'adolescence' covers the whole postnatal period, ranging from weaning (Postnatal Day (PND) 21) to adulthood (PND 60), and is to be considered no longer childhood, but not yet adulthood. It is divided into three different periods: early adolescence (prepubescent or juvenile, PND 21-34), middle adolescence (periadolescent, PND 34-46), and late adolescence (young adult, PND 46-to-59) (Spear, 2000).

### 2.1. The adolescent brain

The brain continues to develop throughout adolescence and into adulthood, undergoing important structural and functional changes in synaptic plasticity (Giedd, 2004; 2008). These changes involve certain neurotransmission systems and hormones, refining some brain areas and pathways. Dynamic processes allow the brain to specialize and sharpen its functions for the specific environmental demands. At a cellular level, there are changes in gray and white matter, appearing to be related to an overproduction of connections in early puberty with rapid pruning in late adolescence, which

reflect a reorganization (Giedd et al., 1999; Sowell et al., 2004; Fields and Stevens-Graham, 2002). This developmental period is full of synaptic plasticity (Chambers et al, 2003), but there is also an imbalance between the increased sensitivity to motivational cues and the still-maturing inhibitory control system (delayed maturation of the prefrontal cortex (PFC)), which leads to an elevated activation of reward-relevant regions and attenuated sensitivity to aversive stimuli, turning out in a behavior biased toward risk and emotional reactivity (Sturman and Moghaddan, 2011).

2.2. Brain maturation stages

There are three main processes covering the brain maturation stages until adulthood (Lenroot and Giedd, 2006): proliferation, where a rapid gray matter growth and new connections occur (Giedd et al., 1999); pruning, where new synaptic connections are established, while others are pruned back to reflect a decrease in the gray matter volume (Bourgeois and Rakic, 1993; Shaw et al., 2008; Tamnes et al., 2010), and lastly, myelination, where myelinated axons speed up the communication among neurons and make more stable connections (Perrin et al, 2009; Sowell et al., 2001). Reviews from Spear (2004; 2007) corroborated that the ontogenetic transition patterns and physiological and behavioral characteristics of adolescents are common among mammalian species.

The adolescent brain experiences great maturity changes, such as a massive loss of gray matter and synapses in the neocortex (Gogtay et al., 2004), the latter being the most characteristic change produced on the PFC. Here, excitatory synapses undergo pruning, decreasing NMDA receptor activity and the extension of glutamatergic stimulation to the cortex (Insel et al., 1990). The balance of excitatory and inhibitory neurotransmission varies between adolescents and adults, suggesting that the increased inhibition is related to the development of the PFC, which promotes greater neural coordination (Sturman and Moghaddam, 2011). The pruning process during adolescence is very specific, resulting in a loss of approximately 50% of the synaptic connections in some regions (Spear, 2013). Myelination and synaptic pruning help to reconfigure and re-wire brain connections into the

adult patterns, contributing to the thinning of the neocortex. Thinning of cortical gray matter regions occurs at the same time as myelination of white matter tracts, decreasing relative gray matter to white matter volume (Tau and Peterson, 2010).

Limbic systems develop earlier than prefrontal control regions (Casey et al., 2008). According to Caseys' model, during adolescence, the individual is biased by an imbalance in the limbic control relative to the prefrontal one, compared to children, for whom these systems are both still developing and, compared to adults, for whom these systems are fully mature. According to this, in emotionally salient situations, the limbic system will win over the control systems, given its maturity relative to the prefrontal system. There is ample supportive evidence from behavioral and human studies in agreement with this model (Galvan et al., 2006; 2007, Hare et al., 2008). Thus, the adolescent brain operates in a promotivational state as a result of a limited inhibitory capacity, poor control regulation, an hyperactivity of the amygdala and DA hyperactivity in the NAcc when processing appetitive stimuli (Rodríguez-Arias and Aguilar, 2012) (Fig 1.)

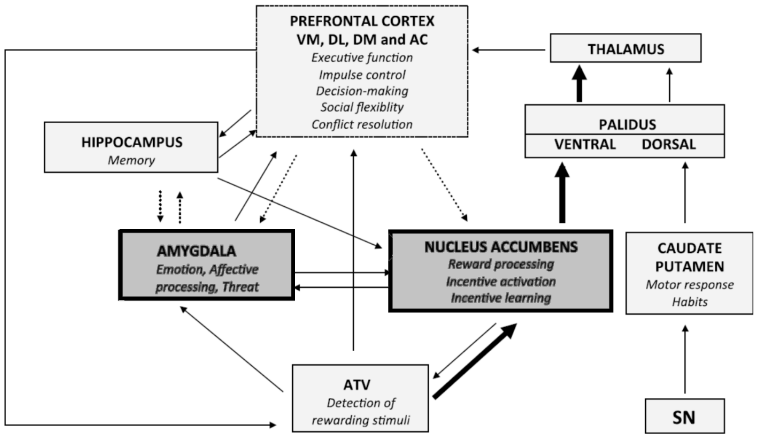


Fig. 1. Neural circuit involved in motivated behavior. Thick lines represent hyperactive brain areas or connections and thin or dashed lines represent brain areas that are more hypoactive in adolescent subjects than in adults (Modified from Rodríguez-Arias and Aguilar, 2012).

All of this provides the opportunity to sculpt the adolescent brain by experiences, nutrient exposure or restriction, or developmental adversities (Gutman and Nemeroff, 2003; Hensch, 2004; Taylor and Poston, 2007). As we mentioned above, adolescents are especially vulnerable to environmental threats, such as stress, drug abuse or inadequate dietary habits (Cruz, 2000; Ifland et al., 2009; Bava and Tapert, 2010). Modifications in the limbic and cortical regions of the adolescent brain lead to behaviors such as hyperphagia and substance abuse, novelty-seeking and risk-taking (Sturman and Moghaddam, 2011; Spear, 2011). Adolescence is generally considered to be a stressful stage of life, and individuals are more likely to perceive events as stressful at this age, exhibiting higher rates of depressed mood, sleep problems, emotional instability and anxiety (Buchanan et al., 1992). They are laid open to a less positive impact from stimuli with moderate to low incentive value, and thus pursue further appetitive reinforcers (Spear, 2000). Novel stimuli, exciting and risky situations and drugs of potential abuse affect the reward circuitry, which is involved in seeking and finding rewards such as food, water, warmth, sexual partners, and other social stimuli (Nesse and Berridge, 1997).

### 2.3. Adolescence and sensitivity to reward

Behavioral sensitivity to rewards has often been described to peak during adolescence and then gradually decline during adulthood (Steinberg et al., 2009, Steinberg 2010). Regarding reward and the dopaminergic system, there is a temporary decrease in the efficacy of the mesolimbic DA projections. Sensitivity to a basic reward such as that of a sweet substance is higher at 11-15 years of age than during late adolescence and emerging adulthood (19-25 years) (Desor and Beauchamp, 1987). This enhanced reward responsivity has been also obtained using simple animal models, finding that adolescent rats were more sensitive than adult ones to the rewarding effects of a wide range of stimuli, such as social interaction, novelty and drugs of abuse (Doremus-Fitzwater et al., 2010). During adolescence, there is a reduced basal rate of DA release with a higher uptake and lower DA release when compared to adults (Stamford, 1989). Interestingly, adolescent

rats have a larger DA storage pool when compared to adults, suggesting that despite the reduced DA release in basal conditions, they can release much more DA if it is stimulated (Laviola et al., 2001). The immaturity of the DA neurotransmission may underlie to the enhanced acute response to psychostimulants in adolescents (Walker and Kuhn, 2008). For example, adolescent rats are more sensitive than adult ones in the cocaine induced CPP and DA release (Badanich et al., 2006). Although the complexity of the human brain and behavior cannot be extrapolated to experimental animals, numerous similarities are found between human adolescents and adolescents of other species. Thus, it is reasonable to support the use of animal models to study the neurochemical and behavioral consequences of feeding on the rewarding effects of drugs of abuse. Drug use during adolescence often predicts an increased likelihood of continued use into adulthood (Arteaga et al., 2010; Merline et al., 2004). This period can foster early experimentation with drugs, as addictive substances are generally more rewarding and less aversive (Schramm-Sapyta et al., 2009). To date, alcohol is the first drug of choice among teenagers. For example, exposure to ethanol (EtOH) binge drinking during this period sensitizes brain regions and developmental processes that are involved in drug addiction behaviors (Pascual et al., 2009) and produces short- and long-term consequences, such as memory impairment and neural cell death in several brain regions (Pascual et al., 2007), which are mostly irreversible (Guerri, 2002). In this line, there is also evidence supporting that the rate of progression to drug dependence in adolescents is unusually fast. Escalation of cocaine use is faster among adolescents than adult users, suggesting a greater addictive potential of cocaine during adolescence than in adulthood (Estroff et al., 1989). Comparing adolescents vs. adults, adolescents show shorter times from first exposure to dependence for alcohol and cannabis, and shorter times between their first and second dependencies (Clark et al., 1998).

## 2.4. Adolescence and dietary habits

Human behavior is always reward-oriented and can lead to pleasurable and positive consequences if accomplished, such as satisfying hunger, etc.

However, reward seeking can be maladaptive and lead to risky decisions with potentially long-term consequences (e.g. drug use, unprotected sex). This risky decision making is very common among adolescents, a period in which the brain reward system is immature (Fareri et al., 2008).

Early adolescence is a key stage during development, where there are new neuronal connections arising in which DA signaling plays a big role (Jia et al., 2013). Consequently, there is a high level of vulnerability to several disorders, such as suffering from stress, substance abuse and dietary deficits. In addition, adolescence is the peak time for the onset of several psychiatric disorders such as schizophrenia, substance abuse disorders and mood disorders, all of them related with an abnormal DA signaling (Paus et al., 2008).

With a growing high-fat "fast food" culture and the prevalence of obesity, nowadays it is worrying how adolescents are constantly being overexposed to this kind of foods. As expected, abnormal dietary habits along with lower levels of physical activity result in an increase in the overweight adolescent population that become obese adults. Apart from the risk of developing obesity-derived cardiovascular diseases such as diabetes, it is important to consider that adolescents are more prone than adults to developing eating disorders, such as anorexia, bulimia and binge eating (Klump, 2013). These behaviors are mediated by environmental and psychosocial factors, such as increased body dissatisfaction, decreased self-esteem, etc. So, this stage of life is a key period to prevent future maladaptive behaviors.

Adolescent rats exhibit the greatest caloric intake relative to their bodyweight throughout their lifespan (Nance, 1983). Likewise, adolescent humans exhibit developmental hyperphagia and have elevated metabolic activity (Ganji and Betts, 1995; Post and Kemper, 1993). Given that the neural substrates that modulate reward of drugs of abuse are the same that regulate other motivational stimuli like food and water, it is possible that the physiological mechanisms that produce this hyperphagia are conducted by the same pathways.

# 3. Food and the reward system

## 3. Food and the reward system

Overeating and obesity are modern global diseases of our society, and both are preventable. For many years, malnutrition was a common problem. Now, according to the last report of the World Health Organization (Fact Sheet 311, WHO 2015), the worldwide obesity rate has more than doubled since 1980, and it now kills more people than undernourishment. In 2014, 39% of the world population over 18 years of age was overweight, and within this, 13% was obese. This problem becomes especially critical in children and young people, who are more vulnerable to inadequate dietary habits, and they are overexposed to high-fat, high-sugar, high-salt, energy-dense foods with lower nutrient quality. In 2013, 42 million children under the age of 5 worldwide were overweight or obese. Regarding these environmental hazards, the rise in obesity rates worldwide has encouraged extensive research to improve our understanding of this problem, namely the excessive intake of food, especially sugary and fatty foods, which have become a serious problem for our society. The major focus of research is to better understand the main homeostatic mechanisms regulating overeating (Kobeissy et al., 2008). Gold (2011) defined 'hedonic eating' as eating based on pleasure rather than energy needs. Recent research shows that hedonic eating modulates neural mechanisms related to reward processing, and therefore, maintains this behavior (For reviews: Avena et al., 2008; Volkow et al., 2013).

Nutrition is crucial to maintain adequate energy stores necessary for survival in all species of the animal kingdom. In mammals, there is a requirement to keep a stable body temperature, and having a high metabolic rate requires constant availability of large amounts of energy storage. This is the reason why mammalian brains have evolved to develop several neuronal systems that drive feeding behavior, and certainly one of the most potent drives for feeding is its rewarding nature (Saper et al., 2002).

In this section, we will examine the homeostatic and hedonic regulation of feeding, in order to better understand the systemic mediators such as leptin or ghrelin. Following that, we will examine the reward mechanisms that give

food its reinforcing properties and, finally, we will explore the interaction between both systems. Thus, to understand the rewarding nature of food better, it is necessary to consider first the different homeostatic sensory mechanisms that support the drive for feeding.

### 3.1. Homeostatic mechanisms of feeding

The brain has an essential circuitry that regulates nutritional status and body stores. When the brain detects alterations in energy stores it activates metabolic and behavioral responses designed to maintain energy balance. Energy homeostasis is controlled mainly by neuronal circuits in the hypothalamus and brainstem, whereas reward and motivation aspects of eating behavior are controlled by neurons in the limbic regions and the cerebral cortex. The hypothalamus is the brain structure involved in controlling the homeostatic status of the organism and it is the main regulating center of food and water intake (Seeley et al., 2003). In 1951, it was reported that lesions produced in this area in rats induced a decrease in eating and drinking (Anad et al., 1951). Electrical stimulation studies showed that exciting this area resulted in an increase in water and food intake (Delgado et al., 1953; Coons et al., 1968). Lately, Hernández and Hoebel (1988) showed that the hypothalamus is not only involved in food and water intake regulation, but it is also involved in reward processes (Hoebel et al., 1988). Leptin and insulin signaling into the hypothalamus regulate the energy balance to maintain bodyweight and food intake. Several nuclei constitute the hypothalamus, such as the arcuate nucleus, the paraventricular nucleus, the lateral hypothalamus, the dorsomedial hypothalamus and the ventromedial hypothalamus (Schneeberger et al., 2014). The lateral hypothalamus contains two groups of neurons that stimulate hunger signals: Neurons that secrete melanin concentrating hormone (MCH) and neurons that secrete orexins.

The arcuate nucleus stands out as the regulating center for energetic homeostasis and contains neurons that are sensitive to leptin signaling. These neurons are divided into two populations: neurons that produce orexigenic (hunger) neuropeptides, such as Neuropeptide Y (NPY) and

Agouti-related protein (AgRP) (Gehlert et al., 1987; Graham et al., 1997) and neurons that synthesize anorexigenics (satiety), such as Cocaine-amphetamine regulated transcript (CART) and Proopiomelanocortin (POMC) (Mercer et al., 2013; Elias et al., 1998). Finally, Cholecystokinin (CCK) and Peptide YY (PYY) are both anorexigenic peptides produced by digestive cells in the gastrointestinal tract. The former was the first gut-secreted peptide to be identified as a satiety factor and it decreases meal size via vagal nerves Liebling et al., 1975; Kraly et al., 1978), while the latter has inhibitory effects on the NPY neurons of the arcuate nucleus. When PYY binds to its receptor, the release of NPY and AgRP is eliminated (Ahima and Antwi, 2008).

Leptin acts on these neurons by inhibiting the orexigenic neurons and stimulating the anorexigenic ones. Therefore, we will outline this circuitry briefly. Leptin was discovered in 1994 (Zhang et al., 1994), finding that the ob gene encoded a hormone that was secreted by the adipose tissue. Blood leptin levels increase when animals are fed and fall when animals are deprived from food (Frederich et al., 1995, Maffei et al., 1995). Leptin receptors have the densest expression in the ventral basal hypothalamus (Mercer et al., 1996; Schwartz et al., 1996), especially the arcuate nucleus, ventromedial, dorsomedial and ventral premammalliary nuclei in the hypothalamus. Furthermore, leptin receptors are also expressed in several extrahypothalamic sites, such as the brainstem. Leptin can push the balance from the NPY/AgRP hunger system to the POMC/CART satiety system and inhibiting orexins and MCH (Fig.2).

Ghrelin is another hormone that acts in concert with leptin, having been discovered more recently (Horvath et al., 2001). It is involved in hunger and meal initiation (Cummings et al., 2001). In 1985, McCormick and co-workers were screening small molecules for their ability to increase growth hormone secretion. The ghrelin receptor is the growth hormone secretagogue receptor 1A (GHSR 1A), which was found to mediate appetite and energy balance, and also activated the neuropeptide Y in the same region as the growth hormone (Guan et al., 1997). Ghrelin is the endogenous ligand

to the GHS receptor and it is synthesized in the stomach when animals are food deprived, as a hunger signal (Kojima et al., 1999). It also increases food intake (Wren et al, 2000) and has pro-obesity effects (Tschöp et al., 2000). Ghrelin receptors are found in the ventromedial hypothalamus and arcuate nuclei (Horvath et al., 2001), but they are also located in other sites that are still being investigated. For example, NPY/AgRP neurons in the arcuate nucleus express ghrelin receptors (Willesen et al, 1999). It exerts its effects on appetite and metabolism by stimulating receptors located in neurons releasing NPY and AgRP (Ahima and Antwi, 2008).

The interaction between these opposing hormones is crucial in the nutritional regulation: raising ghrelin levels as leptin levels fall induces hunger during fasting (Cummings et al., 2001; 2002).

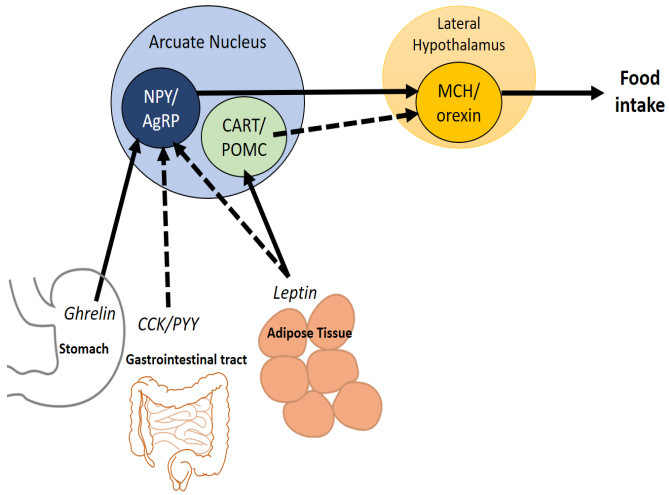


Fig.2. Peripheric and central homeostatic mechanisms of food intake regulation. Thick lines represent excitatory connections and dashed lines represent inhibitory connections.

## 3.2. Hedonic mechanisms of feeding

If feeding was only controlled by homeostatic mechanisms, we would consider it a necessary but unexciting part of existence. When a palatable food is presented, humans and animals go beyond their homeostatic need. Feeding is influenced by pleasure and reward and obtaining food reward motivates consumption (Saper et al., 2002; Zheng and Berthoud, 2007). There are several brain mechanisms of pleasure and incentive motivation for food and food-associated cues related to "wanting" and/or "liking" (Berridge, 1996) and a dysfunction within one or more mechanisms may contribute to eating disorders (Berridge, 2009, Berthoud and Morrison, 2008). Pleasurable effects (liking) of food and other rewards like drugs of abuse are mediated by different mechanisms than those regulating the motivational process of incentive salience (wanting). Thus, continued consumption of palatable food sensitizes the incentive system and makes the reward more wanted than liked and this dissociation progressively increases with the development of addiction (Robinson and Berridge, 2008). Whereas the DA striatal system is mostly (but not exclusively) implicated in 'wanting', the opioid and cannabinoid systems are mainly (but not exclusively) concerned in food 'liking'. In fact, brain-imaging studies in humans have shown that the DA release generated when humans encounter a food cue correlates with their subjective ratings of wanting the food (Volkow et al., 2002). Conversely, the activation of endogenous opioid or cannabinoid receptors seems to stimulate appetite in part by enhancing the 'liking' of the food (for example, its palatability). Although these two mechanisms are separate, they act in concert to modulate eating behaviors (Volkow et al., 2011).

To this day, reward and motivation have been studied in the context of drug addiction (Berke and Hyman, 2000), where brain reward systems allow reinforcement of responses that have no homeostatic value. Some studies point out that food and drug rewards may share commonalities and neural substrates, such as a dopaminergic contribution to food reward from the NAcc (Szczypka et al., 2001). But other neurotransmitters also project into the NAcc, such as opioid agonists, cannabinoid agonists, GABA agonists,

glutamate antagonists all of them playing an important role in regulating feeding behaviors (Reynolds and Berridge, 2001; Stratford et al., 1997; Zhang and Kelley, 2000; 2002). There is also evidence that opioid receptors play key roles in both feeding and reward (Kelley et al., 2002; Volkow et al., 2013), as well as endocannabinoids (Ahima and Antwi, 2008). Both contribute importantly to the reward value of food, including its immediate hedonic impact. For example, a prominent ventral striatal role of opiates in food reward has been extensively examined (Kelley et al., 2002). Given the wide distribution of opioidergic neurons and receptors, opioid pathways in other brain areas such as the hypothalamus may also play a role. Finally, endogenous cannabinoids also potently influence both appetite and reward in rats and humans (Di Marzo et al., 2001; Onaivi et al., 2002). Nonetheless, little is known about their mechanisms of action.

The NAcc and its dopaminergic inputs have been strongly implicated in drug addiction (Di Chiara, 2000). However, this structure has reciprocal interactions with the lateral hypothalamus, and, together, they regulate feeding behavior (Phillipson and Griffiths, 1985, Stratford and Kelley, 1999) (Fig. 3). The lateral hypothalamus projects to the NAcc from neurons containing MCH, which stimulate feeding (Saper et al., 2002) . In addition, the NAcc can disinhibit the lateral hypothalamus neurons through GABAergic projections (Groenewegen et al., 1993). Feeding induced by stimulation of the lateral hypothalamus can be blocked by DA blockade (Phillips and Nikaido, 1975), and conversely, feeding induced by injection of GABA antagonists into the NAcc can be blocked by GABA antagonists in the ventral pallidum, as well as by GABA agonists in the lateral hypothalamus (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999). This mutually reinforcing relationship could explain the motivation for feeding (Fig.3.).

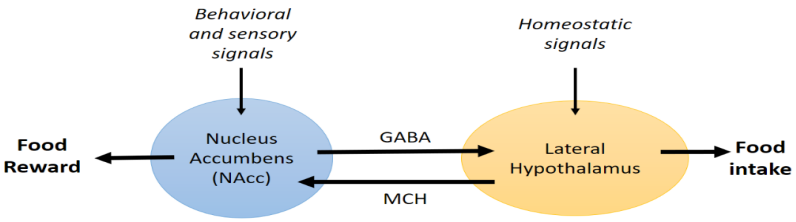


Fig. 3. Relationship between the NAcc and the Lateral hypothalamus regarding motivation for feeding. Effects of reward on feeding drives (GABA) and homeostatic and metabolic influences on reward (MCH) (Modified from Saper et al., 2002).

## 3.3. Interaction between homeostatic and hedonic aspects of feeding

The rewarding effects of food are modulated by homeostatic indicators of satiety. When one is hungry, food is pleasurable, but it may become unpleasant after satiation. Brain imaging studies show changes in the activation of the amygdala and the orbitofrontal cortex that correlate with the changes in self-reports (Small et al., 2001; Zald et al., 2002).

For example, hormones like insulin, leptin and ghrelin, which are hormonal inputs to homeostatic metabolic systems (Figlewicz, 2003), are also involved in the regulation of DA systems (Daws et al., 2011; Niswender et al., 2011). Leptin and ghrelin receptors are expressed in the Ventral Tegmental Area (VTA) (Baladi et al., 2012) (Fig.4).

### 3.3.1. Leptin and ghrelin signaling

Receptors for the anorexigenic hormone leptin are present throughout the hypothalamus and brainstem nuclei and they are functional on DA neurons in the VTA (Hommel et al., 2006; Scott et al., 2009). As previously mentioned, leptin promotes satiety, suppressing the incentive value of food and other rewards by modulating the mesolimbic DA (Figlewicz et al., 2006; Fulton et al., 2000). For example, leptin inhibits responses of sweet-sensitive taste cells in the tongue (Kawai et al., 2000), suggesting that leptin may mediate the hedonic value on food reward at the most peripheral level.

When the intake of high-fat high-sugar diets begins, elevated leptin levels inhibit NPY/AgRP neurons in the arcuate nuclei while stimulating POMC/CART satiety neurons (Pinto et al., 2004; see fig 2). In addition, stimulatory input to orexin neurons in the lateral hypothalamus is inhibited by leptin (Horvath and Gao, 2005).

Leptin directly regulates a population of leptin-receptor expressing VTA DA neurons (Hommel et al., 2006; Fulton et al., 2006), it modulates dopaminergic-dependent measures of food and drug reward, and it normalizes the decreased mesolimbic DA content of leptin-deficient animals (Figlewicz et al., 2006; Roseberry et al., 2007). Leptin action via lateral hypothalamus increases VTA Tirosine hidroxilase (TH) expression and decreases feeding (Leinninger et al., 2009). Several reports have pointed out that leptin regulates not only DA activity but also the opioidergic and endocannabinoid systems. Leptin reverses μ opioid receptor stimulated sucrose feeding in the VTA (Figlewicz et al., 2007). Cannabinoidergic activity in the hypothalamus is directly regulated by leptin levels, as injecting leptin reduces its levels. In addition, leptin-deficient strains exhibit elevated endocannabinoid levels in the hypothalamus (Di Marzo et al., 2001).

Concerning drugs of abuse, it has been suggested that leptin acts as an endogenous antagonist of responses to cocaine (You et al., 2016). For example, leptin attenuates cocaine-induced DA release in the NAcc 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). Leptin receptors in the VTA DA neurons hyperpolarize the DA response, decreasing their action potential by firing frequency and reducing the extracellular DA in the NAcc (Hommel et al., 2006; Krügel et al, 2003).

*To sum up, leptin directly inhibits DA neurons in the VTA, attenuating reward.*

As previously stated, ghrelin is an orexigenic hormone released by the stomach during fasting (Nakazato et al., 2001). Thus, it may be another potential homeostatic signal that could affect food rewards, particularly as its receptors have been identified in midbrain dopaminergic brain regions (Guan et al., 1997). Ghrelin receptor GHS-R1A is also present in VTA, and is implicated in food reward and addiction (Guan et al. 1997). An intracerebroventricular or into-the-VTA ghrelin injection stimulates parameters associated with reward-seeking behavior (Abizaid et al. 2006; Jerlhag et al. 2006, 2007, 2008). Ghrelin/GHSR signaling in the VTA has been identified as a crucial component for food reward, other natural rewards, and drugs of abuse (for review, see Wellman et al., 2013).

Regarding the role of ghrelin on the rewarding properties of food, injecting ghrelin directly into the VTA and NAcc increases food intake in rats (Skibicka et al., 2011). A recent study demonstrated that ghrelin action on the VTA is crucial for the intake of and motivation to obtain palatable/rewarding food (Egecioglu et al., 2010). First, the authors showed how genetic deletion of GHS-R1A or treatment with a GHS-R1A antagonist suppressed the sugary food intake without affecting the standard chow intake. Consistent with this, they also showed that accumbal DA release was absent in GHS-R1A knockout (KO) mice when rewarding food was presented. Secondly, the authors also demonstrated that CPP induced by food is suppressed in rats treated with a GHS-R1A antagonist (Egecioglu et al., 2010). In addition, opioid signaling is necessary to modulate the effects of ghrelin on food reward and intake (Skibicka et al., 2013). Furthermore, functional imaging studies in humans show that peripheral ghrelin administration controls brain responses to food images in reward areas (Malik et al. 2008).

Regarding palatable food, ghrelin activates the mesocorticolimbic DA system via receptors in the VTA and stimulates the intake of palatable food over standard chow (Egecioglu et al., 2010). Likewise, it has been demonstrated that, while NPY stimulates carbohydrate and sugar intake (Lynch et al., 1993; Morley et al., 1987), AgRP and ghrelin stimulate high-fat food consumption (Hagan et al., 2001; Shimbara et al., 2004). Fat decreases

circulating ghrelin levels (Ahrén and Scheurink, 1998), as several reports show that HFDs downregulate ghrelin secretion (Beck et al., 2002; Lindqvist et al., 2005; Bello et al., 2009). Previous reports have associated a reduction of GHSR expression to continuous exposure to fat diet or adiposity (Kurose et al., 2005; Zhang et al., 2013). In addition, ghrelin has a crucial role on binge eating behavior, as GHSR KO mice do not binge on highly palatable foods (Valdivia et al., 2015; King et al., 2016).

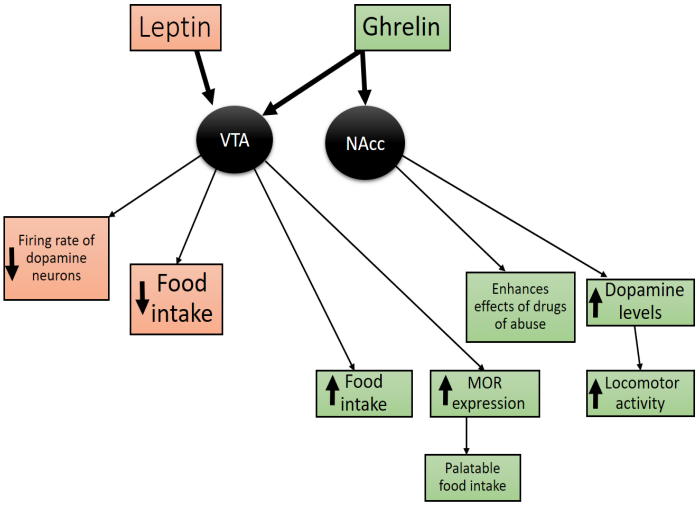


Fig.4. Role of leptin and ghrelin on the reward system (Adapted from Murray et al., 2014).

Ghrelin is also involved in the rewarding effects of other rewards, like drugs of abuse. It has been suggested that ghrelin receptors facilitate the effects of psychostimulants on the activation of DA circuits, increasing the rewarding and locomotor effects of cocaine (Wellman et al., 2005; Abizaid et al, 2011). Moreover, injections of ghrelin increase alcohol consumption (Jerlhag et al., 2009) and facilitate cocaine-induced CPP (Davis et al., 2007).

*To sum up, ghrelin acts not only at the hypothalamus level, but also via VTA, increasing the consumption of palatable food and enhancing the rewarding effects of psychostimulants and alcohol.*

### 3.3.2. Dopaminergic system

Dopamine is a catecholamine neurotransmitter that controls a variety of physiological functions, such as locomotor activity, emotion, cognitive processes, reward, sleep and food intake (Baladi et al., 2012). DA has been the focus of extensive research since it was discovered (Carlsson et al., 1957) and its regulation is also involved in several human disorders, such as Parkinson's disease, ADHD, Tourette's syndrome and drug abuse (Koob and Volkow, 2010; Mink, 2006; Swansson et al., 2007).

There are four major dopaminergic pathways that have been identified: the nigrostriatal, the mesolimbic, the mesocortical and the tuberinfundibular systems (Anden et al., 1964; Dahlström and Fuxe, 1964). Regarding reward, DA acts on the mesocorticolimbic system, projecting from the VTA to the NAcc and to the PFC (Bassareo and Di Chiara, 1997; Di Chiara, 1998). Activation of this system is directly related to the acquisition of hedonic feeding and reward (Kelley, 2004; Volkow et al., 2013). At the same time, these neural pathways are strongly implicated in the reinforcing mechanisms of drugs of abuse.

Eating increases extracellular DA in the NAcc of rats (Hernandez and Hoebel, 1988) with this neurotransmitter being highly responsible for eating behavior. For example, when DA levels are depleted, animals stop feeding and they starve, but when DA levels are restored with L-DOPA, the feeding behavior returns to normal (Sotak et al., 2005; Szczypka et al., 2001). The role of DA in overeating depends on the area that is examined. For example, injections of low doses of DA or dopaminergic agonists in the NAcc increase food intake (Evans and Vaccarino, 1986); while the same injections in the hypothalamus decrease feeding (Yang et al., 1997). After consuming fat or sugar, endogenous DA levels are increased in the NAcc (Hajnal et al., 2004; Rada et al., 2010) and peripheral DA injections have an orexigenic action with HFDs (Rao et al., 2008) as well as carbohydrates like sucrose (Hodge et al., 1994).

Studies in humans show a positive correlation with overeating and binge eating of foods high in fat and sucrose and some alleles for the D2 receptor and the DA transporter (Eny et al., 2009; Shinohara et al., 2004). These findings lead to the hypothesis that subjects with low endogenous DA levels in the NAcc may compensate by overconsuming fat or carbohydrates in order to raise their DA levels to sufficiently high levels to be rewarding (Barson et al., 2012). Some studies show that rats fed on a fat diet decreased extracellular DA in the NAcc (Rada et al., 2010), increased D2 receptor binding (South and Huang, 2008) and decreased DAT density (Huang et al., 2006). In the same line, other authors have reported that obese rats show decreased D2 binding (Johnson and Kenny, 2010), decreased clearance rate (Speed et al., 2011) and DA turnover (Davis et al., 2008).

HFDs impact sensitivity to the behavioral effects of direct-acting D2/D3 agonists. For example, rats on a HFD are more sensitive to quinpirole-induced yawning (Baladi and France, 2010) and locomotor activity (Naef et al., 2011) than rats on a standard diet. The influence of fat on the effects of quinpirole are altered independently of body weight (Baladi et al., 2011). The dopaminergic system and fat intake have a mutual influence. Alterations in D2 receptors have a significant impact on feeding behaviors. For example, bodyweight gain can occur as a side effect of a long-term administration of typical and atypical antipsychotic drugs (Baptista, 1999), though recently it has been reported that there are other non-dopaminergic targets responsible for this weight gain in relation to antipsychotics (Basile et al., 2001; Tardieu et al., 2003).

*To sum up, DA is released when a palatable food is presented. Although the NAcc Shell and DA signaling are the main governing agents of reward-driven consummatory behaviors (Kelley et al., 2005; Smith et al, 2009), both opioid and cannabinoid receptors in the NAcc appear to modulate feeding and reward, as we will see in the next sections.*

### 3.3.3. Opioid system

The endogenous opioid system stands out for its role in analgesia and the modulation of gastrointestinal, endocrine, learning and memory functions. Opioid receptors are most widely expressed in two organs of the body: the brain, especially in the regions implicated in the control of food intake and reward-driven appetite (Ding et al., 1996; George et al., 1994; Will et al., 2003), and the small intestine, where they control gut motility, including bowel movements (Hedner and Cassuto, 1987; Sternini, 2001; Sternini et al., 2004). In addition, they have a critical role in different aspects of addiction, modulating the activity of the mesolimbic system's dopaminergic neurons (Maldonado, 2010). Opioids are involved in a wide range of rewarding stimuli, such as opiate drug reward, affiliative rewards, music/art, sex, humor, earning money and food intake (Carelli, 2002; De Vries and Shippenberg, 2002, Everitt and Robbins, 2005).

Activation of the μ opioid receptor increases DA release in the NAcc by inhibiting GABAergic neurons in the VTA (Kalivas, 1993; Spanagel and Shippenberg, 1992). In standard conditions, these neurons would provide a tonic inhibition of DA neurons, but inhibiting this process results in an increase in DA release (Shalev et al., 2002; Chefer et al., 2009).

Involvement of the central opioid system in the regulation of food intake is well established (Glass et al., 1999; Reid, 1985). The μ opioid receptor pathway plays a major role in the stimulatory effect of high reward food on the mesolimbic DA system (Tanda and Di Chiara, 1998). While DA release in the NAcc is generally associated with the reinforcing effects of food, opioid signaling in this area regulates its palatability and hedonic properties (Cota et al., 2006; Esch and Stefano, 2004). When an opioid μ receptor agonist is administered, the hedonic impact of food is enhanced, as rats exhibit an increased 'liking' reaction in response to an intraoral sucrose administration (Peciña and Berridge, 2005). Kawahara and co-workers (2013) showed that consumption of palatable food without being food-deprived increased DA release in the NAcc via activation of the μ opioid receptor pathway in the VTA, which comprises the Beta-endorphin/μ opioid receptor activation.

This in turn acts on GABAergic interneurons in the VTA, which results in a decrease in GABA release and disinhibition of DA neurons. Several studies have reported that agonists for all three opioid receptor subtypes (μ, δ and κ) increase food intake (Gosnell et al., 1986; Morley and Levine, 1983) and opioid receptor antagonists such as naloxone block the effects of AgRP to increase feeding (Hagan et al., 2001). Specifically, injections of μ opioid receptor agonists directly into the NAcc and the VTA increase feeding of palatable foods (Peciña and Berridge, 2000; Zhang et al, 1998; Figlewicz and Sipols, 2010), whereas an antagonist decreases it (Bodnar et al., 1995) (Fig 5.)

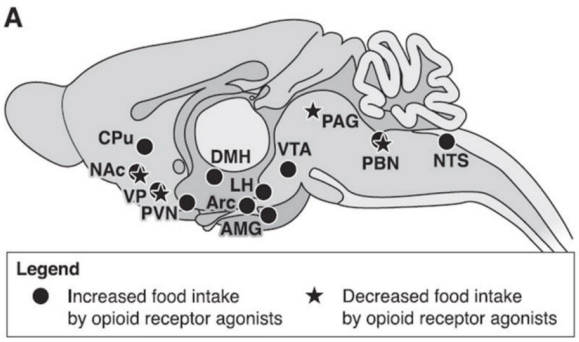


Fig.5. Brain sites where opioid agonists or antagonists modulate food intake (Modified from Le Merrer et al., 2009).

Endogenous opioid receptor activation in brain substrates, such as the NAcc, participates in eating processes via hedonic mechanisms, beyond endocrine and metabolic signals that arise from other structures involved in energy storage (Le Merrer et al., 2009). Food reward is driven by separate mechanisms of liking and wanting, and brain μ opioid receptors contribute to both forms of food reward. Opioid agonists increase the hedonic value of food, making it more pleasurable and palatable (liking), but opioids can also increase the motivational power of food associated cues, making them more relevant and hard to resist (wanting).

Regarding palatable diets, the opioid system regulates hedonic feeding and the intake and response to high-fat or high-sucrose foods (Barbano and Ca-

dor, 2006; 2007; Levine and Billington, 2004). Conversely, access to palatable substances like sucrose and fat increases the antinociceptive effects of morphine (Kanarek and Homoleski, 2000). Continuous access to HFDs induces a significant reduction in μ 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 diet (high sugar + fat) may suppress transcription mechanisms necessary for μ opioid receptor mRNA synthesis, since free access to this diet decreases μ opioid receptor in the VTA, which is increased 48 h after withdrawal from the diet (Martire et al., 2014). Equally, continuous access to a HFD (Vucetic et al., 2011) or cafeteria diets (Ong et al., 2013) induces changes in μ opioid receptor gene expression, decreasing its mRNA in NAcc, although other authors reported no changes (Smith et al., 2002).

*To sum up, opioid receptor μ is increased when a high-fat diet is administered, contributing to the rewarding effects of palatable food and increasing its hedonic value.*

### 3.3.4. Endocannabinoid system

The endocannabinoid system (ECS) is a chemical transmission system that has evolved from the lowest living species to humans (Rodriguez de Fonseca and Schneider, 2008; Mechoulam and Parker, 2013; Lu and Mackie, 2016). The ECS plays a modulating role in the reward/reinforcement circuits of the mesolimbic system and regulates a wide variety of processes, including the immune, cardiovascular, endocrine and nervous system and it is involved in neuronal development, motivation, emotional control and energetic metabolism (Cristino et al., 2014; Mechoulam and Parker, 2013; Lu and Mackie, 2016). Endogenous cannabinoids are substances produced by neurons, including N-arachidonoylethanolamine (Anandamide) and 2-arachidonoylglycerol (2-AG), which can be found in multiple areas of the brain, including the hypothalamus (Pertwee, 2015). On the other hand, the specific endocannabinoid receptors are CB1 and CB2. CB1 receptors are mainly found in the central nervous system and are abundant throughout the cerebral cortex, such as VTA, NAcc, PFC (Freund et al., 2003). Although CB1 receptor

expression in the hypothalamus is relatively lower, the endocannabinoid system (ECS) exerts important functions in this region (Marsicano and Lutz, 2006), which could explain the alterations in food intake. According to this, the hypothalamic ECS plays a regulatory role in controlling food intake and other features of energy metabolism (Cota and Woods, 2005). Regarding the peripheral distribution of CB1, it is expressed in metabolism-related tissues, such as the adipose tissue (Cota et al., 2003), liver (Osei-Hyiaman et al., 2005), skeletal muscle (Cavuoto et al., 2007) and the pancreas (Juan-Picó et al., 2006). Its actions are currently being explored, although it seems to promote lipogenesis and energy storage (Bermudez-Silva et al., 2010).

Several data suggest an important modulatory role for cannabinoid receptors in the expression of feeding behaviors and the NAcc may be one critical site. The ECS is designed to influence every key point of the regulatory network that controls energy homeostasis (Cota and Woods, 2005). The hypothalamic ECS controls food intake by decreasing satiety signals and increasing orexigenic signals (Bermudez-Silva et al., 2010). Furthermore, through interactions with the mesolimbic pathways involved in reward mechanisms, endocannabinoids appear to increase eating motivation, possibly reinforcing the incentive or hedonic value of food (reviewed in Kirkham, 2003). As an example, CB1r expression is upregulated by sucrose intake (Lindqvist et al., 2008), suggesting that sucrose decreases endocannabinoid levels in this brain region. It has been shown that an injection of endocannabinoids or the agonist delta-9-tetrahydrocannabinol (THC) into the NAcc stimulates food intake (Mahler et al., 2007, Verty et al., 2005). Furthermore, CB1 receptor density in the NAcc has been revealed to be inversely related to palatable food consumption, indicating that increased ingestion of calorically dense foods may heighten activation of CB1 receptors in this region and lead to their downregulation (Harrold et al., 2002).

Some neuropeptides and lipids like endocannabinoids, all of them produced in the hypothalamus, appear to have a special role in the positive feedback regulation of fat intake (Barson et al., 2012), as cannabinoids are more linked with dietary fat than with carbohydrate and sucrose intake. Koch

(2001) revealed that THC stimulates a stronger desire for a fat diet than a sweet-fat diet. In addition, HFDs upregulate hippocampal ECS levels and hypothalamic 2-AG, indicating that highly palatable foods may be more satisfying under these conditions (Massa et al., 2010; Higuchi et al., 2012). In another study, Thornton-Jones and co-workers (2007) suggested that a cannabinoid antagonist decreased drinking of a lipid emulsion more than it did on a sucrose solution. HFDs increase CB1 receptor binding and 2-AG levels in the hypothalamus compared to low-fat diets (Higuchi et al., 2011; South and Huang, 2008). Moreover, eating high-fat chow decreases sensitivity to the cataleptic effects of THC (Wiley et al., 2011). An intra-accumbens administration of 2-AG enhanced fat consumption, this effect being attenuated by a CB1r antagonist (Deshmukh and Sharma, 2012). Further supporting the role of CB1r, mice lacking the central CB1r, compared to wildtype mice, exhibit a delayed onset of preference for fat compared to standard chow (Ravinet-Trillou et al., 2004).

In the mesolimbic-mesocortical system, cannabinoids increase the neuronal excitability of the VTA and the release of DA (Lopez-Moreno et al., 2008). Most GABAergic inhibitory interneurons express CB1 presynaptic receptors in abundance, modulating the release of GABA at the synapses (Hájos and Freund 2002; Berghuis et al. 2007). Studies focused on the functional interaction between the GABAergic and EC systems in the NAcc show that administration of morphine or an endocannabinoid agonist such as WIN 55,212-2, as well as heroin SA, leads to a reduction of GABA efflux into the NAcc of rats, which results in an increase in DA release. These effects are reversed by the cannabinoid CB1 receptor antagonist rimonabant (Caillé and Parsons 2006). 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 highly palatable food (Mellis et al., 2007).

All these results suggest that, the endocannabinoids affects appetite through CB1r for specific dietary components. Studies in humans support these findings and additionally show that specific alleles for the CB1 receptor are more common in men and women with higher body fat content (Jaeger et al., 2008; Russo et al., 2007).

*To summarize, CB1 receptors are involved in the rewarding effects of palatable food, specifically in high-fat diets. They increase DA release and promote hunger and food intake. Put together, these studies suggest that feeding can also impact non-dopaminergic systems, such as the cannabinoid and opioid systems.*

# 4. Influence of feeding on drug addiction

## 4. Influence of feeding on drug addiction

Over the past few decades, increased attention has been given to understanding the neurobiological basis of intermittent episodes of behavioral excess. Certain foods (high-fat, sugar-rich) and drugs of abuse activate common dopaminergic systems (Di Chiara and Imperato, 1988; Hernandez and Hoebel, 1988; Wise and Rompré, 1989; Kelley and Berridge, 2002; Berridge et al., 2010; Volkow et al., 2011; Valdivia et al., 2014). There could also be an interaction between food and drugs. For example, there is evidence that the composition of a diet, such as sugar, can affect the intake of drugs of abuse (Avena et al., 2012).

There is an agreement to consider obesity and overeating within the same neurobiological basis as addiction, which considers that the intake of certain types of food resembles drug dependence, behaviorally and neurally (Murray et al., 2014). There is confirmation of tolerance, withdrawal and compulsive food-seeking in animal models of palatable food overeating (Avena et al., 2008). Moreover, lower levels of striatal DA receptors have been found in obese humans, as with the findings observed in patients with drug addiction (Wang et al., 2001).

In addition, it has been claimed that nutritional status is a modulating factor in the development of drug addiction (Avena et al., 2008; Volkow et al., 2013), proposing, for example, that binge-eating may work as a gateway for the development of drug addiction (Degenhardt et al., 2009; Puhl et al., 2011).

Avena and co-workers explored over time four components of addiction such as bingeing, withdrawal, craving and cross-sensitization to prove that sugar was an addictive substance (Avena et al., 2008). They showed how rats with intermittent access to sugar can show a repertoire of behaviors and brain modifications that are characteristic of rats that voluntarily self-administer addictive drugs. For example, sugar dependent rats exhibited an increase of EtOH intake (Avena et al., 2004). Prolonged exposure to sugar-rich diets leads to physical dependence, inducing physical symptoms of withdrawal

when the food is removed (Avena, 2007; 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 HFD led to an increased stress response and the drive to seek palatable food.

Contrasting with the intake of protein or carbohydrates, which are mostly controlled through negative feedback, fat intake appears to be regulated by positive feedback, in which the ingestion of HFDs often stimulates the desire for more fat (Barson et al., 2012; Beck et al., 1990). Evolutionary, this attenuated perception of satiety from fat is adaptive, as fatty foods are energy-dense enough to warrant survival. Human and rodent studies show that consumption of snacks containing fat leads to larger meal sizes and shorter latencies between meals (Marmonier et al., 2000; Warwick et al., 2003). Nowadays, with the abundance of high caloric sources, this mechanism can lead to overeating and health complications.

### 4.1. Animal models of eating disorders

Some authors suggest that it is the way substance/food is consumed, rather than the substance itself, which alters the reward system (Avena et al., 2008; Corwin et al., 2011). Research is mainly focused on two animal models of eating disorders that could change the reward system: the continuous access model and the limited access model. On the one hand, continuous access to HFD (ad libitum) or sugar-rich diets leads to animal models of obesity, while on the other hand, the limited access model may resemble binge eating by its intermittent pattern.

#### 4.1.1. Continuous access: a model of obesity

Obesity has doubled worldwide in the last thirty years, becoming a pandemic (WHO, 2013). As previously mentioned, young people are more prone to develop inadequate nutritional habits such as overeating (Ifland et al., 2009). It is widely assumed that abnormal dietary habits, combined with lower levels of physical activity, result in an increase of overweight adolescents that become obese adults. In fact, in the young population, obesity has increased at an alarming rate, which can be especially problematic (Hurt et al., 2010).

Both drug addiction and obesity can be defined as disorders in which the motivational value of the reinforcer (drug or food, respectively) is abnormally increased in comparison with other pleasurable stimuli (Avena et al, 2012; Volkow et al, 2013). In the last few years, the concept of food addiction has been suggested due to the neuroadaptations that some kinds of foods –those rich in sugars or fats- produce in the brain (Volkow et al., 2013). Preclinical studies have provided robust evidence confirming that free access to a HFD has considerable effects on the brain reward system, producing changes in the dopaminergic system. Ingestion of palatable foods activates dopaminergic neurons within the NAcc and other reward centers (Kelley et al., 2005; Rada et al., 2005; Narayanaswami et al., 2013), thereby decreasing DAT density (Huang et al., 2006). As previously said for animal models, while human studies are non-conclusive, animal studies consistently showed that diet–induced obesity decreases striatal DA concentrations (Davis et al., 2007; Zhang et al., 2013) and TH (Li et a., 2009; Ong et al., 2013).

Studies have pointed out that, due to the common neurobiological pathways that stimulate fat intake and drugs of abuse, there might be an interaction. For example, acute locomotor response to cocaine is enhanced in mice consuming a continuous diet high in fat and/or sucrose (Collins et al., 2015). To date, only two studies have linked a HFD and obesity to cocaine-induced CPP. Morales and coworkers (2012) reported that mice exposed to a continuous HFD that induced obesity showed a decrease in cocaine reward. In the same line, Thanos and co-workers (2010) examined interaction between genetically obese animal strains that eat high-fat chow and sensitivity to the effects of cocaine. Eating high-fat food increased sensitivity to the cocaine-induced CPP in obese-resistant rats. Davis and co-workers (2008) showed the same effect but with amphetamine-induced CPP. Nevertheless, some reports show that exposure to an ad libitum access to a HFD increases the sensitivity of animals to CPP induced by amphetamine (Kuhn et al., 2013) or cocaine (Peleg-Raibstein et al., 2016), which points to the current controversial state. Baladi and co-workers (2015) have recently described that, though sensitization to the locomotor effects of cocaine were

enhanced in adolescent rats fed a HFD, the DA clearance rate in the striatum decreased . Diminished acquisition of cocaine SA and an attenuation of operant responding for sucrose has also been described after continuous access to a fatty diet (Wellman et al., 2007).

Regarding alcohol studies, Pekkanen and Eriksson (1978) described that, after being exposed to a HFD, rats drank more EtOH than controls fed on a balanced diet. On the contrary, Barson and colleagues (2009) demonstrated that rats chronically injected with EtOH show a significant increase in their fat preference. A recent report proved the influence of a HFD on fetal development (Peleg-Raibstein et al., 2016), showing that the offspring of females exposed to a HFD before conception, and during gestation and lactation, preferred alcohol. Carrillo and co-workers (2004) revealed that rats given a single high-fat challenge, or injected with fat, increased alcohol consumption in a two-bottle choice procedure. However, results are controversial, as Much and coworkers (2002) did not find any differences when varying the percentages of macronutrients in the rats' diets

Results until today lead to the reward deficiency hypothesis of obesity, which proposes that reduced DA tone leads to overeating as an attempt to restore striatal DA concentrations (Naef et al., 2015). Perhaps, signals sent out by adipose tissue – leptin being the most likely candidate – control this response (Fulton et al., 2006).

#### 4.1.2. Limited access: a model of binge eating

There are a variety of human disorders that are characterized as intermittent excessive behaviors, such as substance abuse or binge eating (Corwin and Wojnicki, 2006). This addictive-like eating pattern is known as binge eating, which is described as an intermittent, excessive, dysfunctional appetitive behavior that occurs in short periods of time (Puhl et al., 2011). 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 (5$^{th}$ ed., DSM-5; American Psychiatric Association, 2013), and it is not necessarily driven by hunger or metabolic need (Brownley et al., 2007; Davis

et al., 2007). According Avena and co-workers (2009), people usually binge on foods that are rich in fat and sugar, typically high in calories. 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). Discarding clinical population with a binge-eating disorder diagnosis, there is a big number of children and adolescents who binge often without meeting the clinical criteria.

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 those eating standard diet under similar circumstances, and it must be stable and maintained over long periods of time (Corwin and Buda-Levin, 2004). Several animal models of binge-type eating have been proposed (Corwin et al., 2011; Perello et al., 2014), some of which are of special interest given their ability to reveal the transformation from occasional normal behavioral excess to a repetitive and compulsive behavioral excess (Corwin and Wojnicki, 2006).

The protocol proposed by Corwin et al. (1998), the Limited Access Model is a behavioral paradigm without food-deprivation (Corwin, 2004). This protocol provides a limited access to palatable food for 2h, three times a week, on Monday, Wednesday and Friday, producing an escalation of intake, even though animals have always had access to standard chow (Corwin et al., 2011). One of the main characteristics of binge eating in humans is eating in the absence of hunger (Marcus and Kalarchian, 2003; Corwin and Buda-Levin, 2004). Non-deprivation therefore becomes the big advantage of this animal model. Research has shown that as access to palatable food decreases, food consumption increases during the 2h limited access period (Corwin et al., 1998; Corwin, 2004; 2006; Dimitriou et al., 2000; Thomas et al., 2002). Using this paradigm, rats with sporadic brief access to palatable food do not accumulate more body fat than chow controls (Corwin et al., 1998). The intake of fat has been demonstrated to increase or remain relatively stable over periods of 4-8 weeks (Kinzig et al., 2008).

As we mentioned before, even though continuous HFD attenuates cocaine and food reward in the CPP protocol (Morales et al., 2012), only one work addresses the fact that adult rats exposed to a binge-type intake of fat exhibit more robust "addiction-like" behaviors toward a substance of abuse (Puhl et al., 2011). Although no significant differences have been observed, these mice tend to consume more cocaine in fixed ratio training. They also persist in their efforts to obtain cocaine in the face of signaled non-availability, working harder for cocaine in a progressive ratio schedule of reinforcement, and exhibiting more goal-directed behavior toward the cocaine (Puhl et al., 2011). Regarding alcohol studies, only a recent report showed that rats that binged on fat intermittently displayed attenuated acquisition of alcohol intake (Sirohi et al., 2016).

In general, animals that binge on palatable food like sugar exhibit an enhanced locomotor sensitization to psychostimulants and amphetamine (Avena and Hoebel, 2003; Gosnell, 2005). Two recent reports have described the development of locomotor sensitization to cocaine in adolescent mice exposed to restricted HFD, while no response was observed in adult animals (Baladi et al., 2015; Serafine et al., 2015).

Regarding DA, in diet-induced binge eating rodent models, highly palatable foods (fats, sugars and their combination), with restricted access conditions, appear to promote intake responses and result in sustained DA stimulation within the NAcc (Bello and Hajnal, 2010). DA levels increase in the NAcc shell when a food stimulus is novel and palatable, while DA levels increase in the core whenever the food is ingested regardless of palatability or novelty (Bassareo et al., 2002). However, some studies show that binge-associated DA release does not habituate after repeated episodes of consumption (Avena et al., 2006; Rada et al., 2005; Avena et al., 2008). Intermittent access to sugar for 30 days decreases D2R binding in dorsal striatum and increases D1R in dorsal striatum and NAcc (Colantuoni et al., 2001). Finally, studies in humans with binge eating disorder show abnormalities in DA turnover, DA transporter and DA receptors (Bello and Hajnal, 2010).

### 4.1.3. Withdrawal of palatable diets

In addition to the presence of withdrawal symptoms upon cessation of drug use, the other hallmark of substance dependence is an increased drug craving. Similarly, appetitive behavior to palatable foods increases during cessation of such diets. As with drugs of abuse, withdrawal from and craving for specific kinds of foods has also been observed and measured in humans (Rogers and Smit, 2000).

There is extensive evidence of dependence on sugar (Bello et al., 2003; Wojnicki et al., 2007; Avena, 2007; Cottone et al.,2008; Colantuoni et al., 2001), but data regarding the dependence on high-fat food are not so clear. For example, after intermittent access to sugar, forced abstinence induces increased lever pressing for glucose, suggesting an elevated motivation after a withdrawal period (Avena et al 2005). Furthermore, the withdrawal state can induce cross-sensitization behavior with drugs of abuse, as rats that were given intermittent access to sugar and then were forced to abstain exhibit enhanced intake of alcohol (Avena et al. 2004). Pickering and coworkers (2009) reported that obesity-prone animals chronically fed a high-fat high-sugar diet exhibited signs of craving, increased anxiety and symptoms of withdrawal when the diet was drastically interrupted. Several studies have shown that sugar consumption leads to a withdrawal syndrome similar to that occurring after opiate withdrawal (Avena et al., 2009). In addition, several studies have reported 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). Although the method used cannot be considered a model of withdrawal, Le Merrer and Stephens (2006) found that rats consuming sucrose daily showed locomotor cross-sensitization to cocaine in the absence of sucrose.

There are almost no studies evaluating the effect of abrupt cessation of a HFD on the response to drugs of abuse. Teegarden and Bale (2007) did confirm that discontinuation of a HFD led to an increased stress response and the drive to seek palatable food the next day. Two additional studies reported an increase in anxiety levels up to 24 hours after cessation of continuous

access to a high-fat food (Cottone et al., 2009; Sharma et al., 2013). Only one study has evaluated the effect of withdrawal from chronic exposure to HFD on the locomotor response to cocaine in rats, with no changes observed after cessation of the HFD (Baladi et al., 2012).

*To sum up, it seems that fat, cocaine and alcohol are mutually influenced. But in the majority of these studies, high-fat diets are administered to adult animals with very different eating administration patterns.*

# 2. Aims and Hypotheses

## 2. Aims and Hypotheses

As we have seen in the previous section, the current findings of the literature show that ingestion of HFD induces neuroadaptations that alters the reward system, affecting dopaminergic, opioidergic and endocannabinoid pathways, which modifies the response of the animals to the rewarding effects of drugs of abuse. However, further research is essential to understand how dietary factors contribute to the increased vulnerability to drug use, taking into account the eating patterns and the age of exposure to the diet.

The present study endorses the idea of a comorbidity between certain nutritional habits and an increased use of drugs of abuse, suggesting that the type of food and the way it is consumed plays a critical role in the development of substance abuse disorders.

Thus, the main objective of the present Doctoral Thesis is to identify how early exposure during adolescence to inadequate dietary habits interacts with the effects of cocaine and alcohol. To assess this objective, we used as the main methodology two different administration patterns of a high-fat diet, either continuously or intermittently, and we further evaluated its effects on the drug-induced Conditioned Place Preference, as well as the drug – Self-Administration procedure. The knowledge acquired will undoubtedly contribute to taking into account these nutritional factors for the prevention and treatment of substance abuse disorders, especially in the young population.

**Study 1.** The aim of the first study was to evaluate how a continuous exposure to a high-fat diet during adolescence modifies motor and conditioned rewarding effects of cocaine, and whether this sensitivity changes after cessation of this diet. As a second aim, we assessed if the HFD could act as an alternative reinforcer of cocaine. We also evaluated the effects of this fat administration on the opioid, endocannabinoid and ghrelin systems.

Hypotheses

- Animals exposed to a high-fat diet will show a reduction of cocaine-induced CPP.
- Following fat discontinuation, animals will exhibit increased anxiety.
- Following fat discontinuation, animals will exhibit an altered locomotor response to cocaine.
- Following fat discontinuation, animals will be more sensitive to the reinforcing effects of cocaine-induced CPP.
- Animals exposed to a HFD will present changes in opioid, cannabinoid and ghrelin gene expression.
- Animals exposed to a HFD after the CPP procedure will decrease their sensitivity to drug-priming induced reinstatement.

**Study 2.** The aim of the second study was to evaluate the effects of a HFD consumed in a binge pattern during adolescence on the reinforcing effects of cocaine.

Hypotheses

- Animals that binge on fat will exhibit an increased sensitivity to cocaine in the CPP.
- Animals that binge on fat will show enhanced acquisition and reinstatement of cocaine self-administration.
- Discontinuation of bingeing on fat will lead to increased anxiety.
- Discontinuation of bingeing on fat will increase sensitivity to cocaine reinstatement in the CPP paradigm.
- Animals that binge on fat will show changes in their cannabinoid, opioid and ghrelin gene expression.

**Study 3.** The aim of the third study was to evaluate the effects of a HFD consumed in a binge pattern during adolescence on the reinforcing effects of alcohol.

Hypotheses

- Animals that binge on fat will show an enhanced EtOH consumption.
- Animals that binge on fat will exhibit higher motivation to obtain EtOH.
- Animals that binge on fat will show an increased sensitivity to the conditioned rewarding effects of EtOH in the CPP paradigm.
- Animals that binge on fat will show an increased EtOH-induced locomotor sensitization.
- Animals that binge on fat will have an altered MOr, CB1 and TH gene expression after EtOH self-administration.

**Study 4.** The aim of the fourth study was to clarify if after cessation of access to the fat binge, the increased vulnerability to the reinforcing effects of EtOH persists.

Hypotheses

- Animals that binged on fat will present enhanced EtOH consumption 15 days after fat discontinuation.
- Animals that binged on fat will still present a higher motivation to obtain EtOH 15 days after fat discontinuation.
- Animals that binged on fat will show an increased response to EtOH in the CPP paradigm.
- Animals that binged on fat will show increased EtOH-induced locomotor sensitization.

**Study 5.** The aim of the fifth study was to evaluate whether a chronic stress, like being housed on isolation, may modulate the effects that bingeing on fat induces on the conditioned reinforcing effects of cocaine.

Hypotheses

- Isolated animals fed with the standard diet will exhibit an increased sensitivity to cocaine in the CPP.
- Isolated animals that binge on fat will not show increased sensitivity to cocaine, as fat acts as a protection factor against isolation stress.
- Isolated animals will present higher corticosterone levels than grouped animals.
- Isolated animals will present abnormalities in the circulating leptin levels.

**Study 6.** The aim of the sixth study was to evaluate the behavioral profile induced by a HFD consumed continuously or in a binge pattern on locomotor, cognitive and emotional processes. We also evaluated the behavioral consequences of the cessation of fat intake.

Hypotheses

- Spontaneous locomotor activity will be altered in animals fed on fat.
- Continuous access to fat will decrease hippocampal-dependent learning.
- Continuous access to fat will alter the memory process.
- Fat discontinuation will increase anxiety levels.
- Fat consumption will affect social interaction with a conspecific.

# 3.RESULTS

# Study 1.

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

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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

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## 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

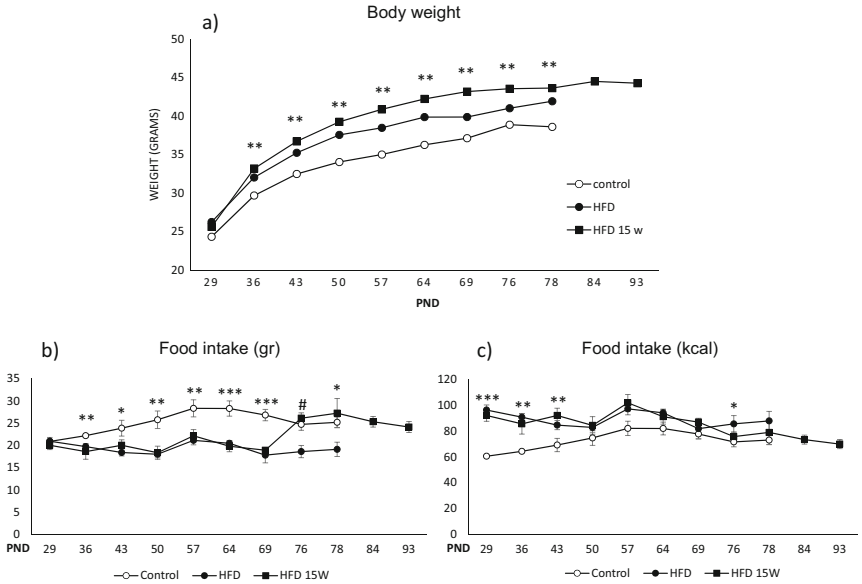


**Fig. 1** **a** Body weight (measured weekly) of animals in the control group, the HFD group, and the HFD 15W group. **b** Food intake in grams during the whole procedure. **c** Food intake in kcal during the whole procedure. Data are represented as the mean (± SEM) amount of body weight measured weekly. 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

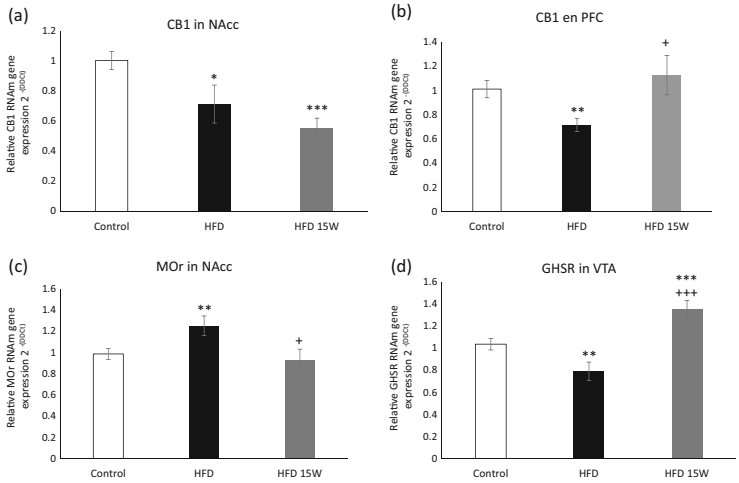


**Fig. 2** Real-time PCR CB1 receptor relative gene expression evaluation in the N Acc (**a**) and PFC (**b**) brain regions of control, HFD, and HFD 15W animals on PND 69 (control and HFD) and PND 84 (15W animals) ($n$ = 10 per group). **c** MOr relative gene expression evaluation in the N Acc brain 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).

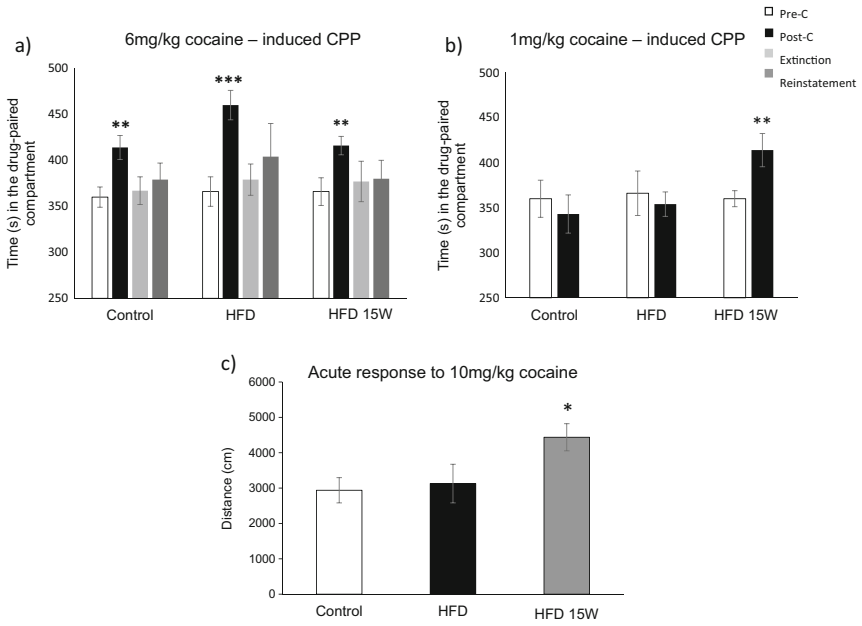


**Fig. 3** **a** CPP induced by 6 mg/kg of cocaine in mice exposed to a continuous HFD. *Bars* represent the time (± SEM) in seconds spent in the drug-paired compartment before conditioning sessions in the preconditioning test (*white bars*), after conditioning sessions in the postconditioning test (*black 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 $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$

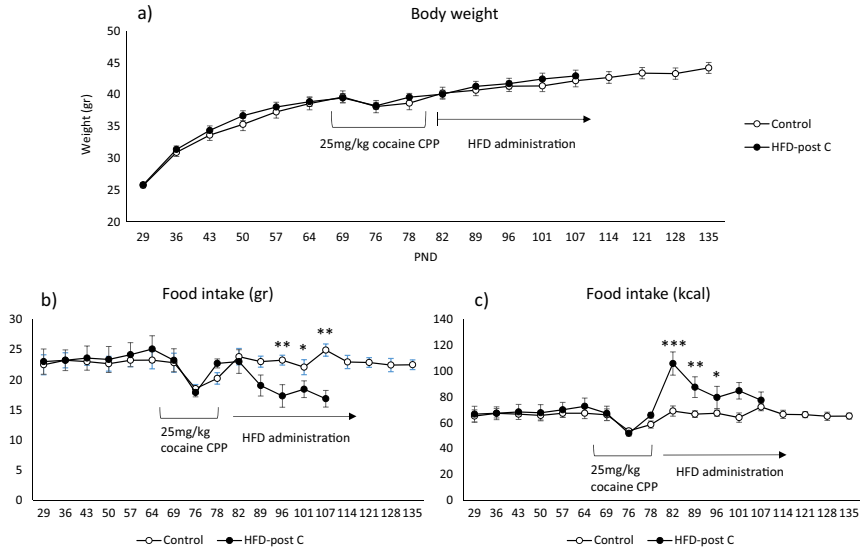


**Fig. 4** **a** Body weight (measured weekly) of animals in the control group and the HFD Post-C group. **b** Food intake in grams during the whole procedure. **c** Food intake in kcal during the whole procedure. Data are represented as the mean (± SEM) amount of body weight measured weekly. HFD and 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

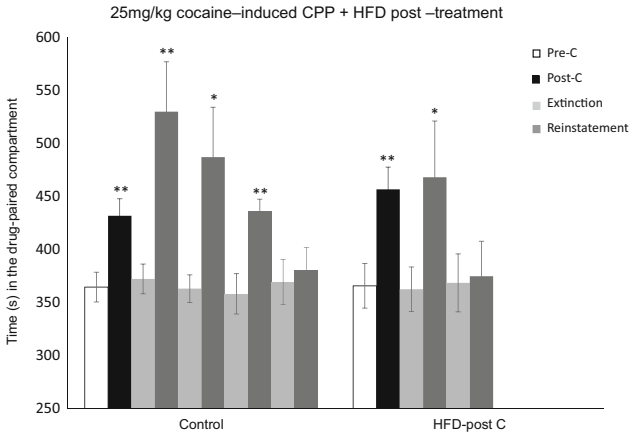


**Fig. 5** CPP induced by 25 mg/kg of cocaine in mice receiving a standard diet. After Post-C, animals in the HFD Post-C group were fed a continuous high-fat diet to explore its effect on extinction and reinstatement. *Bars* represent the time (± SEM) in seconds spent in the drug-paired compartment before 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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# Study 2.

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

**M.C. Blanco-Gandía**, L. Cantacorps, A. Aracil-Fernández, S. Montagud-Romero, M.A. Aguilar, J. Manzanares, O. Valverde, J. Miñarro, M. Rodríguez-Arias.

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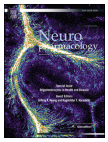

# Effects of bingeing on fat during adolescence on the reinforcing effects of cocaine in adult male mice

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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 2 h, 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.



## 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 and 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

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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 and 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 et al. (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 et al. (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-Arachidonoylglycerol (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 et al. (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 bingeing 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 28 × 28 × 14.5 cm), for 8 days prior to initiating the experimental feeding schedule, at a constant temperature (21 ± 2 °C), with a reverse light cycle (white lights on 19:30–7:30 h).

For CPP, gene expression analysis, corticosterone, ghrelin 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 (Rodríguez-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 h and on at 20.00 h. Animal rooms were controlled for temperature (21±1 °C) 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 (25–26 g) and assigned either a Control (C) diet or HFB (2 h access on Monday, Wednesday and Friday). All groups were fed with the standard diet in their own cages, and 3 days a week they 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 2–3 h 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 1 mg/kg or 6 mg/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 1 mg/kg or 6 mg/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 PND 29 to PND 69), 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 0.5 mg/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-h 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 (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5cm). 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 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

**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 2 h 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 | 70–77 | 79–110 |
|---|---|---|---|---|
| | | 83 (HFD 15w) | 84–91 (HFD 15w) | 93–237 |
| 1st set of mice (Exp 1) (n = 120) | Control (Standard diet)<br>HFB<br>HFB 1w<br>HFB 15w | Elevated Plus Maze | 1 mg/kg CPP<br>6 mg/kg CPP | Reinst. 0.5 mg/kg<br>3 and 1.5 mg/kg |

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

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

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 grey 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).

*2.4.2.1. 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 15 min (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 pre-conditioning 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 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.

*2.4.2.2. 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 min. 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.

*2.4.2.3. Reinstatement of CPP.* 24 h 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, 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®, 5 mg/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 1 h 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 1-h 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 (−10 °C) at levels containing the regions of interest according to Paxinos and Franklin (2001), and were then 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, 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 15w 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 (Fig. 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) [$F(18,606) = 5428$; $p < 0.001$] and [$F(18,606) = 5982$; $p < 0.001$], 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 (Fig. 1d).

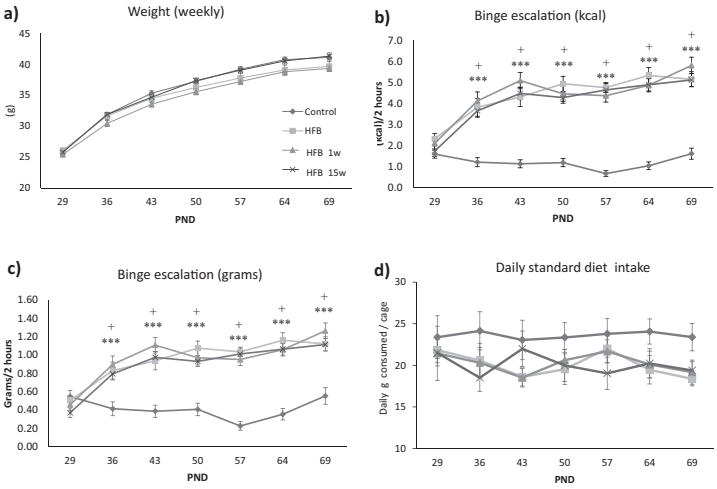


**Fig. 1. (a) Bodyweight of mice over the procedure**. Control mice received a standard diet during the binge sessions and animals in the HFB condition underwent three additional temporary conditions: the HFB group had 2 h access every Monday, Wednesday and Friday throughout the study; the HFB 1w group 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 and g consumed in 2 h 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.

### *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) = 6769; p < 0.01] and [F (2,42) = 8277 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) = 4637; 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).

**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 2 h 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 -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 ++ |
| % 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 |

### *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 1 mg/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) = 3747; 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 0.25 mg/kg cocaine [F (1,11) = 7990; 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 Fig. 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

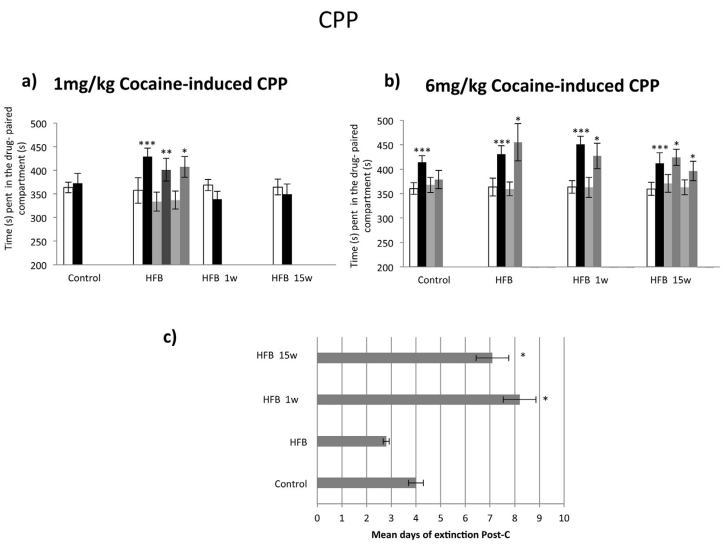


**Fig. 2. (a) Effects of a HFB on adolescent mice in the Conditioned Place Preference**. CPP induced by 1 mg/kg of cocaine in mice exposed to standard diet during the binge sessions (n = 15) (controls) or high fat binge (HFB) with three additional temporary conditions: the HFB group (n = 15) had 2 h 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 min after a priming dose of 0.5 mg/kg and 0.25 mg/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 min 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 s 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.

analysis (see Fig. 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; $\chi^2 = 5828$; $p < 0.05$ and $\chi^2 = 4423$; $p < 0.05$) and the HFB (3 sessions; $\chi^2 = 6114$; $p < 0.05$ and $\chi^2 = 4857$; $p < 0.05$) 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) ($\chi^2 = 11{,}345$; $p < 0.001$). 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 Fig. 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 2–10 ($p < 0.001$) (Fig. 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 (Fig. 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 10-day SA training continue to exhibit drug-seeking behavior in the operant SA chamber, even after a period of cocaine withdrawal.

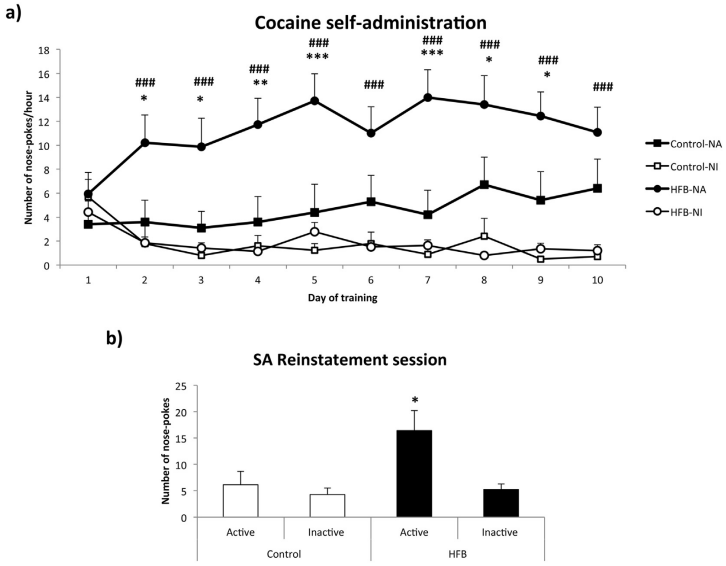


**Fig. 3. (a) Effects of a HFB on adolescent mice in the cocaine self-administration**. Acquisition of cocaine (0.5 mg/kg/infusion) self-administration in mice exposed to a standard diet (control) or high fat binge (HFB) during adolescence. Number of active (NA) and inactive (NI) nose-pokes in 1-h sessions 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-h session. Two-way ANOVA and Bonferroni's post-hoc analysis. *p < 0.05 Active vs. Inactive nose-poke.

### *3.5. Effects of a HFB on circulating leptin, corticosterone, and ghrelin levels and MOr, CB1 and Ghrelin 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 Fig. 4c).

**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 2 h 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 2 h 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 |

## 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.

**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 2 h 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 ** |

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

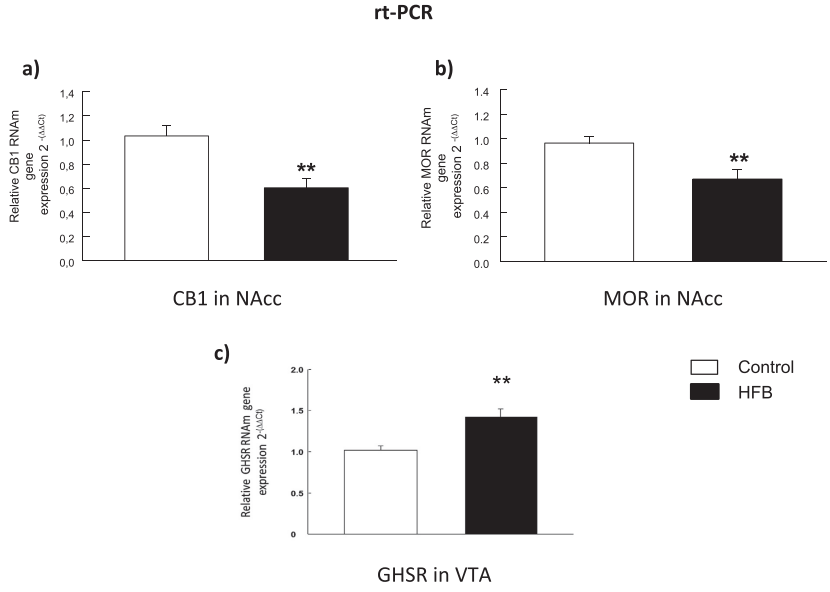


**Fig. 4. Effects of a high fat binge (HFB) on adolescent mice on gene expression in the** Nucleus Accumbens (NAcc). **(a)** Real-time PCR CB1 relative gene expression evaluation in the NAcc brain region of control and HFB groups (n = 9). **(b)** MOr relative gene expression evaluation in the NAcc brain 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.

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 et al. (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 al., 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., 2012), 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 al., 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 36 h 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 et al. (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 et al. (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.

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# Study 2.

## Bingeing on fat increases cocaine reward.

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Editorial: Neuroscience

# Bingeing on fat increases cocaine reward

**M. Carmen Blanco-Gandía and Marta Rodríguez-Arias**

In recent years, rates of overeating have been increasing dramatically, especially among the younger population. Consumption of fast and processed food plays an important role in cardiovascular diseases, diabetes and obesity. Binge eating is one of the most common eating disorders. According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, binge eating is a specific form of overeating, characterized by intermittent excessive eating in a short period of time and marked by feelings of lack of control. Characteristically, binge eating is not driven by metabolic needs and occurs in the absence of food restriction. Foods that are consumed during a binge episode are typically high in calories, fat and/or sugar. Although it is related to obesity, many people who binge eat are not obese, and most obese people do not present binge eating disorders [1]. Many teenagers display this pattern of hedonic eating without fulfilling the clinical criteria for binge-eating disorder. During adolescence, a critical period of brain maturation, individuals are especially vulnerable to harmful influences such as inadequate dietary habits or drug abuse.

The alteration of dopamine brain levels, a neurotransmitter critical to the reward process, is a common neurobiological mechanism implicated in obesity and drug addiction. As with drugs of abuse, the ingestion of palatable foods rich in sugar or fat, activates dopaminergic neurons within reward centers. The escalation of palatable food consumption observed in binge eating mimics what occurs in addiction, since there is a transition from controlled to compulsive intake and subsequent loss of control. In fact, subjects with substance abuse disorders and obese individuals show reduced levels of dopaminergic receptors in the nucleus accumbens, a critical structure of the reward system, as a compensatory response to the excessive dopaminergic neurotransmission [2].

Drug use during adolescence often predicts an increased likelihood of continued use into adulthood [3] and due to the common neurobiological pathways that stimulate fat intake and drugs of abuse, several studies have suggested that high consumption of palatable food could also increase vulnerability to drug use. Epidemiological studies have reported that, in clinical populations there is an overlap between binge-eating disorders and drug addiction [4], as both are characterized by typical addictive processes such as tolerance, withdrawal and compulsive food/drug-seeking. Several studies performed in rodents exposed to high-sugar diets have reported increased effects of cocaine and ethanol [5]. Although less studied, we know that a continuous high-fat diet seems to decrease cocaine reward [6], but restricted access to this diet increases the locomotor effect of cocaine in adolescent mice [7].

In order to better understand the effects of intermittent access to fat during adolescence, we have examined the rewarding effects of cocaine in mice that binged on fat during adolescence [8]. Adolescent mice had limited access to a specific high-fat chow for 2 h, three times a week (on Monday, Wednesday and Friday), while they had constant access to standard chow. We observed an increase in the amount of fat consumed by these animals within this period, and an escalation of intake within the second week of fat exposure. Therefore, the limited access protocol led to the development of fat-bingeing behaviors. However, there was no increase in body weight or leptin levels in the group exposed to fat, although ghrelin was decreased. Leptin and ghrelin are two hormones that influence energy balance. Leptin suppresses food intake and induces weight loss. Ghrelin, on the other hand, is known as the hunger hormone, as it is secreted when the stomach is empty.

To evaluate the rewarding effects of cocaine, we employed the conditioned place preference (CPP), which assesses the role of the environmental cues associated with the drug, and the intravenous self-administration (SA) procedure, which evaluates the hedonic properties of drugs of abuse. Mice that binged on fat during adolescence developed CPP with a low dose that was not effective in regular fed animals, presented a stronger memory of the environmental cues associated with cocaine, and also

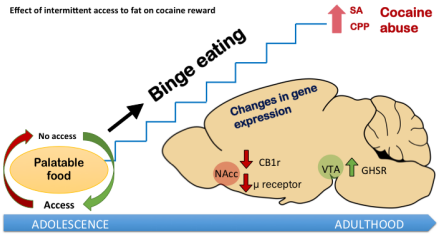


**Figure 1:** Intermittent fat ingestion increases sensitivity to the rewarding effects of cocaine and modifies the opioid and endocannabinoid systems.

relapsed in seeking the drug even after the loss of the drug-associated memory. Comparable results were obtained in the SA procedure. The total amount of cocaine consumed increased in mice exposed to the high-fat diet, and, after fat withdrawal, exposure to a new fat binge reinstated cocaine seeking. Therefore, intermittent fat ingestion increases sensitivity to the rewarding effects of cocaine and also heightens vulnerability to relapse in seeking this drug.

Intermittent fat ingestion, although it does not induce relevant hormonal changes, alters key neurotransmitter systems involved in drug addiction and food ingestion in relevant brain structures that control both processes (Figure 1). Gene expression of cannabinoid CB1 and mu opioid receptors were decreased in the nucleus accumbens of fat fed mice, accompanied by an increased expression of ghrelin receptor in the ventral tegmental area. Thus, high-fat bingeing modulates not only the dopaminergic system, but also the opioid and endocannabinoid systems. This makes animals more prone to seeking and consuming cocaine. Our results suggest that the fat composition of a diet and the way in which fat is consumed plays an important role in determining the sensitivity of an individual drug abuse, particularly during adolescence.

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# Study 3.

## The rewarding effects of ethanol are modulated by binge eating of a high-fat diet during adolescence.

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# The rewarding effects of ethanol are modulated by binge eating of a high-fat diet during adolescence

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ABSTRACT

Binge-eating is considered a specific form of overeating characterized by intermittent and high caloric food intake in a short period of time. Epidemiologic studies support a positive relation between the ingestion of fat and ethanol (EtOH), specifically among adolescent subjects.

The aim of this work was to clarify the role of the compulsive, limited and intermittent intake of a high-fat food during adolescence on the rewarding effects of EtOH. After binge-eating for 2 h, three days a week from postnatal day (PND) 29, the reinforcing effects of EtOH were tested with EtOH self-administration (SA), conditioned place preference (CPP) and ethanol locomotor sensitization procedures in young adult mice.

Animals in the high fat binge (HFB) group that underwent the EtOH SA procedure presented greater EtOH consumption and a higher motivation to obtain the drug. HFB mice also developed preference for the paired compartment in the CPP with a subthreshold dose of EtOH. Independently of the diet, mice developed EtOH-induced locomotor sensitization. After the SA procedure, HFB mice exhibited reduced levels of the mu opioid receptor (MOr) and increased cannabinoid 1 receptor (CB1r) gene expression in the nucleus accumbens (N Acc), and decreased of tyrosine hydroxylase (TH) gene expression in the ventral tegmental area (VTA).

Taken together the results suggest that bingeing on fat may represent a vulnerability factor to an escalation of EtOH consumption.



## 1. Introduction

Adolescence is a period of brain maturation during which individuals are especially vulnerable to environmental threats such as drug abuse or inadequate dietary habits (Cruz, 2000; Schneider, 2008; Bava and Tapert, 2010). Rates of overeating in the last years have grown, affecting mainly the young population (Herpertz-Dahlmann, 2015). Binge-eating is considered a specific form of overeating characterized by intermittent and excessive intake of caloric food in a short period of time (Davis et al., 2007). Many teenagers display this pattern of hedonic eating, which includes eating for pleasure, rather than for metabolic need, without reaching the clinical criteria for binge-eating disorder (Gold, 2011). 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 consumed by control animals under similar circumstances, and must be stable and maintained over time (Corwin and Buda-Levin, 2004).

Results of epidemiologic studies support a bidirectional, positive relation between the ingestion of fat and ethanol (Swinburn et al., 1998; Stickley et al., 2015). Studies in animal models also support this relationship; rats chronically injected with EtOH

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exhibit increased fat preference (Barson et al., 2009), and fat-preferring rats consume more EtOH than water, a pattern that is not seen in carbohydrate-preferring rats (Krahn and Gosnell, 1991). Few studies were performed to elucidate if a high fat diet increases ethanol intake, and those that have been done reported discrepant results. A first report in the 70's found excessive ethanol drinking in rats fed a high-fat diet (Pekkanen et al., 1978). In a more recent study, Carrillo and co-workers (2004) demonstrated that daily overeating of fat over 7 days, a single high-fat meal, or the injection of fat could increase ethanol intake. However, Much et al. (2002) observed that different diets with varying protein and fat composition did not alter ethanol preference with the two-bottle choice.

Consumption of drugs of abuse and hedonic eating, besides sharing a high comorbidity, activates common DAergic pathways (Rada et al., 2005). Acute high-fat diet intake activates dopamine (DA) and the neural pathways involved in reward and motivation processes, (Valdivia et al., 2015). There is now compelling evidence that eating of highly palatable foods causes the same neuroadaptations as drugs of abuse (Grigson, 2002; Hajnal et al., 2008; Pelchat, 2002). In addition to DA, the opioid and endocannabinoid systems also play an important role in the reward process (Wang et al., 2004). Opioid signaling, especially the μ-opioid receptor, regulates the rewarding properties of palatable food, and alterations of this system have been identified in individuals with binge eating disorder (Cota et al., 2006). The endocannabinoid system is also crucial in appetite and reward regulation and modulates the dopamine and opioid systems (Cristino et al., 2014). It has been reported that a high fat diet upregulates endocannabinoid levels (Massa et al., 2010; Higuchi et al., 2012) and that CB1 antagonists reduce binge eating (Parylak et al., 2012).

Few studies have explored how binge eating modulates the intake of drugs of abuse. Binge-eating could act as a gateway for the development of drug addiction (Puhl et al., 2011), and an increase in the sensitivity of adult and adolescent rodents to the rewarding effects of psychostimulants after bingeing on fat has been described by our group and by others (Puhl et al., 2011; Blanco-Gandía et al., 2017). Although some reports suggest that fat intake increases preference for ethanol, a recent report by Sirohi et al. (2016) found that fat binge-fed rats displayed attenuated acquisition of alcohol intake in a preference choice paradigm. Therefore, there is a need to further evaluate the impact of high fat binging on the rewarding effects of ethanol. We have used the limited access model of Corwin et al. (1998), in which animals are allowed ad libitum access to standard food while having limited access to high fat food. The self-administration procedure (SA) has a high degree of validity, as it is a measure of motivation to consume the drug (Moeller and Stoops, 2015). In addition, we assessed vulnerability to the conditioned rewarding effects of EtOH to environmental cues using the Conditioned Place Preference (CPP) paradigm. The CPP is a very sensitive technique that can detect the rewarding effects of subthreshold doses of different drugs by assessing the vulnerability of animals to cue-related rewarding effects of the drugs (Tzschentke, 2007). Finally, given that it has been proposed that behavioral sensitization is linked to the hyperactivation of some of the cerebral pathways related to reward and addiction (Yamamoto et al., 2013), we also assessed locomotor sensitization to EtOH. At the end of the EtOH SA procedure, we also analyzed mu opioid receptor (MOr) and cannabinoid receptor (CB1r) gene-expression in the N Acc. Tyrosine hydroxylase (TH) gene expression was also determined in the VTA, a brain region of the dopaminergic mesolimbic pathway closely involved in the rewarding effects of alcohol consumption (Brodie et al., 1999).

## 2. Materials and methods

### 2.1. Subjects

115 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 6 (cage size 40 × 25 × 22 cm) for 4 days prior to initiating the experimental feeding condition at a constant temperature (21 ± 2 °C), with lights on from 8:00 to 20:00 h, 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.

### 2.2. 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; no sugars added) 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; 20% of carbohydrates are sucrose) 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).

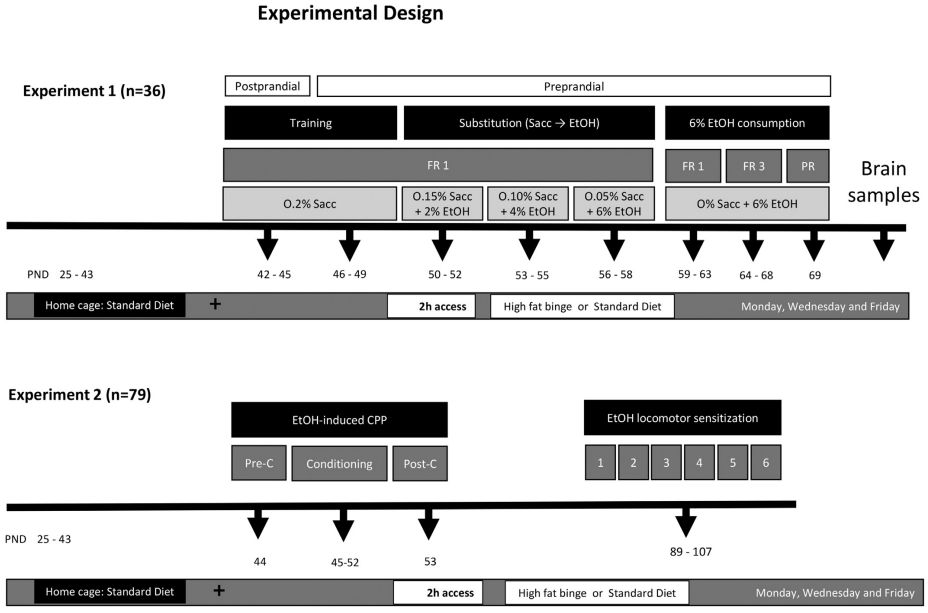
On PND 25, mice were randomly divided into groups with similar average body weight (19–20 g) and assigned either a Control (C) diet or HFB (2 h access on Monday, Wednesday and Friday). All groups were fed the standard diet in their own cages 3 days a week and were exposed to a 2-h binge session in a 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 2–3 h after initiation 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 measured.

An overall description of the experimental procedure with a detailed description of the experimental procedure of the oral EtOH self-administration is provided in Table 1.

### 2.3. Drugs

For the oral self-administration procedure, absolute ethanol (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water. For the CPP and locomotor activity experiments, ethanol (Scharlab S.L., Barcelona, Spain; EtOH), obtained from an initial stock of a 96% v/v solution was diluted at a concentration of 20% v/v in physiological saline (NaCl 0.9% w/v; Sal) and injected intraperitoneally (IP) at a dose of 0.75 (CPP) and 2 g/kg locomotor activity. Control mice were injected IP with the corresponding volume of Sal.

**Table 1**
Experimental Design. Control mice received a standard diet during the binge sessions and animals in the HFB condition had 2 h fat access every Monday, Wednesday and Friday throughout the study.




### 2.4. Apparatus and procedure

#### 2.4.1. Oral ethanol self-administration

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol self-administration was carried out in 12 modular operant chambers (Panlab, Barcelona, Spain). Packwin software (Panlab, Barcelona, Spain) controlled stimulus and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two levers, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active lever delivered 37 μl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period. The inactive lever did not produce any consequence.

To evaluate the consequences of a HFB on the acquisition of oral EtOH self-administration (n = 18 per group), animals underwent an experiment carried out in three phases: training, saccharin substitution and 6% EtOH consumption.

*2.4.1.1. Training phase (8 days).* Two days before initiation of the experiment, access to the standard or fat diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and food allotment was provided 1 h prior to the 1 h session eating to increase the motivation for lever pressing. During the subsequent 3 days, water was provided ad libitum, except during the 1 h period of food access before beginning each session, in which the water bottle was removed from the cages (postprandial). For the following four days, and during the rest of the experiment, food access was provided for 1 h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced weight loss in the mice of around 15% of their free-feeding weight (Navarrete et al., 2012). Mice were trained to press on the active lever to receive 37 μl of 0.2% (w/v) saccharin reinforcement.

*2.4.1.2. Saccharin substitution (9 days).* The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 1998; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac −2% EtOH; 0.10% Sac −4% EtOH; 0.05% Sac −6% EtOH).

*2.4.1.3. 6% ethanol consumption (11 days).* The aim of the last phase was to evaluate the number of responses on the active lever, the 6% EtOH (w/v) intake and the motivation to drink. To achieve this goal, during the last phase, the number of effective responses and EtOH consumption (μl) were measured under fixed ratio 1 (FR1) for 5 daily consecutive sessions, fixed ratio 3 (FR3) (mice have to respond three times on the active lever to achieve one reinforcement) for 5 consecutive daily sessions, and finally, on the day after FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of lever

presses/nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement, according to the previous scale (for example, if an animal pressed the active lever a total of 108 times, this meant it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the breaking point value for this animal would be 40.). All the sessions lasted 1 h, except the PR session, which lasted 2 h.

*2.4.2. Conditioned place preference*

For place conditioning, five identical Plexiglas boxes with two equal size compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a grey central area (13.8 long × 31.5 cm wide × 34.5 high) were employed. The compartments had different colored walls (black vs. white) and distinct floor textures (smooth in the black compartment and rough in the white one). Four infrared light beams in each compartment 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, SA, Spain).

To evaluate the effects of a HFB on the acquisition of EtOH-induced CPP animals (n = 12 per group) underwent an unbiased CPP procedure consisting of three different phases: preconditioning (1 session; Pre-C), conditioning (8 sessions), and preference testing (1 session; post-conditioning, Post-C). On the first day (Pre-C), mice were introduced into the central area of the apparatus with the guillotine doors opened to allow them access to both compartments of the apparatus for 900 s. The time spent by the animal on each side during this period was recorded. Subjects showing a strong unconditioned aversion (<33% of the session time) or preference (>67%) for any cubicle were discarded from the study. The conditioning phase began 24 h after the Pre-C phase. In each group, half of the animals received EtOH in one compartment and the other half in the other compartment, in a counterbalanced manner. After randomly assigning the animals to a conditioning chamber, ANOVA confirmed there were no significant differences between the time spent in the EtOH- and the Sal-paired compartments during the Pre-C phase (data not shown). In the second phase (conditioning), half of the mice were injected i.p. with Sal and the other with EtOH (0.75 g/kg), and were immediately placed in the corresponding conditioning chamber. On alternate days, contingencies were inverted and the animals that had received Sal the day before were injected with EtOH (0.75 g/kg) and those who had received EtOH (0.75 g/kg) were given Sal immediately prior to being introduced into the other conditioning compartment. Subjects underwent a total of 4 pairings for each condition separated by a 24 h interval over an 8-day period. During the 5-min conditioning trial the central area was inaccessible by closing the guillotine doors. The duration of the EtOH pairing was selected because it has been shown that it is induces CPP in mice (Ledesma and Aragon, 2013; Pina et al., 2015). We have previously confirmed that 1 and 2 g/kg of ethanol are effective doses for inducing CPP in standard diet-fed mice; thus, we selected a lower dose - 0.75 g/kg - that, according to previous reports, is ineffective in such mice (Groblewski et al., 2008). The preference test (Post-C) took place 24 h after the last conditioning assay. During Post-C, the guillotine doors separating the two cubicles were removed and the time spent by the untreated mice in each chamber was recorded for 900 s. Subjects underwent binge eating sessions throughout the whole CPP experiment.

To confirm that bingeing on fat affects responsivity only to ethanol and not to all motivational processes and natural rewards, two additional groups of mice (Standard Diet; HFB, n = 15 per group) were conditioned with chocolate (Choco Krispies; Kellogs, Tarragona, Spain). The conditioning procedure was similar to that described by García-Pardo et al. (2015), following the same procedure as the EtOH-induced CPP, but with each pairing session lasting 30 min. To ensure acquisition of CPP, the mice were habituated to the chocolate by allowing them access in their cages over 6 days, removing it 3 days before initiation of conditioning. The animals were deprived of food for 8 h before each conditioning session with chocolate.

*2.4.3. EtOH-induced locomotor sensitization*

To assess the effect of a HFB on the locomotor sensitization elicited by EtOH (2 g/kg), animals (n = 7–8 per group) were injected i.p. and were tested in open-field chambers consisting of four Plexiglas cages (30 cm long × 30 cm wide × 35 cm high) in which locomotor activity was registered by a computerized video-tracking system (Ethovision, Noldus S.A., The Netherlands). Movement of the mouse inside the open-field chambers was recorded and translated automatically by the software to horizontal distance traveled in cm during a 10-min period. The sensitization training protocol involved six trials on alternate days; one trial per day. For the experiment, mice were brought from the vivarium to the experimental chamber 10 min before every session. At the start of each assay, subjects were administered a Sal or EtOH (2 g/kg) injection and immediately placed in the center of the activity enclosure for 10 min. This procedure was selected based on previous reports showing that it evokes locomotor sensitization in mice (Miquel et al., 2003).

*2.4.4. Blood ethanol measures*

Blood ethanol levels were assessed after i.p. administration of 2 g/kg of ethanol. Blood was collected 15, 30 and 60 min after the ethanol challenge. Blood samples were taken by means of the tail-nick procedure, in which the animal is wrapped in a cloth and a 2-mm incision is made at the end of the tail artery. The tail is then massaged until 50 μl of blood is collected in an ice-cold Microvette® CB 300 capillary tube (Sarstedt, Germany). Blood samples were kept on ice, and plasma was separated from whole blood by centrifugation (5 min, 5000 g) and transferred to sterile 2 ml microcentrifuge tubes. The supernatant was then placed in cuvettes with optical properties suitable for use with a spectrophotometer set at 340 nm. Blood ethanol content was enzymatically determined with the NAD-ADH Reagent Multiple Test Vial Kit (Sigma Aldrich S.A.).

*2.4.5. Gene expression analyses. Real time PCR*

Brain sections were cut (500 μm) in a cryostat (−10 °C) at levels containing the regions of interest described by Paxinos and Franklin (2001), and were then 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 TH and μ-opioid receptor mRNA was performed in the Step One Real Time PCR System (Life Technologies, Madrid, Spain). All reagents were obtained from Applied Biosystems and manufacturer protocols were followed. The reference gene 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(-ΔΔCt) method (Livak and Schmittgen, 2001).

### 2.5. Statistics

Data relating to binge intake were analyzed by a mixed ANOVA with one between-subjects variable – Diet, with 2 levels (Control, HFB) - and a within variable – Days, with 8 or 11 levels for adolescence and the SA phase, respectively. Data related to body weight were also analyzed by means of a mixed ANOVA with the same between-subjects variable - Diet - and within variable – Days - but with 12 levels. In order to evaluate if fat intake differed at different points of the experiment, we performed a second mixed ANOVA with one between-subjects variable – Diet, with 2 levels (Control, HFB) - and a within variable – Phase, with three levels (Adolescence, SA Training and EtOH SA). In addition, an ANCOVA with body weight as covariant was performed to compare daily Kcal intake of control and HFB groups. Furthermore, an ANOVA of the Kcal ingested was performed for the HFB group, with a between-subjects variable - Diet - and a within variable – Days - but with 7 levels.

To analyze acquisition of EtOH SA, a two-way ANOVA was performed with Diet (control or HFB) as a between factor and Days (5 levels for FR1 or FR3) as a within factor. A Student's t-test was employed to analyze breaking point values and ethanol consumption during PR.

For the 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 2 levels (Control, HFB for the CPP induced with 0.75 g/kg of EtOH or chocolate), or EtOH dose, with 2 levels (1 and 2 g/kg) - and a within variable - Days, with 2 levels (Pre-C, and Post-C).

Data from the horizontal locomotion (cm) carried out during the second and the sixth days of treatment were analyzed by means of a three-way ANOVA with Diet (Control or HFB) and Treatment (Saline or EtOH) as the between-subjects variables and Days (2 and 6) as the within-subjects variable. 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. Binge escalation

The ANOVA of the kcal intake during each 2-h binge session (Fig. 1a) showed an effect of the variables Diet [$F(1,79) = 13,511$; $p < 0.001$] and Days [$F(6.474) = 18,678$; $p < 0.001$], and the interaction Days x Diet [$F(6,474) = 8770$; $p < 0.001$]. Intake of high-fat food was significantly higher in the HFB group from day 3 onwards ($p < 0.001$ in all cases). These results confirmed the development of a binge pattern of high fat diet intake. Once the SA procedure had initiated, binge sessions lasted 1 h and took place on Monday, Wednesday and Friday, which was the only time animals had access to fatty food. In this case ANOVA also revealed an effect of the variables Days [$F(10,340) = 30,165$; $p < 0.001$)] and Diet [$F(1,34) = 1534,573$; $p < 0.001$] and the interaction Days x Diet [$F(10,340) = 37,973$; $p < 0.001$]. HFB animals exhibited an increased intake of kcal in the 1-h period of food access with respect to the control group ($p < 0.001$). In addition, all the animals showed an increase in their intake as time passed with respect to the first day of restricted access ($p < 0.001$ in all cases). An ANCOVA with body weight as covariant to compare daily Kcal intake of control and HFB groups did not reveal any significant effect. Furthermore, the HFB group consumed significantly less energy from the fat pellets during the 2 h binge than from standard chow over a 24 h period [$F(1,34) = 663.990$, $p < 0.001$]. The amount of Kcal ingested in the form of fat showed a significant increase [$F(1,34) = 14.441$, $p < 0.001$] between the first and subsequent days of bingeing ($p < 0.001$, in all cases), confirming the development of binge-eating behavior (see Fig. 2).

The ANOVA to detect differences between the different phases of the experiment (see Fig. 1b) revealed an effect of the variables Diet [$F(1.34) = 1716,857$; $p < 0.001$] and Phase [$F(2,68) = 1751,431$; $p < 0.001$], and the interaction Diet x Phase [$F(2,68) = 283,953$; $p < 0.001$]. The fat intake of HFB animals continued to escalate during SA training with respect to the adolescent period ($p < 0.001$), and during the EtOH phase with respect to the adolescent period and SA training phase ($p < 0.001$ in both cases). However, in the Control group, kcal consumption only increased during the phases in which access to food was restricted to 1 h per day. In all three phases, HFB animals presented an increased kcal intake with respect to the control group ($p < 0.001$).

In terms of body weight (Fig. 1c), ANOVA revealed an effect of the variables Days [$F(11,363) = 492,663$; $p < 0.001$] and Diet [$F(1,33) = 28,824$; $p < 0.001$] and the interaction Days x Diet [$F(11,363) = 53,054$; $p < 0.001$]. No significant differences in body weight were detected before beginning the SA procedure. From PND 42 onwards HFB mice presented higher body weight than controls ($p < 0.05$ on PND 42 and $p < 0.001$ the rest of days). However, during the SA procedure when access to food was restricted, all animals lost approximately 15% of their body weight. The ANOVA to evaluate differences between the three phases of the experiment (see Fig. 1d) revealed an effect of the variables Diet [$F(1.33) = 60.665$; $p < 0.001$] and Phase [$F(2,66) = 284,527$; $p < 0.001$], and the interaction Diet x Phase [$F(2,66) = 81,760$; $p < 0.001$]. HFB animals weighed significantly more than the control group during phases 2 and 3 (SA training and EtOH SA) ($p < 0.001$). In addition, their body weight increased significantly during the EtOH phase with respect to the adolescent period and SA training phase ($p < 0.001$ in both cases), while the Control group only showed a slightly increase in their body weight during EtOH SA with respect to SA training ($p < 0.001$).

### 3.2. Blood ethanol levels

The ANOVA of the blood ethanol levels did not reveal any statistical difference (Table 2).

### 3.3. Effects of HFB on oral self-administration of ethanol

No differences were found between animals during training or substitution phases, showing that bingeing on fat did not induce any learning deficit (data not shown).

With respect to the effects of bingeing on fat during the FR1 schedule of EtOH SA, the ANOVA for the number of effective responses revealed a significant effect of the interaction Days x Diet [$F(4,132) = 2714$; $p < 0.05$]. Mice in the HFB group responded to a lesser extent on day 2, but to a greater extent on day 4 when compared with controls ($p < 0.01$) (Fig. 3a). With respect to EtOH consumption, the ANOVA revealed a significant effect of the variables Days and Diet ([$F(4,132) = 2514$; $p < 0.05$] and [$F(1,33) = 13,933$; $p < 0.001$], respectively) (Fig. 3b). Mice in the HFB group showed a higher EtOH consumption on days 1 ($p < 0.05$), 2 ($p < 0.001$), 3 ($p < 0.05$), 4 $p < 0.01$) and 5 ($p < 0.05$) with respect to the control group.

During the FR3 schedule, ANOVA revealed a significant effect of the variable Diet [$F(1,33) = 9930$; $p < 0.01$] on EtOH consumption, confirming an increased EtOH SA in animals in the HFB group with respect to the control group on days 6, 7 ($p < 0.05$), 8 and 9 ($p < 0.01$).

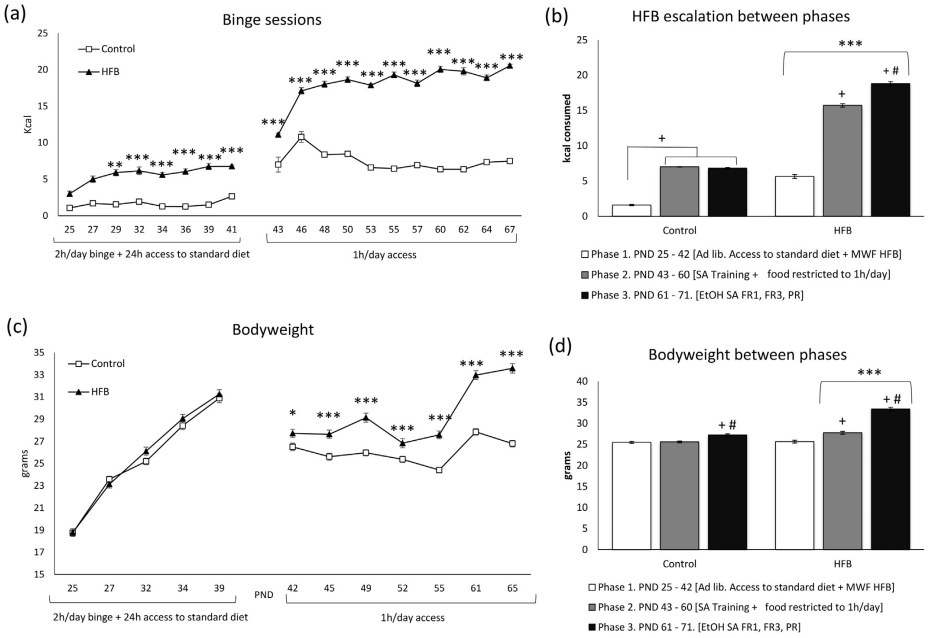



**Fig. 1.** Binge intake, HFB escalation and bodyweight. (a) Intake (kcal) during the 2-h High-fat binge-eating sessions that took place on Monday (M), Wednesday (W) and Friday (F) and during the Self-administration period (1 h access on MWF). The weekly mean (±SEM) amount of kcal consumed during limited access to high-fat food (control group had access to standard food) is stated to confirm the escalation of intake. (b) Mean of HFB escalation for the 3 phases: 1. Adolescence (white bar), where animals had free access to the standard diet and MWF underwent fat binge sessions; 2. SA Training phase (grey bar), during which food was restricted to 1 h/day; and 3. EtOH SA phase (black bar, fat binge sessions on FR1, FR3 and PR schedules). (c) Bodyweight during the whole procedure. (d) Mean bodyweight during the 3 phases. **p < 0.01, ***p < 0.001 significant difference with respect to the control group; + p < 0.001 significant difference with respect to phase 1. #p < 0.001 significant difference with respect to phase 2.

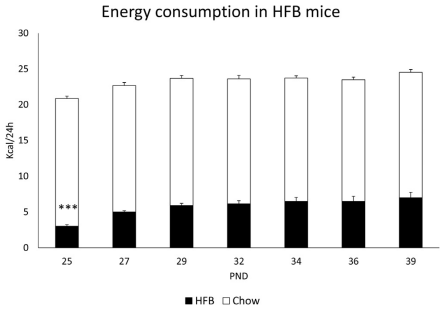



**Fig. 2.** Mean (±SEM) 24 h energy consumption (kcal) per cage (4 mice) in the HFB condition (chow shown by open bars and HFB by solid bars). ***p < 0.001 significant differences with the rest of the days.

During the progressive ratio ANOVA showed an effect of the variable Diet on the number of effective responses to reach the Breaking point [F(1,33) = 9733; p<,001]. Breaking point value was significantly higher in the HFB group (Fig. 3c), and 6% EtOH consumption was higher in the HFB group [F(1,33) = 4771; p < 0.05] (Fig. 3d).

### 3.4. Effects of HFB on the acquisition of EtOH-induced CPP

The ANOVA for the data of the CPP induced by 1 and 2 g/kg of EtOH revealed an effect of the variable Days [F(1,21) = 21,493; p < 0.001], as both groups developed preference for the compartment associated with EtOH.

As can be seen in Fig. 4, HFB modulates the conditioning rewarding properties of EtOH (0.75 g/kg). ANOVA revealed a significant effect of the variable Diet (Control vs HFB) [F(1,22) = 4,72; p < 0.05] and the interaction Diet x Days [F(1, 22) = 5,39; p < 0.05]. Post-hoc comparisons showed that the time spent in the EtOH-paired compartment was significantly higher during the Post-C with respect to the Pre-C in the HFB group only (p < 0.05). Moreover, the time spent in the EtOH-paired chamber during Post-C by the HFB group was also significantly higher than that of the control group (p < 0.05).

For the chocolate-induced CPP (Fig. 7) the ANOVA revealed an effect of the variable Days [F(1,28) = 4318; p < 0.05], as the standard

**Table 2**
Effects of a HFB diet on blood EtOH levels. Mean ± SEM of blood EtOH concentrations (mg/dl) after an acute IP injection of EtOH (2 g/kg) to mice (n = 6 per group).

| | Blood EtOH levels (mg/dl) | | |
|---|---|---|---|
| | 15 min after EtOH injection | 30 min after EtOH injection | 60 min after EtOH injection |
| Standard Diet | 261 ± 19 | 251 ± 20 | 244 ± 11 |
| HFB | 235 ± 22 | 261 ± 19 | 251 ± 25 |

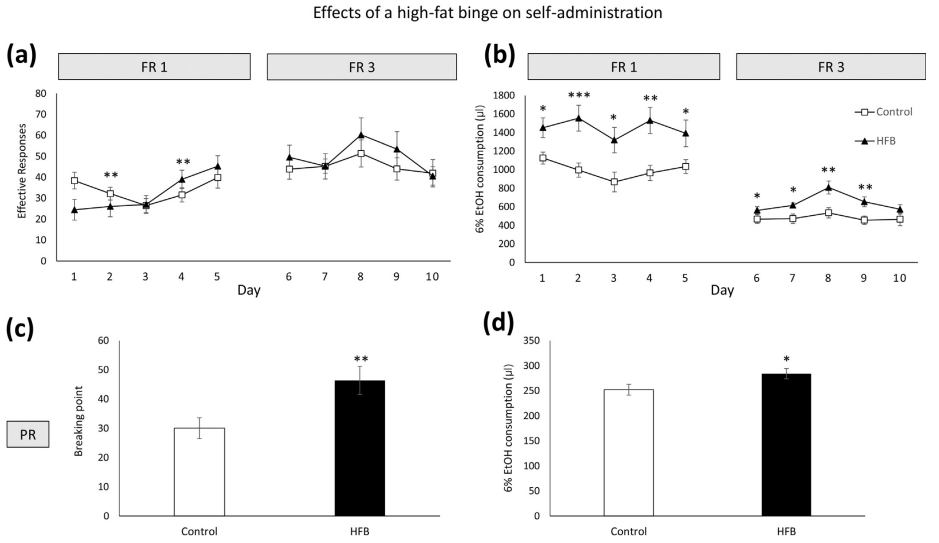



**Fig. 3.** Effects of bingeing intermittently on a high-fat diet on oral EtOH self-administration in OF1 mice (n = 18/condition). The dots represent means and the vertical lines ± SEM of (a) the number of effective responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent mean and the vertical lines ± SEM of (c) the breaking point values during PR and (d) the volumes of 6% EtOH consumption in the PR. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to control mice.

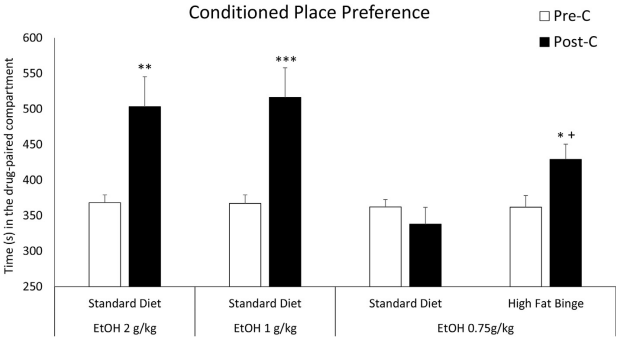



**Fig. 4.** Effects of bingeing intermittently on a high-fat diet on the acquisition of EtOH-induced CPP. Bars represent mean (±SEM) time spent in the EtOH-paired compartment for the different Diet (C or HFB) groups (n = 12 per group) during the Pre-C (white bars) and the Post-C (black bars). *p < 0.05 significantly different compared to Pre- C; + p < 0.05 significantly different compared to control Post-C test.

group developed preference for the Chocolate-paired compartment on the Post-C day ($p < 0.01$), while the HFB group did not.

### 3.5. Effects of HFB on EtOH-induced locomotor sensitization

Fig. 5 displays the effects of HFB on the locomotor-sensitizing effects of EtOH. ANOVA revealed a significant effect of the variable Treatment [$F(1,28) = 12{,}994$; $p < 0.001$] and the interactions Diet x Treatment [$F(1,28) = 4732$; $p < 0.04$], mice treated with EtOH and fed with HFB showed a stronger locomotor response ($p < 0.01$). The ANOVA also showed an effect of the interaction Days x Treatment [$F(1,28) = 4876$; $p < 0.04$]. Locomotion was significantly higher on day 6 than day 2 only in mice treated with EtOH ($p < 0.05$), indicating sensitization to the locomotor effects of EtOH.

### 3.6. Effects of a HFB on MOr, CB1 and TH gene expression

As can be seen in Fig. 6a and b, real-time PCR analyses indicated that exposure during adolescence to a HFB before EtOH self-administration induced a decrease of MOr and an increase of CB1 gene expression values in the N Acc with respect to controls fed a standard diet (Student's t-test, $t = 3.970$, 16 d.f., $p < 0.001$ and $t = -3.820$, 16 d.f. $p < 0.01$ respectively). In terms of the VTA (Fig. 5c), HFB animals exhibited a decrease of TH gene expression with respect to controls. (Student's t-test, $t = 3.3033$, 16 d.f. $p < 0.01$).

## 4. Discussion

The current study demonstrates, for the first time, the strong interaction between bingeing on fat during adolescence and the rewarding effects of ethanol. First, our mice developed a binge-like consumption of fat despite having continuous access to standard food. Second, animals that binged intermittently on fat during adolescence exhibited a 6% higher EtOH consumption in the oral SA paradigm and showed increased motivation to obtain the drug with a progressive ratio schedule. Third, mice were more sensitive to the conditioned rewarding effects of subthreshold doses of EtOH (0.75 g/kg) that were not effective in standard animals. Fourth, HFB mice exhibited changes with respect to the control group in gene expression of CB1, MOr and TH after EtOH SA. These results lead us to suggest that bingeing on fat during adolescence contributes to the development of a specific vulnerability to alcohol abuse.

The model employed in the present study uses intermittent and limited access to palatable food to induce bingeing and escalation of intake without energy restriction. In accordance with previous results obtained in our laboratory and by other groups, we have observed that the limited access protocol leads to the development of fat-bingeing behaviors (Corwin et al., 1998; Corwin, 2004; Wojnicki et al., 2008; Blanco-Gandía et al., 2017). The animals allowed intermittent access to fat increased their intake of food and kcal from the third binge session onwards. However, the HFB group consumed significantly less energy from the fat pellets during the 2 h binge than from the standard chow over a 24 h period.

Our results showed no differences in blood EtOH levels between standard diet and HFB groups when 2 g/kg were injected ip. It is possible that stomach and intestinal fat content interfered with the absorption of ethanol from the gut in the SA experiment, as it was consumed orally. However, we would like to underline that animals performed all the behavioral tests (SA, CPP, and sensitization) before the high-fat binge eating session on the day in question; therefore, no fatty food was in their stomach or intestine when they performed the tests.

During the self-administration procedure, when feeding was restricted to 1 h per day, HFB mice continued to show higher kcal consumption and body weight than controls. Moreover, kcal in these mice was higher during the SA EtOH period than during SA training. This suggests that the increase in fat intake despite the administration of EtOH was also a source of kcal, which may have been due to a positive relation between the ingestion of fat and ethanol, as hinted at by previous reports (Swinburn et al., 1998; Stickley et al., 2015).

Mice fed HFB showed higher EtOH consumption under both FR1 and FR3 ratio fixed schedules. A possible learning deficit can be ruled out, as both groups acquired and maintained a stable operant response. However, while HFB mice maintained a stable and regular consumption of EtOH, control mice displayed a more linear truncated trend. On the one hand, we have observed that, even when there were no differences in the number of effective

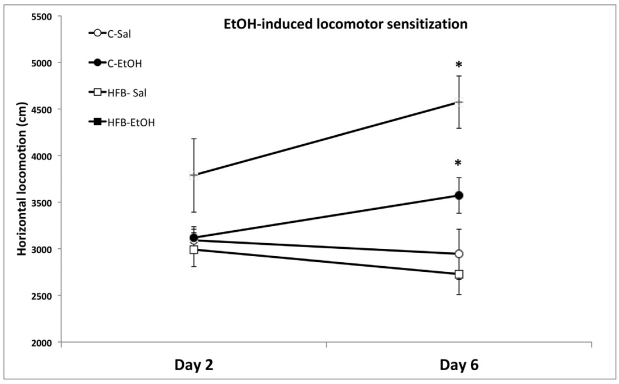



**Fig. 5.** Effects of bingeing intermittently on a high-fat diet on EtOH-induced locomotor sensitization. Values represent mean (±SEM) locomotor activity (cm in 10 min) for mice ($n = 7–8$ per subgroup) previously exposed to a standard or HFB diet and treated with Sal or EtOH (2 g/kg) immediately before being introduced into the open field on 6 alternate days. For the statistical analyses, only data for day 2 and day 6 were considered. *$p < 0.05$ significantly different from Day 2.

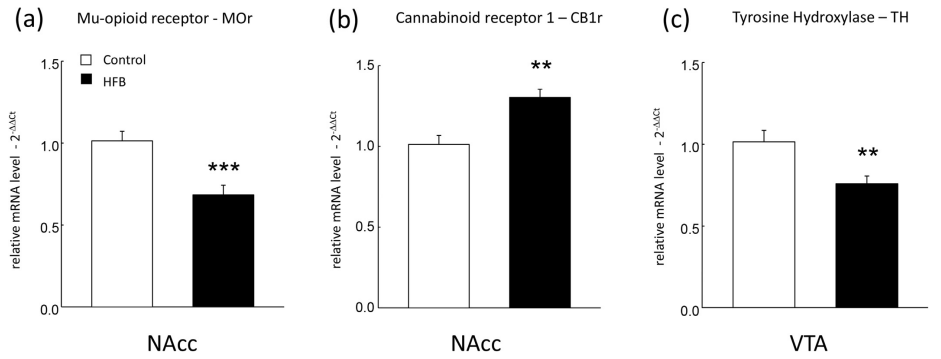



**Fig. 6.** Effects of a HFB on gene expression in the N Acc and VTA. (a) Real-time PCR MOr relative gene expression evaluation in the N Acc brain region of control and HFB groups. (b) CB1 relative gene expression evaluation in the N Acc brain region of the two groups. (c) TH relative gene expression evaluation in the VTA brain region of the two groups. The columns represent mean and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the N Acc or VTA of OF1 mice (n = 9 in all groups). **p < 0.01, ***p < 0.001 values of HFB mice that differ significantly from their corresponding controls.

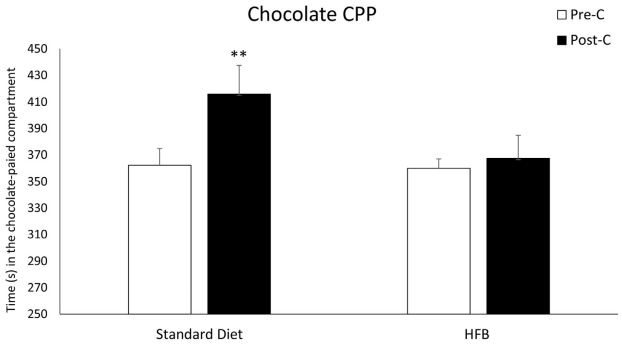



**Fig. 7.** Effects of bingeing on a high-fat diet on the acquisition of Chocolate-induced CPP. Bars represent mean (±SEM) time spent in the Chocolate-paired compartment for the different Diet (C or HFB) groups (n = 15 per group) during the Pre-C (white bars) and the Post-C (black bars). **p < 0.01 significantly different compared to Pre- C.

responses, the HFB group consumed more EtOH than the standard diet group, which means that control animals did not drink all the EtOH they gained access to by pressing the lever. In addition, a higher progressive ratio response was seen in HFB mice, which may suggest an increased motivation and compulsivity to obtain the drug, as higher breaking points in the PR schedule were paralleled by higher rates of lever pressing by the HFB mice. Further studies are needed to assess changes in motivation due to HFB.

A key point to take into consideration is that the SA procedure requires food restriction in order to induce learning of the operant task and promotes EtOH consumption. The procedure we employed has been validated in previous studies (Rodríguez-Arias et al., 2016). It is known that DA accumulates in the presynaptic terminals of the N Acc during starvation, promoting higher DA release when a reinforcer is administered (Pothos et al., 1995). However, in the present study, food restriction was applied to both control and HFB mice, and both groups showed equal learning of the operant task. We observed escalation of fat consumption in the HFB group during FR1 and FR3 schedules of EtOH self-administration, with said animals consuming more fat and more EtOH than control animals during this phase. In line with this, several studies have pointed to a common mechanism underlying diet preference and oral intake of EtOH. The development of rat lines with a high preference for the rewarding aspects of alcohol show higher fat intake by these animals (presumably indicative of higher fat preferences) in diet self-selection situations (Forsander, 1988). Moreover, fat-preferring rats have been shown to consume significantly more EtOH than carbohydrate-preferring counterparts (Krahn and Gosnell, 1991), results corroborated by Barson et al. (2009), who reported a significant increase of fat preference in rats chronically injected with EtOH. Thus, the effects of and preference for drugs may be bidirectionally influenced.

In a pioneering study in 1978 using EtOH in a free choice consumption procedure, Pekkanen and Eriksson described that rats fed a 4-week fatty diet with a high percentage of energy drank more EtOH than controls fed a balanced diet. More recently, Carrillo et al.

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**Conflicts of interest**

None.

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# Study 4.

## Ethanol consumption is enhanced 15 days after fat binge eating interruption

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*In preparation*

# Ethanol consumption is enhanced 15 days after fat binge eating interruption

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**Abstract**

There is a high comorbidity between alcohol abuse and eating disorders, especially in the young population. We have previously reported that bingeing on fat during adolescence increased the rewarding effects of ethanol (EtOH). The aim of the present study was to clarify if, after cessation of binge eating, this vulnerability to EtOH persisted. We employed OF1 mice that binged on fat during adolescence (PND 25- 39) and tested them 15 days after the last access to a high-fat binge (HFB) using the self-administration paradigm, the conditioned place preference (CPP) and the locomotor sensitization to ethanol. Our results showed that after 15 days from the last binge session, animals in the HFB 15W group displayed increased ethanol consumption and higher motivation to obtain ethanol. Moreover, there were no effects in the CPP but HFB 15W animals exhibited increased locomotor response to ethanol. The present work sustains our previous study that demonstrates that the compulsive intake of fat leads to a gateway to other addictive disorders, such as alcohol consumption, and the effects produced by bingeing on fat have long lasting consequences, even when access is interrupted.

**Key words:** binge eating, fat, ethanol, conditioned place preference, self-administration, fat withdrawal

## 1. Introduction

Adolescence is a developmental period full of synaptic plasticity (Spear, 2000; Chambers et al., 2003), in which individuals become especially vulnerable to environmental threats, such as stress, drug abuse or inadequate dietary habits (Cruz, 2000; Schneider, 2008; Bava and Tapert, 2010). In this period of brain maturation alcohol can have a negative impact on its structure and function (Harper and Matsumoto, 2005), producing short and long-term consequences, such as memory impairment and neural cell death in several brain regions (Pascual et al., 2007), which are mostly irreversible (Guerri, 2002). On the other hand, we know that to date, alcohol is one of the first drugs of choice among teenagers. Substance abuse in early stages of life is linked with a wider rate of drug abuse and dependence in adulthood (Brown et al., 2008, Nasrallah et al., 2009).

Drugs and hedonic eating share common dopaminergic pathways, and a great number of studies have demonstrated the comorbidity that exists between them (Gold et al., 2003; Hudson et al., 2007; Davis and Carter, 2009). Today we know that eating for pleasure, and not for metabolic need, affects dopamine release and the neural pathways that are involved in reward and motivation processes, which in turn further reinforces this type of eating behavior (Avena et al., 2008; Volkow et al., 2013; Murray et al., 2014). Particularly, binge-eating is considered a specific form of overeating that in recent years has been studied and deliberated as an addictive behavior that mimics that of drugs of abuse (Corwin and Wojnicki, 2006). It is characterized by a dysfunctional appetite, which is manifested by an intermittent, excessive intake of caloric food. Many teenagers exhibit this kind of hedonic eating (Gold, 2011), which covers eating for pleasure, rather than for metabolic need, without meeting the clinical criteria for a binge-eating disorder. Moreover, studies in animals have confirmed that adolescent rats are more prone to binge-eating than adults (Bekker et al., 2014). Nevertheless, it has been proposed that binge-eating, as a maladaptive behavior, could work as a gateway for the development of drug addiction (Degenhardt et al., 2009; Puhl et al., 2011), and it has already been reported to be the case with sugar

(Avena et al., 2004), where rats that binge on sugar consumed more EtOH than those with an *ad libitum* access to sugar. We have recently confirmed that bingeing on fat increases EtOH consumption and the conditioned rewarding effects of EtOH (Blanco-Gandía et al., *under review*).

As with drugs of abuse, withdrawal from and craving for specific kinds of foods has also been observed and measured in humans (Rogers and Smit, 2000). There is extensive evidence of dependence on sugar and withdrawal (Bello et al., 2003; Wojnicki et al., 2007; Avena, 2007; Cottone et al., 2008; Colantuoni et al., 2001). For instance, rats given intermittent access to sugar and then forced to abstain exhibit enhanced intake of alcohol (Avena et al. 2004). However, data regarding the dependence of high-fat food are scarce. We have previously reported that 2 weeks after the abrupt interruption of continuous access to fat, animals present higher anxiety levels, thus confirming a state of withdrawal (Blanco-Gandía et al., 2017a). In addition, these mice were more sensitive to the conditioned rewarding effects of cocaine. With respect to the consequences of an abrupt cessation of fat bingeing, we observed an increase in anxiety 15 days after the last fat binge session, with normal response to cocaine-induced CPP but increased response in cocaine SA (Blanco-Gandía et al., 2017b). Therefore, ours and other studies suggest that withdrawal from high-fat diet can induce cross-sensitization behavior with drugs of abuse

The aim of the present study was to evaluate if the previously reported effects of HFB are long-lasting and remain after fat discontinuation. To that end, mice were exposed to a HFB pattern during adolescence and 2 weeks after cessation of this diet, EtOH self-administration (SA), conditioned place preference (CPP) and locomotor sensitization were evaluated. We employed the limited access model from Corwin and co-workers (1998), in which animals escalate in their intake of a high-fat diet (binge) with an intermittent (Monday, Wednesday, Friday) and limited (2h) access. These animals are satiated and develop a binge-eating pattern on palatable food, as they have ad libitum access to standard chow and limited access to high-fat food. Although current studies with high-fat diets are scarce, we

hypothesized that, based on previous results on cocaine (Blanco-Gandía et al., 2017b), animals that binged on fat would still be more vulnerable to the rewarding effects of ethanol after a period of fat discontinuation.

## 2. Materials and Methods

### 2.1. Subjects

A total of 100 male mice of the OF1 strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28 x 28 x1 4.5cm) for 5 days prior to initiating the experimental feeding condition, at a constant temperature (21±2°C), lights on from 8:00 to 20:00, 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 present study.

### 2.2. Drugs

For the oral self-administration procedure, absolute ethanol (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) ethanol solution that was equivalent to a 7.6% (v/v) ethanol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water. Ethanol (Scharlab S.L., Barcelona, Spain; EtOH), obtained from an initial stock of a 96% v/v solution was diluted at a concentration of 20% v/v in physiological saline (NaCl 0.9% w/v; Sal) and injected intraperitoneally (IP) at the dose of 0.75 and 2 g/kg for the CPP and the locomotor activity experiments respectively. Control mice were injected IP with the corresponding volume injections of Sal.

## 2.3. Apparatus and Procedure

### *2.3.1 Experimental Design*

In this study, we employed 3 sets of animals. Each set was composed by 2 groups: control and HFB 15W. One set performed the SA procedure, the second set underwent the CPP and a third set carried out the locomotor sensitization test.

Control mice in each set were fed with a standard diet in their cages and during the binge sessions. Animals in the HFB condition received standard diet in their home cages but had 2h access every Monday, Wednesday and Friday (MWF) to high-fat food until 15 days before the behavioral tests (HFB 15W). During the SA procedure, animals were restricted to only 1h access to standard food per day and HFB 15W did not binge anymore. The food restriction schedule produced weight loss in mice of around 15% from their free-feeding weights (Navarrete et al., 2012).

A thorough description of the experimental procedure is detailed on Figure 1.

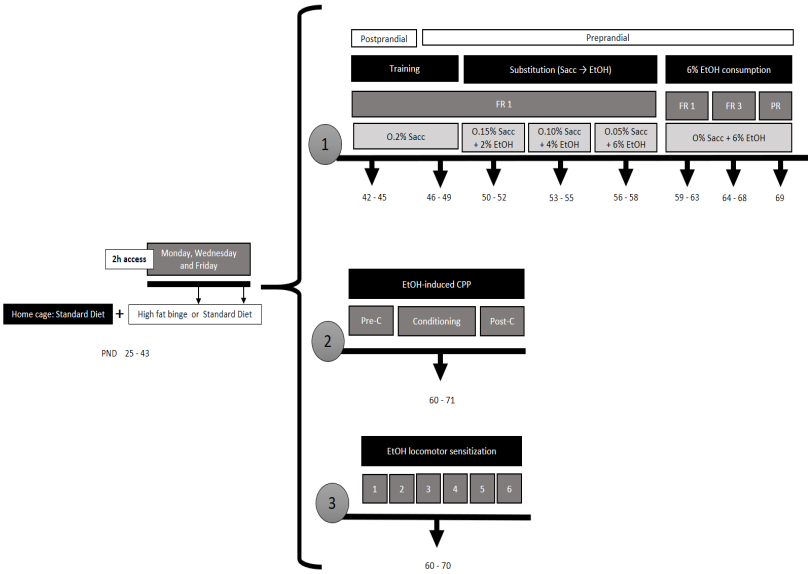


Figure 1. Experimental design

### *2.3.2. Feeding Conditions*

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

Mice were acclimated for 5 days before initiating experiments. Then, they were randomly divided into two groups with similar average bodyweight (25-26g) and assigned either to: Control (C) or to a high-fat diet binge 15 days withdrawal (HFB 15W), which consisted in 2h access on MWF. All groups were fed with the standard diet in their own cages and, 3 days a week, all groups had 2h access to the binge session in an individual plastic cage, the control group having standard diet, and the HFB 15W group having the high-fat diet. Water was freely available at all times.

Binge sessions started on PND 25 and finished on PND 39, every MWF for 2h. On PND 42 animals began the Training Phase and on PND 59 the 6% EtOH consumption phase. The escalation in consumption of the high-fat diet from the first week access (PND 25-29) and the last week before the tests (PND 39-43) shall confirm that there was a significant escalation and therefore a binge-eating pattern. Mice and the standard diet food in their home cage were weighed every MWF throughout the study.

*2.3.3. Oral ethanol self-administration*

This procedure is based on that employed by Navarrete et al., (2014). The set was composed by animals with a standard diet during the binge sessions (n=10) and animals in the HFB 15W condition (n=20) with a 2h access every MWF to high-fat food until 15 days before the 6% EtOH consumption phase (HFB withdrawal group).

Oral ethanol self-administration was carried out in 5 modular operant chambers (Med Associates, Inc.) and Med-PC IV software controlled stimuli and fluid delivery and recorded the operant responses. These chambers had two small holes with adjacent photocells to detect nose-poke responses. Active nose-poke delivered 37 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, followed by a 6s time out period. Inactive nose-pokes did not have any consequence.

The experiment was carried out in three phases: training, saccharin substitution and 6% EtOH consumption.

***Training phase (8 days)***

Two days before beginning the experiment, standard diet was restricted to only 1h access per day. Before the first training session, water was deprived for 24h, and food allotment was provided 1h prior to the 1h session to increase the motivation for lever pressing. During the subsequent 3 days, water was provided ad libitum except during food access for 1h before beginning each session, in which the water bottle was removed from the cages (postprandial). The following four days and during the rest of the experiment, food access was provided for 1h after the end of each daily session and water was available ad libitum to avoid EtOH consumption due to thirst (preprandial). The food restriction schedule produced weight loss in mice of around 15% from their free-feeding weights (Navarrete et al., 2012). Mice were trained to nose-poke to receive 37 µl of 0.2% (w/v) saccharin reinforcement.

***Saccharin substitution (9 days)***

The saccharin concentration was gradually decreased as the EtOH concentration was gradually increased (Roberts et al., 1998; Samson, 1986). Each solution combination was set up to three consecutive sessions per combination (0.15% Sac -2% EtOH; 0.10% Sac -4% EtOH; 0.05% Sac -6% EtOH).

***6% ethanol consumption (11 days)***

The aim of the last phase is to evaluate the number of responses on the active nose-poke, the 6% EtOH (w/v) intake and the motivation to drink it. To achieve this goal, during the last phase, the number of effective responses and EtOH consumption (µl) were measured under fixed ratio 1 (FR1) for 5 daily consecutive sessions, fixed ratio 3 (FR3) (mice have to respond three times on the active nose-poke to achieve one reinforcement) for 5 daily consecutive sessions, and finally on the subsequent day to FR3, a progressive ratio (PR) session was completed to establish the breaking point for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). The response requirement to achieve reinforcements was escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward EtOH consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses that they performed to achieve one reinforcement, according to the previous scale (for example, if an animal nose-pokes a total of 108 times, this means that it has been able to respond a maximum of 40 consecutive times for one reinforcement. Therefore, the breaking point value for this animal would be 40). All the sessions lasted 1h except the PR session, which lasted 2h.

*2.3. 4. Conditioned Place Preference*

For place conditioning, 8 identical Plexiglas boxes with two equally sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a grey central area (13.8 long × 31.5 cm wide × 34.5 high) were employed.

The compartments had different colored walls (black vs. white) and distinct floor textures (smooth in the black compartment and rough in the white one). Four infrared light beams in each compartment 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, SA, Spain).

To evaluate the consequences of a HFB withdrawal on the acquisition of EtOH-induced CPP (n= 15 per group), animals were subjected to an unbiased CPP procedure that consists in three different phases: preconditioning (1 session; Pre-C), conditioning (8 sessions), and preference testing (1 session; postconditioning, Post-C). In the first day (Pre-C), mice were placed on the central area of the apparatus with the guillotine doors opened to give them access to both compartments of the apparatus for 900s. The time spent by the animal in each side during this period was recorded. Subjects showing a strong unconditioned aversion (<33% of the session time) or preference (>67%) for any cubicle were discarded from the study. The conditioning phase began 24h after the Pre-C. In each group, half of the animals received EtOH in one compartment and the other half in the other in a counterbalanced manner. After assigning the animals to a conditioning chamber randomly, an ANOVA confirmed that there were no significant differences between the time spent in the EtOH- and the Sal-paired compartments during Pre-C (data not shown). In the second phase (conditioning), half of the mice were injected with Sal and the other half with EtOH (0.75 g/kg) and were placed immediately afterwards on their corresponding conditioning chamber. On alternate days, the contingencies were inverted and the animals that had received Sal the day before were injected with EtOH (0.75 g/kg) and those who had received EtOH (0.75 g/kg) were given Sal just prior to introducing them into the other conditioning compartment. Subjects received a total of 4 pairings for each condition separated by a 24h interval during 8 days. For every of the 5-min conditioning trial, the central area was inaccessible by closing the guillotine doors. The duration of the EtOH pairing was selected because it has been shown that it is effective to induce CPP in mice (Ledesma

and Aragon, 2013; Pina et al., 2015). We decided to administer the dose of 0.75 g/kg because pilot studies from our laboratory and previous published reports have shown that doses of EtOH below 1 g/kg are unable to produce CPP in standard mice (Groblewski et al., 2008). The preference test (Post-C) took place 24h after the last conditioning assay. During Post-C, the guillotine doors separating the two cubicles were removed and the time spent by the untreated mice in each chamber was recorded for 900s. Subjects continued their binge eating sessions throughout the CPP experiment.

*2.3.5. EtOH-induced locomotor sensitization*

To assess the effect of a HFB withdrawal on the locomotor sensitization elicited by EtOH (2 g/kg), animals (n= 10 per subgroup) were tested in open-field chambers that consisted of four Plexiglas cages (30 cm long × 30 cm wide × 35 cm high) in which locomotor activity was registered by a computerized video-tracking system (Ethovision, Noldus S.A., The Netherlands). Movement of the mouse inside the open-field chambers was recorded and translated automatically by the software to horizontal distance traveled in cm during 10 min. On the habituation day, mice were able to spend 10 minutes of exploration in the chambers without drug in order to eliminate the novelty effects and set a locomotor baseline. The sensitization training protocol involved six trials on alternate days, one trial per day. For the experiment, mice were taken from the vivarium and brought to the experimental chamber 10 min prior to every session. At the start of each assay, subjects were given a Sal or EtOH (2 g/kg) injection and immediately placed in the center of the activity enclosure for 10 min. This procedure was selected based on previous reports showing that it is able to evoke locomotor sensitization in mice (Miquel et al., 2003).

**2.4. Statistics**

Data relating body weight and intake of binges were analyzed by a one-way ANOVA with a within variable PND with 13 levels (PND 25 to 69) in the case of bodyweight and 7 levels for the binge sessions: PND 25, 27, 29, 32, 34, 36, 39; in which animals were weighed and commenced the binge session.

To analyze acquisition of EtOH SA, a two-way ANOVA was performed with Diet (control or HFB 15W) as a between factor and Days (5 levels for FR1 or FR3) as a within factor. A Student's t-test was employed to analyze breaking point values and ethanol consumption during PR. For the 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 2 levels (Control, HFB 15W). Data from the horizontal locomotion (cm) carried out during the first and the sixth days of treatment were analyzed by means of a three-way ANOVA with Diet (Control or HFB 15W) and Treatment (Saline or EtOH) as the between-subjects variables and Days (1 and 6) as the within-subjects variable. Subsequent Bonferroni post – hoc tests were calculated when required. Data are presented as mean ± SEM. Analyses were performed using SPSS v22.

## 3. Results

### 3.1. Body weight and binge escalation

The ANOVA of the body weight (Figure 2) revealed an effect of the variable Day [$F(12,336)=374.951$; $p<0.001$] and the interaction Days x Diet [$F(12,336)=4,995$; $p<0.001$]. All the animals increased their bodyweight throughout the experiment ($p<0.001$, with respect PND 25). During the SA procedure when access to food was restricted, all animals lost approximately 15% of their body weight. No significant differences in body weight were detected between groups in the whole experiment.

To confirm the binge pattern of intake of a high-fat diet, all animals escalated properly in every session (Fig 3). The ANOVA showed an effect of the variable Diet [$F(1,28)=115,247$; $p<0.001$]. Mice in the HFB 15W had higher intake of high-fat food during the binge sessions with respect to the control group. Likewise, our results revealed a significant difference of the variable Days [$F(6,168)=20,333$; $p<0.001$] and the interaction Days x Diet [$F(6.168)=12,419$; $p<0.001$]. The HFB 15W showed an escalation in the intake of high-fat food kcal from day 1 onwards ($p<0.001$ all days).

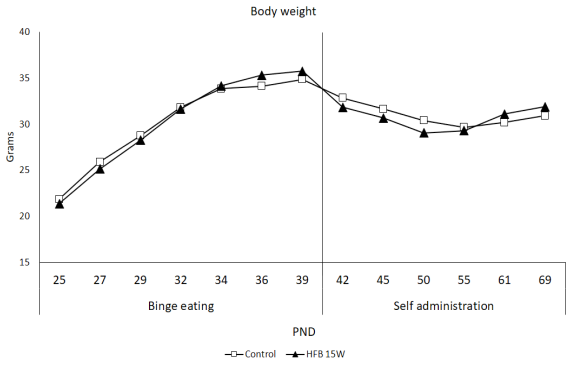


Figure 2. Bodyweight during the whole procedure.

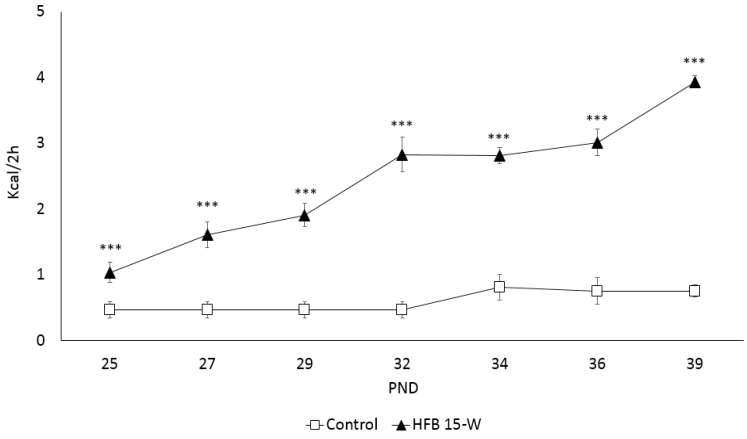


Figure 3. Intake (kcal) of the high-fat binge-eating sessions that took place on MWF with 2h access in all groups. Represented is the mean (± SEM) amount of kcal consumed in 2 hours of limited access to high-fat food (control group had access to standard food) to confirm the escalation of intake. ***p<0.001 significant difference with respect the control group.

### 3.2. Oral ethanol self-administration

No differences were found between animals during the training or the substitution phases, meaning that bingeing on fat did not induce any learning deficit (data not shown).

With respect to the long-lasting effects of a HFB (See Figure 4), in the FR1 schedule the ANOVA revealed a significant effect of the variable Days on the effective number of responses [$F(4,100)=4,018$ $p<0.01$] and EtOH consumption [$F(4,100)=6,243$ $p<0.001$] (Figure 4a and 4b), with significant differences on day 1 compared to day 3 ($p<0.01$), 4 ($p<0.01$) and 5 ($p<0.05$). There was also an effect of the variable Diet [$F(1,25)=7,486$; $p<0.01$], and the interaction Diet x Days [$F(4,100)=3,324$ $p<0.001$] on EtOH consumption. Mice of the HFB 15W group exhibited an increased oral SA of EtOH (6%) with respect to the control group (Figure 3b) in days 3 ($p<0.01$) 4 ($p<0.01$) and 5 ($p<0.001$).

In the FR3 schedule, the ANOVA revealed a significant effect of the variable Days on the effective responses [$F(4,100)=3,114$ $p<0.05$] and indicated a significant effect of the interaction Days x Diet [$F(4,100)=3,013$; $p<0.05$] on EtOH consumption. Bonferroni post-hoc analyses showed higher EtOH intake in the HFB 15W group in days 3, 4 and 5 ($p<0.001$ in all cases).

Finally, analyses of the PR showed an effect of the variable Diet [$F(1,23)=5,938$ $p<0.05$). Breaking point values were significantly higher among animals in the HFB 15W group (Figure 4c), although 6% EtOH consumption was similar in both groups (Figure 4d).

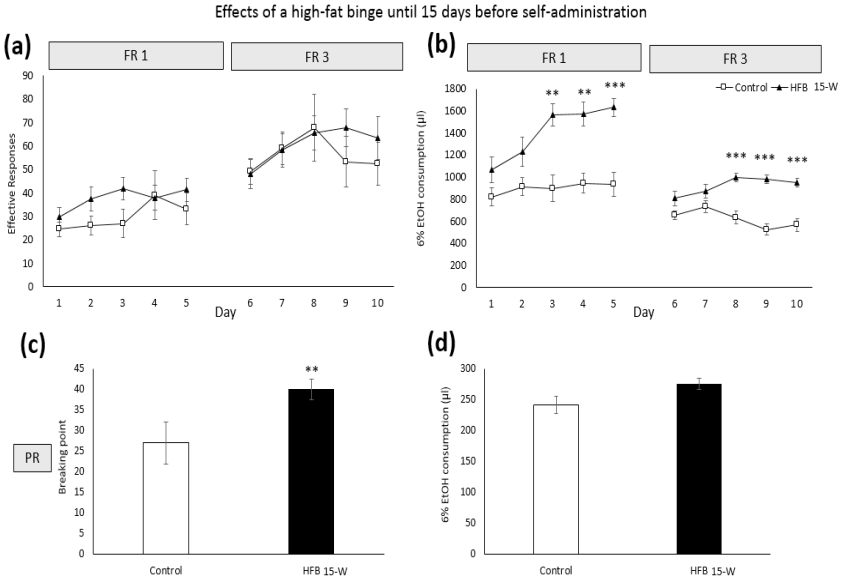


Fig. 4. Analysis of oral EtOH self-administration in OF1 mice (n=30). The dots represent means and the vertical lines ± SEM of (a) the number of effective responses and (b) the volume of 6% EtOH consumption during FR1 and FR3. The columns represent mean and the vertical lines ± SEM of (c) the breaking point values during PR and (d) the volumes of 6% EtOH consumption during PR. **p<0.01; ***p<0.001 values from HFB 15W mice that are significantly different from control mice.

### 3.3. Effects of a High-Fat Binge until 15 days before the acquisition of EtOH-induced CPP

As can be seen in Figure 5, the HFB 15W did not have an effect on the conditioning properties of EtOH (0.75 g/kg). The time spent in the EtOH-paired chamber did not differ from the Pre-C on the Post-C day in any of the groups.

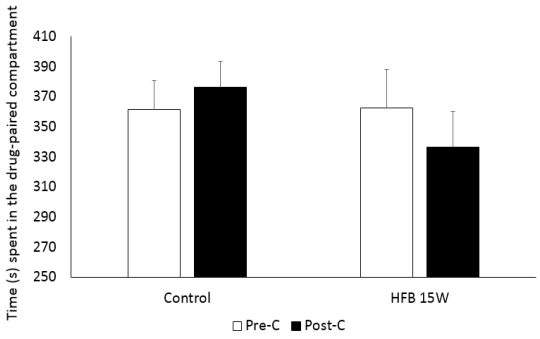


Fig. 5. Effects of bingeing intermittently on a high-fat diet until 15 days before the acquisition of 0.75g/kg EtOH-induced CPP. Bars represent mean (±SEM) of time spent in the EtOH-paired compartment for the different Diet (C or HFB 15W) groups (n= 15 per group) during the Pre-C (white bars) and the Post-C (black bars).

### 3.4. Effects of a High-Fat Binge until 15 days before the EtOH-induced locomotor sensitization

Figure 6 displays the locomotor-sensitizing effects of EtOH. The ANOVA indicated a significant effect for the Treatment [$F(1, 36)= 39.096$, $p<0.001$], Days [$F(1,36)= 28.050$, $p<0.001$], and an interaction Days × Treatment [$F(1,36)= 73.239$, $p<0.001$]. The post-hoc revealed that EtOH-induced sensitization occurred in both conditions, since the locomotor activity of both groups significantly increased on day 6 with respect to day 1 ($p<0.001$). In addition, the acute locomotor activity response to EtOH was significantly higher in the HFB 15W group than the rest of the groups on day 1 ($p<0.05$).

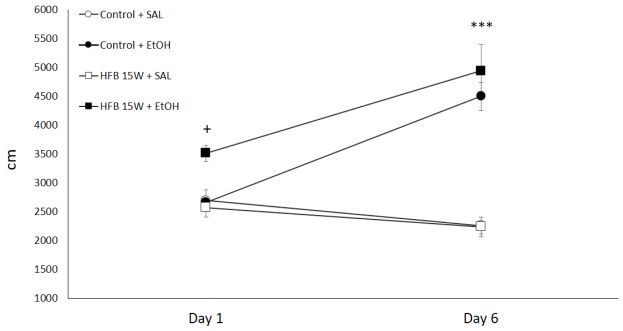


Fig. 6. Effects of bingeing intermittently on a high-fat diet until 15 days before of EtOH-induced locomotor sensitization. Values represent mean (±SEM) of locomotor activity (cm in 10 min) for mice (n= 10 per subgroup) previously exposed to a standard or HFB diet and treated with Sal or EtOH (2 g/kg) immediately before being introduced in the open field during 6 alternate days. *** $p<0.001$ significantly different from the Day 1; + $p<0.05$ significantly different from Sal on day 1.

## 4. Discussion

The current study showed for the first time that bingeing on fat during adolescence induces long-lasting consequences on the rewarding effects of EtOH. Here, we have assessed how animals that binged intermittently on fat during adolescence and were abstained from fat 2 weeks before the beginning of the behavioral procedures exhibited a higher EtOH consumption in the oral SA paradigm and showed an increased motivation to obtain the reward in the progressive ratio schedule. Although mice exposed to HFB 15W were not more sensitive to EtOH-induced CPP, they still presented higher motor response to this drug.

In a previous report (Blanco-Gandía et al., *in press*), we studied the effects that bingeing on fat during adolescence induces on the rewarding effects of EtOH while the animals are still bingeing. We observed that animals in the HFB group that underwent the EtOH SA procedure presented greater EtOH consumption and a higher motivation to obtain the drug. HFB mice also developed preference for the paired compartment in the CPP with a subthreshold dose of EtOH, and independently of the diet, mice developed EtOH-induced locomotor sensitization. In the present study, we have confirmed that part of these effects are long-lasting despite the end of the fat consumption.

Regarding the oral SA, under the FR1 and FR3 schedules HFB 15W mice exhibited an increase in EtOH consumption and a higher number of effective responses with respect to the control group. Both groups similarly acquired and maintained stable operant responding, therefore discarding a possible learning deficit. The HFB 15W group exhibits an escalation in EtOH consumption along the days, both in FR1 and in FR3, which could be explained by the absence of the other reinforcer, since motivation is enhanced after a period of abstinence (Weiss, 2005). We also observed a higher progressive ratio response in the HFB 15W mice, which demonstrates an increased motivation and compulsivity to obtain the drug. These results are in the same line as Avena and colleagues (2004), using food rich in sugar. They reported that rats with an intermittent access to sugar consumed more

EtOH in a two-bottle choice paradigm. In combination with our results, these studies point to the idea of comorbidity between binge-eating disorders and EtOH intake, suggesting that it is not only the type of food, but also the way it is consumed: intermittently and compulsively.

Similar to what occurs during withdrawal from drugs of abuse, stress-related pathways are also involved in the withdrawal of palatable food (Puhl et al., 2011). Withdrawal from access to a cafeteria or high-fat diet induced an abnormal function of the HPA axis with a significant increase in CRF and basal corticosterone levels (Teegarden and Bale, 2007; Sharma et al., 2013; Martire et al., 2014). Conversely, eating sucrose or fat attenuates the stress levels reducing limbic CRF (Teegarden and Bale, 2007; Christiansen et al., 2011), which is the main force behind drug reinstatement and relapse. This could explain that animals in a fat binge withdrawal period replace this lack of pleasure with other rewards like ethanol.

However, we did not observe any difference regarding the rewarding effects of a subthreshold dose of EtOH like 0.75g/kg on the CPP paradigm. Previous studies point to the fact that doses below 1g/kg are unable to produce CPP in standard mice (Groblewski et al., 2008). Although we observed an increase in the conditioned rewarding effects of EtOH when bingeing on fat occurs during the whole procedure (Blanco-Gandía et al., *in press*), no effect was detected 2 weeks after the last binge. Although previous exposure to a high-fat diet increases the sensitivity of animals to CPP induced by amphetamine (Kuhn et al., 2014) or cocaine (Blanco-Gandía et al., 2017a and b), this was not the case for EtOH. Different mechanisms modulate self-administration and CPP, pointing out that cessation of bingeing on fat deregulates different neurobiological processes in the reward system. The CPP procedure aims to evaluate the relevance of the environmental cues associated with the drug (Tzschentke, 2007), based on the change from initially neutral environmental cues to conditioned stimuli with secondary motivational properties (Aguilar et al., 2009). On the other hand, the SA paradigm evaluates the direct primary reinforcing effects of drugs according to the effort made by the animal to obtain the drug. Fifteen days after cessation of bingeing on fat, mice showed

normalized development of the conditioned environmental cues, while the primary reinforcing properties of EtOH are still increased.

It is well known that behavioral sensitization has been considered one of the key components in drug addiction (Didone et al., 2016). After bingeing on fat during adolescence, we reported 2 weeks later the development of sensitization to the locomotor effects of EtOH in controls as well as mice exposed to fat. However, the acute locomotor response to EtOH in the HFB 15W group on day 1 is increased with respect the control group. This result is similar to that observed in mice while bingeing on fat during the whole procedure (Blanco-Gandía et al., *in press*). Some studies have already described the increased locomotor effects of psychostimulants in animals fed on a high-fat diet (Baladi et al., 2012 and 2015), although another study found that only continuous access to fat, but not an intermittent pattern, induced locomotor cocaine sensitization (Serafine et al., 2015).

Except for the lack of effect on the CPP, our results showed that having a history of fat bingeing induced almost the same effects as the actual bingeing of fat. We have previously detected modifications of the dopaminergic, opioid and endocannabinoid systems after being exposed to HFB. A similar exposure to HFB than those used in the present study induce a decrease in gene expression of CB1 receptor and mu opioid receptor in the nucleus accumbens (N Acc) (Blanco-Gandía et al., 2017b). A remaining question to resolve is whether these changes persist after the removal of HFB. We know that the modifications induced by continuous access to fat (a decrease in CB1 receptor gene expression in the NAcc and prefrontal cortex with an increase on mu opioid receptor in the NAcc) are only partially restored after cessation of fat (Blanco-Gandía et al., 2017b). Although gene expression of mu receptor normalizes, the CB1 receptor in the NAcc remains decreased. A recent study of Carlin and co-workers (2016) found that female mice exposed during adolescence to a high-fat diet showed an increase in dopamine transporter expression but a decrease tyrosine hydroxilase expression in the ventral tegmental area accompanied by a decrease in D2 receptors in the NAcc, and neither of these changes normalized after 4 weeks on standard

chow. However, other changes as the decrease expression of dopaminergic D1 and D2 receptors in the prefrontal cortex or the decrease of D1 receptors in the NAcc were completely restored. Therefore, these results suggest that it is possible to reverse some of these changes with a 4-week standard chow replacement, but not all of them.

This study suggests that early compulsive experiences can affect later behaviors in life. The animal model of bingeing on fat resembles what occurs with bulimia nervosa, which consists of daily fasting followed by bingeing, usually on high-fat and/or sugar rich food (Gendall et al., 1997). Moreover, patients with eating disorders also have an increased rate of substance abuse, such as alcoholism (Vaz-Leal et al., 2015). Therefore, our results indicate that an intermittent access to high-fat food can be sufficient to induce long-lasting changes in the brain that leads subjects to an increased EtOH consumption and vulnerability to its reinforcing properties. Results of the current study might have clinical implications, as they suggest that even when adolescents interrupt their bingeing behavior, their neurobiological pathways are not completely recovered.

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# Study 5.

## Housing conditions modulate the reinforcing properties of cocaine in adolescent mice that binge on fat.

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Abstract: Binge eating is a specific form of overeating characterized by intermittent, excessive eating. To date, several studies have addressed the effects that bingeing on fat has on the rewarding effects of drugs of abuse, but they have found contradictory and highly variable results. Housing conditions could modulate these results, as most studies employ isolated animals to measure the exact amount of food that is ingested. The aim of this study was to evaluate the effects of housing conditions on the response of mice to cocaine, modulated by bingeing on a high-fat diet during adolescence.
After 40 days of binge-eating for 2h, three days a week (PND 29-69), the reinforcing effects of a non-effective dose of cocaine (1 mg/kg) was evaluated using the conditioned place preference (CPP) paradigm. The anxiolytic profile using the elevated plus maze and circulating leptin and corticosterone levels were also assessed.
Our results show a significant escalation in the consumption of a high-fat diet between the first and the last week in both types of housed mice. Among the grouped mice, only those exposed to high-fat binge (HFB) developed CPP. Conversely, isolated mice fed with standard diet were more sensitive to the rewarding effects of a subthreshold dose of cocaine than those fed with HFB. Plasmatic leptin levels were elevated in both groups that developed CPP. Although isolated animals presented higher corticosterone levels with respect to the grouped ones, anxiety levels did not differ. Therefore, our results highlight the importance of housing conditions on the effects that a high-fat diet exerts on cocaine reward.

8th June 2017

Please find enclosed the manuscript "Housing conditions modulate the reinforcing properties of cocaine in adolescent mice that binge on fat" by M. Carmen Blanco-Gandía, Sandra Montagud-Romero, María A Aguilar, José Miñarro, and Marta Rodríguez-Arias

This work has not been published previously and is not under consideration for publication elsewhere. The authors have no possible conflict of interest in the carrying out and reporting of this research. All the authors have made a substantial contribution for the conception and design of the study (J Miñarro, MA Aguilar, and M Rodríguez-Arias), acquisition, analysis and interpretation of the data (C Blanco-Gandía, S Montagud-Romero, and M Rodríguez-Arias) and drafting the article and revising it (C Blanco-Gandía, J Miñarro, MA Aguilar, and M Rodríguez-Arias). All the authors have approved the version to be submitted.

The experimental protocol has been approved by an Institutional Review Committee for the use of animal subjects. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. All the efforts were made to minimize animal suffering and to reduce the number of animals used.

We look forward to hearing from you,

Sincerely yours,

Dr. Marta Rodríguez-Arias

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# Housing conditions modulate the reinforcing properties of cocaine in adolescent mice that binge on fat.

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**Highlights**

- Housing modulates the fat diet binge effects on cocaine reward
- Leptin is elevated in animals that develop Conditioned Place Preference
- High-fat binge decreases sensitivity to cocaine reward in isolated animals
- Corticosterone is high in isolated animals compared to grouped, but HFB decreases it.

**Abstract**

Binge eating is a specific form of overeating characterized by intermittent, excessive eating. To date, several studies have addressed the effects that bingeing on fat has on the rewarding effects of drugs of abuse, but they have found contradictory and highly variable results. Housing conditions could modulate these results, as most studies employ isolated animals to measure the exact amount of food that is ingested. The aim of this study was to evaluate the effects of housing conditions on the response of mice to cocaine, modulated by bingeing on a high-fat diet during adolescence.

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Our results show a significant escalation in the consumption of a high-fat diet between the first and the last week in both types of housed mice. Among the grouped mice, only those exposed to high-fat binge (HFB) developed CPP. Conversely, isolated mice fed with standard diet were more sensitive to the rewarding effects of a subthreshold dose of cocaine than those fed with HFB. Plasmatic leptin levels were elevated in both groups that developed CPP. Although isolated animals presented higher corticosterone levels with respect to the grouped ones, anxiety levels did not differ. Therefore, our results highlight the importance of housing conditions on the effects that a high-fat diet exerts on cocaine reward.

**Highlights**

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**Keywords:** binge eating, fat, social isolation, conditioned place preference, leptin, corticosterone

## 1. Introduction

Adolescence is a period of brain maturation marked by structural alterations in many limbic and cortical regions, related to major changes in emotional and cognitive functions. Drug use during this critical period of development often predicts an increased likelihood of continued use into adulthood [1, 2, 3.]. Currently, there is a growing high-fat "fast-food" culture and prevalence of obesity, particularly among adolescents [4, 5, 6.]. Palatable food, like drugs of abuse, causes an increase in dopamine release that is linked with its pleasurable effect. Recent research shows that hedonic eating, defined by Gold [7] as eating based on pleasure rather than energy needs, affects neural mechanisms connected with reward and maintains this behavior [6]. Binge eating is described as an intermittent, excessive, dysfunctional appetitive behavior that occurs in short periods of time [8]. Statistics suggest that binge eating is more common than other eating disorders [9], and there is evidence of a clinical overlap between binge-eating disorders and drug addiction [10, 11].

Although some results point to the fact that nutritional status is a modulating factor for the development of drug addiction [6, 12], and high-fat diets may work as a gateway for the development of drug addiction [8, 13], other studies did not find the same results or found the opposite [14, 15, 16]. Therefore, many studies to date are contradictory and the results are highly variable. Two factors can explain these divergent results, the continuous vs binge fat intake and the housing conditions.

There are few studies of high-fat feeding that employ grouped animals, even if this condition is the only that provides the most valuable and translational results. For example, Loebens and Barros [17] observed that a high-fat diet decreased the withdrawal effects of cocaine. Although in the Morales and co-workers [16] study, continuous access to fat decreased the preference for cocaine in the CPP paradigm, we have previously reported that grouped mice exposed to continuous access to a high-fat diet showed an increase on the conditioned rewarding effects of cocaine only when the diet is abruptly interrupted [18]. In addition, exposure to HFB in

grouped mice increased cocaine-induced CPP during and after this fat exposure [19].

Most studies use isolated rodents, in order to control individual food intake and bodyweight. For example, France's group employs isolated animals in all their studies, demonstrating that animals with a continuous access to fat are more sensitive to the locomotor effects of cocaine [20, 21] and methamphetamine [22]. Other groups have also demonstrated that prenatal exposure to high-fat diets lead to a higher vulnerability to the rewarding effects of ethanol or amphetamine in the individually-housed offspring [23]. With respect to binge-eating, Puhl and co-workers [8] observed that isolated adult rats bingeing on fat were more vulnerable to the rewarding effects of cocaine self-administration. Other studies have found the opposite, showing that obesity-prone rats fed on a high-fat diet were less sensitive to the rewarding effects of cocaine [24]. Equally, an impaired acquisition of the self-administration has been observed in isolated rats with ad libitum access to a high-fat diet [14, 15].

Isolation is considered one of the most potent stressors, in both humans and animals [25 – 27], as it can lead to behavioral, anatomical and neurochemical changes that remain during adulthood [26, 28]. Adolescence is a critical period for social experiences, and the interactions between social factors and addictive behavior have been well documented in human and animal studies, suggesting that impaired social attachment during early development can enhance the susceptibility to drug addiction [29 – 31]. Isolation in an early stage of life produces an increase in self-administration of low doses of cocaine and amphetamine [32 - 36], enhancing motivation for cocaine in the progressive ratio schedule [36]. A recent study shows that isolated rats evoke a greater dopamine release than grouped rats in response to cocaine, suggesting an enhanced cocaine reinforcement [37]. In addition, several studies have shown that social isolation is related to increased adiposity, as isolated mice exhibit augmented plasma leptin levels [38-40].

In light of these previous reports, we hypothesized that the different reported results regarding the effects of high-fat feeding on drugs of abuse may be due, at least in part, to the different housing conditions used in these studies. The aim of

this study is to explore how these two factors may modulate the reinforcing effects of cocaine. To achieve this objective, mice housed in groups or in isolation will be fed only with a standard diet or exposed to a high-fat binge during adolescence. The rewarding effects of cocaine will be evaluated using the Conditioned Place Preference (CPP) paradigm. As it is well known that housing conditions can affect anxiety [41], we also evaluated anxiety-like behaviors with the Elevated Plus Maze (EPM) and measured circulating corticosterone and leptin levels.

## 2. Methods

### 2.1. Subjects

A total of 60 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and then, half of them were housed under standard conditions in groups of 4 and the other half was isolated. All the experimental procedures are in agreement 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 present study.

### 2.2. Drugs

For CPP, animals were injected i.p. with 1 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S. A. Madrid, Spain) that was diluted in physiological saline. The cocaine dose of 1 mg/kg to induce CPP was based on previous studies [42] where it was shown to be a sub-threshold dose to induce CPP in naïve mice.

### 2.3. Procedure and Apparatus

#### 2.3.1. Feeding Conditions

Our feeding procedure is based on the limited access model described by Corwin et al. [43], in which non-food-deprived animals with sporadic and limited access to high-fat food developed 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 high-fat food will be referred to as high-fat binge (HFB).

On PND 29, in each of the housing conditions, 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 2h 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, and their intake on the standard diet in their home cage was also measured.

### 2.3.2. Experimental Design

Animals developed a binge-eating pattern from PND 29 to 69 with a total of 18 binge-eating sessions, and then behavioral tests were started. On PND 69 animals performed the EPM, and from PND 70 to 77 they were conditioned with 1mg/kg cocaine. After the procedure, blood samples were taken in order to measure plasmatic corticosterone and leptin levels. An overall and more detailed description of the experimental procedure is provided in Table 1.

<table>
<tr><th></th><th></th><th>29 -68</th><th>69</th><th>70-72</th><th>73-76</th><th>77</th><th>78-118</th><th>119</th></tr>
<tr><td rowspan="2">Isolated</td><td>Control</td><td rowspan="4">Binge exposure</td><td rowspan="4">Elevated Plus Maze</td><td rowspan="4">Pre-C Phase</td><td rowspan="4">Conditioning Phase 1mg/kg cocaine</td><td rowspan="4">Post-C Phase</td><td rowspan="4">Extinction and Reinstatement phase</td><td rowspan="4">Blood Samples</td></tr>
<tr><td>HFB</td></tr>
<tr><td rowspan="2">Grouped</td><td>Control</td></tr>
<tr><td>HFB</td></tr>
<tr><td></td><td></td><td colspan="7">Standard or High Fat Binge sessions every Monday, Wednesday and Friday for 2h all the procedure</td></tr>
</table>

Table 1. Experimental procedure

### 2.3.3. Elevated Plus Maze

The EPM consisted of two open arms and two enclosed arms and a central platform, elevated 45cm above floor level. 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 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 are generally used to characterize the anxiolytic effects of drugs [44, 45]. For more details, see the previously described protocol [46].

2.3.4. Conditioning Place Preference

For Place Conditioning, we employed twelve identical Plexiglas boxes with two equally sized compartments separated by a gray central area. 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). The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as previously described [47] and consists of three phases. In the Pre-Conditioning phase (Pre-C, 3 days), mice of 69 PND were allowed access to both compartments of the apparatus for 15 minutes (900 s). Half of the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. In the conditioning phase (conditioning, 4 days), animals received an injection of vehicle/cocaine immediately before being confined to the vehicle/cocaine-paired compartment for 30min. After an interval of 4h, they received again an injection of cocaine/vehicle immediately before being confined to the corresponding compartment for 30min. Last, in the post-conditioning phase (Post-C, day 8), the time spent by the untreated mice in each compartment during a 900s 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.

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 the time spent in the drug-paired compartment during the extinction session and Pre-C test values in two consecutive sessions.

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. 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.

#### 2.3.5. Determination of plasma leptin and corticosterone levels

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

### 2.4. Statistics

Data relating to body weight and binge intake were analyzed by a mixed ANOVA with two between-subjects variables –Diet, with two levels (Control, HFB); Housing, with two levels (Isolated, Grouped)– and a within variable –Days, with seven levels (PND 29, 36, 43, 50, 57, 64 and 69). The EPM and plasma leptin and corticosterone data were analyzed by a two-way ANOVA with two between variables –Diet, with two levels (Control, HFB) and Housing, with two levels (Isolated, Grouped). For CPP, the time spent in the drug-paired compartment was analyzed by means of a mixed analysis of variance (ANOVA) with two between variables –Diet and Housing– 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. 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.

## 3. Results

### 3.1. Bingeing on fat and body weight

Results obtained in the statistical analysis of body weight revealed an effect of the variables Days [$F(6,336) = 977,413$; $p<0.001$], Housing [$F(1,56)=21,786$; $p<0.001$], and the interaction Days x Housing [$F(6,336)=24,425$; $p<0.001$). Although all animals increased their weight along the weeks, from PND 36 onwards, grouped mice showed higher body weight than isolated animals ($p< 0.001$). There was not an effect of the variable Diet, meaning that bingeing on fat does not make individuals weigh more than controls (Figure 1a).

An escalation in the intake of the high-fat diet (see Fig. 1b) was confirmed by the ANOVA, which revealed a significant difference of the variable Diet [$F (1.56) = 28.902$; $p<0,001$], and the interaction Days x Diet [$F(6.336)= 37.812$; $p<0.001$). HFB groups exhibited a significant augmented Kcal intake of high-fat diet with respect to controls on PND 50, 57, 64 and 69 ($p<0.001$). The interaction Housing x Diet also showed a significant effect [$F (1.56) = 5.775$; $p<0.05$], as both control and HFB isolated groups showed greater intake than their respective grouped mice ($p<0.01$).

Fig.1

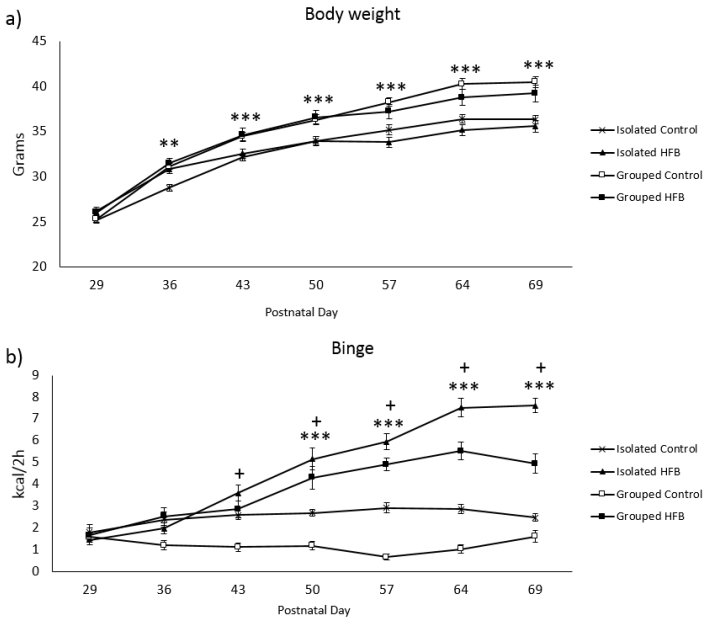


**Figure 1. (a)** Bodyweight of mice over the procedure. Mean (± SEM) amount measured weekly of control and HFB animals' bodyweight (n=15 per condition) **p<0.01; ***p<0.001 significant difference between grouped vs. isolated mice **(b)** Binge sessions. Intake (kcal) in the 2h high-fat binge-eating sessions (control group had access to standard food) 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 or standard food, stated here weekly to confirm the escalation of intake. **p<0.01; ***p<0.001 significant difference from both HFB groups with respect to the control groups. + p<0.001 overall significant difference with respect to PND 29, showing escalation with respect to the first day of binge.

### 3.2. Elevated Plus Maze

There were no significant differences in anxiety levels between any of the groups (Table 2).

Table 2. Effects of housing and diet on anxiety evaluated in the Elevated Plus Maze.

| | Isolated | | Grouped | |
|---|---|---|---|---|
| | Control | HFB | Control | HFB |
| Time in open arms | 140 ± 6 | 132 ± 16 | 127 ± 12 | 120 ± 8 |
| % Time in open arms | 58 ± 2 | 54 ± 6 | 54 ± 4 | 55 ± 2 |
| Time in central platform | 49 ± 4 | 56 ± 7 | 59 ± 6 | 60 ± 4 |
| Time in closed arms | 102 ± 5 | 103 ± 10 | 100 ± 9 | 101 ± 5 |
| Entries in open arms | 22 ± 1 | 24 ± 3 | 26 ± 7 | 27 ± 2 |
| % Open entries | 52 ± 2 | 53 ± 4 | 61 ± 4 | 60 ± 2 |
| Entries in closed arms | 22 ± 3 | 22 ± 3 | 18 ± 4 | 21 ± 5 |
| Total entries | 44 ± 4 | 46 ± 5 | 44 ± 5 | 50 ± 4 |

### 3.3. Cocaine-induced Conditioned Place Preference

Results of the cocaine-induced CPP in animals receiving a dose of 1mg/kg cocaine are presented in Figure 2a. The ANOVA for the time spent in the drug-paired compartment revealed an effect of the interaction Days x Housing x Diet [$F(1.51) = 8.038$; $p < 0.01$]. Grouped mice exposed to HFB spent more time in the drug-paired compartment in Post-C than in Pre-C ($p<0.001$). On the contrary, cocaine-induced CPP was observed only in isolated mice fed on a standard diet ($p<0.05$). These groups required 2 and 8 sessions respectively for the preference to be extinguished. The Kaplan-Meier analysis (see Figure 2b) revealed that the isolated control group required more time to achieve extinction (8 sessions) than the grouped HFB group (2 sessions; $\chi^2 = 12.620$; $p<0.001$). A priming dose of 0.5 mg/kg cocaine reinstated the preference in both groups (Grouped HFB: Student's t-test, $t=-4.149$, 11 d.f. $p<0.01$; isolated control: Student's t-test, $t=-2.507$, 13 d.f. $p<0.05$). After 1 extinction session in both groups, reinstatement with a priming dose of 0.25 mg/kg cocaine was also achieved (Isolated control: Student's t-test, $t=-5.667$, 13 d.f. $p<0.001$; grouped HFB: Student's t-test, $t=-3.076$, 11 d.f. $p<0.01$).

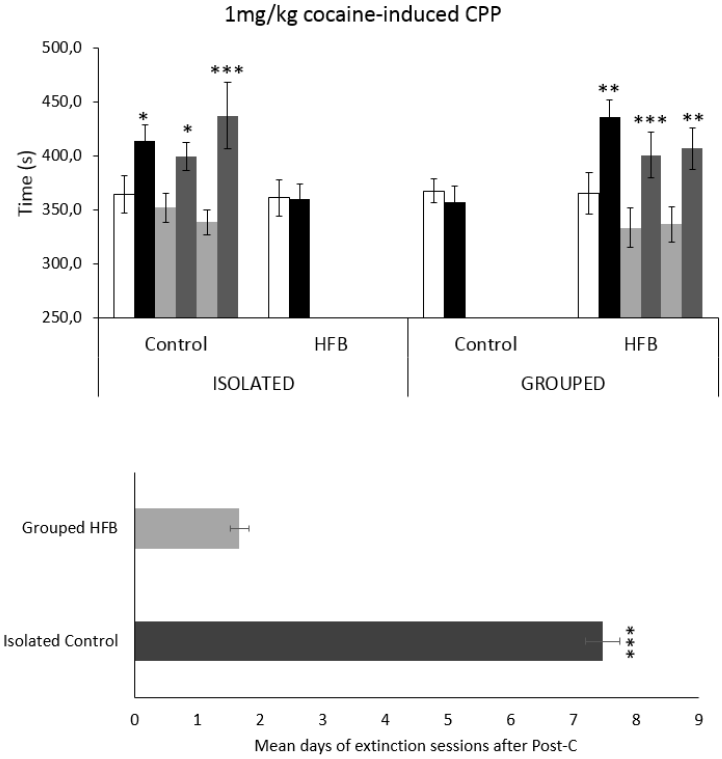


**Figure 2 (a)** CPP induced by 1 mg/kg of cocaine in isolated and grouped mice exposed to a HFB. Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white), post-conditioning (black), the last extinction session (light grey) and reinstatement (dark grey). The reinstatement test was evaluated 15 minutes 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)** 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<0.001 with respect to grouped HFB mice.

### 3.4. Effects of a HFB on circulating leptin and corticosterone levels

With respect to circulating leptin levels (see Fig.3), the ANOVA showed an effect of the interaction Housing x Diet [$F(1,34)=44.639$; $p<0.001$]. Grouped HFB mice presented an increase in leptin levels with respect to their control group ($p<0.01$). On the contrary, mice in the isolated control group exhibited significant increases of leptin with respect to the isolated HFB animals ($p<0.001$) and the grouped control group ($p<0.001$).

Regarding circulating corticosterone levels (see Fig 4), the ANOVA showed an effect of the variable Housing [$F(1.28)=35,792$; $p<0.001$] and Diet [$F(1.28)=5,054$; $p<0.05$]. Isolated mice presented higher corticosterone levels than grouped mice ($p<0.001$). On the other hand, mice fed on a HFB showed lower corticosterone levels than controls, due to the effect of fat in the isolated mice ($p< 0.05$).

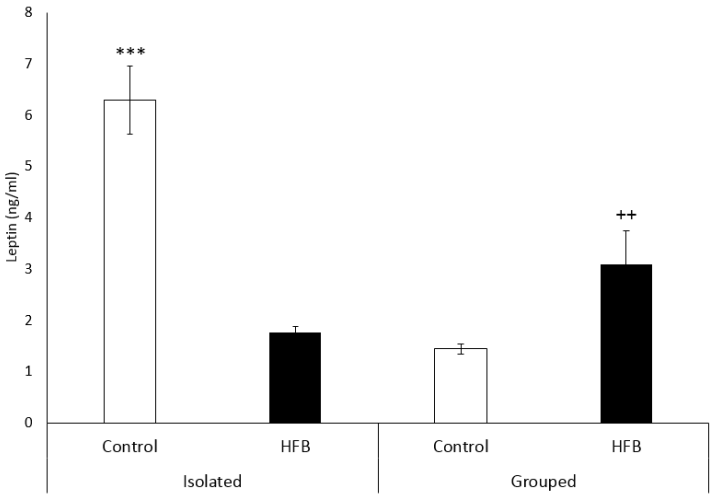


**Figure 3.** Effects of HFB on adolescent grouped and isolated mice in the circulating leptin levels. Data are presented as mean values ± S.E.M. (ng/ml) ***$p<0.001$ as regards isolated HFB and grouped control mice; ++ $p<0.01$ grouped HFB with respect to grouped control mice.

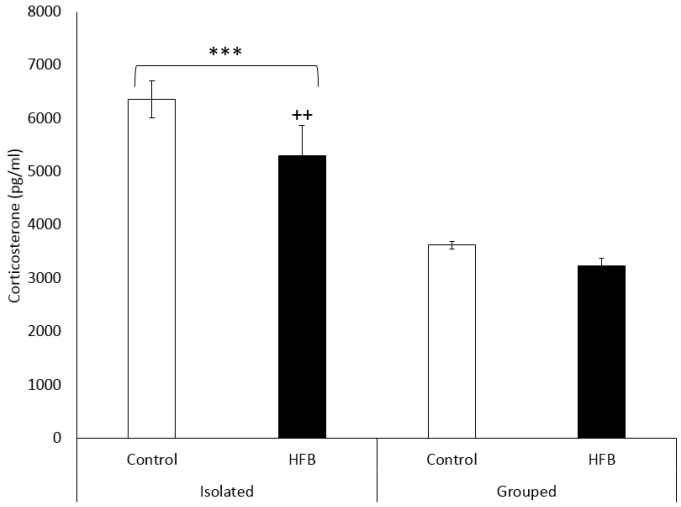


**Figure 4.** Effects of HFB on adolescent grouped and isolated mice in the circulating corticosterone levels. Data are presented as mean values ± S.E.M. (pg/ml) ***p<0.001 with respect to grouped mice ++ p<0.01 with respect to isolated control mice.

## 4. Discussion

The present study demonstrates for the first time that housing conditions modulate the influence that bingeing on fat during adolescence exerts on the conditioned rewarding properties of cocaine. Only grouped animals that binge on fat developed CPP with a subthreshold dose of cocaine. On the contrary, isolated mice bingeing on fat did no develop cocaine-induced preference. However, isolated control animals fed on a standard diet showed CPP with this non-effective cocaine dose. Interestingly, circulating leptin levels were significantly higher only in the two groups of mice that developed cocaine-induced CPP. Although isolated mice presented higher levels of corticosterone, no differences in anxiety levels were observed among isolated and grouped mice.

As we have previously reported, grouped mice exposed to a high-fat binge developed CPP with a non-effective cocaine dose (1mg/kg) [19]. Moreover, this preference was reinstated with very low priming doses (up to 0.25 mg/kg of

cocaine), which suggests a greater sensitivity to the reinstatement of the extinguished preference by the cocaine-priming dose. However, the results obtained in isolated mice show the complete opposite. Isolated mice bingeing on fat did not develop CPP, but the isolated controls fed on standard diet did show CPP with the 1mg/kg cocaine dose. Like grouped HFB mice, isolated controls reinstated the CPP with the very small cocaine priming doses, needing more sessions than grouped HFB mice to extinguish the preference.

These interesting results could be due to a different exposure to the high-fat diet. However, this is not likely a suitable explanation. Animal models of bingeing need to demonstrate large intakes within defined brief periods, with similar environmental conditions being provided for control animals [48]. The model used in the present study, in which animals are never food-deprived, renders the model more relevant, as it relies on intermittent limited access to palatable food to drive escalations in intake [49]. We have observed, in agreement with previous reports, that mice exposed to HFB developed fat-bingeing behavior [19, 43, 50, 51]. There was a significant increase in the amount of kcal ingested by isolated or grouped HFB mice in every binge session from the second week of exposure to fat. In addition, isolated HFB animals presented a significant increase of fat consumption with respect to their grouped counterparts. This result would support the hypothesis that palatable food is used as compensation during periods of stress [52]. High levels of stress can change eating patterns and increase consumption of palatable foods, increasing the incentive salience of this type of reward [53]. Although, several reports showed that social isolation promotes obesity [38, 54], isolated HFB mice presented lower bodyweight than grouped HFB animals. One possible explanation could be that isolated mice showed higher locomotion, as previous studies have reported [55]. Although stress has been associated with increased binge eating [56], this effect is different in lean individuals as compared to obese ones [57], being exacerbated in obese individuals.

In addition, social isolation has been associated with hyperleptinemia [38- 40], a result that is confirmed in our study. In previous reports, we observed that grouped mice exposed to HFB showed higher leptin levels than grouped mice exposed to standard food [19], an increase that reached statistical significance in

the present study. However, we also observed that isolated mice fed on standard food showed higher plasma leptin levels than isolated HFB mice. Circulating leptin levels are involved in satiety signaling and food intake [58, 59], being also a modulator of stress-induced eating [60]. Although isolated animals weighed less than the grouped ones, isolated control animals had significantly higher circulating leptin levels. Glucocorticoids increase plasma leptin levels [61]. Therefore, stress can produce permanent changes in the organism's metabolism [62], which could explain the alterations observed on the leptin levels in isolated control animals. Studies in patients under glucocorticoid therapy suggest a resistance to the effects of endogenous leptin, even when plasma levels remain high [63, 64]. Exposure to HFB in isolated mice can act as an anxiolytic, decreasing the stress levels and therefore reducing the leptin levels. Supporting this hypothesis, corticosterone levels in isolated mice were blunt in those fed on HFB.

To sum up, isolated and grouped mice developed bingeing on fat, and although isolated mice showed higher kcal fat intake, they weighed less than grouped HFB animals. As expected, leptin levels increased in grouped mice exposed to fat but no increases were observed in isolated HFB mice, despite isolated control mice showing a powerful increase in leptin levels.

A second explanation of the results obtained in the CPP is based on the influence of social isolation during adolescence on shaping cognition and emotion [65, 66]. Both bingeing on fat and social isolation can modify the rewarding system and turn an individual into a vulnerable target to drugs of abuse. Social isolation during adolescence is a potent stressor for rodents [26, 66], and only 72h of isolation are sufficient to increase plasma corticosterone levels [68]. Confirming this effect, we have observed an increase in corticosterone levels in both isolated groups of mice, which is also in line with previous studies performed in our laboratory using social stress [69]. On the other hand, anxiety did not increase in the isolated animals, as it has been previously described [70]. Several social isolation studies have utilized EPM tests and the results have been inconsistent, although an anxiogenic-like effect has been observed in a novelty-induced hypophagia test [71]. On the other hand, previous studies have also reported that bingeing on fat or sugar does not

affect anxiety levels in the EPM [72, 73], even though an anxiolytic effect of leptin injections has been reported [74].

The increased response to cocaine observed in the control isolated groups is explained by the known effect that post-weaning social isolation causes in the neural substrates of reward and motivation [30, 75]. Social isolation induces higher oral intake of ethanol [76], morphine [77] and cocaine [78, 79]. The studies performed in isolated mice exposed to high-fat diets showed discrepant results. After continuous access to fat, an impaired acquisition of cocaine self-administration was described [15], and obesity-prone rats fed on a high-fat diet were less sensitive to the rewarding effects of cocaine [24]. In addition, isolated animals fed on a chronic high-fat diet were less susceptible to the cocaine effects in the open field test [74]. However, isolated rats exposed to HFB showed a non-significant increase of cocaine self-administration [8]. In isolated HFB mice, an increase was not observed in the conditioned rewarding effects of cocaine exhibited by grouped HFB animals and by isolated control mice. One possible explanation for these results is that, in isolated animals, bingeing on fat prevents the increased vulnerability induced by isolation, as fat could act as a "comfort food" that reduces the stress produced by isolation. For example, daily access to a sucrose-containing drink decreases the plasma corticosterone response to restraint stress in male rats that have been exposed to a chronic variable stress paradigm [80]. In this line, we observed that exposure to HFB diminished the increment in corticosterone levels induced by isolation. Interestingly, the isolated mice exposed to HFB did not show an increase in leptin levels. Leptin directly regulates a population of leptin-receptor expressing dopaminergic neurons in the ventral tegmental area [81, 82], and therefore, modulates dopaminergic-dependent measures of food and drug reward. Although HFB only partially counteracts the increase in corticosterone induced by isolation, it could lessen leptin increase. There are several studies that obtained comparable effects of high-fat diets. Several studies propose that highly palatable food rich in sugar or fat can reduce the HPA axis responses [83, 84]. Maniam and co-workers [85] reported that a diet high in fat and sugar reversed the anxiety symptoms induced by limited nesting in male rats. More interestingly, Arcego and co-workers [86] have shown

that both social isolation during the prepubertal period and exposure to a high-fat diet affects memory and BDNF levels in the prefrontal cortex of rats, but when both manipulations were combined, these impairments were prevented.

## 5. Conclusion

As described in previous sections, both stress-responsive hormones and metabolic factors influence brain dopaminergic transmission and drug sensitivity. These adaptations might promote an increased intake of palatable food, potentiating brain reward system activity. Housing conditions are as important as dietary conditions, and they must be taken into consideration in preclinical research. The present results contribute to the understanding of the effects of housing conditions and stress on the modified response to drugs of abuse and highlight the modifications that a high-fat diet can induce in animals, depending on their housing conditions.

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# Study 6.

## Intermittent vs. continuous access to a high fat diet during adolescence: Behavioral profile.

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*In preparation*

# Intermittent vs continuous access to a high fat diet during adolescence: Behavioral profile

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**Abstract**

Over the past few years, the effects of eating a high-fat diet as a model of obesity on cognitive functions have been widely studied, especially on the hippocampus. However, there are different patterns of high-fat consumption, such as the continuous form or the binge-eating pattern. Additionally, there are no studies to date that evaluate the effects of cessation of any of these high-fat diet patterns on cognitive and anxiety behaviors. Thus, the aim of the present study was to evaluate the effect of the exposure to a high-fat diet in an intermittent or continuous form during adolescence on cognition and anxiety and the consequences of the interruption of this type of intake. Male mice received either a standard diet or a high-fat diet during 40 days in two different patterns: intermittently or continuously. In addition, two more groups with intermittent or continuous access interrupted their fat exposure 15 days before behavioral tests. Afterwards, mice were evaluated on the Elevated Plus Maze, Open Field, Object Recognition test, Passive Avoidance, Hebb Williams Maze and the Social Interaction test. Our results show few effects in animals that binge on fat, apart from hyperlocomotion and aggressive behaviors. Animals continuously eating fat present marked spatial learning deficits as well as increased attack behaviors with conspecifics. Discontinuation of fat, either in a binge or a continuous pattern, leads to an increase in anxiety levels.

**Keywords:** Learning, high fat, memory, anxiety, social behavior

## 1. Introduction

Over the past few years, the overconsumption of palatable high-fat and sugar-rich foods has increased dramatically in the adolescent population, contributing to overeating and obesity (WHO, 2015; Herpertz-Dahlmann, 2015; Volkow et al., 2013). Adolescence is a special vulnerable developmental period, in which several cognitive, behavioral and biological changes are developed (Pickles et al., 1998, Bava and Tapert, 2010). Young people are more prone to develop inadequate nutritional habits, such as overeating and eating disorders (Ifland et al., 2009). The overindulging on this kind of diet leads to increased body weight, progression into obesity and development of metabolic and cardiovascular disorders (Bruce-Keller et al., 2009). Additionally, it is well known that there is a high comorbidity between overweight and obesity and cognitive deficits and psychiatric disorders (Swanson et al., 2011; DiLeone et al., 2012; Leffa et al., 2015).

Several studies have reported that there is a strong relationship between high-fat diets (HFD) and cognitive impairment, such as in learning and memory processes (Alencar et al., 2010). These cognitive impairments may be mediated by mechanisms involving insulin, leptin and inflammatory pathways (for review: Cordner and Tamashiro, 2015). Impairments in cognition have been related with obesity in humans and animals, noting that there are inflammatory markers in the hippocampus that affect memory processes (Morris et al., 2015). Focusing on adolescence, recent studies point to the fact that juvenile exposure to a HFD impairs spatial discrimination learning and decreases neurogenesis, having a great impact on the hippocampal function (Boitard et al., 2012; Valladolid-Acebes et al., 2013). Animal models also confirmed that, after consuming a HFD during a long period, there are notable cognitive deficits, especially in spatial performances such as the Morris Water Maze (Heyward et al., 2012; McNay et al., 2010) and the novel object recognition (Del Río et al., 2016).

However, recent studies focused on different patterns of high-fat consumption, such as the limited and intermittent form, namely *binge eating*. Binge eating is characterized by a dysfunctional appetite manifested by an intermittent,

excessive intake of caloric foods in a short period of time (Gold, 2011). In this kind of models, animals have a limited access to the HFD only for a few days a week (e.g. Monday, Wednesday and Friday) during a limited period of time (Corwin et al., 1998). It is crucial to note that, although binge eating is related to obesity, most people who binge on food are not overweight, and most obese people do not present binge-eating disorders (Hudson et al., 2007). There are no studies that focus on the behavioral and cognitive consequences of bingeing on a HFD.

In previous studies, we found that binge eating on fat increases the rewarding effects of cocaine (Blanco Gandía et al., 2017a). Mice exposed during adolescence to an intermittent and limited access to fat self-administered more cocaine and were more sensitive to the conditioned rewarding effects of this drug. Moreover, we observed similar results with ethanol (Blanco-Gandía et al., *in press*). However, no such rewarding increment was observed in mice continuously exposed to fat (Blanco-Gandía et al., 2017b). We also observed that abrupt cessation of fat, despite normalizing metabolic alterations such as leptin levels or body eight, induced an increased sensitivity to cocaine in mice continuously exposed to fat. Therefore, any of these patterns of fat intake produces a dysregulation in the brain reward system that is reminiscent of that neuroadaptations occurring in drug abuse and dependence (Avena et al., 2008; Kenny, 2011; Stice et al., 2013). People experience a highly rewarding feeling when they eat palatable food, even in the absence of hunger (Gold, 2011), which makes it important to distinguish between hedonic and homeostatic signals in the regulation of food intake (Lutter and Nestler, 2009).

Consequently, in evaluating the sensitivity to drugs of abuse we observed different results depending on the model of HFD employed, whether access was continuous or in a binge pattern. Both models have great translational value, since they represent two types of fat ingestion in young people: those who become obese, and those who intermittently binge on fat and do not become overweight. The aim of the present work was to evaluate the behavioral and cognitive differences between animals receiving these

two different patterns of fat administration. Although literature shows that continuous access to fat induces cognitive deficits, no studies have evaluated the effect of intermittent access to fat. In addition, there are certain traditional symptoms of substance withdrawal that can also be observed and measured when individuals abstain from certain foods (Teegarden and Bale, 2007). To date, there are no studies that evaluate the behavioral effects of cessation of a HFD on cognitive behaviors. Therefore, it is of great interest to elucidate the behavioral profile of these two eating patterns and their withdrawal after cessation of fat intake. To address this issue, we employed adolescent mice, which received either a standard diet, or had continuous or intermittent access to a HDF for 40 days until they became young adults. In addition, two more groups were exposed to an intermittent or continuous access to fat until 15 days before the behavioral tests. We evaluated the spontaneous locomotor activity using the open field test, cognitive performance with the object recognition test, passive avoidance with the Hebb-Williams maze, and anxiety and social behavior with the elevated plus maze (EPM) as well as the social interaction test. In addition, circulating leptin levels were measured.

## 2. Material and Methods

### 2.1. Subjects

A total of 120 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28x28x14.5cm) 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 19:30-7:30h) 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 present study.

### 2.2. Feeding Conditions

Two different types of diets were used in this study. Standard diet (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9kcal/g) was used to feed the control group, and high-fat diet (TD.06415, 45 kcal % fat, 36 kcal % carbohydrates and 19% kcal protein; 4,6 kcal/g) was used for the continuous access and binge groups. Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to as the standard diet and the high-fat diet from this point forward.

There were 2 groups with sporadic limited access to high-fat food and two more groups with a continuous access to high-fat food. The limited access procedure is based on the Limited Access Model described by Corwin et al. (1998), in which non-food deprived animals with sporadic limited access to high-fat food develop binge-type behaviors. Water was freely available all the time.

### 2.3. Experimental Design

Mice were acclimated for 8 days before initiating experiments. Then, they were randomly divided into 5 groups (n=15/condition) with similar average bodyweight (25-26g) and assigned either to: Control (C), Binge (B), Binge withdrawal (BW), Continuous Access (CA) and Continuous access withdrawal (CAW). The Control group was the same for both the continuous access and the binge groups, as previous studies from our laboratory have demonstrated that controls that binge on standard diet and controls with ad libitum access to standard diet show the same behavioral outcomes (Blanco-Gandía et al., 2017a). Animals in the Binge groups had 2h access to the high-fat diet on Monday, Wednesday and Friday and were fed *ad libitum* with the standard diet in their home cages. This binge was provided 2-3h after the beginning of the dark phase. Animals in the withdrawal groups (BW, CAW) were switched to standard food 15 days before the initiation of the behavioral tests. The following tests were performed on PND 69 (for control, B, and CA) and PND 84 (for BW and CAW) in this order: Elevated Plus Maze,

Spontaneous Locomotor activity, Object Recognition Test, Passive Avoidance test, Social Interaction and Hebb Williams Maze. In addition, another set of animals (n=9/condition) was employed to determine circulating leptin levels on PND 69 and 84 (withdrawal groups). A more detailed description is provided in Table 1.


Table 1. Experimental design

## 2.4. Apparatus and Procedure

### *2.4.1. Body weight and circulating leptin levels*

Animals were weighed once a week throughout the study, and their intake of standard diet in their home cage was also measured.

Plasma leptin levels were measured with an ELISA kit from B-Bridge International (Cupertino, CA, USA) following the manufacturer's instructions. The sensitivity of the test is 0.2. All samples were run in duplicate.

### *2.4.2. Spontaneous locomotor activity*

Spontaneous locomotor behavior of mice was quantified in an open field for a period of 1 hour. The open field test was performed in an opaque plastic box (30x30x15cm) open at the top. The animal was placed in the box and the activity was recorded automatically by an automated tracking

control (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The parameters studied were total distance traveled (cm), and velocity (cm/s).

*2.4.3. Object Recognition*

The novel object recognition was used to assess recognition memory (Broadbent et al., 2004). It was performed as described (Ennaceur and Delacour, 1988; Prickaerts et al., 2002). The apparatus consisted of an open box (24 x 24 x 15) located in a testing room with constant illumination. The objects used were two small river stones (A) and a small non-toxic plastic toy (B), heavy enough to prevent displacement. On the day before the test, the habituation day, mice were allowed to explore the box (with no objects) for 2 min. On the day of the test, a training session (T1) was followed by a test session (T2) after a 1-min interval. Each session (T1 and T2) lasted 3 min.

For T1, mice were placed in the middle of the box faced away from the two identical stones (AA) arranged in the center of the testing box for 3 minutes. Afterwards, mice were removed from the box and returned to their home cages. One of the stones was changed to one small toy (non-familiar object). After the retention interval of 1 minute outside of the testing box, mice were reintroduced into the box for T2. Object exploration was defined as the orientation of the animal's snout towards the object, within a range of 2 cm or less from the object. Running around the object or sitting on it was not recorded as exploration. Objects were washed with ethanol after each individual trail to equate olfactory cues. The basic measures in the object recognition test were the times spent by the rats to explore an object during T1 and T2 (Prickaerts et al., 2002). *e1* and *e2* are measures of the total exploration time of both objects during T1 and T2, respectively. *d1* was considered as an index measure of discrimination between both the new and familiar objects. The basic measure in the object recognition test was the discrimination index, calculated as [D.I. = (tnovel − tfamiliar)/(tnovel + tfamiliar) × 100%].

*2.4.4. Passive Avoidance Test*

For the passive avoidance test, a step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed. This cage is made of Perspex sheets and divided into two compartments (15 cm × 9.5 cm × 16.5 cm each one). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

Passive avoidance tests were carried out following the procedure described in Aguilar et al. (2000). On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60 s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h and 7 days later following the same procedure but without the shock. The maximum step-through latency was 300 s.

*2.4.5. Hebb Williams Maze*

The maze we used in our experiment is made of black plastic and is 60 cm wide x 60 cm long x 10 cm high. It contains a start box and a goal box (both 14 cm wide x 9 cm long), which are positioned at diagonally opposite corners. The maze contains cold water at a wading depth (15°C, 3,5cm high), while the goal box is stocked with fresh dry tissue. Several maze designs are produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and the motivation of water escape to be measured.

The procedure followed was based on that employed by Galsworthy et al. (2005), in which mice must navigate the maze and cross from the wet start box to the dry goal box in order to escape the cold water. Animals underwent a 5 min habituation period (dry sand, no barriers) on day 1 and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently submitted to mazes 1, 5, 3, 4 and 8 on separate days, on which 8 trials took place (see Ravinovitch and Rosvold, 1951 for all maze designs). The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box. The following measurements were recorded: total latency score (the sum of the latencies in all the problem trials in each maze); and error scores, for which a similar total was used (where "error" was considered as entering the error zone specified by Galsworthy et al. (2005).

*2.4.6 Elevated Plus Maze*

The 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 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 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).

*2.4.7. Social Interaction*

This test consisted of confronting an experimental animal and a standard opponent in a neutral cage (61 × 30.5 × 36 cm) for 10 min following 1 min of adaptation prior to the encounter. Standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulfate solution 1 day before testing (Smoothy et al., 1986). This kind of mouse induces an attack reaction in its opponent but does not outwardly provoke or defend itself, since it cannot perceive a pheromone that is present in the urine of the experimental animals and functions as a cue for eliciting aggressive behavior in mice with a normal sense of smell (Brain et al., 1981; Mugford and Nowell, 1970). Behavior was videotaped under white illumination. The videotapes were analyzed using a custom-developed program (Brain et al., 1989) that facilitates estimation of times allocated to different broad functional categories of behavior –body care, digging, non-social exploration, exploration from a distance, social investigation, threat, attack and avoidance/flee – each of which is characterized by a series of different postures and elements. A more detailed description can be found in Rodríguez-Arias et al. (1998).

**2.5. Statistics**

Data relating to body weight were analyzed by a mixed ANOVA with one between-subjects' variable – "Diet", with 5 levels (Control, B, BW, CA, CAW) and a within variable "Days" with 7 levels: Week 1, 2, 3, 4, 5, 6, 7. Data related to leptin levels were analyzed by a one-way ANOVA with a between variable "Diet" with 5 levels: control, B, BW, CA and CAW.

Data relating to social interaction, object recognition, the elevated plus maze and the open field test were analyzed by a one-way ANOVA with a between variable "Diet" with 3 levels: Control, B, BW or Control, CA, CAW. In addition, in the social interaction test, non-parametric Kruskal–Wallis tests were used to assess the variance of the behavioral measures in the different

groups according to the times allocated to threat and attack. Subsequently, appropriate paired comparisons were carried out using Mann–Whitney U tests to compare behaviors following the different treatments.

The passive avoidance test was analyzed by a two-way ANOVA, with the same between variable Diet with three levels -Control, B, BW or Control, CA, CAW-, and one within variable with three levels -training day, test 24h and test 7 days-.

The data from the Hebb-Williams maze were analyzed by a two-way ANOVA with one between subject variable Diet, and one within subject variable -Maze, with five levels. Individuals unable to complete the task within the time limit scored maximum latencies.

Bonferroni adjustment was employed for post hoc comparisons. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v22.

## 3. Results

### Body weight and circulating leptin levels

The ANOVA revealed a significant effect of the variable Diet [F(4,70)=12,671; p<0.001, showing that animals in the CA and CAW groups exhibited an increased body weight with respect to the standard diet group (p<0.001) and the B and BW groups (p<0.01). There was also an effect of the variable Days [F(6,420)=1305,069; p<0.001], as all animals had an increase in body weight every week. Finally, there was a significant effect of the interaction Diet x Days [F(24,420)=9,302; p<0.001], showing that from week 2 onwards, mice in CA and CAW groups showed an increased body weight with respect to Standard Diet, B and BW mice (p<0.001).

Regarding leptin levels (see Fig 2.), there was an effect of the variable Diet [F(4,40)=3,712; p<0.01], as animals in the CA group presented higher leptin levels than the Standard diet, B and BW groups (p<0.05).

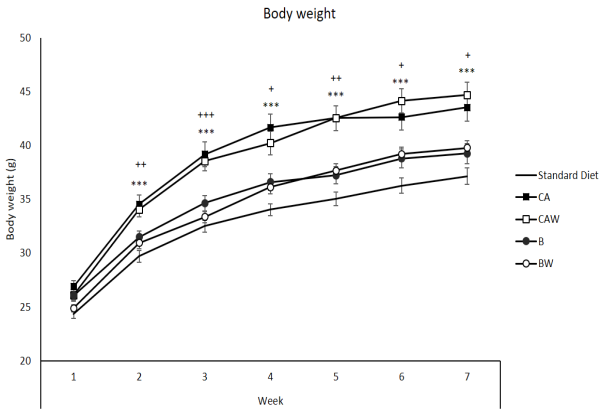


Figure 1. Weekly body weight of animals in the standard diet, CA, CAW, B and BW groups. Represented is the mean (± SEM) amount of body weight measured weekly. ***p<0.001 significant difference from CA and CAW with respect to the standard diet group. + p<0.05, ++ p<0.01, +++ p<0.001 significant difference from CA and CAW with respect to the B and BW groups.

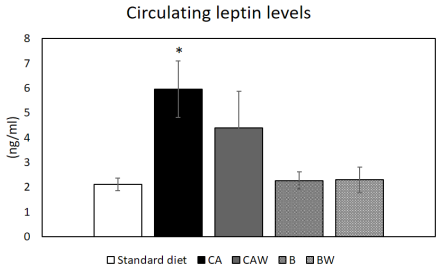


Figure 2. Circulating leptin levels of animals in the standard diet, CA, CAW, B and BW groups. Represented is the mean (± SEM) amount of leptin levels measured on PND 69 in Standard diet, CA and B; and on PND 84 on CAW and BW. *$p<0.05$ significant difference from CA with respect to the Standard Diet, B and BW groups.

## 3.1. **Behavioral profile in mice that binged on fat during adolescence**

*Open Field: spontaneous locomotor activity*

The ANOVA showed an effect of the variable Diet on Distance and Velocity [$F(2,42)= 4,831$; $p<0.01$] and [$F(2,42)=5,190$; $p<0.01$]. Animals in the B group exhibited an increased distance traveled (Fig. 3a) and an increased velocity (Fig. 3b) with respect to the Standard Diet group ($p<0.01$ in both). No differences were found between B and BW animals.

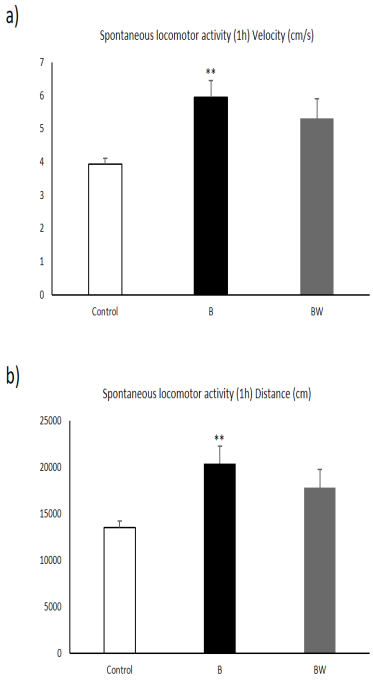


Figure 3. Effects of a high-fat diet in an intermittent pattern on adolescent mice on Velocity (3a) and Distance (3b) in the Open field test. Data are presented as mean values ± S.E.M. **p<0.01 with respect to the control group.

*Cognitive profile*

**Object Recognition**

The data from the object recognition test are presented in Table 1. No differences in d1 or D. I. were observed between the groups.

| | ***e1* (s)** | ***e2* (s)** | ***d1* (s)** | **D.I. (%)** |
|---|---|---|---|---|
| **Control** | 11 ± 0.5 | 11 ± 0.8 | 5 ± 0.4 | 50 ± 2 |
| **B** | 9 ± 0,5 | 9 ± 0,7 | 4 ± 0,4 | 47 ± 4 |
| **BW** | 9 ± 0,6 | 9 ± 0,6 | 4 ± 0,4 | 49 ± 3 |

Table 2. Effects of a high-fat diet in a binge (intermittent) pattern on adolescent mice in the Object Recognition Test. e1 and e2 are measures of the total exploration time of both objects during T1 and T2, respectively. d1 was considered as an index measure of discrimination between both the new and familiar. The basic measure in the object recognition test was the discrimination index, calculated as [D.I. = (tnovel – tfamiliar)/(tnovel + tfamiliar) × 100%].

**Passive Avoidance test**

Results from the passive avoidance test are represented in Figure 4. The ANOVA revealed an effect of the variable Days, as all groups presented longer step-through latencies in both 24h and 7 days test sessions than in the training session [F(2,84)=202,177; p<0,001]. There were significant differences at 24h and 7 days with respect to the training day (p<0.001). All groups took more time to enter the dark compartment in both test days, with no differences between groups.

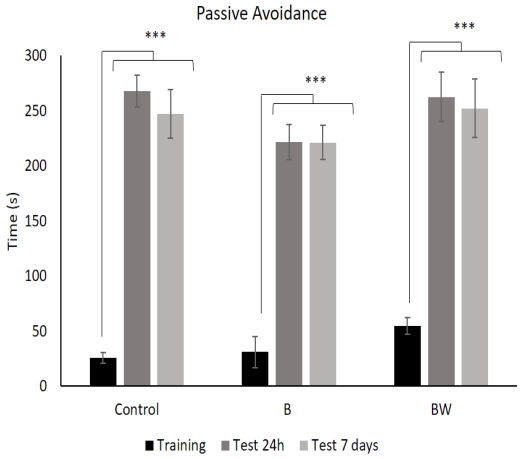


Figure 4. Effects of a high-fat diet in a binge pattern on adolescent mice on the time taken to enter the dark compartment in the Passive Avoidance Test, 24h and 7 days after training. Data are presented as mean values ± S.E.M. ***p<0.001 with respect to the training day.

**Hebb-Williams Maze**

The ANOVA for the total latency score (see Figure 5) revealed an effect of the variable Maze [F (4,168=4,124; p<0.01]. Animals require more time to reach the goal in maze 5 with respect to mazes 1 (p<0.05), 3 (p<0.01) and 8 (p<0.01). The ANOVA for the total number of errors revealed no significant differences.

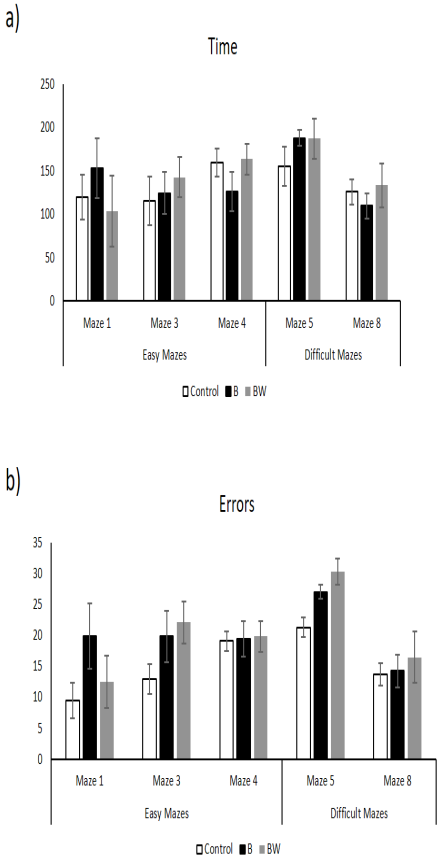


Figure 5. Effects of a high fat diet in a binge pattern on adolescent mice on the total latency score to reach the goal in the 8 trials (a) and errors (b) in the Hebb-Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). Data are presented as mean values ± S.E.M.

*Anxiety profile after intermittent fat consumption*

**Elevated Plus Maze**

The ANOVA (see Table 3) showed an effect of the variable Diet on Time and percentage of time spent in open arms [F (2,45) =13,489; p<0,001] and [F (2,45) =15,479; p<0,001]. BW animals spent less time (p<0.001) and percentage of time in open arms than control and Binge mice (p<0.001).

There is also an effect of the variable Diet on the number of entries in open arms as well as the percentage of open entries [F(2,45) = 6,769; p<0.01] and [F (2,45) = 8,277 p<0.001). BW animals presented lower number of entries in open arms with respect to the B group (p<0.01) and lower percentage of open entries than the control and binge groups (p<0.01 in both).

| | Control | B | BW |
|---|---|---|---|
| Time in open arms | 125 ± 13 | 117 ± 8 | 58 ± 10 ***+++ |
| % Time in open arms | 54 ± 5 | 53 ± 3 | 27 ± 4 ***+++ |
| Entries in open arms | 27 ± 3 | 31 ± 2 | 20 ± 2 ++ |
| % Open entries | 63 ± 5 | 60 ± 3 | 43 ± 4 **++ |
| Total entries | 42 ± 2 | 53 ± 4 | 45 ± 3 |

Table 3. Effects of a high fat diet in an intermittent pattern on adolescent mice in the EPM. Data are presented as mean values ± S.E.M. **p<0.01; ***p<0.001 with respect the control group ++ p<0.01; +++p<0.001 with respect to the Binge (B) group.

**Social interaction**

The data for the different types of behavior evaluated in the social interaction test are presented in Table 4. The ANOVA revealed an effect of the variable Diet on Non-social exploration [$F(2,40)=8,586$; $p<0.001$], social investigation [$F(2,40)= 9,635$; $p<0.001$], and mean time of social investigation [$F(2,40)= 20,569$; $p<0.001$]. B and BW mice spent less time in non-social exploration ($p<0.01$ and $p<0.001$ respectively), more time in social investigation ($p<0.01$ and $p<0.001$ respectively), and an increased mean time in each social investigation approach ($p<0.05$ and $p>0.01$ respectively) with respect to the control group. Additionally, the Kruskall-Wallis analysis showed a significant effect with respect to the time spent in threat ($\chi 2$ (df = 2, p = 0.05) = 6.216) and attack ($\chi 2$ = (df = 2, p = 0.01) = 8.296). The Mann–Whitney U test revealed that mice in the B and BW groups spent more time in threat ($p<0.05$ and $p<0.01$ respectively) and attack ($p<0.01$ and $p<0.001$ respectively) than controls.

| | Control | B | BW |
|---|---|---|---|
| Body care | 4 ± 1 | 4 ± 2 | 6 ± 2 |
| Digging | 10 ± 2 | 7 ± 2 | 6 ± 2 |
| Non-Social Exploration | 567 ± 2 | 553 ± 5 | 547 ± 5** |
| Exploration from a distance | 2 ± 0.4 | 3 ± 0.2 | 2 ± 0.3 |
| Social Invest | 15 ± 1 | 23 ± 2* | 26 ± 3*** |
| Threat | 1 ± 0.8 | 5 ± 2* | 6 ± 2 ** |
| Attack | 0.4 ± 0.3 | 6 ± 3 * | 6 ± 2** |

Table 4. Effects of a continuous high-fat diet in adolescent mice on the means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behavior from the social interaction test. Differences from control group *p<0.05, **p<0.01, ***p<0.001

## 3.2. Behavioral profile in mice with a continuous access to a high-fat diet during adolescence

### Open Field: spontaneous locomotor activity

No differences were found between standard diet animals and continuous access animals.

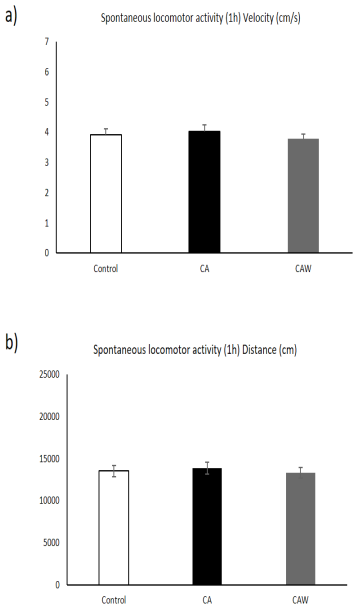


Figure 6. Effects of a continuous high-fat diet on adolescent mice on Velocity (6a) and Distance (6b) in the Open field test. Data are presented as mean values ± S.E.M.

*Cognitive profile*

**Object Recognition**

The data from the object recognition test are presented in Table 5. No differences in D. I. were observed between groups.

| | *e1* (s) | *e2* (s) | *d1* (s) | D.I. (%) |
|---|---|---|---|---|
| Control | 11 ± 0.5 | 11 ± 0.8 | 5 ± 0.4 | 50 ± 2 |
| CA | 9 ± 0.6 | 10 ± 0.7 | 5 ± 0.6 | 51 ± 5 |
| CAW | 8 ± 0.5 | 8 ± 0.7 | 4 ± 0.6 | 51 ± 4 |

Table 5. Effects of a continuous high fat diet in adolescent mice in the Object Recognition Test. e1 and e2 are measures of the total exploration time of both objects during T1 and T2, respectively. *d1* was considered as an index measure of discrimination between both the new and familiar. The basic measure in the object recognition test was the discrimination index, calculated as [D.I. = (tnovel − tfamiliar)/(tnovel + tfamiliar) × 100%].

**Passive Avoidance test**

Results from the passive avoidance test are represented in Figure 7. The ANOVA revealed an effect of the variable Days and Days x Diet, [$F(2,84)=202.536$; $p<0.001$] and [$F(4,84) = 2,738$; $p<0.05$] respectively. All groups presented longer step-through latencies in both 24h and 7 days test sessions than in the training session ($p<0.001$). However, the CA group presented a shorter step-through latency on the 7 days test session ($p<0.05$), with respect to the control group.

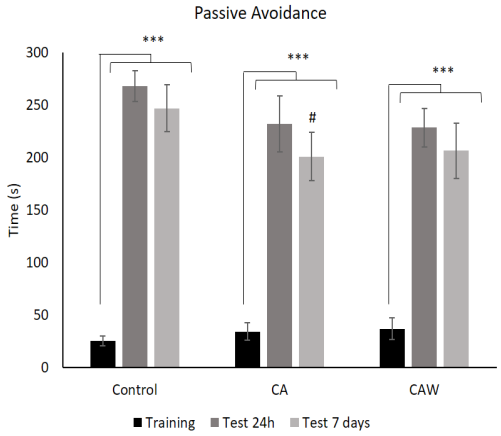


Figure 7. Effects of a high-fat diet in a continuous pattern on adolescent mice on the time taken to enter the dark compartment in the Passive Avoidance Test, 24h and 7 days after training. Data are presented as mean values ± S.E.M. ***$p<0.001$ with respect to the training # $p<0.05$ with respect to the control group.

## Hebb-Williams Maze

The ANOVA for the total latency score to reach the goal in the 8 trials (see Figure 8) revealed an effect of the variable Maze [$F(4,164=6,029$; $p<0.001$], Diet [$F(2,41)=5,139$; $p<0.01$] and the interaction of Maze x Diet [$F(8,164=2,356$; $p<0,05$]. Mice of the CA group employed more time to reach the goal in mazes 5 and 8 with respect to the control ($p<0.01$ and $p<0.05$ respectively) and the CAW groups ($p<0.01$ in both mazes). The ANOVA for the total number of errors revealed no significant differences.

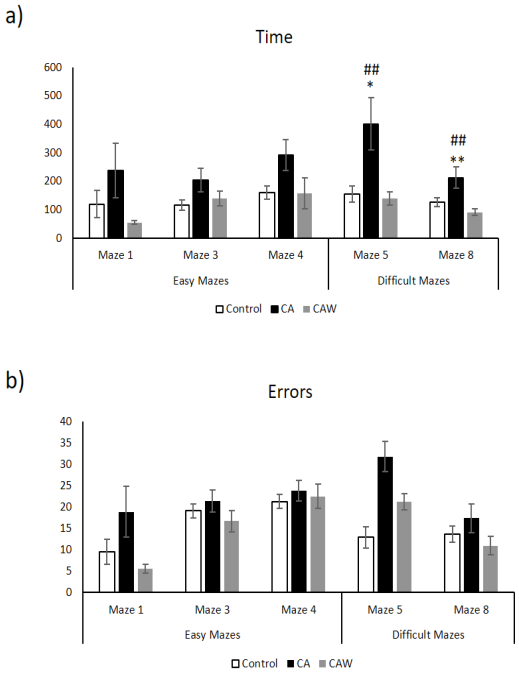


Figure 8. Effects of a high-fat diet in a continuous pattern on adolescent mice on the total latency score to reach the goal in the 8 trials of the Hebb-Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). Data are presented as mean values ± S.E.M. *$p<0.05$, **$p<0.01$ with respect to the C group and ## $p<0.01$ with respect to the CAW group.

*Anxiety profile after continuous fat consumption*

**Elevated Plus Maze**

The ANOVA (see Table 6) showed an effect of the variable Diet on Time and percentage of time spent in open arms [F (2,42) =11,901; p<0,001] and [F (2,42) =12,957; p<0,001]. CAW animals spent less time and percentage of time in open arms than control and CA mice (p<0.001).

There is also an effect of the variable Diet on the number of entries in open arms and also the percentage of open entries [F(2,42) = 5,456; p<0.01] and [F (2,42) = 7,938 p<0.001]. CAW animals presented lower number of entries in open arms with respect to the CA group (p<0.01). Regarding the percentage of open entries, CAW animals presented lower entries than the control group (p<0.01) as well as their corresponding diet group CA (p<0.01).

| | Control | CA | CAW |
|---|---|---|---|
| Time in open arms | 125 ± 13 | 130 ± 11 | 54 ± 13 ***### |
| % Time in open arms | 54 ± 5 | 59 ± 4 | 27 ± 6 ***### |
| Entries in open arms | 27 ± 3 | 35 ± 3 | 21 ± 4 ## |
| % Open entries | 63 ± 5 | 65 ± 4 | 44 ± 5 *## |
| Total entries | 42 ± 2 | 57 ± 6 | 44 ± 7 |

Table 6. Effects of a continuous high-fat diet in adolescent mice in the EPM. Data are presented as mean values ± S.E.M.*p<0.05; **p<0.01; ***p<0.001 with respect to the control group. # p< 0.05; ## p<0.01; ### p<0.001 with respect to the CA group.

**Social Interaction**

The data for the different types of behavior evaluated in the social interaction test are presented in Table 7. The ANOVA revealed an effect of the variable Diet on social investigation [$F(2,42)= 3.216$; $p<0.05$] and attack [$F(2,42)= 4.506$; $p<0.01$]. CAW mice showed more time spent in social investigation ($p<0.05$) with respect to the control group. Additionally, the Kruskall-Wallis analysis showed a significant effect with respect to the time spent in attack ($\chi 2$ = (df = 2, p = 0.05) = 5.923). The Mann–Whitney U test revealed that mice in the CA spent more time in attack than controls ($p<0.05$).

| | Control | CA | CAW |
|---|---|---|---|
| Body care | 4 ± 0.8 | 3 ± 1 | 5 ± 2 |
| Digging | 10 ± 2 | 7 ± 1 | 9 ± 2 |
| Non-Social Exploration | 567 ± 2 | 557 ± 5 | 559 ± 4 |
| Exploration from a distance | 2 ± 0.4 | 2 ± 0.3 | 3 ± 0.5 |
| Social Invest | 15 ± 1 | 18 ± 2 | 21 ± 2* |
| Threat | 1 ± 0.8 | 6 ± 2 | 3 ± 2 |
| Attack | 0.4 ± 0.3 | 7 ± 3* | 2 ± 1 |

Table 7. Effects of a continuous high-fat diet in adolescent mice on the means of accumulated times (in seconds) with SEM allocated to different categories of spontaneous behavior from the social interaction test. Differences from the control group *p<0.05

## 4. Discussion

The present work compared for the first time the behavioral profile that exposure to a different pattern of HFD during adolescence induced in the motor, cognitive and anxiety profile of mice. The main result of this study is that intermittent vs continuous fat intake induced different behavioral profiles. Overall, our results revealed that early exposure during adolescence to an intermittent binge access to high-fat food produces mainly an increase in locomotor activity, with a pro-social but aggressive related behavior, such as threat and attack. On the other hand, continuous access to fat induced obesity, with increased bodyweight and circulating leptin levels. These mice also displayed impairment in memory tasks in the passive avoidance test and in hippocampal-dependent learning, requiring more time to reach the goal in the difficult mazes of the Hebb-Williams Maze. Continuous access to fat also lead to an increase in aggressive behaviors in the social interaction test. Most of these alterations are normalized after 2 weeks of fat cessation, except for the changes in social behaviors. In addition, discontinuation of any type of fat administration produces an increase in anxiety in the EPM.

The results of the present study are in agreement with our own and other laboratories showing that only animals in the CA and CAW group showed an obesity profile, the former also presenting higher plasmatic leptin levels (Ahrén and Scheurink, 1998; Lin et al., 2000; Wellman et al., 2007; Morales et al., 2012; Blanco-Gandía et al., 2017b). In contrast, bingeing on fat did not produce any increase in body weight or leptin levels, confirming that sporadically overeating did not induce obesity (Corwin et al., 1998).

Mice that binge on fat during the whole procedure show a significant increase in the distance traveled and velocity in the Open Field test. Conversely to our results, Rosetti et al. (2014) reported that female rats given chow pellets for 5 days and palatable food for 2 days over 7 consecutive weeks presented bingeing behavior and displayed reduced locomotor activity in the open field. In addition, although the study is not comparable with ours, Bake et al (2014) observed that male mice exposed to HFB increased rearing during the 2h period leading up to the scheduled feeding while locomotor activity

started to increase 1h before indicating food anticipatory behaviors. It is worth to mention that other studies report that food restriction induces hyperactivity (Altemus et al., 1996). However, in our model, animals that binge on fat intermittently also have continuous access to standard chow. In a recent study, Valdivia et al (2015) show that limited accesses to HFD induces an activation of different sub-populations of the ventral tegmental area dopamine neurons and accumbens neurons that is, in general, more pronounced than the activation observed after a single HFD consumption event. Therefore, all the results suggest that bingeing on fat could induce an increase in motor activity due to dopamine stimulation. This hyperlocomotion was completely normalized 15 days after the last binge on fat, pointing out that the neurobiological changes underlying this alteration are restored when binge behavior finished.

On the other hand, continuous access to fat did not induce locomotor changes, which is in line with other studies that did not report changes in the open field test after 8 weeks of high fat diet treatment (Del Río et al., 2016). However, other obese mouse models, such as *ob* (mice lack of leptin or its receptors that develop hyperinsulinemia, obesity and hyperphagia) and *agouti* (overexpression of this peptide, which is orexigenic, stimulating feeding and leading to obesity), tend to exhibit lethargic behavior and decreased activity (Erickson et al., 1996).

***Cognitive behavioral profile***

Several studies have shown that in diseases like obesity, where leptin levels are higher than normal, cognitive functions such as memory and learning can be affected (Valladolid-Acebes et al., 2013; Shanley et al., 2001; Naderali et al., 2009). In agreement with these results, we observed an impairment in learning and memory among the mice continuously exposed to fat, but not so on the mice that binged on fat.

Memory was slightly impaired in CA mice in the passive avoidance test 7 days after the training. Although they remembered the training and took significantly longer time to cross the dark compartment, this time was

shorter than in controls. These results have been previously reported in several studies (Hwang et al., 2010; Sharma et al., 2015)

CA mice also showed deficits in hippocampal dependent learning. The Hebb-Williams Maze is a very sensitive test to detect any spatial learning, providing mazes with different degrees of difficulty. Previous reports have found that continuous access to fat induces deficits related to spatial abilities that directly involve the hippocampus (Valladolid-Acebes et al., 2013). Our results showed that these learning deficits arise when the maze becomes more challenging, since animals in the CA group take longer to reach the goal in mazes 5 and 8, categorized as difficult mazes (Vidal-Infer et al., 2012). High-fat diets are deleterious for the hippocampus structure and function (Greenwood and Winocur, 2005) as well as for synaptic plasticity and neurogenesis (Lindqvist et al., 2006). Our results are consistent with studies that show how juvenile exposure to a HFD impairs spatial discrimination learning and decreases neurogenesis, having a profound impact on the hippocampal function (Boitard et al., 2012; Valladolid-Acebes et al., 2013). Metabolic effects of high-fat diet exposure, like insulin resistance, general metabolic dysfunction, and increased fat mass have been identified as factors that may contribute to impaired cognitive function (Uranga et al., 2010; Schwartz et al., 2013; Luchsinger et al., 2004). Regarding the withdrawal group in the continuous access pattern, some studies have shown that high-fat diet consumption during adolescence impairs learning and memory when they become adult, and this effect is not reversed by further caloric restriction or bodyweight (Valladolid-Acebes et al., 2011). In our work, we did not detect such impairments when access the high-fat diet was interrupted, as the withdrawal groups behaved like the control group. It is worth noting that, although CAW mice's bodyweights stayed similar to when they had access to fat, their leptin levels decreased to normal parameters.

Conversely, no differences were found in the object recognition task for CA or CAW mice. Previous studies have shown an impaired discrimination index in the object recognition task after receiving a continuous high-fat diet for 4 and 8 weeks (Del Río et al., 2016). Perhaps the composition of the diet

or the different strain of mice could explain these discrepancies. Finally, no alterations in cognitive functions were observed in B or BW mice.

***Anxiety and social profile***

As we previously reported, bingeing on fat or having continuous access does not affect the anxiety levels (Blanco-Gandía et al., 2017a and b), and other studies have reported likewise (Rosetti et al., 2014; Bocarsly et al., 2011).

Only mice withdrawn from any of the fat administration schedules showed increased anxiety (CAW and BW groups). These mice spent less time and percentage of time in the open arms of the maze, and made less entries and percentage of entries in the open arms, in comparison to mice fed with the standard diet or consuming fat (CA and B groups). Therefore, BW and CAW exhibited a clear anxious behavior. These results are in agreement with previous results of our laboratory (Blanco-Gandía et al., 2017a and b).

Although fat consumption did not affect the EPM, social behaviors are deeply modified. Mice continuously consuming fat showed an aggressive profile with higher attack behavior. This aggression disappeared after the cessation of fat (CAW group), being substituted by an increase in positive social behaviors. The behavioral profile of mice intermittently exposed to fat also showed an aggressive profile, but in that case, it was accompanied by an increase in social behaviors. In addition, this profile did not change after the cessation of fat, as B and BW presented similar behaviors.

Our results are in the same line as previous studies that showed higher aggression in obese rats in the social interaction test (Buchenauer et al., 2009). In this study, diet-induced obesity mice presented an increase in aggressive behaviors. These authors suggest that obese mice exhibited a higher level of emotional arousal, as they found that these animals also presented elevated corticosterone levels compared to lean littermates. Although some studies have pointed out the impact of overfeeding on the HPA axis in a vulnerable early period (Boullu-Ciocca et al., 2005), we did not find increased corticosterone levels in those fed with fat (Blanco-Gandía et

al., 2017a). It would be interesting to do further studies with these variables. In the social interaction test, mice are exposed to a novel and brightly-lit environment with an unknown opponent, which is perceived by the animals as a high aversive condition (Kask et al., 2001). Thus, maybe these animals reacted to the conspecific and showed aggressive behaviors as a defensive mechanism.

## 5. Conclusion

Our study showed that the pattern of fat administration modulates the effects on mice's motor activity, cognition, anxiety and social behaviors. Although any type of fat intake increased aggressive behaviors, those mice exposed continuously to fat presented a decrement in cognitive functions with an impairment of memory and learning functions. However, no cognitive effects were observed in the mice intermittently exposed to fat. We also observed that cessation of fat, either continuously or intermittently, induced an increased anxiety.

These results highly contrast with those reported regarding the effects of a high-fat diet on the rewarding properties of cocaine or ethanol. We found that animals that binge on fat are more sensitive to drugs of abuse like cocaine and alcohol (Blanco-Gandía et al., 2017a; Blanco-Gandía et al., *in press*), even after fat discontinuation. However, our previous studies reported that animals with continuous access to fat did not present increased sensitivity to drugs of abuse while they were feeding on fat, but they presented an enhanced response when fat discontinuation occurred (Blanco-Gandía et al., 2017b).

To sum up, our results pointed out that exposure to a rich high-fat diet during adolescence induced deep alterations in the brain functions. However, the way this diet is ingested determined the nature and the extent of these behavioral changes.

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# 4.GENERAL CONCLUSIONS

## 4. General Conclusions

The main aim of the present work was to study the influence of a HFD during adolescence on the rewarding effects of cocaine and ethanol. Currently, there is a wide variety of results in the literature but in which the food is consumed. Obesity and overeating share the same neurobiological basis with addiction to substances of abuse, and they resemble dependence both neurally and behaviorally. The present work is mainly focused on two animal models of eating disorders, which based on the previous literature, seem to alter the reward system: the continuous access model and the binge eating model. The continuous access to a HFD leads to animal models of obesity, while the limited and intermittent access to a HFD resembles binge eating disorders. In the same way as drugs of abuse, withdrawal symptoms and craving for specific kinds of foods have been observed in humans (Rogers and Smit, 2000), therefore we also evaluated if the cessation of these two models of fat administration could induce withdrawal symptoms. This experimental design allowed us to discriminate between two ways of modulating the rewarding effects of drugs of abuse. We employed adolescent mice, due to their particular behavioral and neurobiological characteristics, as well as their increased vulnerability to eating disorders and substance abuse.

To study the influence of a HFD on the vulnerability to drugs of abuse, we measured the impact of the environmental cues and the direct rewarding action and motivation to consume the drug, which together represent, along with the sensitization response, a complete approximation to the study of addiction using animal models. In the CPP procedure, a primary motivational stimulus (drug) is used as an unconditioned stimulus, which after repeated exposure, turns environmental cues that were initially neutral into secondary motivational properties, becoming a conditioned stimulus (Tzschentke, 2007; Aguilar et al., 2009). When the animal is exposed to the context in which the substance was given, it spends more time in the drug-paired compartment (if the stimulus is appetitive). On the other hand, the SA paradigm is an experimental model that evaluates the primary reinforcing effects of drugs according to the effort made by the animal to obtain the drug.

In this model, the animal performs an operant response, such as pressing a lever to obtain the drug reinforcement.

The most important result of the present doctoral thesis is to show that the effects of a HFD significantly vary depending on the eating pattern employed. In general, our results showed that a continuous HFD mainly causes large effects at a metabolic level and few at the level of drug sensitivity, while binge eating has little metabolic effects but a larger impact on the reinforcing effects of drugs, such as cocaine and alcohol. A novel contribution in the present work has been to study the effect of the cessation of a HFD on the vulnerability to drugs, which has been scarcely studied.

***Continuous HFD during adolescence***

Continuous access to fat leads to significant increases in body weight and leptin levels as well as a decrease in ghrelin plasmatic levels. These changes were normalized after 15 days of HFD cessation **(Study 1)**.

The behavioral profile of these mice was deeply affected **(Study 6)**. Although HFD did not induce effects on motor activity or anxiety, social behavior and cognitive functions were impaired. When interacting with a non-aggressive conspecific, mice continuously exposed to HFD showed higher aggressive responses, in line with previous studies (Buchenauer et al., 2009). Moreover, obesity can be particularly problematic in children's and adolescents' cognitive functions, as this developmental period is crucial for the maturation of the hippocampus (Spear, 2000). When executing a spatial learning task like the Hebb-Williams Maze, we observed that animals in a HFD required more time to reach the goal in the mazes considered more difficult, revealing that their learning process had been deteriorated. These results are consistent with previous studies that show how juvenile exposure to a HFD impairs spatial discrimination learning and decreases neurogenesis, having a profound impact on the hippocampal function (Boitard et al., 2012; Valladolid-Acebes et al., 2013). In addition, a slight

detriment in memory was also observed in these mice.

Conversely to the profound effect that continuous HFD induces on social and cognitive functions, it does not affect the rewarding effects of cocaine. Based on previous studies, we expected to find that continuous exposure to a HFD would reduce the conditioned rewarding effects of cocaine, but we did not observe any alteration using an effective or a subthreshold dose of cocaine. However, we confirmed that HFD acts as an alternative reinforcer reducing cocaine seeking during the extinction of the CPP **(Study 1)**. Animals exposed to a HFD extinguish the conditioned memories faster and showed less sensitivity to reinstate the preference. Our results are in line with several human studies that report hyperphagia and weight gain after cessation of drug abuse (Edge and Gold, 2011), supporting the concept of "addiction transfer," in which one addiction is replaced by another (Chechlacz et al., 2009). Further studies using additional protocols may be of interest to clarify this hypothesis.

**To sum up, our results suggest that a continuous HFD intake induces significant metabolic changes, learning deficits, and impairment in social behaviors. However, HFD did not modify the vulnerability to the conditioned rewarding effects of cocaine. Moreover, chronic fat intake during cocaine removal acts as an alternative reward.**

***Bingeing on HFD during adolescence***

Previous studies show that many people who binge are not obese, and most obese people do not present binge eating disorders (Hudson et al., 2007). Our study confirmed this statement, as animals that binged on fat did not differ in the body weight gain with respect to mice fed with a standard diet, also in agreement with previous reports (Corwin et al., 1998). In concordance, leptin levels are not particularly affected by bingeing on fat. In line with previous studies showing that ghrelin secretion is downregulated

by HFD, mice bingeing on fat showed low ghrelin levels (Beck et al., 2002; Lindqvist et al., 2005; Bello et al., 2009). However, we observed that isolation modulates the effect of HFB, thereby inducing the opposite effects to those seen in grouped animals **(Study 5)**. Most studies that use the binge eating protocol employ isolated rodents in order to control individual food intake and bodyweight (Baladi et al., 2015; Serafine et al., 2015; McGuire et al., 2011). However, the comparison of the modulation that housing conditions exert on the previously described effects of HFB has not been previously done. We observed that bingeing on fat seems to act in isolated animals as a comfort food during this period of stress (Dallman et al., 2005). Our results show that it is necessary to consider the housing conditions of the animals, as it can have a big influence on the final results and lead to misinterpretations.

As opposed to mice that took HFD continuously, animals that binged on fat presented less behavioral alterations. HFB mice are more active and exhibited more social-aggressive behaviors than the standard diet group **(Study 6)**. However, these animals are more sensitive to the rewarding effects of cocaine and ethanol. HFB mice self-administered more cocaine or ethanol and were more sensitive to the conditioned rewarding effects of both drugs **(Studies 2 and 3).**

How can we explain the contrasting results induced by a HFD depending on the administration schedule? The escalation on a high-fat food mimics what occurs with drug abuse (Zernig et al., 2007), since it goes from a controlled intake to a compulsion and loss of control (Goeders et al., 2009; Perelló et al., 2014). Recent work from Valdivia and co-workers (2015) show how satiated animals eat for pleasure and reward, without a homeostatic purpose, as occurs in our study. During this process, dopaminergic neurons in the NAcc core are increasingly activated as the escalation occurs. According to these authors, repeated exposure to palatable food in a limited and intermittent manner produces neuroadaptations that activate the dopaminergic system persistently, in line with what occurs after chronic administration of drugs of abuse (Valdivia et al., 2015; Steketee and Kalivas, 2011). Studies conducted

with other types of food, such as sugar, sweet-fat or vegetable-fat mixtures also modify dopamine turnover, giving support to this hypothesis (Avena et al., 2009). Thus, we consider that bingeing on fat, presented in a limited and in an intermittent schedule, induces the development of sensitization to the rewarding effects of cocaine and ethanol.

**To sum up, our results provide behavioral evidence that bingeing on high-fat food during adolescence increase vulnerability to cocaine and ethanol consumption in mice. Once the neural mechanisms mediating addiction have been activated, the individual becomes more vulnerable to developing other compulsive and abusive behaviors.**

***Withdrawal from HFD exposure***

Prolonged exposure to rewarding stimuli such as palatable foods or drugs of abuse can lead to dependence, and when the stimulus is removed, the manifestation of physical symptoms of withdrawal appear (Avena, 2007). Two weeks after the abrupt cessation of HFD administration, either continuous of in a binge pattern, mice showed an increase in anxiety and higher corticosterone levels, typical signs of the withdrawal syndrome. No studies to date have evaluated the effects of cessation of a HFD on cognitive and learning behaviors. Animals in a fat withdrawal period did not show the alterations observed as cognitive impairments or hyperlocomotion, although social behavioral changes persisted **(Study 6)**.

Conversely to metabolic and behavioral normalization, animals withdrawn from fat after binge administration still showed an increased self-administration of ethanol and cocaine **(Study 2 and 4)**. Nevertheless, these animals did not develop preference for subthreshold doses of cocaine or alcohol anymore. However, mice withdrawn from continuous fat intake showed an increased response to the conditioned rewarding and motor effects of cocaine **(Study 1)**. Our data are in line with those evaluating the effects of sugar-rich diets, in which animals forced to abstain from sucrose

present an enhanced response to ethanol, methamphetamine and cocaine (Avena et al., 2004; Avena and Hoebel, 2003; Gosnell, 2005). As we can see in Table 6, 15 days after the last fat administration, leptin and ghrelin levels are normalized. Some studies have reported that leptin attenuates cocaine-induced-increases in DA levels in the NAcc and reduces the ability to establish a cocaine CPP (You et al., 2016).

| | HFD | HFD W | HFB | HFB W |
|---|---|---|---|---|
| LEPTIN | ↑ | = | = | = |
| GHRELIN | ↓ | = | ↓ | = |

Table 1. Effects of HFD on leptin and ghrelin plasmatic levels

Our results, therefore, pointed out that some neuroadaptations induced by HFD persist after the cessation of the ingestion, highlighting the long-lasting effect of this diet on the reward system. Nutritional manipulations in rodents have shown to affect the long-term functioning of the DA system (Kelley and Rowan, 2004; Teegarden et al., 2009). Teegarden and co-workers reported that withdrawal from a HFD can induce deep alterations on the reward systems, such as a reduction in the dopaminergic signal in mice previously exposed to a HFD (Teegarden and Bale, 2007; 2008; Teegarden et al., 2009). In addition, the increased corticosterone levels after fat discontinuation create an aversive state after cessation of palatable food, which provokes further compulsive intake when palatable food becomes available again (Cottone et al., 2009; Koob and Zorrilla, 2010). In agreement with this hypothesis, we observed that after fat withdrawal, exposure to a new fat binge reinstated cocaine seeking in the self-administration procedure **(Study 1)**.

**Exposure to HFD during adolescence induces neuroadaptations that will be expressed after abrupt cessation of fat consumption during adulthood, increasing anxiety levels and enhancing sensitivity to cocaine or ethanol.**

***Effects of HFD on opioid and cannabinoid systems***

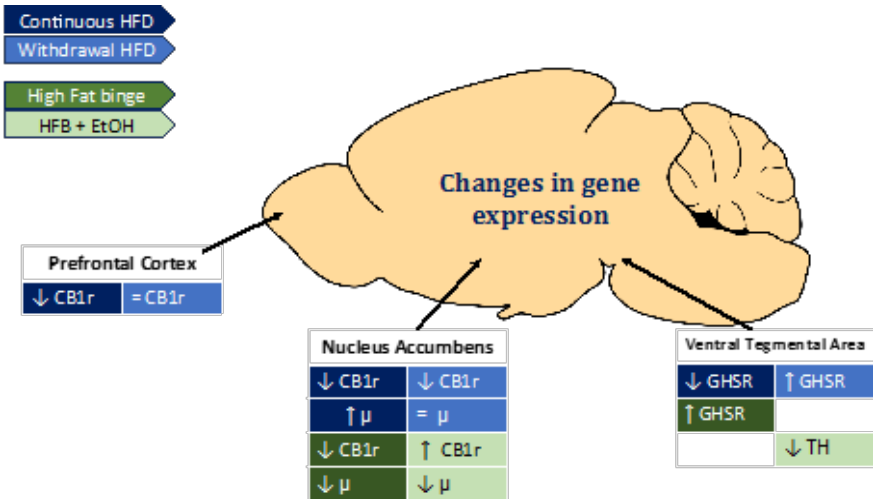


Figure 7. Changes in gene expression induced by HFD

Finally, we showed that HFD administration during adolescence induced changes in the opioid, and cannabinoid systems. With respect to the μ opioid receptor gene expression, continuous *vs* binge HFD induced opposite changes in N Acc. While continuous access increases mu receptor gene expression, binge eating decreases it. On the contrary, CB1 receptor gene expression is similarly affected by HFD. A decreased gene expression is observed in the N Acc and PFC after either continuous or binge HFD administration. More importantly, most of these changes persisted even after fat discontinuation. Therefore, the altered response to the rewarding effects of cocaine and ethanol are also accompanied by changes in opioid and cannabinoid systems. Beyond the particular role that each of these neurotransmitter system plays in the response to cocaine or ethanol, our

results demonstrated that fat ingestion induced persistent modifications of these systems that could be responsible, at least partially, for the increase in the rewarding effects of cocaine and ethanol.

### *Translational value of the study*

Children and teenagers are the main target of the processed food industry, which invests a lot of money in external signals towards young people, such as gifts included in cereal boxes, bright colors, and animated pictures etc., inciting them to demand and consume their products. At the psychological level, palatable food is an easily available reward, which plays a role of self-medication and a resource for raising one's mood or reducing stress (Sinha and Jastreboff, 2013; Cartwright et al., 2003).

Our results provide behavioral and neurobiological evidence that the intake of palatable food during an early stage of life modifies the vulnerability to substance abuse. The present study suggests nutritional patterns to be an important variable to take into consideration when treating psychostimulant and alcohol disorders. It should be mandatory to know, when treating substance use disorder patients, their nutritional habits, social relationships, cognitive function, etc. to be able to help them not only on their drug abuse problem, but to change their eating habits and life style.

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