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Involvement of the endogenous opioid and cannabinoid systems in addictive like-behaviours

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Universitat Pompeu Fabra
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Alla mia famiglia,
che mi ha insegnato a
viaggiare in direzione
“ostinata e contraria”.

Nothing would be more tiresome than eating and drinking if God had not made them to a pleasure as well as a necessity.

Voltaire

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Abstract

The increase incidence of obesity and eating disorders represents a major health problem in developed countries. The low rate of success of treatments to prevent or reverse obesity, and overeating that causes it, highlights the important behavioural alterations that are associated to this disease. These alterations seem to be mediated by modifications in the reward circuits that mimic changes occurring during addictive behaviour. Moreover, like drugs of abuse, obesity is associated with abnormal intake habits when maintaining diet that can endure vulnerability to relapse. In the present thesis, we have first investigated the involvement of the endogenous opioid system in the neurobiological mechanism underlying drug and food reinstatement, as a potential therapeutic target in these behavioural disorders. Moreover, we have investigated the relationships between overeating and behavioural addiction. Indeed, we have demonstrated that repeated operant training with palatable food promotes behavioural alterations, as well as epigenetic, proteomic and structural plasticity changes in the reward circuit reminiscent to those observed with drugs of abuse. Finally, we identified the cannabinoid receptor 1 and the delta opioid receptor as common neurobiological substrates underlying these alterations.

Resumen

El aumento de la incidencia de la obesidad y de los trastornos de la alimentación representa un importante problema de salud en los países desarrollados. La baja tasa de éxito de los tratamientos disponibles para prevenir o revertir la obesidad y el fácil acceso a la comida obesogenica que lo causa, destacan la necesidad de encentrar dianas terapéuticas eficaces. Las importantes alteraciones conductuales que se asocian a esta enfermedad parecen estar mediadas por modificaciones en los circuitos de recompensa que imitan los cambios que ocurren durante el comportamiento adictivo. Por otra parte, al igual que las drogas de abuso, la obesidad se asocia con hábitos de ingesta anormales que pueden incrementar la vulnerabilidad a la recaída de búsqueda de comida. En la presente tesis, hemos investigado primero la implicación del sistema opioide endógeno en el mecanismo neurobiológico que subyace a la recaída del comportamiento de búsqueda de drogas y comida como una posible diana terapéutica en estos trastornos del comportamiento. En segundo lugar, hemos investigado las relaciones entre la sobre ingesta de comida palatable y la adicción conductual. De hecho, hemos demostrado que el entrenamiento operante repetido con comida palatable promueve alteraciones de la conducta, así como cambios epigenéticos, proteómicos y de plasticidad estructural en el circuito de la recompensa que recuerdan a los observados con las drogas de abuso. Es destacable señalar que hemos identificado el

receptor cannabinoide 1 y el receptor delta opioide como sustratos neurobiológicos comunes que subyacen a estas alteraciones.

Abreviations

2-AG 2: arachidonoylglycerol

AEA: anandamide

AMPK: 5' adenosine monophosphate-activated protein kinase

AgRP: agouti-related peptide

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ARC: arcuate hypothalamic nucleus

BED: binge-eating disorders

cAMP: cyclic adenosine-5'-monophosphate

CART: cocaine- and amphetamine- regulated transcript

CB1R: cannabinoid receptor type 1

CB2R: cannabinoid receptor type 2

CCK: colecistoquinina

CRF: corticotropin-releasing factor

CRH: corticotrophin-releasing hormone

D1-4R: dopamine receptor type 1-4

DA: dopamine

DMH: dorsomedial hypothalamic nucleus

DOR: delta opioid receptor

KOR: kappa opioid receptor

DSM: Diagnostic Statistical Manual of Mental Disorders

ECS: endocannabinoid system

EPSP: post synaptic potential

FTO: fat mass and obesity associated gene

GABA: γ-aminobutyric acid

GLP-1: glucagon-like peptide 1

GPR: G-protein coupled receptor

HCP: hippocampus

ICD-11: International Statistical Classification of Diseases and Related Health Problems 11th Revision

LHA: lateral hypothalamic area

LTD: long-term depression

LTP: long-term potentiation

MCH: melanin-concentrating hormone

MDMDA: 3,4-methylenedioxy-methamphetamine

mGluR1-8: metabotropic glutamate receptor 1-8

MOR: mu opioid receptor

MSH: melanocyte stimulating hormone

NAc: nucleus accumbens

NMDAR: N-methyl-D-aspartate receptor

NPY: neuropeptide Y

PDYN: prodynorphin

PENK: proenkephalin

PFC: prefrontal cortex

PKA: protein kinase A

POMC: proopiomelanocortin

PP: pancreatic polypeptide

PVH: paraventricular hypothalamic nucleus

PYY: peptide YY

THC: Δ9-tetrahydrocannabinol

TRPV1: transient receptor potential cation channel subfamily V

VMH: ventromedial hypothalamus

VTA: ventral tegmental area

YFAS: Yale Food Addiction Scale

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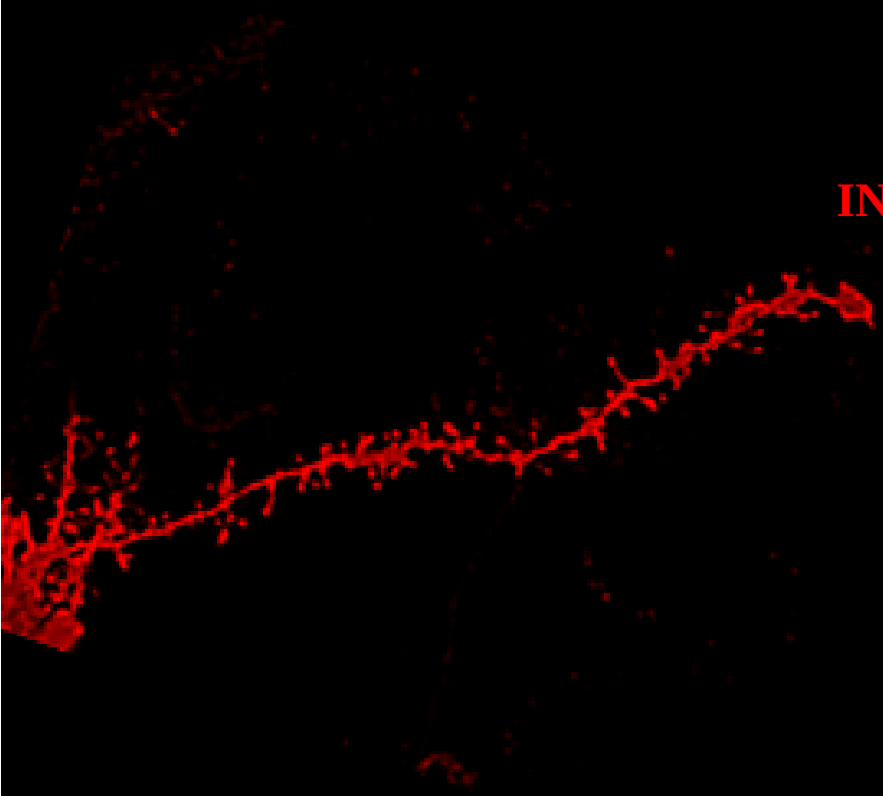


INTRODUCTION

1. ADDICTION DISEASE

1.1. Addiction and relative diseases

Addiction is a multifactorial brain disease that affects behavioural responses, in which neurobiological changes promoted by chronic exposure to a drug of abuse lead to compulsion to seek and take the drug, loss of control over intake despite consciousness of its negative consequences and relapse even after long periods of abstinence (American Psychiatric Association, APA 2013). Addiction is complex and has multifaceted symptoms, associated with dysfunctions in motivational, emotional, learning and behavioural control (Goldstein & Volkow 2011). A current triadic model proposes that the pathophysiology of addiction involves interactions between the drug, the environment and the vulnerable phenotype (Kreek et al. 2002). Indeed, genetic and environmental factors contribute to the development and progression of this maladaptive pattern of drug use leading to social and physical impairments (APA 2013).

Clinically, this brain disease is diagnosed by several psychiatric manuals, such as the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) of the APA or the *International Classification of Diseases*, 11th Revision (ICD-11; World Health Organization, WHO). Important revisions to the definitions and diagnosis of addiction were recognized in various versions of DMS during the last years. In particular, the DMS-III (1980) considered mainly the physical effects (tolerance and withdrawal) produced by the long-

term exposure to drugs of abuse to diagnose addiction, using a physiological dependence approach ("assumption drug centered"). In the DSM-IV (1994), the term addiction was referred also to the psychological changes that the drug use can lead, with a psychological dependence approach (“assumption individual centered”) and in this line other 5 diagnostic criteria related to the loss of control of consumption were added. Indeed, tolerance and withdrawal were no longer necessary criteria to diagnose drug dependence and the diagnosis was given only when the patient met at least three of the seven criteria (see Table 1). In the last DSM-5 edition (2013), the term addiction was also applied to compulsions that are not substance-related, taking into account the behavioural aspect of this disease, with an approach “behavioural centered”. In this line, gambling disorder was added as the only diagnosable condition in a new category of behavioural addictions since it resembles substance use disorder to a certain extent. In this last version, addiction combines the DSM-IV categories of substance abuse and substance dependence into a single disorder, named substance use disorder. Each specific substance is addressed as a separate use disorder, but nearly all substances are diagnosed based on the same overarching criteria (APA 2013). In addition, DSM-5 includes criteria to specify the severity of substance use disorders, which results from merging the previous lists of dependence and abuse criteria into a single list of 11 criteria (see Table 1). Considering the pattern of drug-use and the related-symptoms of pathological intake, the threshold for substance use disorder diagnosis is graded by the number of criteria met: 0–1, unaffected;

DSM-III criteria for substance abuse (1980)	DSM-IV criteria for substance abuse (1994)	DSM-5 criteria for substance use disorders (SUDs) (2013)	Rat model criteria of cocaine addiction (2004, 2010) and mice model of eating addictive-like behaviour (2015)
All of these three criteria should be accomplished	At least one of these four criteria	At least two of the eleven criteria: (0-1 unaffected; 2-3 mild; 4-5 moderate; 6 or more severe)	
1. Disturbance of social or occupational functioning	1. Recurrent failure to fulfill major role obligations	1. Recurrent failure to fulfill major role obligations	
2. Pattern of pathological use	2. Recurrent substance use in physically hazardous situations	2. Recurrent substance use in physically hazardous situations	
	3. Recurrent substance-related legal problems.		
3. Impairment in social or occupational functioning due to substance use	4. Continued substance use despite persistent or recurrent social or interpersonal problems	3. Continued substance use despite persistent or recurrent social or interpersonal problems	
DSM-III criteria for substance dependence (1980)	**DSM-IV criteria for substance dependence (1994)**		
One out of these two criteria	Three out of these seven criteria		
1. Tolerance	1. Tolerance	4. Tolerance	
2. Withdrawal	2. Withdrawal	5. Withdrawal	
	3. The substance is often taken in larger amounts or over a longer period than intended	6. The substance is often taken in larger amounts or over a longer period than intended	1. Persistence to response (criteria 3-4 in DSM-IV and 6-7 in DSM-5)
	4. Persistent desire or unsuccessful efforts to cut down	7. Persistent desire or unsuccessful efforts to cut down	
		8. Craving	
	5. Considerable time spent in obtaining the substance or using, or recovering from its effects	9. Considerable time spent in obtaining the substance or using, or recovering from its effects	2. Motivation (criteria 5 and 6 in DSM-IV and 9-10 in DSM-5)
	6. Important social, work, or recreational activities given up because of use	10. Important social, work, or recreational activities given up because of use	
	7. Continued use despite knowledge of problems caused by or aggravated by use	11. Continued use despite knowledge of problems caused by or aggravated by use	3. Resistance to punishment (criteria 7 in DSM-IV and 11 in DSM-5)

Table 1. Comparison of the diagnosis items of drug use related disorders in DSM-III, DSM-IV and DSM-5, with their corresponding criteria measured in the rat or mouse model of addiction (modified from Piazza & Deroche-Gamonet, 2013).

2–3, mild; 4–5, moderate; ≥6, severe disorder. Classifications are categorical, i.e. a criterion is positive when present, independent of its intensity. To avoid the mislabelling of patients as dependent or addicted, tolerance and withdrawal do not count when the individual develops physiological dependence while adhering to a prescribed regimen (Compton et al. 2013).

Therefore, transition to addiction was initially defined as the appearance of changes in drug effects (mainly tolerance and withdrawal), and it is currently described by changes in the modality of drug-taking from controlled drug use to loss of control. Thus, the occasional but limited use of a drug is clinically distinct from escalated drug use, loss of control over limiting drug intake, and the emergence of chronic compulsive drug-seeking that characterize addiction (Koob & Volkow 2010). Indeed, not all occasional drug users will became addict. For instance, out of 100 people initiating cocaine use, 15–17 will develop addiction (Anthony et al. 1994). It is estimated that in the last years, drug use disorders affected around 27 million people (UNODC, World Drug Report 2015). No efficient cure for substance use disorders actually exists, and the available therapies are usually able to alleviate only withdrawal symptoms, but remain largely unsuccessful to promote control over drug intake (National institute of drug of abuse, NIDA 1999). Indeed, relapse rate range between 50% and 90% for most of the drugs within the first year of treatment (Gonzales et al. 2010; Powell et al. 2010). This high rate of relapse to drug use after abstinence represents a major clinical problem, and understanding

the neurobiological basis of relapse constitutes a primary challenge of drug addiction research.

1.2 Neurobiological substrate of addiction

Current views recognize that drug addiction is based on pathological changes in brain functions produced by repeated pharmacological insult of drugs of abuse to specific brain circuits (Kalivas & O'Brien 2008). Drug-induced alterations in neurotransmitter systems disrupt the neuronal functions of the brain structures that compose the mesocorticolimbic system, and ultimately produce cognitive and emotional dysfunctions (Koob & Volkow 2010). In this line, drug addiction affects many areas of the brain reward system including nucleus accumbens (NAc), ventral tegmental area (VTA) and ventral pallidum, memory/learning-conditioning circuits including amygdala and hippocampus (HCP), motivation /drive systems including orbitofrontal cortex, subcallosal cortex, dorsal striatum (ST) and motor cortex, inhibitory control/executive function including inferior frontal cortex (PFC), orbitofrontal cortex and anterior cingulate cortex and stress reactivity including habenula and amygdala (Volkow et al. 2011; Volkow & Baler 2013). This network of interacting circuits contributes to the complex set of pathological behaviours underlying addiction.

An altered dopamine (DA) signalling has been implicated in all stages of drug addiction, from induction to maintenance and relapse. Initial work on the DA role in drug reward focused on the

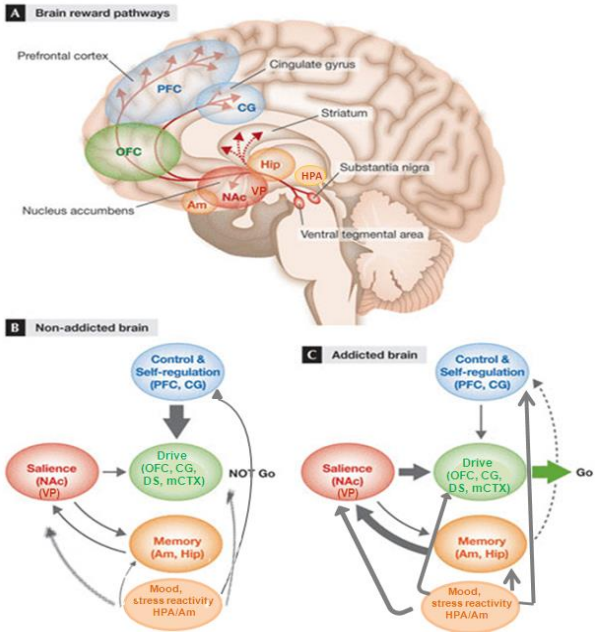


Figure 1. Model of brain circuits implicated in drug addiction a) Schematic and oversimplified sagittal view of the main brain structures involved in addiction. **b-c)** Model proposing a network of interacting circuits in the non-addicted and addicted brain: reward (red: located in the NAc and VP); motivation (green: located in OFC, DS, CG and mCTX); memory (gold: located in the Am and HCP); and executive control (blue: located in dorsolateral PFC, anterior CG, and inferior frontal cortex). In addition, these circuits also interact with circuits involved in the regulation of mood and stress reactivity (orange: HPA and Am); **b)** in non-addicted brain these circuits are balanced and the result is a proper inhibitory control and decision making; **c)** in the addicted brain the enhanced expectation value of the drug in the reward, motivation and memory circuits overcomes the control circuit, favoring a positive-feedback loop initiated by the consumption of the drug and perpetuated by the increased activation of the motivation/drive, memory and stress reactivity circuits. PFC, prefrontal cortex; CG, anterior cingulate cortex; OFC, orbitofrontal cortex; DS dorsal striatum; mCTX, motor cortex; Am, amygdala; Hip, hippocampus; NAc, nucleus accumbens; VP, ventral pallidum; HPA, habenula (adapted from Lee et al. 2012).

mesolimbic DA pathway. Indeed, the DA projection from the VTA of the midbrain to the NAc is the major substrate of reinforcement for both natural rewards and addictive drugs (Schultz 1997). Addictive drugs directly or indirectly trigger exaggerated, but transitory increases in extracellular DA in the NAc. This increase positively correlates with the intensity of "pleasure" that subjects experience when taking drugs (Volkow et al. 2010).

However, other DA pathways, such as the mesostriatal DA neurons in substancia nigra projecting to dorsal ST also contribute to drug reward (Wise 2009). Indeed, the nigrostriatal and mesolimbic DA systems have close anatomical relationships. Due to the proximity between substantia nigra and VTA (Wise 1981), these two areas have several functional overlaps. Thus, DA neurons of the substantia nigra respond similarly to rewards than DA neurons of the VTA (Apicella et al. 1991). Interestingly, the DA involvement in reward does not seem to equate only with hedonic pleasure, mediated in part by endogenous opioids and cannabinoids. Indeed, DA appears to encode prediction of rewards, imprinting incentive value to reinforcers and learning of reward associations by conditioning processes through modulation of subcortical and cortical brain regions (Volkow et al. 2012).

Conditioning is one of the initial neuroadaptations that follows exposure to drugs and involves DA phasic signalling, predominantly though activation of DA receptor 1 (D1R), and synaptic changes in N-methyl-D-aspartate (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) (Volkow et al. 2012). These conditioned responses are belived to

underlie the intense desire for the drug that occurs when addicted subjects are exposed to drug cues that triggers the compulsive use of the drug. This hypothesis is supported by evidence showing that blockade of D1R or D2R activity in NAc shell and core prevents acquisition of conditioned learning tasks (Di Ciano et al. 2001). In the same direction, injection of D1R agonist or antagonist, respectively, enhances or decreases retention of memory in rodents (Morris et al. 2003), indicating also an important modulatory role of DA system in memory consolidation in the HCP. In addition, repeated experiences to a given stimulus lead to the shift of DA neurons activation from the presentation of the reward to the environmental cue that predicts its appearance. Therefore, it has been postulated that DA activity provides a predictor signal for the learning of stimulus through reward associations. Thus, DA cell firing is enhanced in response to the prediction of reward and not to reward itself, whereas DA cells fire decreases if the expected reward fails to materialise (Schultz 1997).

DA signalling also modulates motivation to seek the drug through the regulation of several target regions including NAc, orbitofrontal cortex, dorsal ST and amygdala. Specifically, DA signalling is implicated in the motivation cost required to complete tasks that demand high levels of effort (Salamone et al. 2007). Dysregulation of the DA signalling is not only associated with an enhanced motivation (Volkow & Li 2005), but also contributes to the shift from impulsive to compulsive drug intake through the modulation of circuits involved in decision making and executive functions (Goldstein & Volkow 2002). Indeed, the PFC plays a crucial role in

inhibitory control, and an impaired PFC function is linked to the poor control and high compulsivity seen in addiction (Volkow & Baler 2013). Indeed, a decreased PFC activity associated with the reduction in striatal D2R levels has been reported during drug addiction (Volkow et al. 2007; Thanos et al. 2007). The decrease in D2R results in a hypofunctioning of the reward system, and "addicted" individuals compensate for decreased DA availability consuming large amounts of rewarding substances. This improper cortico-striatal DA signalling could underlie the impulsive behaviour and loss of control over drug intake (Volkow et al. 2000). Addictive drugs share the common feature to enhance DA activity and release in the mesocorticolimbic circuit by mimicking the effects of natural rewards. The potency of drugs as well as the mechanism, by which they increase DA, differs for the various drug classes. Indeed, addictive drugs can be distinguished into three main groups according to the way that they activate DA neurons in the mesocorticolimbic system (Lüscher & Ungless 2006).

Class I:

Drugs that bind to G protein–coupled receptors. This group includes the opioids (e.g. morphine, heroin, through mu opioid receptors, MOR), cannabinoids (e.g. Δ9-tetrahydrocannabinol, THC), through cannabinoid receptor 1, CB1R), and gamma-hydroxy butyrate, through $GABA_B$ receptors). These drugs, acting on G protein-coupled receptors that are of the Gi/o family, inhibit mainly gamma-aminobutyric acid (GABA) neurons in the VTA. As GABA neurons act as local inhibitory interneurons in the

VTA, their inhibition leads to a net disinhibition of DA neurons and increase DA release.

Class II:

Drugs that interact with ionotropic receptors or ion channels. This group includes nicotine (acetylcholine receptors), alcohol ($GABA_A$ and other receptors), and benzodiazepines ($GABA_A$ receptors). Mechanisms of these drugs are less clear but in general have a combined effect: inhibit GABA terminals in the VTA (e.g alcohol) and also directly modulate DA neurons activity in the VTA (e.g. nicotine) leading to enhanced release of DA.

Class III:

Drugs that target monoamine transporters. This group comprises cocaine, amphetamines, and amphetamine derivates such as methylenedioxymetamphetamine (MDMA, ecstasy). Whereas all other drugs directly act on VTA neurons, these drugs block the re-uptake of DA, or stimulate non-vesicular release of DA in their projecting axons, causing an accumulation of extracellular DA in target structures, such as the NAc.

As previously reported, addictive drugs also modulate other neurotransmitter/neuromodulator systems such as the opioid, the glutamatergic, the GABAergic and the endocannabinoid system (ECS), among others which also play key roles in drug rewarding effects (Koob & Le Moal 2008a).

The glutamatergic system plays an important role in drug addiction (Kalivas 2009). In particular, glutamatergic signalling in the mesocorticolimbic system, via its ionotropic (AMPAR, NMDAR and kainate receptor) and metabotropic (mGluR1-8) receptors, has been considered an essential substrate for neuronal plasticity, learning and memory mechanisms involved in different aspects of the addictive process, such as reinforcement learning, drug sensitization and craving/relapse (Tzschentke & Schmidt 2003). Indeed, glutamatergic blockade in different parts of the mesocorticolimbic system prevents the acquisition of the operant self-administration behaviour to obtain natural rewards or drugs of abuse (Kelley et al. 1997; Kotlińska & Biała). These effects may rely on the capacity of the glutamatergic transmission to activate DA cells in the VTA and the following DA release in the NAc (Karreman et al. 1996). The glutamatergic system is also a key substrate for drug relapse (Knackstedt & Kalivas 2009). Imaging studies in human addicts have described that the presentation of the drug-related cue promotes drug relapse associated with increased glutamatergic activity in several brain structures, such as the amygdala and the PFC (Everitt and Wolf 2002).

The GABAergic transmission is also involved in the mechanisms underlying drug addiction. GABA is the major inhibitory signalling in the central nervous system through the activation of its ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. In the VTA, the GABAergic system regulates DA transmission and modulates a variety of drug-related reinforcement and reward behaviours, through pre and post synaptic actions (Steffensen et al. 2009). Thus,

stimulation of the $GABA_B$ receptor prevents the reinforcing effects of cocaine (Shoaib et al. 1998), methamphetamine (Bartoletti et al. 2004), nicotine (Paterson et al. 2008) heroin, morphine (Assadi et al. 2003) and ethanol (Maccioni et al. 2007). Similarly, stimulation of $GABA_A$ modulates the reinforcing effects of benzodiazepines (Shinday et al. 2013), barbiturates (Winger et al. 1975), cocaine, amphetamine (Meririnne et al. 1999), and ethanol (Davies 2003). Indeed, cocaine self-administration (Negus et al. 2000; Goeders et al. 1993; Barrett et al. 2005; Karler et al. 1995) and cocaine and amphetamine-induced conditioned place preference (Meririnne et al. 1999; Reynolds et al. 2003; Rush et al. 2004) are inhibited by agonists or positive modulators of $GABA_A$. Moreover, systemic administration of $GABA_A$ antagonists such as bicuculline and picrotoxin and the inverse agonist Ro 15-4513, reduces ethanol self-administration (Chester & Cunningham 2002). Similarly, $GABA_A$ receptor antagonist SR 95531 administrated into specific brain regions such as central nucleus of the amygdala, bed nucleus of the stria terminalis, and shell of the NAc, also reduces ethanol responding in a free-choice operant task (Hyytiä & Koob 1995). The effect of $GABA_A$ agonists on ethanol self-administration is less clear (Chester & Cunningham 2002) and may be region-specific, since site-specific injections of muscimol into dorsal and median raphe nuclei (Tomkins et al. 1994) or into NAc (Hodge et al. 1995) increase and decrease ethanol self-administration respectively. However, it is not clear why the administration of both, a $GABA_A$ receptor agonist (Hodge et al. 1995) and a $GABA_A$ receptor antagonist (Hodge et al. 1995) into the NAc reduces ethanol self-

administration. This result may depend on the affinity to a specific $GABA_A$ subunit. Indeed, knockout mice lacking the delta subunit of the $GABA_A$ receptor consumed less ethanol compared with wild-type mice in a free-choice operant procedure (Mihalek et al. 2001). Furthermore, $GABA_A$ agonists, like benzodiazepines, also improve the manifestations of the withdrawal symptoms to ethanol (Prater et al. 1999; Miller & Gold 1998) and psychostimulants (Miller & Gold 1998). In this line, the activation of both GABA receptors diminishes the reinstatement of alcohol (Malcolm 2003) and other drugs of abuse such as nicotine (Lubbers et al. 2014; Fattore et al. 2009) and cocaine (Torregrossa & Kalivas 2008).

The ECS and the opioid system also exert a common role in the neurobiological mechanisms underlying drug addiction, as reported in chapters 3 and 4.

In summary, behavioural dysfunctions in addictive disorders have been mainly related to drug-induced alterations in DA and glutamatergic systems in the mesocorticolimbic circuit. However, other neurochemical systems, such as the GABAergic, endogenous cannabinoid and opioid system also play important roles in the addictive process. A remaining critical issue not yet fully clarified is to understand how these alterations lead to persistent physiological changes in the different brain reward areas leading to the development and maintenance of addiction.

1.3 Genetic and environmental influence: effects on vulnerability to addiction

Addiction disorder is a complex disease mainly resulting from interactions between genetic and environmental factors combined with drug induced changes in the brain. Other factors also influence the vulnerability to addiction as the gender and the individual comorbidity with other mental disorders. Gender is an important factor influencing addiction. Indeed, men have more probability than women to become addicted, possible due to hormonal differences (Mitchell & Potenza 2015) and progesterone may attenuate drug-rewarding effects (Quinones-Jenab & Jenab 2010). In the same line, drug addiction is often developed in individuals suffering from psychiatric disorders, such as depression, anxiety or schizophrenia. The high prevalence of these comorbidities does not mean that one condition causes the other, even if one appears first. In fact, there are at least three scenarios that should be considered:
1) Mental illnesses can lead to drug abuse. Patients may abuse drugs as a form of self-medication. For example, the use of tobacco products by patients with schizophrenia is believed to lessen the symptoms of the disease and improve cognition (AhnAllen et al. 2015);
2) Drugs of abuse may bring to another mental illness. The increased risk of psychosis in vulnerable marijuana users suggests this possibility (Caspi et al. 2005);
3) Both drug use disorders and other mental illnesses could be caused by shared risk factors such as genetic vulnerabilities, and/or

environmental triggers, like early exposure to stress or trauma (Nee 2013).

Moreover, the involvement of similar brain regions is also a determinant factor for the comorbidity of these diseases (NIDA, 2010).

It is estimated that the risk to progress from recreational use to substance related disorder is around 15-17% for cocaine, 31% for tobacco (Anthony et al. 1994) and 10% for cannabis users (Lopez-Quintero et al. 2011). Therefore, risk factors influencing the transition to addiction are of fundamental relevance. Addiction as a polygenic disease hinges a vast number of genes able to influence brain development, neurotransmitter systems, drug metabolic pathways, neuronal circuitry and behavioural patterns (Drgon et al. 2010) that could contribute to brain changes leading to transition from controlled to compulsive drug use. Indeed, several polymorphic variations have been demonstrated to affect the addiction vulnerability (Bierut et al. 2006; Greenwald et al. 2013; Heath & Picciotto 2009). An example is the genetic variant of DA family receptors (Le Foll et al. 2009). Since DA is a critical modulator in the reward area, it is not surprising that many facets of the addiction phenotype are influenced by genetic variability in the DA system. In a similar way, polymorphisms of the CB1R (Zhang et al. 2004) or the MOR and DOR (LaForge et al. 2000) might contribute to the predisposition to engage in addictive behaviours.

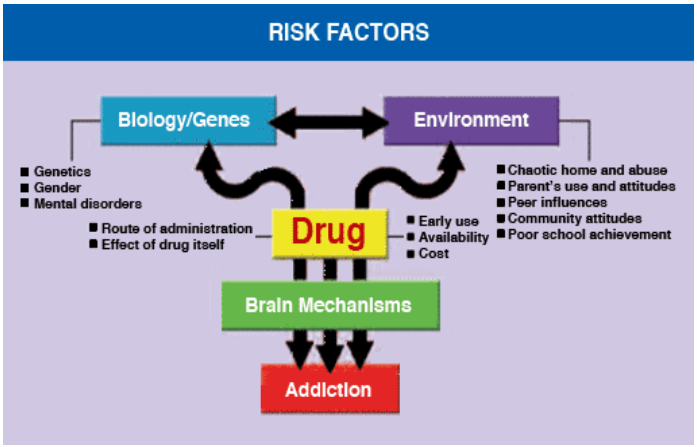


Figure 2. Risk factors in addiction disease. The overall risk for addiction is impacted by the biological makeup of the individual and the surrounding social environment with a consequent influence of brain development and function, which in turn will influence behaviour (NIDA, 2014).

Addiction is not only due to the risk conveyed by genes themselves, but also the added impact of the environment on these genes. Environmental risk factors are conditions in person's surroundings that could predispose an individual to become addicted to drugs. A person may have many environments of influence such as the community, family, school, and friends. The risk of addiction can develop in any of these domains. Thus, environmental conditions influence the expression of genes and could contribute to the initiation of addictive drug use and to the transition from controlled use to addiction. Indeed, human behaviour is the product of intricate networks involving thousands of genes working in the concert with multiple developmental and environmental events. In this line,

epigenetic regulations represent important adaptive changes linked with a dynamic environment that have emerged as critical elements for understanding how chronic drug exposure is connected to long lasting neuronal changes (Robison & Nestler 2011). Indeed, chronic cocaine and alcohol treatments activate and repress many genes such as FosB, cyclin-dependent kinase 5, and brain-derived neurotrophic factor, where their epigenetic dysregulation, at the chromatin level, contributes to the development and maintenance of addiction (Biliński et al. 2012; Levine et al. 2005). Similarly, repeated exposure to heroin leads to a DNA hypermethylation on the gene promoter of MOR which, in turn, follows a reduced transcription and consequently decreased MOR protein levels (Nielsen et al. 2012). In addition, intermittent subcutaneous nicotine administration in rats increases the expression of D1R mRNA in PFC, and this increase seems related to changes in histone H4 acetylation of the D1R gene promoter (Gozen et al. 2013).

In summary, addiction is viewed as a multifactorial and complex disease resulting from a combination of genetic and environmental factors that influence brain functions and behaviours (Hamer 2002). Understanding the various contributions of these factors will help to develop more effective prevention and treatment interventions for this disease.

1.4 Different stages of the addiction process

The occasional use of a drug is clinically distinct from compulsive drug seeking and uncontrolled intake that characterize addiction.

The switch from an occasional use to a compulsive habit is a key diagnostic feature of addiction and this only emerges after a prolonged substance use history. This transition to addiction is composed by several steps leading to changes in the modality of drug-taking from controlled drug-use to loss of control (Piazza & Deroche-Gamonet 2013). The transition to addiction involves neuroplasticity changes in some brain structures that may begin with modifications in the mesolimbic DA system and a cascade of neuroadaptations from ventral to dorsal ST and orbitofrontal cortex and eventually dysregulations of the PFC, cingulate gyrus and extended amygdala (Koob & Volkow 2010).

This transition results from three sequential and independent phases: (1) initial drug use, when a drug is voluntary taken due to its hedonic effects, (2) drug harmful use, when a drug is chronically consumed to avoid negative emotional state and finally (3) drug addiction, when drug intake becomes compulsive and uncontrolled (Koob & Nestler 1997; Koob & Volkow 2010).

Initially, the individual learns to take drugs during his recreational activities, and the primary reinforcing effects of the drug is the major reason of the initial drug use (Piazza & Deroche-Gamonet 2013). This sporadic drug use activates the same brain substrates that mediate the positive reinforcing effects of natural rewards, i.e. increased DA release in the NAc shell (Di Chiara & Imperato 1988).

The repeated and increasing pattern of drug intake leads the drug use extremely likeable, due to the DA release (Bassareo & Di Chiara 1997) and the motivational effects of drugs related to

adaptations that also include the DA system (Hooks et al. 1992; Anagnostaras & Robinson 1996). Sensitization of the DA transmission in the NAc is considered a crucial factor of the neurobiological mechanisms leading to transition to addiction (Piazza & Deroche-Gamonet 2013). Another well-documented mechanism is the impaired PFC function, leading to reduced ability to inhibit excessive drug taking (Volkow et al. 2011). The increased activity of DA neurons in the NAc is consistent with an enhanced desire to take drugs, whereas the impairment of the PFC function is mainly related to increased impulsivity (Jentsch & Taylor 1999). Afterwards, drugs progressively shift from being strongly wanted to also strongly needed. The establishment of sustained drug intake induces an allostatic state associated with a less sensitive reward system that needs stronger stimulation to achieve the same level of reward and this state will progressively bring the non-drug state out of the comfort zone. Accordingly, a negative emotional state that includes dysphoria, anxiety or even physical withdrawal symptoms appears when the drug is not available. This negative reinforcement promotes even more the consumption of the drug (Koob 2009; Hyman et al. 2006; Kosten & George 2002). The mechanism underlying the negative reinforcement is hypothesized to derive from dysregulation of key neurochemical elements involved in reward and stress functions within the basal forebrain structures involving the ventral ST and extended amygdala (Koob 2009). This stage includes not only a diminished reward neurotransmission, such as decreased DA and serotonin levels in the ventral ST, but also activation of brain stress systems, such as corticotropin

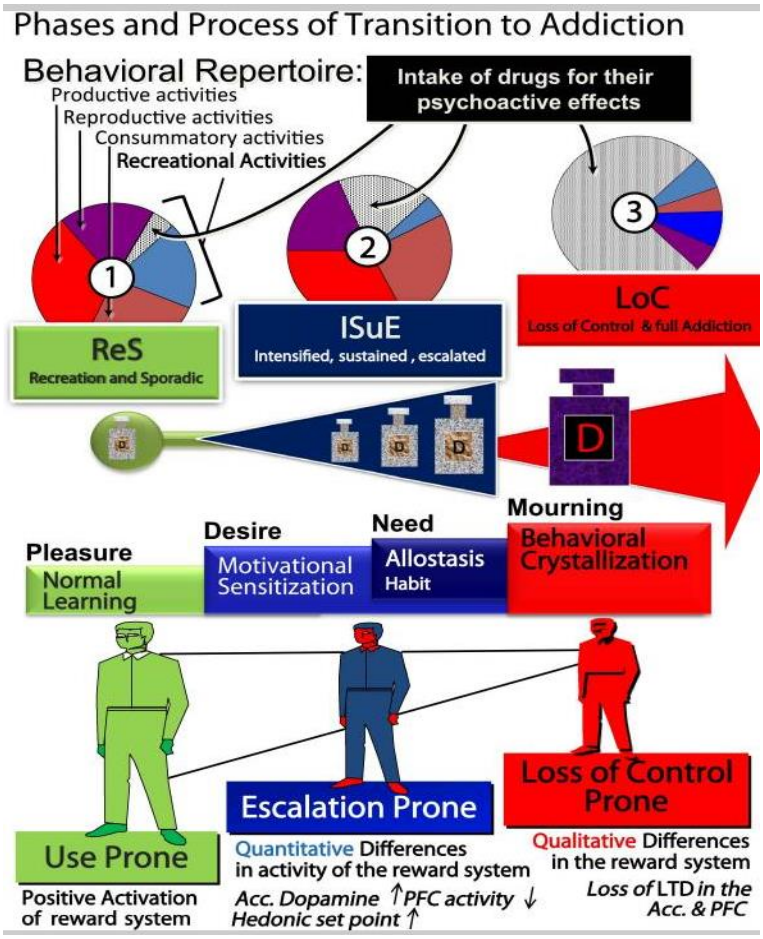


Figure 3. Phases of the transition to addiction: (1) Recreational, sporadic (ReS) drug use, in which drug intake is sporadic and still one among many recreational activities of the individual. (2) Intensified, sustained, escalated (ISuE) drug use, in which drug intake intensifies and frequent and becomes the principal recreational activity of the individual; although some decreased societal and personal functioning start appearing, behaviour is still largely organized. (3) Loss of control (LoC) of drug use and full addiction that results in disorganization of the addict's behaviour; drug-devoted activities are now the principal occupations of the individual (Piazza & Deroche-Gamonet 2013).

-releasing factor (CRF) in the extended amygdala that regulates the induction of anxiety-like behaviours (Koob 2009). Indeed, the hypothalamic-pituitary-adrenal axis and the brain stress system mediated by CRF are dysregulated by chronic administration of all major drugs of abuse, with a common response of elevated adrenocorticotropic hormone, corticosterone, and amygdala CRF during acute withdrawal to all the prototypical drugs of abuse (Delfs et al. 2000; Olive et al. 2002). Although these mechanisms are shared substrates for the majority of drugs of abuse, others appear to be drug-specific. Indeed, increased sensitivity of opioid receptor transduction mechanisms is revealed in the NAc during opiate withdrawal (Stinus et al. 1990), decreased GABAergic and increased NMDA glutamatergic transmission during alcohol withdrawal (Weiss et al. 1996), and differential regional changes are reported in nicotine receptor functions (Dani & Heinemann 1996). In addition, the somatic manifestations of the withdrawal symptoms are different depending on the drug used.

Moreover, the sustained drug use induces compulsive seeking-behaviours and loss of control of drug intake, a persistent state in which drug use escapes control even when serious negative consequences ensue (Hyman & Malenka 2001). The behavioural alteration is characterized by a loss of long term depression (LTD) of synaptic plasticity in reward areas (Kasanetz et al. 2013) mediated by NMDAR-dependent LTD and accompanied by the appearance of an impaired mGluR 2/3-mediated LTD in the cortex of addicted rats (Piazza & Deroche-Gamonet 2013). Moreover, modification in synaptic plasticity has been reported to occur

quickly in the VTA, then in the NAc and later in the PFC (Lüscher & Malenka 2011). This loss in synaptic plasticity and the fault in maintained adaptive behavioural responses to changes in environmental contingencies (Neiman & Loewenstein 2013) produce a kind of crystallized behaviour around one unique goal, the compulsive drug seeking and intake (Piazza & Deroche-Gamonet 2013). More studies are needed to understand the genetic/epigenetic, cellular, and molecular mechanisms that mediate the transition from occasional drug use to the loss of behavioural control over drug-seeking and drug-taking.

1.5 Relapse

Persistent drug overstimulation causes long-lasting cellular, molecular and neurochemical adaptations in the brain that seem to be involved in the high vulnerability to relapse after cessation of drug use (Kalivas & Volkow 2011; Kalivas 2009). Relapse can be defined as the return to drug-seeking and drug-taking behaviour after a period of abstinence. The high rate of relapse to drug use is recognized as the most difficult clinical problem in the treatment of addiction (O'Brien 1997).

Three main stimuli have been identified to trigger relapse in humans: (1) the reexposure to drugs of abuse (Everitt et al. 2003), (2) drug-associated environmental cues (Carter & Tiffany 1999b) and (3) stress (Shaham et al. 2000; Shalev et al. 2000). Neuronal mechanisms underlying drug priming-, cue- or stress-induced relapse involve different brain areas and neurotransmitters.

Functional brain imaging studies in humans (Daglish & Nutt 2003; Goldstein & Volkow 2002) have demonstrated an interconnected set of cortical and limbic brain regions in associative learning underlying relapse. Indeed, learned associations that occur during the process of repeated drug use can later manifest as trigger factors in relapse to renewed drug-seeking and drug-taking behaviour. The relapse of drug seeking and taking by re-exposure to drugs of abuse

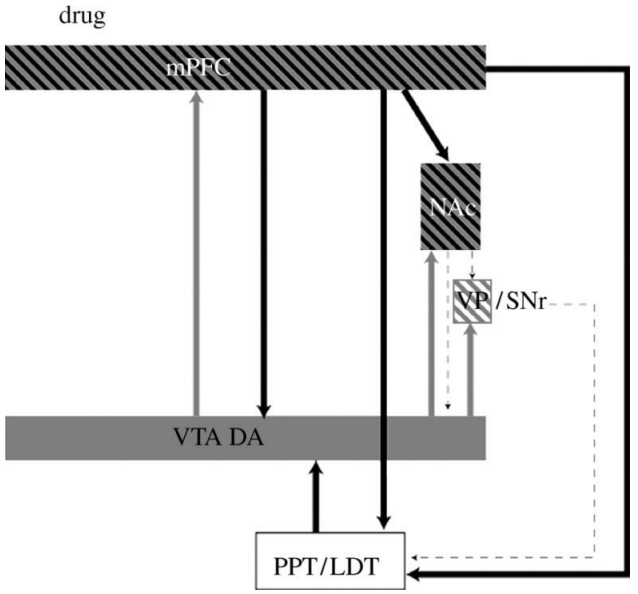


Figure 4. Diagram showing the primary circuits and neurotransmitters implicated in drug-induced reinstatement. VTA, ventral tegmental area, cell body regions of mesocorticolimbic DA pathway; NAc, nucleus accumbens; medial PFC, medial prefrontal cortex; VP/SNr, ventral pallidum/substantia nigra reticulata; PPT/LDT, peduncular pontine and laterodorsal tegmental nuclei. Grey, DA; black, glutamate (Stewart 2008).

is a robust phenomenon in humans. Neuroanatomical studies indicate that relapse depends on the re-activation of the mesocorticolimbic system toward the reward. Indeed, circuits and main neurotransmitters implicated in drug-induced relapse were identified. These include DA projections from the VTA to the NAc and medial PFC, and glutamatergic inputs from medial PFC to the VTA, the peduncular pontine, the laterodorsal tegmental nuclei and the NAc. There is general agreement that activation of the DA pathway from the VTA to the medial PFC contributes significantly to the reinstatement of drug seeking (McFarland & Kalivas 2001; Sun & Rebec 2005; Capriles et al. 2003). Indeed, increased DA release in the medial PFC appears to promote cocaine seeking by stimulating the glutamatergic projection from the medial PFC to the NAc (McFarland et al. 2003; Park et al. 2002). Accordingly, cocaine, amphetamine or DA administered into the dorsal PFC reinstates cocaine seeking (McFarland & Kalivas 2001; Park et al. 2002). The VTA projects to the dorsal medial PFC and, in turn, this region projects back directly to the VTA or indirectly through the peduncular pontine and laterodorsal tegmental nuclei (Kalivas & McFarland 2003; Shaham et al. 2000). The mesocorticolimbic DA system plays a critical role in drug-induced reinstatement. Activation of these midbrain DA neurons by local morphine infusions (Di Chiara & North 1992) or other compounds known to increase the firing rates of DA neurons such as NMDA (Karreman et al. 1996) reinstates heroin and cocaine seeking (Stewart 1984; Vorel 2001). In addition, reversible inactivation of the VTA blocks cocaine priming-induced reinstatement of drug seeking (McFarland

& Kalivas 2001). Medial PFC glutamate and VTA DA projections to the NAc serve as the final common pathway for all events inducing relapse (Kalivas & McFarland 2003). In this context, the NAc core, but not the shell, is primarily responsible for modulating cocaine priming-induced reinstatement of drug seeking (Kalivas & McFarland 2003; McFarland & Kalivas 2001). Indeed, reversible inhibition of the core by $GABA_A$ and $GABA_B$ agonists attenuates cocaine-seeking behaviour induced by a cocaine prime (McFarland & Kalivas 2001). In addition, the ventral portion of the HCP, known as the ventral subiculum, is innervated by DA projections from the VTA and stimulation of the ventral subiculum activates DA cell bodies in the VTA and subsequently leads to increased DA transmission in the NAc (Legault et al. 2000). Interestingly, electrical stimulation of the ventral subiculum reinstates cocaine or amphetamine seeking in rats (Taepavarapruk & Phillips 2003; Vorel 2001). At present, little is known about the role of DA or glutamate in brain areas other than the VTA, NAc and medial PFC in drug-induced reinstatement. Inactivation by $GABA_A$ and $GABA_B$ agonists of the ventral pallidum attenuates cocaine-induced reinstatement, whereas this GABAergic blockade of the substantia nigra, central and basolateral nuclei of the amygdala and the mediodorsal thalamus has no effect (McFarland & Kalivas 2001; Schmidt et al. 2005).

Contexts or environmental stimuli that are repeatedly associated with the drug consumption are known to promote compulsive drug taking and craving and are a primary trigger of relapse (Carter & Tiffany 1999a; Shalev et al. 2002; See 2002). Discrete stimuli such

as odours and sounds, among others, can have similar effects. The mechanisms underlying this kind of relapse include activation of mGluR2, mGluR3, D1R, CB1R, and MOR, all of which contribute to relapse of heroin, cocaine, alcohol, and food seeking induced by

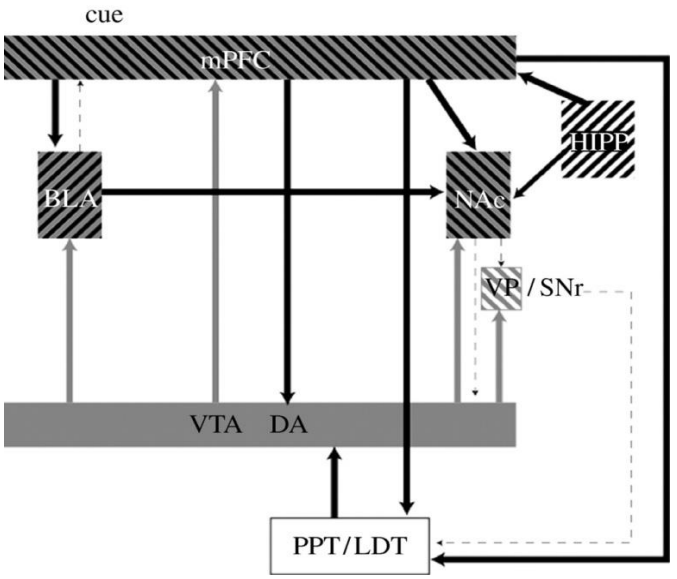


Figure 5. Diagram showing the primary circuits and neurotransmitters implicated in cue-induced reinstatement. VTA, ventral tegmental area, cell body regions of mesocorticolimbic DA pathway; NAc, nucleus accumbens; medial PFC, medial prefrontal cortex; VP/SNr, ventral pallidum/substantia nigra reticulata; PPT/LDT, peduncular pontine and laterodorsal tegmental nuclei; BLA, basolateral amygdala; HIPP, HCP. Grey, DA; black, glutamate, (Stewart 2008).

environmental contexts (Crombag et al. 2008; Nair et al. 2009; Ciccocioppo et al. 2001). Principal regions associated with relapse induced by environmental contexts are the basolateral amygdala, the HCP, the medial PFC and the NAc core. Indeed, the DA inputs

from the VTA to basolateral amygdala, medial PFC and NAc, and glutamatergic inputs from the basolateral amygdala and PFC to the NAc and the VTA respectively, are involved in this kind of relapse (Bossert 2004; Bossert et al. 2006; Fuchs et al. 2007). Cue-induced relapse primarily involves enhanced glutamate transmission from the dorsal medial PFC to the NAc (Kalivas et al. 2003). Indeed, previous animal studies have suggested an important role of the NAc core in this type of reinstatement (Di Ciano et al. 2008; Di Ciano & Everitt 2004). Furthermore, the dorsomedial PFC sends direct and indirect projections to the ventral striatopallidal pathways, that may allow planning and executing an appropriate motor response (Salinas & McGaugh 1996), and to VTA and the raphe nuclei (Uylings & van Eden 1990). In turn, DA projections from the VTA to the NAc, medial PFC, and basolateral amygdala acting on D1R would be able of stimulating glutamate projection neurons in the amygdala and medial PFC (See et al. 2001; Sun & Rebec 2005; Alleweireldt et al. 2006; See 2009), whereas DA acting on both D1R and D2R in the NAc shell (Anderson et al. 2003; Bachtell et al. 2005; Schmidt et al. 2006) could act in concert with AMPAR to reduce GABAergic transmission to the ventral pallidum (O'Connor 2001; Torregrossa et al. 2008). In addition, basolateral amygdala and dorsal HCP are key structures involved in the reconsolidation of drug context memories (Wells et al. 2011) and HCP and amygdala inputs to the NAc can modulate reward-related behaviours. Moreover, the projection from the basolateral amygdala to the dorsomedial PFC (Pitkanen 2000) may allow the amygdala to provide relevant information to the PFC about the

affective significance of drug-associated stimuli. The PFC integrates this information and guides purposeful behaviour relevant to the salience of drug-associated stimuli (Everitt & Robbins 2000). Previous research indicates that the dorsomedial PFC, basolateral amygdala and HCP exhibit neuronal activation concomitant with cocaine-seeking behaviour in a cocaine-paired environment (Neisewander et al. 2000), and lesions of these structures impair place conditioning, a task dependent on context-reward learning (Tzschentke & Schmidt 1999; Ferbinteanu & McDonald 2001; Meyers et al. 2003). However, inactivation of the HCP abolished contextual reinstatement, but failed to alter explicit cue-induced reinstatement of cocaine-seeking behaviour (Fuchs et al. 2005).

Stress leads to state-related changes in brain reward circuits resulting in a greater sensitivity to the reinforcing properties of drugs particularly in vulnerable individuals (Piazza & Le Moal 1998). Several stressors have been reported to reinstate drug seeking behaviour and different systems mediate this behaviour.
The reward system is the first system involved, principally through glutamatergic projections from the PFC to the VTA, the NAc, basolateral amygdala, the peduncular pontine and the laterodorsal tegmental nuclei, and DA projections from the VTA to several brain structures such as PFC, NAc and basolateral amygdala. Thus, stress induces activation of the DA projections from VTA to medial PFC (Thierry et al. 1976). This activation involves D1R which, in the medial PFC, is expressed on pyramidal cells promoting excitability primarily by increasing NMDAR-mediated effects (Lewis &

O'Donnell 2000; Seamans et al. 2001). Moreover, glutamatergic projections from dorsal medial PFC to NAc core (Sesack et al. 1989) and potentially GABAergic projections from NAc shell to VTA (the 'direct' striatal pathway) (Zahm et al, 2001) and to ventral pallidum (the 'indirect' pathway) have also a role in this reinstatement. Other different pathways are also involved in stress-induced reinstatement. Thus, several studies have reported the involvement of the adrenergic neurotransmission from neurons in the lateral tegmental nucleus to the bed nucleus of the stria terminalis and central amygdala nucleus, as well as the CRF projection from the central amygdala to the bed nucleus of the stria terminalis (Shaham et al. 2000). Moreover, CRF projections from the bed nucleus of the stria terminalis to VTA (Rodaros et al. 2007) are also involved in stress-induced reinstatement of drug seeking. The bed nucleus of the stria terminalis is also activated by adrenergic projections (Aston-Jones et al. 1999). The bed nucleus of the stria terminalis and central amygdala are both considered components of the extended amygdala, which is a continuum of interconnected nuclei that also includes the shell of the NAc and ventromedial aspect of the ventral pallidum, and has been postulated to function as an integrated structure in modulating emotional responses (Zahm & Heimer 1993; Koob et al. 1993). CRF system is known to be activated in response to stressors and to mediate a wide variety of physiological and behavioural responses to stress, including fear and anxiety (Schulkin et al. 2005; Davis 2006). Indeed, CRF can be released into the VTA in a stress condition (Wang et al. 2005), which points to an interaction

between the CRF-containing cell groups and the DA neurons in the VTA, providing a possible pathway for CRF stress activation to

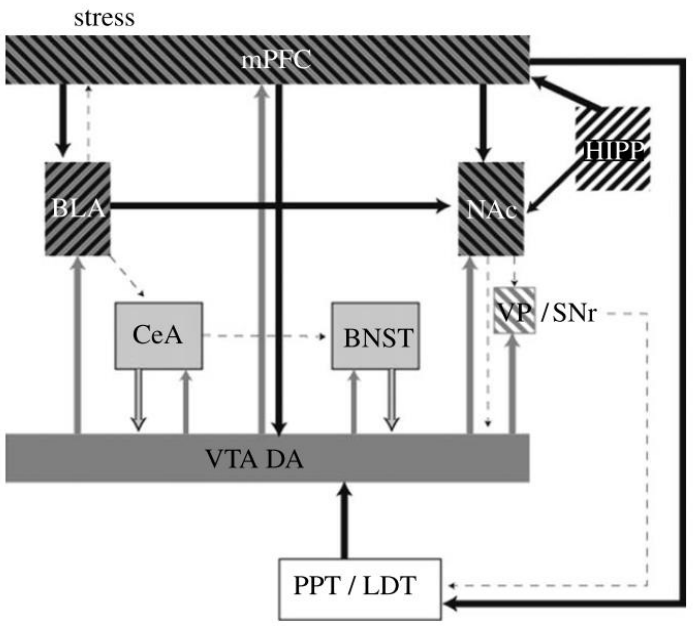


Figure 6. Diagram showing the primary circuits and neurotransmitters implicated in stress-induced reinstatement. VTA, ventral tegmental area, cell body regions of mesocorticolimbic DA pathway; NAc, nucleus accumbens; medial PFC, medial prefrontal cortex; VP/SNr, ventral pallidum/substantia nigra reticulata; PPT/LDT, peduncular pontine and laterodorsal tegmental nuclei; BLA, basolateral amygdala; HIPP, HCP; CeA, central nucleus of the amygdala; BNST, bed nucleus of the stria terminalis. Dark grey, DA; black, glutamate; light grey, CRF (Stewart 2008).

modulate seeking behaviour (Prasad et al. 1995; Piazza & Le Moal 1996). Furthermore, alpha 2-adrenergic agonists, such as clonidine, have been found to reduce stress-induced relapse to drug seeking (Erb et al. 2000; Shaham et al. 2000) suggesting that noradrenergic

activation also mediates stress-induced drug seeking and relapse (Shaham 1996; Erb et al. 1998). In addition, hypocretin/orexin signalling is also critically involved in relapse of drugs-seeking behaviour, such as nicotine. Hypocretin 1 reinstates nicotine-seeking through a mechanism independent of corticosterone releasing factor activation (Plaza-Zabala et al. 2013). Furthermore, hypothalamic arginine vasopressin and its V1b receptor, plays also an important role in response to stress. High arginine vasopressin mRNA levels have been found in medial/basolateral amygdala during stress-induced reinstatement of heroin seeking-behaviour. Thus, enhanced amygdalar arginine vasopressin expression may be related to individual vulnerability to reinstatement of drug-seeking in stress conditions (Zhou et al. 2015). Similarly, substance P may play a role in addictive behaviours, including stress-induced relapse, due to its interaction with specific areas of the reward system, such as the VTA, amygdala, bed nucleus of the stria terminalis and NAc (Commons, 2010). The cholinergic projections from the NAc to the basolateral amygdala contain the substancia P receptor, NK1 (Bell et al. 1998), suggesting that this receptor can contribute to the consolidation of emotionally-motivated learning underlying relapse (McGaugh 2004). In addition, substance P likely interacts with the CRH system in mediating stress-activated relapse, since substance P and NK1 receptors are co-localized with central CRH and CRH receptors (Commons, 2010). Moreover, KOR/dynorphin system participates in stress-induced reinstatement of cocaine seeking (Mantsch et al. 2015) due to its critical involvement in the modulation of the GABAergic transmission in VTA. Furthermore,

GABA receptors have been extensively investigated in diminishing stress responses. In this line, benzodiazepines attenuate the withdrawal symptoms of psychostimulants, opiates, and alcohol (Paine et al. 2002), which activate stress systems and are a primary initiator of relapse (Breese et al. 2005). Relieving these symptoms may decrease the probability of a relapse event (See & Waters, 2010). In addition, leptin has also a role in stress-induced reinstatement of heroin seeking, although this effect since limited to relapse promoted by food deprivation as a stressor event (Shalev et al. 2001).

1.6 Animal models to study drug addiction

Animal studies have been crucial in understanding the pathophysiology of drug addiction. Although animal models of addiction do not entirely emulate the human condition, important features of the drug addiction process can be reliably measured in animal studies with having variables controlled. The validity of animal models is typically assessed using three validation criteria: face, construct and predictive validity (Sanchis-Segura & Spanagel 2006). Face validity indicates that a model recapitulates important anatomical, biochemical, neuropathological or behavioural features of a human disease (Nestler & Hyman 2010). Construct or etiologic validity refers to the fact that biological mechanisms underlying the disorder are similar in both humans and animals. In the ideal situation, researchers would achieve construct validity by recreating in an animal the etiologic processes that cause a disease in humans

and thus replicate neural and behavioural features of the illness (Nestler & Hyman 2010). Predictive or pharmacological validity signifies that a model responds to treatments in a way that predicts the effects of those treatments in humans (Nestler & Hyman 2010).

Regarding the validity in the drug addiction field, traditional self-administration procedures have firmly established that drugs of abuse function as reinforcers in animals (Bozarth 1990). Studies on contingent and noncontingent drug intake in animals have already provided important advances and have contributed to understand the mechanisms involved in the initiation and maintenance of drug consumption. However, drug intake is just the first step of the complex addiction process and it is becoming evident that other factors are involved in the development of this disease. Indeed, addiction is influenced by genetic predisposition, environmental risk factors, and the interaction between both variables. A strong effort has been devoted to increase the knowledge about the molecular, cellular, and behavioural adaptations regulating these interactions to understand the mechanisms mediating addictive-like behaviour.

Different models have been used to characterize the neurobiological substrates underlying the rewarding effects of drugs of abuse, the aversive aspects of drug withdrawal as well as some long-lasting behavioural alterations associated to repeated drug exposure. Examples of models able to measure these specific features include the intracranial electric self-stimulation techniques, place conditioning methods and self-administration parading among others (Sanchis-Segura & Spanagel 2006).

1.6.1 Intracranial self-stimulation

The intracranial self-stimulation procedure provides unique ways to investigate the anatomical basis of reward and motivation and is an important tool for the assessment of the reward-facilitating and withdrawal effects of drugs of abuse (Vlachou et al. 2011). Several brain sites support intracranial self-stimulation, including the lateral

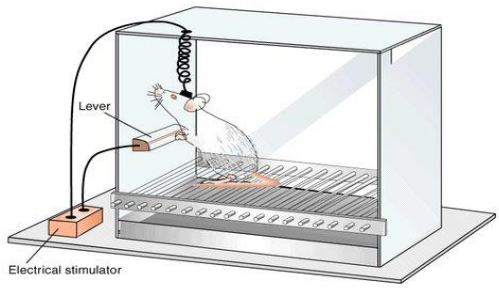


Figure 7. A simplified example of intracranial self-stimulation in rats. Animals are implanted with electrodes into a specific brain site of the reward circuit. The functioning of the brain reward circuit is often assessed by measuring the minimal electrical current intensity for which animals will perform an easy response, such as pressing a lever, to receive the stimulation. The minimal current intensity that the subject is willing to self-deliver is the reward threshold.

hypothalamus, medial forebrain bundle, and VTA among the sites that produce the most vigorous intracranial self-stimulation responding (Olds & Milner 1954). In this model, animals previously implanted with intracranial electrodes into specific regions of the brain reward pathways are trained to maintain operant behaviour to obtain an electric pulse through these electrodes. During these

sessions, the threshold of the minimal current needed to promote intracranial self-stimulation is estimated. Typically, rewarding stimuli, such as drugs of abuse decrease the self-stimulation threshold, whereas aversive drugs or stimuli, such as drug withdrawal, elevate the threshold for self-stimulation (Markou et al. 1993).

1.6.2. Place conditioning

Conditioned place preference is a procedure for assessing the rewarding efficacy of drugs using a classical Pavlovian conditioning paradigm. In this paradigm, the animal is exposed to a drug or non-drug treatment that has appetitive or aversive properties in a previously neutral environmental context. Following several pairings of the unconditioned stimulus with the distinct environmental cues (conditioned stimulus), only the presence of the context will evoke approach or avoidance behaviour (conditioned response) (Tzschentke 2007).

The simplest version of the place conditioning apparatus consists of two environments that can differ in characteristics, such as colour, texture, pattern, in which the animal explore freely both compartments. One of the environments is paired with the drug and the other with the vehicle administration. After conditioning sessions, animals, in a drug-free state, are allowed to explore freely both environments, and the time spent in the drug-paired environment is considered an index of the rewarding value of the drug. The preference for one environment over the other confers

information about the motivational state created by the drug. A drug with rewarding properties will typically induce place preference, whereas a drug with aversive effects or withdrawal from chronic

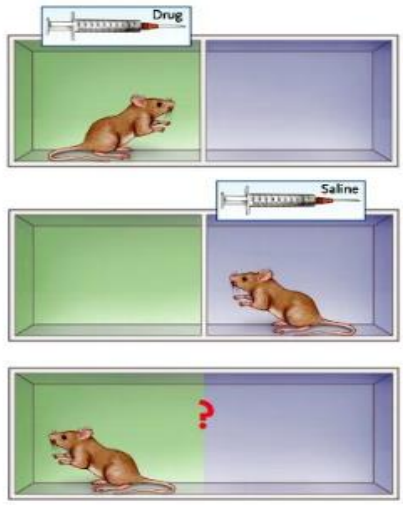


Figure 8 Place conditioning. Mice are placed in two discrete environments, and are then injected with a drug in one environment and with saline in the other environment. In a drug-free state, the animal is allowed access to both environments, and the amount of time spent in each environment is recorded. A positively reinforcing effect of the drug is apparent if the mouse spends more time in the environment in which the drug was administered (place preference) than in the one in which saline was administered (adapted from Camí & Farré, 2003).

drug administration will mainly produce place aversion. Although drug consumption in humans can induce conditioned approach/avoidance to specific drug-related stimuli, conditioned place preference and conditioned place aversion are not intended to model any particular feature of human behaviour (Sanchis-Segura & Spanagel 2006).

These paradigms mainly represent an indirect measure of the rewarding or aversive effects of a drug by measuring the response of the animal towards the conditioned stimulus and can be also used to model relapse by re-exposing the animal to this paradigm that mimics drug associated-cues (Aguilar et al. 2009).

1.6.3. Operant drug self-administration

The operant model of self-administration is considered to be the most reliable and predictive model of drug use in rodents in the addiction field. Indeed, it mimics human drug seeking/taking and the neurobiological substrates related to drug effects appear to be similar in humans and experimental animals (Sanchis-Segura & Spanagel 2006). This animal model is widely used in the preclinical research to directly evaluate the primary reinforcing effects of drugs of abuse as well as the relapse after periods of abstinence.

In drug self-administration procedures, the reinforcer can be delivered by different routes of administration. The most common routes of administration are intravenous and oral, but intracerebroventricular, intracranial, inhalation, intragastric and intramuscular routes have also been used. Studies commonly use the route of administration that is most similar to the route used in humans for that particular drug. In this procedure, animals learn to perform an instrumental response to obtain the reinforcer in experimental chambers provided with an active and an inactive lever/nose poke. Responding on the active lever/nose poke will activate a pump/dispenser delivering the reinforcer. Active

manipulandum pressing can be paired with unconditioned stimuli, such as a light or a tone, which improves learning of the operant behaviour. Activation of the inactive malipulandum will have no consequences, but will provide important control procedures for nonspecific motor and motivational actions, such as increases in exploratory activity and locomotion. The use of different schedules of reinforcement can provide information of the reinforcing properties of the drug. Reinforcement is known as behaviour which tends to be repeated and strengthened, thus it increases the probability of the behaviour being expressed. The simplest schedule

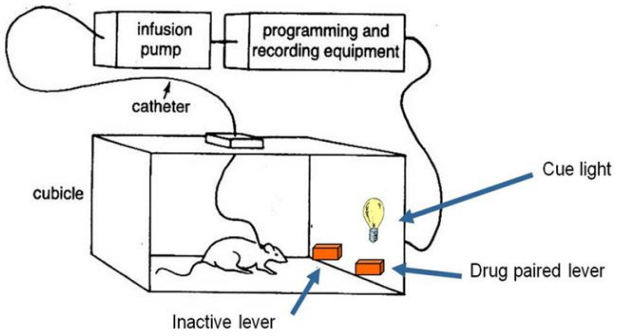


Figure 9. Intravenous self-administration procedure for drugs. Animal implanted with an intravenous catheter in the jugular vein and trained to self-administer drug. The chamber is provided with two levers/nose pokes. The number of responses in each lever/nose poke will be recorded by equipments. Responses on the active lever/nose poke will deliver a drug infusion and activate a light (cue), while responses of the inactive lever/nose poke will have no consequences.

of continuous reinforcement used is the fixed ratio schedule, where the drug is delivered each time a preselected number of responses is completed. After each reinforcer delivery, a time-out period

(usually 10 sec) occurs in which operant responses are not rewarded. This period aimed to avoid any potential drug overdoses and can also serve to evaluate impulsive-like behaviour (Diergaarde et al. 2009). Additional information about the motivational effects for a drug reward can be obtained by using a progressive-ratio schedule, in which the required ratio to deliver a drug increases following an arithmetic progression. The work requirement is raised until responding ceases. This maximum work level, the "breaking-point", refers to the highest response rate accomplished to obtain a single reinforcer. Thus, the "breaking-point" is considered to be a measure of the motivation for the drug and can be compared between drugs to assess relative reinforcing efficacy or strength.

After the acquisition and maintenance of drug self-administration behaviour in operant conditioning chambers, extinction procedures can provide measures of the persistence of drug-seeking behaviour in the absence of response-contingent drug availability. When the reinforcing element is no longer present, first there is an increase of the response (burst pattern) followed by a gradual reduction in operant responses results in eventual cessation or "extinction" of the operant behaviour (Yan & Nabeshima 2009). Extinction testing sessions are identical to training sessions except that no drug is delivered and the environmental cue is not presented after responding in active manipulandum. Resistance to extinction and high responding rate on active manipulandum are related to high persistence to seek the drug. Interestingly, the resistance to extinction in mice is similar to the case in human addicts (Childress et al. 1993; Gilpin et al. 1997; McKay et al. 2001), although the

possible mechanism underlying the resistance to extinction in mice remains unclear (Yan & Nabeshima 2009).

The reinstatement model has been widely used to study relapse in animals. Reinstatement refers to the reinitiating of drug seeking in animal after the extinction of the previous drug administration (Shalev et al. 2002). After the extinction of drug-reinforced behaviour, the ability of drug priming, drug-associated stimuli, and stress to trigger reinstatement of drug-seeking behaviour can be evaluated (Yan & Nabeshima 2009).

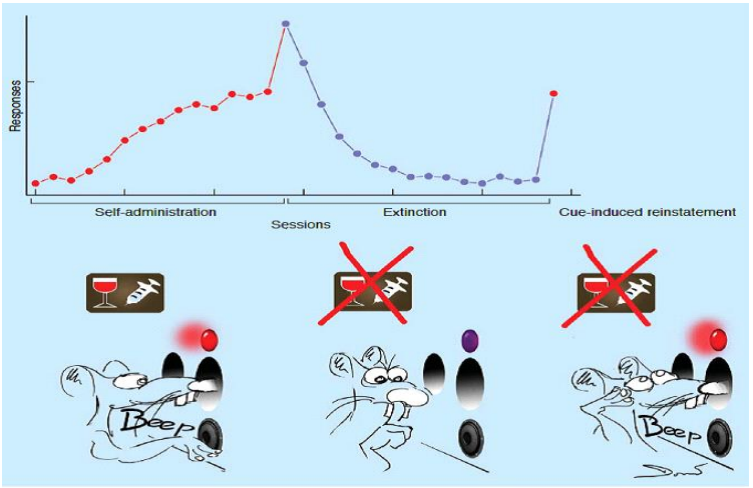


Figure 10. A typical cue-induced reinstatement experiment. The reinstatement model of relapse is divided in 3 phases: acquisition of self-administration behaviour, extinction and reinstatement. The cartoon above depicts a typical cue-induced reinstatement procedure (De Vries & Schoffelmeer 2005).

Drug priming effects on reinstatement have been reported in cocaine-, amphetamine-, heroin-, ethanol-, MDMA- and nicotine-trained animals (Chiamulera et al. 1996; Lê et al. 1998; Self &

Nestler 1998; McClung et al. 2010; Daza-Losada et al. 2009), among others.

Reinstatement induced by environmental stimuli (cue) associated to drug administration has also been described. There are different types of drug associated experimental cues: "discrete cues" paired with drug infusions during self-administration training (See 2002), as the cue-light stimuli; "discriminative cues" that become predictors of drug availability during the acquisition of the drug self-administration behaviour (Weiss 2005); and "contextual cues" associated with drug delivery as familiar environment in the Skinner box (Crombag & Shaham 2002). In drug-associated cues or conditioned reinforcers, responses on the active lever result in the presentation of a brief stimulus (light or tone) followed by drug delivery. This previously neutral stimulus can acquire conditioned reinforcing properties and evoke drug-seeking behaviour in experimental animals. Indeed, subsequent re-exposure after extinction to drug-associated stimuli produces strong recovery of responding at the active lever in the absence of any further drug availability (Yan & Nabeshima 2009). Cue-induced effect on reinstatement has been reported in cocaine-, amphetamine-, heroin-, ethanol-, nicotine-, MDMA-, cannabinoid- and food-trained animals (Ball & Slane 2014; Fattore et al. 2010; Martín-García et al. 2009; Soria et al. 2008; Shaham et al. 2003; Ball et al. 2007), among others.

Stress-induced reinstatement of drug seeking has been most often induced by intermittent footshock stimuli, pharmacological agents

that induce stress (e.g. yohimbine), and food deprivation (Shalev et al. 2010). Stressor, such as electric footshock, could generate an internal condition partly similar to the state induced by drugs of abuse, which would reinstate drug-seeking behaviour in absence of further drug availability (Ahmed & Koob 1997; Shaham et al. 2003; Yan & Nabeshima 2009). Stress-induced reinstatement has been reported in cocaine-, amphetamine-, morphine-, heroin-, ethanol-, food- and nicotine-trained animals (Ahmed & Koob 1997; Economidou et al. 2006; Erb et al. 1998; Ghitza et al. 2006; Plaza-Zabala et al. 2012; Shaham 1996; Cruz et al. 2010; Shaham et al. 2003), among others.

i. Drug addiction model

The voluntary intake of drugs of abuse is a behaviour largely conserved throughout phylogeny (Deroche-Gamonet et al. 2004). The possibility of studying these behaviours in other species than humans has helped to understand the neurobiological basis of drug taking (Nestler 1997). Several symptoms of the addictive-like behaviour have been shown to occur in experimental animals, such as escalation of drug use, cognitive deficits, resistance to extinction, increased motivation for drugs, preference for drugs over nondrug rewards, and resistance to punishment (Belin & Deroche-Gamonet 2012; Deroche-Gamonet et al. 2004). Although first attempts to develop models of sustained and escalated drug use occurred in the mid-seventies (Johanson et al. 1976; Bozarth & Wise 1985), models with several facets of the addiction disorder have just recently

appeared (Deroche-Gamonet et al. 2004; Belin & Deroche-Gamonet 2012; Vanderschuren & Everitt 2004). These models considered interindividual differences in drug responses and the concept of vulnerability was introduced for the first time in the preclinical research. Researchers attempted to capture facets of the addiction-like behaviour by the operational translation of the seven diagnostic criteria of the DSM-IV. These seven items were grouped into three behavioural pillars considered the hallmarks of substance dependence, able to represent completely the loss of control over drug intake (Deroche-Gamonet et al. 2004). Indeed, the items 3 and 4 of DSM-IV (see Table 1) indicate a difficulty to limit drug use, the items 5 and 6 indicate a high motivation for the drug and the item 7 refers to the drug use maintained despite its negative consequences. Thus, these points can be reproducible in animal models and correspond respectively to: 1) *Persistence in drug-seeking*, measured by the persistence of drug seeking during a period of signalled drug nonavailability. 2) *Motivation for the drug*, measured by the breakpoint in the progressive ratio schedule of reinforcement 3) *Resistance to punishment*, measured by the persistence of the animals' responding when the drug delivery was associated with a punishment, an electric footshock (Deroche-Gamonet et al. 2004). In humans, the diagnosis of addiction is performed by counting the number of diagnostic criteria met by a subject. A similar approach was used in rats after long-term drug exposure, by scoring them for each of the three addiction-like behaviours. Different and extreme subpopulations according to the number of positive criteria met were found and the group which

reached all three criteria was defined as "addicted". A prominent percentage of rats, around the 20%, showed loss of control over drug-intake (Piazza & Deroche-Gamonet 2013). The addict rats did not differ significantly from the no addict rats in terms of initial rates of cocaine self-administrated (Belin et al. 2008; Deroche-Gamonet et al. 2004). However, they showed escalation of cocaine intake when given long access to the drug and a high vulnerability to relapse (Belin et al. 2009) that can be greatly reduced by a pre-treatment with a mGluR2/3 agonist (Cannella et al. 2013), confirming the contribution of glutamatergic mechanisms to drug addiction. At the neurobiological level, addicted rats are characterized by an impairment in synaptic plasticity in the ventral ST (Kasanetz et al. 2010) and in the medial PFC (Kasanetz et al. 2013), suggesting that addiction, at least to cocaine, is associated with impaired fronto-striatal connectivity in rats. These findings are also in agreement with a demonstration that altered synaptic plasticity in the prelimbic cortex supports compulsive drug-seeking behaviour in rats (Chen et al. 2013). The relationships between different dimensions of the seeking behaviour traits and stages of addiction, from vulnerability to drug self-administration initiation to compulsive intake were also reported. It was argued that seeking behaviour in rats predicts vulnerability to use cocaine, and high impulsive seeking behaviours predict vulnerability to shift from controlled to compulsive cocaine use, that is, addiction (Belin & Deroche-Gamonet 2012).

This model of addiction may help to identify the neuropharmacological and molecular mechanisms underlying

individual addiction vulnerability and provide an important advancement to better approximate the physiological and behavioural aspects of drug addiction in humans.

1.7 Neuronal plasticity

Brain is a sophisticated information processing and storage system. Neurons accomplish these processes by integrating internal and external inputs and by modifying their morphology and/or functioning in response to these stimuli. Storage of information by changes in brain structures and functions is known as plasticity. More specifically, plasticity can be defined as the ability of neural circuitries to undergo adaptations consequent to experience which, in turn, influence the behaviour. Plasticity mechanisms include the: (a) modification of the strength or the efficacy of synaptic transmission (synaptic plasticity) and (b) modification in dendritic complexity associated to the growth of new synaptic connections or the pruning away of existing ones (structural plasticity) (Malenka 2003).

1.7.1 Synaptic plasticity

Synaptic plasticity is the cellular phenomenon by which synapses can undergo permanent changes in their properties consequent to specific patterns of activity. Synaptic plasticity can be divided in:

1) Short-term plasticity that allows a rapid and reversible modulation of synaptic transmission strength (Deng & Klyachko 2011);

2) Long-term plasticity, involving changes that last for hours to underpin learning and memory (Martin et al. 2000);

3) Homeostatic plasticity of synapses and neurons to maintain appropriate levels of excitability despite continuous occurrence of short- and long-term plasticity (Pérez-Otaño & Ehlers 2005).

Synaptic plasticity may encompass a long-lasting strengthen or weaken in the efficacy of synaptic transmission, referred as long-term potentiation (LTP) and long-term depression (LTD) respectively. LTP and LTD have been found to occur in neurons that release various neurotransmitters, although the most common neurotransmitter involved is the glutamate, the principal excitatory neurotransmitter in the brain.

LTP that persistently enhances the synaptic strength is often measured in terms of the magnitude of excitatory post synaptic potential (EPSP) enhancement at a given time-point after induction. LTP is widely studied in the CA1 region of the HCP (Bliss & Collingridge 1993; Reymann & Frey 2007) involved in memory storage. Long-term plasticity at glutamatergic synapses is mediated by NMDA-receptor. Glutamate ionotropic receptors (NMDAR, AMPAR and kainate) present on the postsynaptic membrane are the initial triggers for the ensuing postsynaptic calcium (Ca^{2+}) signalling mechanism responsible for the induction of LTP. Receptors allow the transduction of electrical events at the postsynaptic membrane into chemical signals which, in turn, activate both pre and postsynaptic mechanisms to generate a persistent increase in synaptic strength. Glutamate binding to the AMPAR leads to a sodium (Na^{+}) influx into the postsynaptic

compartment leading to depolarization, removing Mg^{2+} that plays a role as blocker of the NMDAR. The removal of Mg^{2+} blockade causes NMDAR opening, which promotes and Ca^{2+} and Na^{+} conduct into the cell. Ca^{2+} influx activates several important signalling pathways involving different protein, kinases and phosphatases (Figure 11). One of the kinases activated is $Ca^{2+/}$calmodulin dependent protein kinase II, which is known as the memory molecule. The Ca^{2+} influx through NMDAR also activates the adenyl cyclase, which generates cAMP in the postsynaptic compartment. This second messenger triggers a series of downstream signalling mechanisms, which function in LTP maintenance by the activation of the protein kinase A (PKA) that regulates gene expression. PKA can modify transcription by phosphorylating several transcription factors, one of which is the cAMP response element binding protein. The final effect of this process is the rapid insertion into the postsynaptic membrane of AMPARs that is the major mechanism underlying LTP expression. In addition, a mechanism that requires a retrograde signal (perhaps NO) spreading from the postsynaptic region to the
presynaptic terminal is considered to be partly involved in the modulation of the neurotransmitter release (Purves et al. 2001). The activation of mechanisms that require the synthesis and expression of new proteins and/or genes, leads to the stabilization of these synaptic changes for hours, days or weeks (Malenka & Bear 2004). A measure of postsynaptic changes in synaptic strength is evaluated

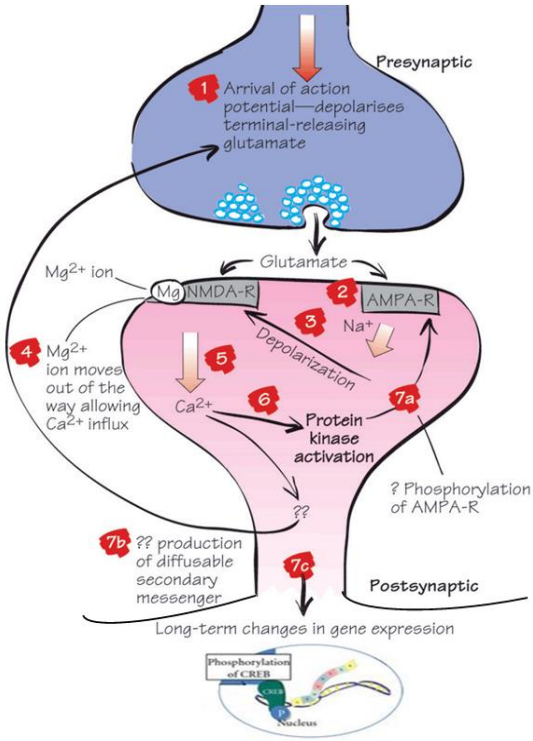


Figure 11. Schematic diagram of the induction and expression of NMDAR-dependent LTP observed in the rodent brain: 1) glutamate is released from activated pre-synaptic neurons, 2) glutamate binds with both AMPAR and NMDAR, 3) binding opens AMPAR channels leading to the entry of Na^+ that depolarizes post synaptic neurons, 4) binding opens gate of NMDAR channels but Mg^{2+} still blocks channels. Sufficient depolarization from this AMPAR opening plus other EPSPs drives Mg^{2+} out, 5) Ca^{2+}entry through open NMDAR channel, 6) Ca^{2+}entry activates Ca^{2+} second-messenger pathway, 7a) second-messenger pathway promotes phosphorilation and insertation of additional AMPAR into post-synaptic membrane, increasing its sensitivity to glutamate, 7b) second-messenger pathway also triggers release of retrograde paracrine (likely nitric oxide) that stimulates long-lasting increase in glutamate release by presynaptic neuron, 7c) second-messenger modifies transcription by phosphorilation of several transcription factors, such as cAMP response element binding protein.

by the ratio between AMPARs and NMDARs in electrophysiological studies. The ratio is defined as the peak

synaptic AMPAR current relative to the peak synaptic NMDAR and indicates that the modification in synaptic transmission is caused by enhanced or decreased AMPAR transmission respect to the NMDAR transmission.

Another subclass of glutamate receptors, mGluRs, is also involved in LTP induction (Bashir et al. 1993). Specifically, activation of mGluRs1 or mGluRs5 produces LTP-like effects by increasing $Ca^{2+/}$calmodulin dependent protein kinase II levels and phosphorylation of AMPAR receptor GluR1 subunits, which in turn promotes an enhancement of AMPAR number and channel conductance (Delgado & O'dell 2005; Jia et al.1998).

The inverse mechanism of LTP is the LTD. LTD can be defined as a long lasting decrease in the synaptic response of neurons following a long patterned stimulus (Collingridge et al. 2010). LTD occurs principally when postsynaptic cells are depolarized to reverse LTP at saturated synapses and to reduce circuit excitability, but LTD could also occur at synapses that are not potentiated. Although a major form of LTD is mediated by NMDARs, the ultimate direction of change in synaptic efficacy is brought by changes in AMPAR function (Collingridge et al. 2010). Ca^{2+} influx through the NMDAR is crucial for the induction of both LTP and LTD. LTD is triggered by postsynaptic Ca^{2+} entry after activation by presynaptic stimulus. If the postsynaptic depolarization is weak, it cannot activate NMDARs completely. The partial removal of Mg^{2+} block results in the reduction of Ca^{2+} entry. This mechanism activates particular phosphatases, such as calcineurin and protein

phosphatase (PP1) with the final effect of AMPARs dephosphorylation and their removal from the post-synaptic membrane, thus resulting in weakens synaptic transmission and LTD induction. Since the induction of LTD is controlled by the postsynaptic glutamate receptors, presynaptic components are required as retrograde messengers to modulate the transmission, such as endocannabinoids (Bliss & Cooke 2011). Upon stimulation, endocannabinoids are released from postsynaptic neurons and travel across the synaptic cleft to activate CB1R on presynaptic terminals, resulting in depression of synaptic transmission. Another form of LTD depends on mGluR1. Glutamate binding to mGluR initiates a signalling cascade, which involves the breakdown of the membrane lipid phosphoinositol 4, 5–bisphosphate by phospholipase C to the important signalling molecules inositol trisphosphate and diacylglicerol able to induce calcium mobilization. This leads to the activation of the calcium sensitive kinase protein kinase c that, in turn, dephosphorylates AMPAR and causes receptor internalization resulting in weaken synaptic transmission. Activation of mGluR1 could be involved in opposed downstream signalling pathways that may both enhance or depress the synaptic transmission. However, synaptic depression mediated by mGluR1 has been the most commonly observed (Delgado & O'dell 2005). Indeed, mGluR-mediated EPSPs/EPSCs are only evoked following high stimulus intensity or brief high frequency stimulation (Anwyl 1999). mGluR1 activation can lead to either LTP or LTD, depending on the frequency and intensity of stimulation (Bortolotto et al. 1994).

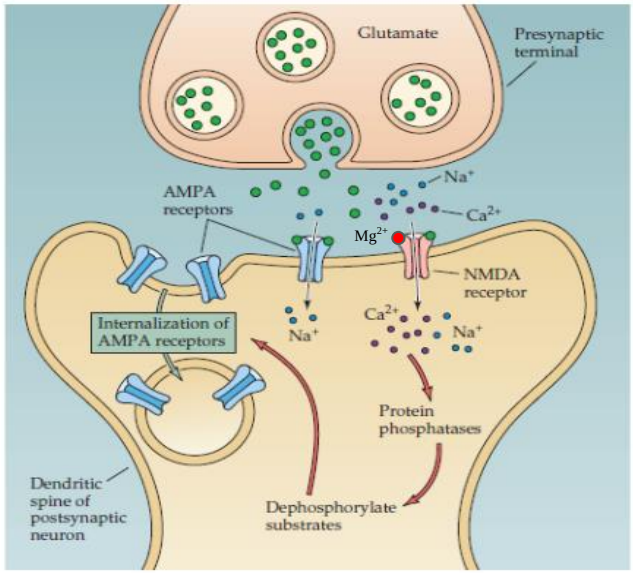


Figure 12. Diagrams of the induction and expression of LTD in the rodent HCP region. NMDAR-dependent LTD is triggered by Ca^{2+} entry through postsynaptic NMDAR channels, leading to the increase in the activity of the protein phosphatases. The primary expression mechanism involves internalization of postsynaptic AMPARs and a down regulation of NMDARs by an unknown mechanism (adapted from Isaacson, 2013).

1.7.2 Structural plasticity

Persistent changes in behaviour and psychological functions are due to the reorganization of synaptic connections (structural plasticity) that remove or create new connectivity patterns between neurons in relevant brain circuits. Functional plasticity and structural plasticity are strikingly associated. Indeed, LTD promotes spines shrinkage or synapse elimination (Bastrikova et al. 2008). In contrast, induction of LTP has been associated with an increase in spine turnover, characterized by enhancement in spine growth or even in newly

formation or also in spine elimination (Engert & Bonhoeffer 1999; Nägerl et al. 2004; De Roo et al. 2008). At the molecular level, LTP or LTD trigger changes in signalling pathways that lead to the re-organization of cytoskeletal proteins, such as actin and transynaptic adhesion molecules (Kasai et al. 2003). Rho GTPases and their downstream effectors have an important role in regulating the cytoskeleton, and consequently in regulating spines and dendritics morphology in response to extracellular stimulation. The morphological adaptation induced by LTP or LTD includes changes in the size of body cells, dendritic three arborizations or spines number and shape, affecting different partners and taking place on different time scales (minutes to days).

High morphological variability of dendritic spines reflects the different stage of maturation of excitatory synapses (Bourne & Harris 2008). Small spines are recognized as immature spines with low stability and easily turned into other spines or eliminated (Kasai et al. 2002). Conversely, large spines, mainly referred to mushroom type of spines, are associated with mature, stable spines that have been strengthened through a process of activity- or plasticity-mediated enlargement. Small spines are less likely than larger ones to contain smooth endoplasmatic reticulum, spine apparatus and have few or no polyribosomes. In comparison, most of the larger spines contain smooth endoplasmatic reticulum, spine apparatus, polyribosomes and have larger post-synaptic density than small ones (Nimchinsky et al. 2002). As the size of post-synaptic density correlates with the number of post-synaptic receptors, large spines

have shown to contain more AMPARs and to be more sensitive to glutamate stimulation than small spines (Takumi et al. 1999).

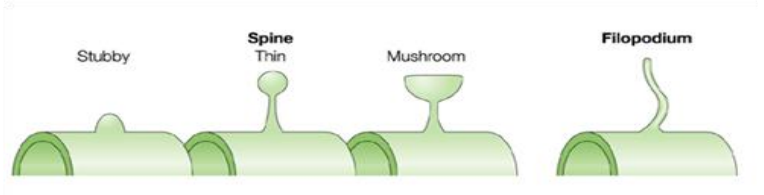


Figure 13. Morphological classification of dendritic spines. They are commonly classified into four different categories: stubby, thin, mushroom or filopodia. Stubby spines are devoid of a neck and are particularly prominent during postnatal development. Thin spines are most common and have a thin, long neck and a small bulbous head, whereas mushroom spines are those with a large head. Lastly, dendritic filopodia are typically longer, normally have no clear head, and often represent immature spines (Yuste & Bonhoeffer 2004).

Other types of spines have been reported. Thin spines are elongated and stable structure with a small bulbous head and are relatively young, with newly formed synaptic structure (Bourne & Harris 2007a). Another type of protrusion is named filopodia, usually characterized by the absence of enlargement at the tip. Filopodia protrusions are believed to represent precursors of dendritic spines (Petrak et al. 2005; Toni et al. 2007) and they have been mainly seen during early stages of neurodevelopment although their function remains largely unclear as the majority of filopodia just appear and disappear without transforming into spine synapses (Zuo et al. 2005; De Roo et al. 2008). They seem to be implicated in

synaptogenesis mechanisms due to their elongated morphology that can facilitate axodentritic synaptic contacts (García-López et al. 2010).

1.7.3 Drug-induced neuronal plasticity

Despite their chemical diversity and individual molecular targets, the major substrates of drugs of abuse are hypothesized to be molecular and cellular mechanisms that underlie long-term associative memories in several forebrain circuits mainly involving the ventral and dorsal ST and PFC, which receive inputs from midbrain DA neurons (Hyman et al. 2006). The basolateral amygdala and NAc core are also key structures within limbic cortical-striatal circuitry where reconsolidation of cue-drug memories occur (Theberge et al. 2010). Thus, addictive drugs increase plasticity mechanisms involved in learning and memory processes in these circuits responsible for reward. A single exposure to several drugs of abuse triggers LTP of AMPAR-mediated currents at excitatory synapses into VTA DA neurons (Niehaus et al. 2010). Accordingly, AMPAR/NMDAR ratio is significantly increased in the VTA in response to single exposure of different drugs of abuse, which seems to reflect an initial form of adaptive synaptic plasticity for all addictive drugs (Mameli et al. 2011). Hence, drug-evoked changes in the VTA may constitute an initial step in many of the behavioural changes that define addiction. These initial changes in the VTA are confirmed by the fact that pharmacological inhibition of NMDAR in the VTA blocks drug-

evoked synaptic plasticity, behavioural sensitization (Dunn et al. 2004), and acquisition of morphine-conditioned place preference (Harris et al. 2004). Moreover, prolonged cocaine self-administration reduces excitatory synaptic responses in the NAc shell (Schramm-Sapyta et al. 2006) associated with an inability to elicit LTD in both the core and the shell of the NAc (Martin et al. 2006). In this context, only a modest proportion of rats develop behaviours analogous to human addicts following prolonged cocaine self-administration while all animals develop impaired LTD in the NAc (Kasanetz et al. 2010). Interestingly, LTD was impaired in all animals two weeks following the cessation of cocaine self-administration, although the ability to generate LTD slowly recovered only in "non-addicted" animals. In contrast, "addicted" animals expressed persistently impaired LTD (Kasanetz et al. 2010). Therefore, long-lasting impairment of LTD induced by chronic cocaine intake results in a persistent deficit in synaptic plasticity that may contribute to the transition to addiction.

Drug-induced synaptic adaptation in the NAc occurs also during cue-induced reinstatement of cocaine seeking after protracted withdrawal (Grimm et al. 2001). Increased AMPAR surface expression and AMPAR/NMDAR ratio associated to a consistent increase in excitatory synaptic strength in NAc are reported during prolonged withdrawal periods (Russo et al. 2010; Lüscher & Malenka 2011). However, internalization of AMPAR cell surface in the NAc (Boudreau et al. 2007) and decrease in the AMPAR/NMDAR ratio associated to a reduction of excitatory

synaptic strength in the NAc shell have been shown after priming- and cue-induced reinstatement (Rothwell et al. 2011).

Synaptic adaptations are not the only mechanisms by which drugs of abuse can modify the reward DA system circuitry to cause long-lasting behavioural changes. Indeed, addictive drugs activate complex intracellular signalling cascades, including transcription factors, that change intrinsic membrane excitability and modify dendrite and spine structures (Kalivas 2009). Indeed, neuroplastic changes after repeated drug use lead to a reorganization of mesocorticolimbic synaptic connectivity due to adaptations that counteract chronic brain insults, but their undesirable counterpart is the subsequent increase of drug consumption (Hyman et al. 2006). Many studies have shown that chronic administration of most drugs of abuse (mainly amphetamine, cocaine, nicotine and morphine) induces structural plasticity changes in reward areas (Russo et al. 2010). Thus, repeated exposure to psycostimulants and nicotine has been associated with changes in postsynaptic AMPAR and the consequent increase of total denditric spine density in the NAc, VTA and medial PFC (Robinson & Kolb 2004). Conversely, chronic exposure to opiates decreases the number of denditric spines in the same areas (Robinson et al. 2002) and decreases the soma size of the VTA DA neurons (Mazei-Robison & Nestler 2012). Indeed, chronic morphine induces changes in phosphatidylinositol 3′-kinase signalling that contribute to the morphological modifications of the soma size. This global change in the VTA DA architecture could participate in the decreased DA release in the NAc (Mazei-Robison & Nestler 2012). These

morphological changes vary in parallel with drug-induced behavioural changes and their extent seems to be correlated to the magnitude of the "addictive state". In fact, rats with a longer cocaine exposure dramatically escalate their drug intake, present high motivation and show an increase in spines density in the NAc core (Ferrario et al. 2005). For the majority of the drugs, these structural changes have been maintained long after the discontinuation of chronic drug administration (Kolb et al. 2003; González-Forero et al. 2004).

In summary, changes in strength of synaptic connections and in structural spine morphology have been demonstrated in neural reward circuits after repeated administration of drugs of abuse and might contribute to maladaptive learning of addictive behaviours. A clear understanding of the molecular substrates that mediate these adaptations could help in ravelling new targets for the development of efficient therapies for drug addiction.

2. EATING ADDICTION: LESSON LEARNED FROM DRUG ADDICTION

2.1 Common insights with drug addiction

Empirical and experimental evidence indicates that certain individuals can develop maladaptive patterns of consuming behaviours that are essential for survival, including food intake (Gold et al. 2009). Indeed, the decision to eat is not only influenced by the internal state of the caloric equation of calories intaken and the calories expended, but also by non homeostatic factors, such as food palatability. Palatable food is very pleasurable and is readily overconsumed despite the resulting health consequences. This behaviour is due to the intrinsic rewarding effects of foods. Indeed, a food rewarding experience contributes to motivation to repeat the experience and, under certain circumstances or in vulnerable individuals, results in the development of loss of control over food intake. This maladaptive pattern of food overconsumption resembles in certain aspects that undertaken by individuals using drugs of abuse. Indeed, several studies suggest commonalities between overeating and drug addiction, such as reinforcement effects of rewards, motivation and external cues to eat or use drugs, among others. Similar to drugs, the reinforcing effects of food are mediated by its ability to increase DA levels in the limbic system. Indeed, palatable food activates the brain reward circuitry through fast sensory inputs and through slow post-ingestive consequences,

such as raising glucose concentration in blood and brain, whereas drugs activate these same pathways directly or indirectly (Koob & Volkow 2010) stimulating DA release in the NAc (Di Chiara & Imperato 1988; Kilts et al. 2001). However, the magnitude of the DA response to food is much smaller than the DA response to drugs (Pandit et al. 2011). The increase of DA release due to the repeated stimulation of reward pathways triggers neurobiological adaptations that may make the behaviour compulsive leading to further loss of control over intake (Pelchat 2009). Interestingly, decreased D2R levels in the ST have been reported in obese individuals (Wang et al. 2001). These findings suggest that low DA activity could be the mechanism of vulnerability to obesity as individuals with fewer D2Rs have to eat more in order to experience the rewarding properties of food intake. The improper striatal regulation by D2R signalling is associated with decreased activity in prefrontal regions involved in salience attribution (orbitofrontal cortex), inhibition (anterior cingulate cortex), and decision making (dorsolateral PFC) (Volkow et al. 2007) that could underlie the enhanced incentive motivational value of drugs or food and the difficulty in resisting their consumptions (Volkow et al. 2008). In addition, impairments in orbitofrontal cortex and anterior cingulate cortex are associated with impulsive and compulsive behaviours. Thus, impaired modulation of DA in these regions is likely to contribute to the shift from impulsive to compulsive patterns of drug or food intake (Goldstein & Volkow 2002).

Molecular and functional interactions between the homeostatic and reward pathways in food intake regulation have been reported. Specifically, several hormones and neuropeptides involved in energy homeostasis influence the DA reward pathway (Volkow et al. 2013a), such as glucagon-like peptide-1 (GLP1) (Alhadeff et al. 2012), ghrelin (Abizaid et al. 2006), leptin (Figlewicz et al. 2003), insulin (Figlewicz et al. 2008), orexin (Fadel & Deutch 2002) and melanocortin receptors (Davis et al. 2011). Consistent with preclinical studies, imaging studies have also shown that anorexigenic peptides (e.g., insulin, leptin, peptide YY, PYY) decrease the sensitivity of the brain reward system to food reward, whereas orexigenic peptides (e.g., ghrelin,) increase this sensitivity (Volkow et al. 2011).

2.2 Food intake control

Availability of palatable food is a crucial environmental factor promoting overeating. In this context, the term of "non-homeostatic feeding" refers to eat for pleasure, as opposed to "homeostatic feeding", where food intake is restricted to satisfy biological needs (Pandit et al. 2011). However, homeostatic and hedonic neural circuits are closely interlinked, and both respond to metabolic signalling. The control of food intake and energy metabolism is therefore a complex process that depends on the ability of the brain to receive and integrate a wide range of external and internal signals in order to produce appropriate responses in terms of food intake, energy expenditure and metabolic activity (Williams et al, 2001).

2.2.1 Homeostatic regulation of food intake

The homeostatic control of appetite is mediated by the biological need to maintain body's energy stores, which is achieved by increasing the motivation to eat following depletion of stores. It requires reciprocal communication between peripheral organs that provide information about the nutrient status and energy stores of the body, and the brain that integrates all this information and advises about the availability of food in the external environment (Berthoud 2006; 2007).

i. Central regulation

Central regulation of food intake is an organized mechanism involving humoral signals and afferent neuronal pathways mainly acting in hypothalamic neuronal circuits, and descending commands using vagal and spinal neurons (Palkovits 2003). Hypothalamic nuclei regulate an enormous number of homeostatic functions and, among them, of particular importance are the control of hunger and satiety closely related to energy balance control. The hypothalamus is organized in anatomically discrete neuronal nuclei that form interconnected circuits via axonal projections. The arcuate nucleus (ARC), paraventricular nucleus (PVH), dorsomedial hypothalamic nucleus (DMH), lateral hypothalamus nucleus (LHA) and ventromedial hypothalamus nucleus (VMH) are among the most relevant hypothalamic sites modulating the homeostatic processes (Williams & Elmquist 2012). These nuclei integrate hormonal

(insulin, leptin, ghrelin and others), nutrients signalling and neuronal inputs from different peripheral locations (mainly stomach, liver, pancreas, muscle and white and brown adipose tissue) (Figure 14). The LHA functions as a hunger center while the VMH functions as a satiety center. Indeed, the LHA contains subpopulation of orexin and melanin-concentrating hormone (MCH) neurons promoting feeding behaviour (Li et al. 2014), while the VMH contains a high density of oxytocin receptors that negatively regulate energy balance acting to reduce feeding and increase energy expenditure (Noble et al. 2014). The ARC contains functionally discrete populations of neurons, such as the orexigenic (neuropeptides, NPY and agouti-related peptide, AgRP) and anorexigenic neuropeptides (proopiomelanocortin, POMC; the precursor of melanocyte peptides stimulating hormone, α-, β-, γ-MSH, adrenocorticotropic hormone, and cocaine- and amphetamine- regulated transcript, CART) (Elias et al. 1998), that indicate the nutritional status (Harrold et al. 2012). The PVH integrates signals from many neuronal pathways that regulate energy intake. These pathways include NPY/AgRP and POMC/CART neurons of the ARC and orexin neurons of the LHA area (Schwartz 2000). The DMH receives circadian information from the suprachiasmatic nucleus and senses leptin and other feeding signalling (Bellinger & Bernardis 2002). Hypothalamus activates these orexigenic/anabolic or anorexigenic/catabolic processes in response to energy homeostasis (Schwartz et al. 2000).

- Orexigenic/anabolic pathway: it is activated in response to a low energetic state and produces the release of orexigenic peripheral signals, such as ghrelin, stimulates NPY and AgRP expression within the ARC, and the subsequent release of MCH and hypocretins by the lateral hypothalamus. These neuronal responses lead to the sensation of hunger and the motivation to seek food that initiates a feeding episode.
- Anorexigenic/catabolic pathway: the release of anorectic peripheral signals, such as insulin and leptin, stimulates the activity of POMC and CART expressing neurons within the ARC. This activation promotes the release of anorexigenic neurotransmitters, such as corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone and oxytocin, leading to increase metabolic rate and promote satiety, which finish the episode of eating.

Moreover, the dorsal vagal complex that consists of the dorsal motor nucleus, the area postrema, and the sensory nucleus of the tractus solitarius have been classically associated with feeding behaviours owing to the vago-vagal reflex, linking the central nervous system with the peripheral tissues (Schwartz et al. 2000). Subnuclei of the nucleus tractus solitarius are the first central neurons to process ingestion-related vagal afferent signals, overall from the gastrointestinal tract. Indeed, the ingestion of food gives rise to mechanical and chemical stimulation of the gastrointestinal tract and to the secretion of a variety of hormones, such as leptin,

cholecystokinin (CCK) and GLP-1, among others, that mediated the control of energy status and meal consumption (Berthoud 2008; Vrang et al. 2007). These events activate vagal afferent neurons, which projections stimulate the nucleus tractus solitarius and central–visceral afferent pathways, including neurons located in the medulla, pons, hypothalamus and ventral forebrain. CCK has also a

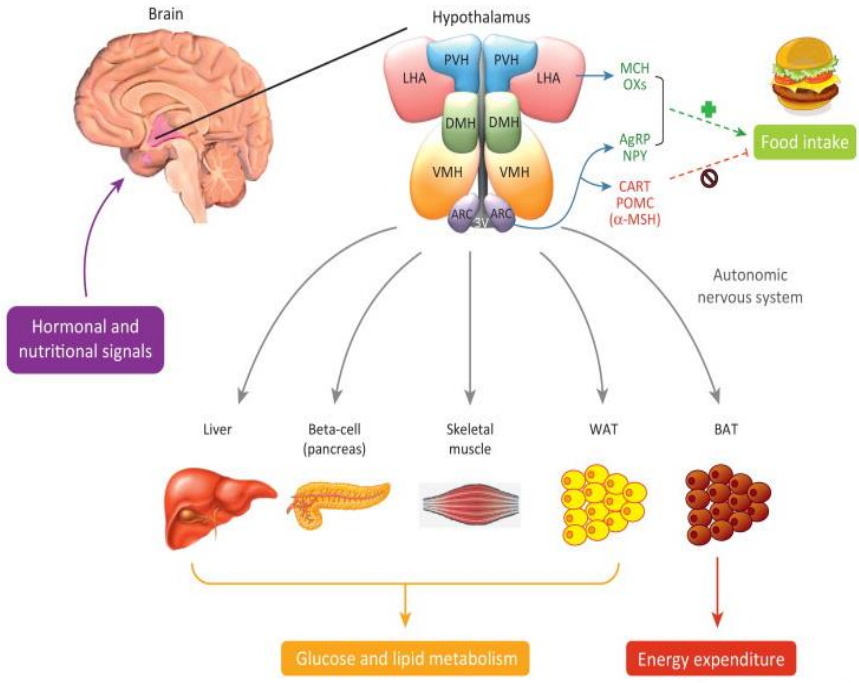


Figure 14. Hypothalamic regulation of body energy balance and metabolism. Specific nuclei in the hypothalamus respond to alterations in food availability, energy stores and nutritional requirements and communicate hormonally and via the autonomous nervous system to elicit functional changes in a range of organs/tissues including the liver, pancreatic cell, muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) (adapted from López et al. 2013).

direct effect on the brainstem, acting locally via the activation of CCK receptor 1 on nucleus tractus solitarius neurons, with the final effect of suppressing food intake (Preedy et al. 2011; Hisadome et al. 2011). Central processing of these signals is widely regarded as the primary determinant of the reduced food intake that follows meal consumption (Grill & Hayes 2009).

ii. Peripheral regulation

Gut and fat-derived hormones are released in response to homeostatic and hedonic signals facilitating the regulation of feeding by providing feedback information about energy consumption. These hormones include leptin, insulin, glucagon, adiponectin, ghrelin, pancreatic polypeptide (PP), CCK, PYY and GLP-1, among others (Banks 2006; Yu et al. 2006).

Leptin, mainly derived from adipocytes, circulates in concentrations proportional to body fat content (Maffei et al. 1995). Leptin concentration is decreased by food restriction and restored by refeeding. Centrally and/or peripherally administration of leptin decreases food intake, increases energy expenditure and causes weight loss, whereas deficiencies in leptin are associated with obesity (Friedman & Halaas 1998). Leptin directly affects melanocortin neurons in the ARC of the hypothalamus and the anorectic effects of leptin are generally attributed to signal transduction in the hypothalamus (Zhang et al. 2005). Recent studies demonstrate that leptin can also influence feeding behaviour

via activation of its receptor on VTA DA neurons (Billes et al. 2012).

Insulin is produced by β cells in the islets of Langerhans in the pancreas and also circulates in the bloodstream in proportion to body fat content (Bagdade et al. 1967). Insulin acts directly in the liver to regulate the synthesis of glucose in post prandial condition and stimulates the increase of glucose uptake in most peripheral tissues. At the central level insulin acts on K_{ATP} channels in hypothalamic neurons to control hepatic glucose production. The activation of these channels normally restrains hepatic gluconeogenesis (Pocai et al. 2005). In contrast to this critical role that happens mainly during meals, insulin also provides an ongoing message to the brain proportional to the total body fat. In this line, insulin reduces food intake by acting in the mediobasal hypothalamus (Woods et al. 2006; Gerozissis 2004). Indeed, insulin receptors are highly expressed in the ARC and here, they are co-expressed with the anorexigenic neuropeptides POMC and CART, as well as with the orexigenic neuropeptides NPY and AgRP (Benoit et al. 2002). Changes in the expression of these hypothalamic neuropeptides have been regarded as the pivotal mechanism mediating insulin anorexigenic effects via inhibition of NPY/AgRP neurons and activation of POMC neurons (Plum et al. 2006).

Glucagon hormone is synthesized and secreted from α-cells of the islets of Langerhans (Kieffer & Habener 1999). Glucagon metabolic functions are in many respects opposite to those of insulin. The most prominent physiological role of glucagon is to regulate

glucose blood levels. It stimulates glucose production via hepatic glycogenolysis or gluconeogenesis, thereby helping maintain euglycemia during states of rapid glucose utilization or fasts, respectively. Pancreatic glucagon is also secreted as food is ingested, and provides a satiety signal reaching the brain via sensory axons of the vagus nerve and leading to termination of the meal (Geary 1990).

Adiponectin is a hormone that modulates a number of metabolic processes, including enhanced glucose use and regulation of fatty acid oxidation in the skeletal muscle and liver (Díez & Iglesias 2003) via receptor-dependent activation of the 5′-AMP–activated protein kinase (AMPK) (Lancaster & Febbraio 2011; Yamauchi et al. 2002). This hormone is exclusively secreted from adipose tissue and is inversely correlated with body fat percentage in adults (Ukkola & Santaniemi 2002). In the central nervous system, adiponectin stimulates food intake via direct activation of AdipoR1 in the ARC (Kubota et al. 2007).

Ghrelin is mainly synthesised by endocrine cells in the stomach. Ghrelin levels increase before expected meals and rapidly decrease after food intake, which suggests a role in meal initiation (Williams & Cummings 2005). Ghrelin generally increases food intake by modulating the expression of hypothalamic peptides after binding its receptor in the hypothalamus. Indeed, peripheral and intracerebroventricular ghrelin injections increase food intake, whereas chronic ghrelin administration induces obesity (Korbonits 2004).

PP is secreted from the pancreas and its level is directly proportional to the caloric load consumed. At the peripheral level, PP inhibits further food intake by modulating the rate of gastric emptying during meal. On the other hand, at central level it presumably modulates gastrointestinal function via stimulation of NPY receptors, such as Y5 or Y4 receptors in the dorsal vagal complex, including the area postrema, nucleus tractus solitarius and dorsal motor nucleus of the vagus (Whitcomb et al. 1997). Activation of these receptors produces a moderate increase in food intake. Thus, PP seems to be an anorexigenic signal in the periphery and an orexigenic signal in the central nervous system. However, the mechanism of the regulation of food intake induced by PP remains to be determined (Katsuura et al. 2002).

Meal termination and satiety factors include CCK, mainly released from the gastrointestinal tract, which besides controlling gall bladder contraction, pancreatic secretion, and gut motility, also inhibits food intake via brainstem neurons (Chaudhri et al. 2006). Endocrine cells in the distal intestine produce PYY and GLP-1, which both inhibit feeding (Chaudhri et al. 2006; Druce & Bloom 2006). PYY is released into the circulation in response to the meal composition and in proportion to the calories ingested (Adrian et al. 1985). PYY slows the gastric emptying, increases efficiency of digestion and nutrient absorption after a meal, and inhibits food intake. Peripheral administration of PYY reduces food intake in rats (Chaudhri et al. 2006).

GLP-1 enhances insulin sensitivity (Miki et al. 2005) and inhibits gastric acid secretion and gastric emptying, suppresses glucagon

release and promotes an increase in pancreatic β-cell mass (Edvell & Lindström 1999). Due to these effects, the acute infusion of GLP-1 in humans reduces both appetite and food intake (Verdich et al. 2001).

2.2.2 Hedonic control of food intake

Environmental cues, rewarding stimuli and emotional factors play an important role in food intake in humans, which may override homeostatic requirements during periods of relative energy abundance by increasing the desire to consume foods (Berthoud 2006). In this context, food palatability represents an important component involved in liking, wanting, and learning to acquire food, and each of these aspects has separate but overlapped neuropsychological substrates.

The hypothalamus is highly connected to crucial brain areas involved in the hedonic pleasure, emotion and memory and these structures are strongly activated in response to the presentation of food stimuli (Kelley, Baldo & Pratt 2005; Volkow et al. 2012). Even though the mesolimbic pathways are responsible for the reward control of feeding behaviour, the orbitofrontal cortex regulates gustatory, olfactory, visual, somatosensory and sensory functions, such as taste and smell, and has an important role in reward related feeding (Rolls 2011).

Brain sites playing the most prominent role in the hedonic control of food intake include the NAc, amygdala, PFC, VTA and HCP. These sites process appetitive and rewarding aspects of eating,

including palatability and pleasure that are arguably the most powerful motivators of food intake.

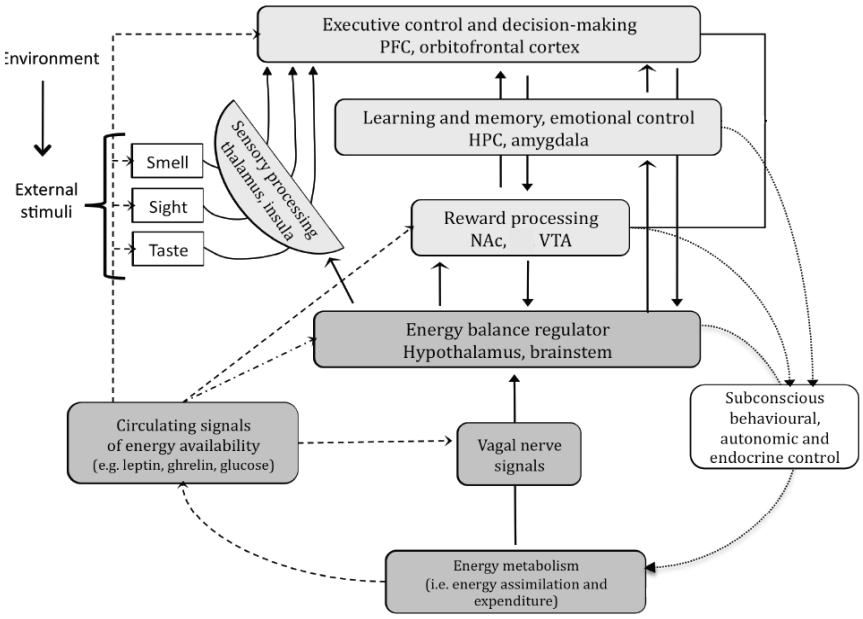


Figure15. Interaction of the homeostatic and hedonic system in the control of food intake. Schematic diagram showing neural systems and flow of information involved in the control of food intake and regulation of energy balance. The traditional regulatory circuitry using neural and hormonal feedback from the internal milieu acting on hypothalamus and brainstem is shown on the bottom (dark-grey boxes). Sensory and cortico-limbic brain areas used for processing information from the environment are shown in the upper half (light gray boxes). The broken lines with open arrows on the left indicate modulation of sensory, cognitive, and reward processes by circulating signals of fuel availability, such as leptin, ghrelin, and glucose. The full lines/open arrows indicate modulation by nutritionally relevant neural signals such as taste and visceral sensory information, as well as signals originating from the hypothalamus. Full lines/closed arrows represent neural interconnections, and dotted lines/full arrows represent subconscious behavioural, autonomic, and endocrine output/effector pathways (adapted from Zheng & Berthoud 2007).

These neuronal circuits are involved in learning, allocating attention and effort toward food rewards, setting the incentive value of stimuli in the environment and integrating information about energy stores and gut contents with information about food availability (Dagher 2009).

DA reward pathway from VTA to NAc is responsible not only for motivating food consumption, but also for the pleasurable feeling that eating produces (Wang et al. 2009). In this line, a distinction between `wanting', more associated with DA, and `liking', more associated with opioids, is often made (Berridge 2009). Liking refers to the hedonic value, palatability or pleasurable feeling associated with food, while wanting is considered to be a desire that stimulates goal-directed behaviour to obtain the food and is therefore often regarded as motivation. These two components often overlap and depend on each other.

Other mesocorticolimbic structures, different from the NAc, have shown to tightly control eating behaviour. In particular, the projections from PFC, amygdala and HCP to hypothalamus play an important role in cognitive suppression of metabolic satiation signals (Berthoud 2002). The amygdala influences reward-related food intake since a reciprocal connection between the central nucleus of the amygdala and the NAc is involved in opioid-mediated eating (Kim et al. 2004) and connections between the basolateral amygdala and forebrain regions appear to play a role in cue-potentiated feeding and in assessing food palatability (Petrovich et al. 2007; Balleine et al. 2003). Moreover, HCP and PFC are also key structures mediating feeding. Indeed, food sated rats with HCP

lesions showed increased appetitive behaviour (Davidson et al. 2007) and alteration of PFC activity in rodents, which causes hyperphagia and enhances preference for sweet palatable foods (Mena et al. 2011).

Metabolic signalling can also directly act on the brain reward circuit adding a motivational meaning to the energetic feeling of hunger and satiety. Indeed, anorexigenic peptides decrease the sensitivity of the brain reward system to the hedonic effects of food, whereas orexigenic peptides increase this sensitivity (Zheng et al. 2007). A close relationship between these signals and DA activity has been reported (Domingos et al. 2011). Thus, the activation of the leptin receptor in VTA DA neurons (Figlewicz et al. 2003) stimulates the intracellular JAK-STAT pathway leading to a reduction in DA firing rate and a decrease of food intake (Hommel et al. 2006).

In summary, increasing evidence underlines the important contribution of the mesocorticolimbic circuit in the control of eating behaviour and dysregulation in this circuit is likely to contribute to the physiopathology of some eating disorders.

2.3 Eating disorders

According to the DSM-5, eating disorders are characterized by a persistent disturbance of eating or eating-related behaviour resulting in the altered consumption of food that significantly impairs physical health, psychosocial function and emotions. Individuals suffering from eating disorders typically have unusual concern about body image and weight as well. Eating disorders affect

several millions people, most often women between the ages of 12 and 35. They are associated with a wide range of adverse psychological, physical, and social consequences. While past findings have described the causes of eating disorders as primarily environmental and sociocultural, new studies have uncovered evidence of a prevalent genetic/heritable aspect. Indeed, numerous studies have demonstrated a possible genetic predisposition toward eating disorders (Mazzeo & Bulik 2009). Epigenetic mechanisms leading to environment-alter gene expression seem to be also implicated (Frieling et al. 2010). These mechanisms will be discussed in section 2.4.3. Eating disorders are difficult to treat with many remissions and recurrences (Berkman et al. 2007; Shapiro et al. 2007) and, at the present, the treatment consists mainly in cognitive behavioural therapy while the pharmacotherapy still remains a challenge.

Obesity and eating disorders share some similarities. Indeed, some forms of obesity are driven by an excessive motivation for food and by the inability to restrain from eating despite the desire to do so. In this line, standard interventions based on promoting lifestyle changes to decrease excessive food consumption and increase physical activity are effective in normalizing body weight if followed rigorously, but they are difficult to sustain (Volkow & O'Brien 2007). These issues highlight the behavioural underpinning in obesity disease and lead to suppose that obesity is not only a metabolic disorder, but also a mental and behavioural alteration (Volkow & Wise, 2005). In agreement with this assumption, a growing body of evidence has documented comorbidity between

obesity and other psychiatric alterations, including mood and anxiety disorders.

In spite of the partial overlap between eating disorders and obesity, these disorders also differ in several aspects. Firstly, obesity is the consequence of varying interactions between individual and environmental factors resulting in a diversity of obesity phenotypes (Marcus & Wildes 2009). Some obesity phenotypes may be caused by mental disorders, such as those generally associated to binge-eating disorder (BED). However, not every person who is obese or overweight has an eating disorder. In fact, the vast majority of obese individuals do not have BED and, although BED is generally associated with overweight, not every person suffering from BED will become obese (Bruce & Wilfley 1996; Telch & Agras 1994). Moreover, excluding the subset of those meeting diagnostic criteria for BED, most obese people do not show signs of dramatic abnormalities in eating behaviour similar to those described in anorexia, bulimia or BED. Indeed, eating disorders are generally characterized by an acute sense of loss of control during binge-eating episodes, whereas some forms of obesity may be associated to a more generalized pattern of uncontrolled eating. Mostly, obese individuals present lower responsiveness to internal satiety cues and higher responsiveness to external food cues that are likely to confer vulnerability to overeating in environmental conditions where the food supply is palatable and accessible (Carnell & Wardle 2008; O'Rahilly & Farooqi 2008). In addition, individuals with eating disorders show distress and feelings of guilt and disgust after food consumption (Ziauddeen et al. 2012), and these feelings sometimes

evolve in “inappropriate” compensatory behaviours (DSM-5). In contrast, these feelings do not necessarily characterize obese patients.

Currently, obesity is not included in the eating disorder category of the DSM-5 due to the little evidence in support that obesity is a mental disease. Indeed, there are wide ranges of genetic, behavioural and environmental factors that vary across individuals contributing to the development of obesity. Thus, obesity is a heterogeneous condition with a complex and incompletely understood etiology that cannot be considered per se in the psychiatric nomenclature. The relationship between obesity and numerous psychiatric disorders highlight both the heterogeneity of obesity and the limitations inherent in descriptive diagnostic categories.

2.3.1 Conceptualization in the DSM-5

Different types of eating disorders have been reported. Bulimia nervosa and anorexia nervosa are the most common specific forms of eating disorders. Other main eating disorders include BED and other atypical disorders that did not fit entirely in the previous categories and received a diagnosis of “specified feeding or eating disorder”. According to the DSM-5, eating disorder symptoms resemble to those types typically endorsed by individuals with substance use disorders, such as craving and compulsive use. These similarities may reflect the participation of similar neural systems, including those involved in regulatory self-control and reward.

Indeed, neuroimaging studies suggest altered function of reward circuitry in patients with eating disorders (Harrison et al. 2010). Thus, this similar symptomology can support the inclusion of eating disorders as addiction disorders. However, empirical evidence suggests that other patterns of food consumption more properly share salient features with substance use disorder. Indeed, the concept of "being addicted to food" has developed progressively and become popular in recent years. However, the existence of eating addiction as a clinical disorder that leads to loss of control over food intake is still under critical view due to the fact that food is essential to survival and, as a result does not figure in the DSM-5 at present. Nevertheless, behavioural similarities between eating addiction and the recognized eating disorders have been found, together with many shared psychological and biological risk factors (Davis et al. 2011). In this line, compulsive food consumption underlying eating addiction also characterizes binge eating episodes, which generally occur in bulimia, BED and in binge eating/purging type of anorexia nervosa. These similarities are consistent although they are only partial, as later discussed for BED. Indeed, the addiction model used to understand eating disorders (Szmukler & Tantam 1984; Marrazzi & Luby 1986) has been widely criticized for being over simplistic and for lack parsimony in its explanations of eating disorder symptoms. Moreover, the addiction model does not distinguish between BED, bulimia and anorexia, three brain disorders with different etiology and symptoms. Thus, although the exploration of eating addiction is relatively new, distinguishing between eating addiction as a

disorder different from anorexia, bulimia and BED may be helpful in identifying the mechanisms underlying the development, maintenance, prevention, and treatment of problematic eating.

2.3.2 Eating disorder currently recognized in medical manuals

The following eating disorders are specified as mental disorders in standard medical manuals, such as ICD-11 and DSM-5:

i. Anorexia nervosa

This disorder is characterized by food restriction, odd eating habits or rituals due to an obsessive fear of gaining weight and an unrealistic perception of low body weight. Indeed, patients with anorexia usually experience “fat phobia" that influences their self-evaluation and/or brings them in denial of the seriousness of their current low body weight (APA, 2013). The disease manifests itself in two distinct forms: a restricted type or a binge eating/purging type (Pinhas et al. 2011). In the first case, the weight loss is accomplished primarily through dieting, fasting and/or excessive exercise. Individuals with anorexia nervosa tend to exhibit high activity levels, as well as mental alertness, during their weight loss from food restriction (Casper et al. 1991; Klein et al. 2007). This in turn drives them to engage in excessive exercises, creating a detrimental positive feedback/reward cycle (Klein et al. 2007). In the second case the individual has engaged in recurrent episodes of binge-eating or purging behaviour, characterized by the loss of

control over food intake followed by laxative abuses or vomiting. Anorexia nervosa is most commonly found in young women with 18 being the average onset (Fairburn & Harrison 2003).

Because no single factor has been shown to be either necessary or sufficient for causing anorexia nervosa, a multifactorial model might be most appropriate (Connan et al. 2003), even thought risk factors are associated with the individual temperament, environmental, genetic and psychological aspects. Indeed, the onset of this disorder is often associated with stressful environmental events during life. Biological and/or genetic component influences are also reported in many cases. A recent genome-wide association study confirmed several risk genes in the mesocorticolimbic system involved in anorexia (Brown et al. 2007). Genes coding for serotonin (5-HT) and DA are implicated in the altered reward modulation in people with anorexia (Kaye et al. 2009). Indeed, low levels of 5-HT and DA metabolites are found in the cerebrospinal fluid of these patients (Kaye et al. 1984). Modifications in the expression of genes coding for 5-HT1A, 5-HT2A receptors and 5-HT transporter have also been reported in the cortex and dorsal raphe of anorexic patients (Bailer et al. 2005; Kaye et al. 2005). Moreover, evidence supports the involvement of polymorphisms within gene encoding DOR on human chromosome 1 in the genetic susceptibility to anorexia, especially to the restricting type (Brown et al. 2007; Bergen et al. 2003). Indeed, a disturbance in DOR gene can facilitate the development of addictive-like behaviour to fasting and exercise (Rask-Andersen et al. 2010).

Psychological aspects are also crucial in the development of this disorder. Indeed, an association between major depression and anorexia nervosa has been widely reported in clinical studies (Wade et al. 2000). Additionally, patients with anorexia nervosa show comorbidities with intellectual disability and dissociative disorders (Mammen et al. 2007), and demonstrate elevated levels of suicide, making anorexia nervosa a deadly mental illness (Wade et al. 2000).

ii. Bulimia nervosa

This disorder is characterized by recurrent binge-eating followed by compensatory behaviours such as purging (self-induced vomiting), excessive use of laxatives/diuretics, or excessive exercise. Bulimia nervosa shares similar behavioural substrates with anorexia although the framework of both diseases is different. Indeed, anorexics are underweight, whereas bulimics tend to be normal or overweight, due to the calories consumed during binges. Moreover, anorexia is characterized by restriction of eating, the opposite behaviour observed in bulimia. Furthermore, the DSM-5 explicitly requires for a diagnosis of bulimia nervosa a subjective sense of loss of control over eating to define a binge-eating episode (APA, 2013). Bulimia nervosa also shares many clinical symptoms with BED, even though, according to the DSM-5, they have distinct diagnostic criteria, such as lack of compensatory behaviour (e.g., self-induced vomiting or purging) in BED that differentiates these two disorders.

The neurobiological mechanisms in this disease are still not understood. It has been suggested that bulimia nervosa is the behavioural manifestation of a decreased activity of serotonin and/or DA transmission (Steiger 2004; Galla et al, 1995). Bulimia commonly begins in adolescence or young adulthood. Comorbidity with mental disorder is common in individuals with bulimia nervosa and an increased frequency of bipolar and depressive disorders has been reported. Indeed, nearly 87% of patients with bulimia have moderate to severe depressive symptoms (Stunkard et al. 2006) and significantly elevated risk for mortality (2%) by committing suicide has been reported (DSM-5).

iii. Binge Eating Disorder (BED)

BED is characterized by recurrent episodes of consumption of large quantities of food accompanied with a sense of loss of control over eating, as described by DSM-5 (APA, 2013). DSM-5 criteria specify that individuals must experience at least three of the following impaired control behavioural indicators for the diagnosis of BED: (1) eating rapidly, (2) eating until uncomfortably full, (3) eating large amounts of food when not hungry, (4) eating alone due to embarrassment, (5) feeling depressed, disgusted, or guilty after overeating. An occurrence of excessive food consumption must be accompanied by a sense of lack of control that is reported as the inability to refrain from eating or to stop eating once started. The type of food consumed during binges varies across individuals and for a given individual across time. BED appears to be characterized

more by an abnormality in the amount of food consumed than by craving for a specific nutrient.

Epidemiological studies indicate that BED is much more common in the general population than other eating disorders. It mainly occurs in normal/weight/overweight and obese individuals (Bruce & Wilfley 1996; Spitzer et al. 1993), although it could also be present in anorexia nervosa (Swanson et al. 2011). A positive correlation has been observed between BED and psychological distress, depression, interpersonal problems and low self-esteem (Telch & Agras 1994). Moreover, these patients have higher lifetime rates of panic and personality disorders compared with individuals that do not meet these criteria (Yanovski et al. 1993). Women are 1.5 times more likely than men to be diagnosed with BED (Spitzer et al. 1993).

Pharmacotherapy research of bulimia nervosa and BED is currently in early stages. Fluoxetine is the only medication with regulatory approval in bulimia nervosa, and no medication has been approved for BED. Many of the available pharmacotherapy studies in bulimia nervosa and BED are limited by small sample size, high placebo response and dropout rates, and unclear generalizability of findings to real world clinical situations (McElroy et al. 2012).

iv. Other specified feeding or eating disorder

This category includes eating or feeding disorder that does not meet full DSM-5 criteria for anorexia, bulimia, or BED, but they cause significant distress or impairment in social and occupational areas.

It is important to note that this category is not an indication of a less severe eating disorder, simply a different expression of symptoms. Examples of these eating disorders include individuals with atypical anorexia nervosa, who meet all criteria for anorexia nervosa except being underweight, despite substantial weight loss; atypical bulimia nervosa, who meet all criteria for bulimia nervosa except that bulimic behaviours are less frequent or have not been ongoing for long enough; purging disorder; and night eating syndrome.

2.3.3 Eating disorders not currently recognized in clinical manuals of psychiatric disease.

i. Obesity

Obesity is a complex metabolic disorder defined as an excess of accumulation of fat in adipose tissue to an extent that can negatively affect the person's health (James et al. 2001; Garrow 1988). The most common method for defining obesity is the Body Mass Index (BMI) strongly associated with adiposity and obesity-related morbidity, and category thresholds have been established. Indeed, overweight is commonly set in a BMI range between 25 and 29.9 and obesity in a BMI of 30 or above (James et al. 2001; WHO, 2004). The weakness of this definition is that it does not distinguish muscle weight from fatness and does not account for the wide variation in body fat distribution (James et al. 2001; WHO, 2004). About 13% of the adult population in the world (11% of men and

15% of women) was obese in 2014 and this percentage will tend to increase (WHO; 2014).

The etiology of this disease is multifactorial involving a multitude of genetic, neural, physiological hormonal, nutritional, social, and psychological factors, which can interact to promote weight gain under numerous conditions (Bray & Champagne 2005). In many cases obesity is conceptualized as the imbalance of energy-intake and energy-expenditure. However, the cause appears much more complex. Indeed, food availability, environmental cues and alterations in dietary patterns with a prevalence of energy-dense fat and sweet foods contribute to the high prevalence of obesity (Rolls 2011; Young & Nestle 2002). The evidence strongly confirms that the rewarding properties of palatable food appearing to override homeostatic processes. Sedentary activities, by reducing energy expenditure, also have high influences in obesity (Zhang & Wang 2004). Overconsumption of food along with a sedentary lifestyle is an association appropriate for ensuring of weight gain.

A crucial importance in the obesity framework is the combination of genetic and environmental factors that are likely to interact in diverse ways among individuals (Freedman & Stern 2004). Important candidate gene variants are the polymorphisms in the "fat mass and obesity-associated" (FTO) locus that seem to confer risk of obesity through increasing energy intake and reducing satiety (Hetherington & Cecil 2010). In addition, monogenic mutations have been discovered in genes that play essential roles in the appetite control, food intake, and energy homeostasis, primarily in those coding for the hormone leptin, the leptin receptor, pro-

opiomelanocortin, and the melanocotin-4 receptor, among others (Hu 2008). Moreover, variations of genes involved in DA neurotransmission, such as D2R Taq I A1 allele (Blum et al. 1996), as well as the gene coding for CB1R (Schleinitz et al. 2010) have also been associated with obesity. Other candidate genes have been studied and the number of genes involved in this disorder continues to expand. Polymorphisms of genes implicated in the adipocyte metabolism such as the gene of fatty acid binding protein 2 (Shabana & Hasnain 2015), insulin induced gene 2 (Liu et al. 2015), Niemann-Pick protein, type C1 for the intracellular lipid transport, MAF involved in adipogenesis and insulin-glucagon regulation (Hofker & Wijmenga 2009) could also play a role in the development of obesity. In addition, polymorphisms of the glucocorticoid receptor have also been associated to fat accumulation, particularly in the central abdomen, and obesity (Cellini et al. 2010). Other possible candidates for the genetic substrate of obesity include genes coding for apoliproteins, adrenergic receptors, insulin, insulin receptors, insulin-like grow factor, glucose transport proteins and CCK, among others (Bouchard 1994).

Another important cause factor affecting the development of obesity is stress (Pickering 1999). Data support the notion that humans experiencing stress seek comfort by consuming palatable foods that are high in fat and sugar (Pecoraro et al. 2004). These types of foods have powerful reinforcing properties and repetitive rewards can alleviate feelings of anxiety and discomfort (Dallman et al. 2005), representing important factors to promote obesity (Bray

2004). Other pathophysiological factors contribute to the obesity prevalence. Indeed, the number of depressive episodes positively predicts the risk of developing obesity, suggesting that mood is the main driver of emotional eating (Björntorp 2001; Scott et al. 2008). In turn, obesity has been considered an important risk factor for mortality and morbidity because it exacerbates the deleterious effects of other diseases. Indeed, obesity is associated with many comorbidities, such as cardiovascular diseases, type II diabetes mellitus and certain cancers (Bray 2004), and increases the risk of stroke, hypertension, and dyslipidemia (Must et al. 1999; Must & Strauss 1999).

Several neurochemical systems have been reported to be involved in the mechanisms underlying obesity. As already reported, the endogenous opioid, DA and ECS have a crucial role in the development of obesity. However, treatment options for obesity remain quite limited. Lifestyle changes in the form of dieting and/or exercise do not generally produce sustainable weight loss (Leblanc et al. 2011), whereas effective psychological therapies, such as cognitive behavioural therapy, cannot easily be delivered on a mass scale (Wing et al. 2006) and long-term results are disappointing. Bariatric surgery is much more effective in terms of weight loss, comorbidity reduction and enhanced survival (Kral & Näslund 2007; Sjöström et al. 2007). However, these procedures tend to be reserved for the morbidly obese considering the concerns about perioperative mortality, surgical complications and the frequent need for reoperation (Field et al. 2009). Pharmacological agents that induce weight loss may reduce appetite or increase satiety, reduce

the absorption of nutrients, or increase energy expenditure. In the past, drug therapies available have included thyroid hormone, dinitrophenol and amphetamines, followed by amphetamine analogues such as aminorex, fenfluramines (Ioannides-Demos et al. 2005), CB1R antagonist as rimonabant and the 5-HT and noradrenalin (NA) uptake inhibitor, sibutramine. Unfortunately, those were withdrawn for the market due to an important risk of adverse consequences (Janero et al. 2011). Other agents have been trialled though only orlistat (a gastrointestinal lipase inhibitor) was approved for long-term use ($\geq$ 24 weeks) in obese patients (Ioannides-Demos et al. 2011). It does not directly act on appetite as other obesity pharmacotherapies, rather it decreases fat absorption by binding to pancreatic lipase, the principle enzyme that hydrolyses triglycerides (Padwal & Majumdar 2007). Liraglutide and exenatide, GLP-1 analogues, were developed for the treatment of type 2 diabetes associated to obesity (Vilsbøll et al. 2007). Liraglutide was approved in the 2014 by Food and Drug Adminisration agency and demonstrated beneficial weight loss in obese patients by increasing the secretion of leptin, which results in suppressed appetite, decreased energy intake and a delay in gastric emptying (Astrup et al. 2009). In the same line, metformin is the first-line drug of choice for the treatment of type 2 diabetes that can also be used in overweight and obese patients since contributes to weight loss. Lorcaserin is a selective 5-HT2 receptor agonist used as a weight-loss drug approveted by Food and Drug Administration in 2012. The activation of 5-HT2 receptors in the hypothalamus is

supposed to activate POMC production and consequently promotes weight loss through satiety mechanisms (Helmut 2010).
A number of polipharmacological treatments have been reported to be effectively 'polytherapies', such as Contrave® and Qnexa®. Contrave® is a fixed-dose combination of naltrexone and bupropion. Qnexa® formulation contains doses of the amphetamine-analogue phentermine that are 1/10 to 1/2 the doses used for obesity and doses of topiramate that are 1/16 to 1/4 the doses used as an anticonvulsant (Bello & Campbell 2012).

ii. Eating addiction

Certain pathologic patterns of food consumption bear a striking resemblance to substance use disorders, associated with increased risk for comorbidity complications and relapse. The commonalities existing between compulsive eating and drugs of abuse, such as behavioural alterations, external cue-control of appetite or excessive motivation for reinforcement suggest that compulsive food consumption could be conceptualized as a mental addictive-like disorder. In this view, eating addiction is defined as a compulsive overeating syndrome accompanied by strong craving and extreme difficulty in abstaining from palatable food, which leads to a high risk of relapse (Davis et al. 2013). The idea that a person can be addicted to food has recently gotten more approval and may play an important role in obesity epidemic. However, normal-weight people exposed to high-fat, high-calorie foods may also be vulnerable to eating addiction, suggesting that there is considerable variation in

responsiveness to "unhealthy" food environments (Hetherington & Cecil 2010). This observation emphasizes the importance of avoiding simple use of BMI as a general marker for compulsive overconsumption. At present, it is discussed whether or not these specific patterns of food consumption should be viewed as addictive processes. In this line, opinions in favour and against the existence of eating addictive-like behaviour are currently discussed. Indeed, several models reject the eating addiction concept underlining the fact that food is essential to survival and it is normal to eat repeatedly and to look forward to eating for pleasure. These opinions also highlight the matter that eating depends on different peripheral and central factors, while drug addiction depends just on central factors.

Conversely, other authors support the eating addiction construction highlighting that core components of addiction, across substances and reinforcing behaviours are reported in overeating (see Table 1). However, controversies concerning the possible classification of eating addiction as a "chemical" (substance-based) or a "behavioural" (non substance-based) addiction (Ifland et al. 2015; Albayrak et al. 2015) under the current DSM-5 criteria have been recently reported. Regarding chemical addiction, several models propose that some foods, containing specific "substances" such as high-fat and/or sugar, are capable of promoting addiction-like behaviour and neuronal changes under certain conditions (Corwin & Grigson 2009) similar to those promoted by an addictive drug such as nicotine, alcohol among others. Accordingly, the use of the term *"food addiction"* appears appropriate in this context. Indeed,

the word “food” connotes the use of a substance that engages addictive processes.

In contrast to chemical addiction, other models conceptualize food overconsumption as a behavioural addiction in predisposed individuals under specific environmental circumstances (Hebebrand et al. 2014). The term “*eating addiction*”, similar to compulsions that are non substance-related (i.e. gambling), more properly underscores the behavioural addiction to eating (Hebebrand et al. 2014). In this context, the use of the term *“food addiction”* has been criticized because it appears more like a passive process which simply befalls an individual and does not emphasize on the behavioural component.

Although the concept of eating addiction has received considerable attention from the popular media, it is still not clinically recognized in the DSM-5 and other manuals.

In summary, the question of whether eating addiction is a valid concept is still subject of debate. Although findings provide support for eating addiction as a clinically relevant phenomenon, more scientific research is needed to its acceptance in clinical settings. Another point to be clarified is whether eating addiction should be considered as a substance use or as a behavioural disorder and thus diagnosed with the substance use disorder criteria or with those used to diagnose gambling.

2.4 Eating addiction: perspective

2.4.1 Chemical addiction or "food addiction"

Labelling a food or nutrient as addictive implies that it possesses an inherent property with the capacity of making susceptible individuals addicted to it. As already reported, chemical addiction would refer to a kind of food, or its constituent, that engages specific neuronal mechanisms and produces behavioural adaptations comparable to those engendered by drugs. The growing legitimacy of food addiction concept has been heavily influenced by the premise that hyper-palatable foods have the potential to foster excessive consumption and a state of dependence (Davis & Carter 2014). Past evidence in animal models reports that food can trigger addictive processes, although it should be essential to learn which constituent of foods might be responsible. Food is nutritionally complex and it could be difficult to suppose that under normal physiological circumstances humans crave specific foods to ingest a specific "substance". In this view, several studies have focused on some specific nutrients such as sugar, fat and salt that, akin to addictive-substances, can alter the brain reward system. However, addiction to a particular substance or a nutrient profile, including high sugar content or combinations of high sugar and high fat, have been previously described only in animal studies (Colantuoni et al. 2001; Berner et al. 2008; Ifland et al. 2009). Previous rodent models of addiction to food have typically used behavioural paradigms based on analogues inspired on substance dependence criteria (DSM IV) (de Jong et al. 2012). Indeed, maladaptive forms of

eating behaviours using sugar and high palatable food have been shown in the conditioned place preference test and in operant self-administration paradigms (de Jong et al. 2013; Velázquez-Sánchez et al. 2015). Compulsive food-seeking were evaluated in these models (Johnson & Kenny 2010; Avena et al. 2008) by measuring the animal motivation for palatable foods despite facing potentially harmful consequences. Indeed, an increased motivation to obtain a sucrose reward under a PR schedule after chronic exposure to high-fat and high-sucrose choice diet was shown (Morgan et al. 2006). Conditioned aversion paradigm has been used to show that palatable food seeking can become resistant to punishment (Johnson & Kenny 2010; Latagliata et al. 2010). Models of reinstatement of extinguished palatable food-seeking behaviour induced by cues stimuli have also been described in mice (Martín-García et al. 2011) and rats (Ghitza et al. 2007).

Sugar and fat seem to alter the brain reward system in a similar way in rats (Avena et al., 2008; Carrillo et al., 2003). Thus, changes in DA, acetylcholine and opioid levels in sugar–bingeing rats are similar to those observed with some drugs of abuse (Avena et al. 2009). A similar addictive-like state may emerge with fat. Indeed, a binge of fat also modifies the DA and enkephalin system activity (Liang et al. 2006). However, these models cannot yet go beyond relating addiction to broad categories of high-fat, high-sugar or hyperpalatable foods and it is still unknown whether a particular concentration of nutrient(s) might engender the addictive process (Ziauddeen & Fletcher 2013). Evidence in rats showing preference for salty foods has also been reported (Bertino & Tordoff 1988).

However, the role of salt in directly increasing the rewarding value of food is relatively unexplored. Some studies have described a link between salt intake and activation of reward brain areas in rodents, although little evidence exists about the intrinsic reinforcing properties of salt (Tekol 2006). Recent studies described that rats exposed to "cafeteria diet" (composed of numerous nutrient combinations) also develop compulsivity toward palatable food, indicating that is not a single substance which possesses reinforcing properties (Johnson & Kenny 2010). Netherless, there is not currently evidence at present that these nutrient components or very simple combinations of them can elicit a substance use disorder in humans.

In summary, past evidence in rodents suggests that addictive-like behaviour can be manifested toward foods composed by specific nutrient components, such as fat, sugar or salt (Kaplan 1996). However, there is insufficient evidence to label fat, sugar or salt as addictive as drugs of abuse, according to current DSM-5 diagnostic criteria, although these nutrient components have rewarding properties and are highly palatable.

2.4.2 Behavioural addiction or "eating addiction"

Addictive-like responses may be attributed not just to substances but also to behaviours. Thus, the recent DSM-5 has acknowledged the existence of behavioural addiction for the first time. In this edition, the conceptual model of "substance" addiction has been replaced for an increasing emphasis on the "behaviour" of

substance use rather than the chemical properties of the substances themselves (Gawin 1991). This change leads to an overlap in psychological characteristics between 'chemical' and 'behavioural' addictions, and the common properties of addictive substances or activities. In this line, eating addiction might be viewed mainly as a specific form of behavioural addiction and could be categorized alongside conditions like gambling addiction, especially when the psychological compulsion to eat is driven by the positive feelings that the brain associates with the act of eating. Eating addiction patterns could be the result of learning rather than a substance-driven form of addiction.

Researchers have stressed the behavioural component of this disorder proposing that the addictive pattern of eating may spring by the way in which the food is consumed, rather than its sensory and nutritional properties. Thus, palatable food is typically considered "forbidden" due to the high calorie content. This could indeed lead to a restricted pattern of consumption of the high-energy food that may engage addictive processes (Corwin & Grigson 2009). In fact, evidence suggests that the state of prohibition could stimulate even more the consumption of palatable food (Pelchat 2009).

As eating addiction, other excessive behaviours related to internet, sex, exercise, and shopping have been considered for the inclusion. However, none was deemed to have sufficient evidence for the identification as a mental health problem (Potenza 2014). Currently, pathological gambling is the only one listed in the newly labelled "non-substance-related disorders" category (DSM-5). Gambling

behaviour was included because it activates similar reward systems that are targeted by drugs of abuse, and because it produces behavioural symptoms that overlap with those produced by substance use disorders.

In summary, the DSM-5 currently does not allow the classification of an "eating addictive disorder" within the diagnostic category substance-related and addictive disorders. However, similar to other behaviours, eating could become an addiction in predisposed individuals under specific environmental circumstances. Conversely, there is little evidence that humans can develop a specific nutrient use disorder. More evidence is also needed to confirm the existence of eating addictive like-behaviour in humans before its consideration as formal disorders.

2.4.3 Diagnosis of "food addiction" in human

The Yale Food Addiction Scale (YFAS) (Gearhardt et al. 2009b) can be viewed as the first questionnaire to assess the severity and frequency of symptoms of dependence in relation to individual food consumption based on similarities between certain aspects of overeating and the DSM-IV criteria for substance dependence (APA, 1994). This questionnaire was create in 2009 and it is to date use in expectation to clarify the concept of eating addiction disorder and to eventually come up with stringent diagnostic criteria according to those of the DSM-5. YFAS consists of a series of 25 questions which address individual's eating habits during the past 12 months. Even though it is based on substance dependence

criteria, the YFAS demonstrated good validity with other measures of eating problems and clearly focuses on the assessment of eating behaviour and not on substance based addiction (Hebebrand et al. 2014). The questionnaire includes items related to foods containing different substances and a diagnosis of food addiction as a substance use disorder is therefore not possible using this questionnaire (Hebebrand et al. 2014). However, further modifications for the YFAS diagnosis of eating addiction are needed to better capture the cognitive and behavioural aspects of eating addiction.

The YFAS criteria have been used to explore the prevalence of food addiction in obese subjects (Davis 2013b), in clinical population with and without BED (Gearhardt et al. 2013; Lent et al. 2014) and in a population of below normal weight (Flint et al. 2014). Studies using YFAS scale found prevalence rates of food addiction of about 5-10% in people of normal weight and about 15-25% in obese participants (Meule 2012; Meule 2011). To date, several studies have found substantial comorbidity between BED and YFAS food addiction. Indeed, 56.8% of people with BED met YFAS criteria for food addiction (Gearhardt et al. 2013).

2.4.4 Individuals vulnerabilities and risk factors of eating addiction

Multiple factors are implicated in the propensity to develop eating addictive-like behaviours. These factors include genetic predisposition, environmental risk factors, age, comorbidity with

other mental disorders and negative emotions. Individual differences seem to play an important role in eating addiction. As previously described, genetic variations involved in drug addiction such as polymorphisms of the CB1R and MOR could also be predisposing factors leading to eating addiction. Other genetic factors could also play a role in the personality traits that are associated with increased overconsumption, such as novelty seeking and impulsiveness (Albayrak et al. 2012). On the other hand, environmental factors including food availability and advertising also have an important impact on the development of eating addiction. Availability is important since people cannot become addicted to something that they cannot find. Moreover, the repeated exposure to cues, such as advertising, may condition craving and the exposure to these environmental factors at a young age appears to have an important impact on the development of the addiction process (Picherot et al. 2010). As previously explained, the interaction of individual vulnerability and environmental factors is also crucial in the development of the eating addict phenotype.

An interesting phenomenon that remains to be elucidated is the differential gender implication in eating addiction. Indeed, compulsive food consumption affects females more often than males (Davis 2013a; Liang et al. 2013). In spite of this prevalence, drug addiction seems to principally affect men. It is hypothesized that such differences are due to hormonal reasons since estrogens can modulate DA signalling and thus drug and food response (Roth et al. 2004).

In addition, patients with psychiatric comorbidity with mood, anxiety, conduct disorders and depression have a high risk to engage in this behaviour (Gearhardt et al. 2012).
As previously described in the case of obesity disease, physiological stressors such as overloaded work, interpersonal issues or self-pride increase the motivation to engage in overeating behaviours. Indeed, stressors have been associated with an increased high-fat food intake or extra snacking between meals (O'Connor et al. 2009).

2.4.5 Clinical implication

i. Eating addiction and BED

BED shares many characteristics with addictive behaviours (Gold et al. 2009) and scientific literature supports the addiction conceptualization of this eating disorder. Loss of control becomes a crucial part in the diagnosis of BED. Indeed, several diagnostic/research criteria for the bingeing-related eating disorders approximate the criteria for substance use disorders, such as diminished control over intake, high motivation, continued use despite negative consequences and diminished ability to cut down (Davis & Carter 2009; Gearhardt et al. 2009). Moreover, BED and addiction share neuronal correlate, such as similar patterns of DA activity in response to cue stimuli (Avena et al. 2008; Schienle et al. 2009). Like eating addiction, BED also occurs in normal weight individuals albeit less frequently than in overweight or obese individuals (Hebebrand et al. 2014).

Despite similarities, BED and addiction also differ in important concepts. First, the loss of control over consumption occurs differently in BED and addiction. Indeed, BED diagnosis specifies that an episode of out-of-control over eating could occur during a discrete period of time, referring to a limited food consumption period during the day. Conversely, the impaired control in eating addiction is not necessarily experienced during a single episode of overeating, but it could persist throughout the day and does not necessarily include eating binges. In addition, binge-eating episode occurs at least once a week according to the DSM-5, whereas the loss of control over drug intake in addiction could occur with high frequency of consumption during days and weeks. In this line, addict individuals spend an abnormal amount of time in using the drug, as well as in obtaining and recovering from the effect of the drug. All these items have not been demonstrated in BED, in which the substance-focused activities could occur infrequently and the patient does not spend a great deal of time in obtaining and using food (Gearhardt et al. 2009). Moreover, addiction diagnosis places a greater emphasis on the contribution of the substance (addictive potential of drug) while BED diagnosis does not consider specific types of food consumed, but merely the amount (Gearhardt et al. 2011; Gearhardt et al. 2014). Clinical studies reveal that although only 1-4% of the population meets diagnostic criteria for BED, episode of uncontrolled eating are also seen in nonclinical populations (Gearhardt et al. 2009b) suggesting that eating addiction may also be linked to other patterns of eating behaviours that are not associated with BED. Clarifying whether the two

conditions are sufficiently different in etiology and clinical course is an important step to warrant classification as separate pathological eating disorders.

ii. Eating addiction and Obesity

Obesity is not considered at present a mental illness and is not included in the psychiatric manuals (e.g. DSM). However, there are robust associations between some types of obesity and a number of psychiatric disorders such as BED, depression, bipolar disorder and schizophrenia (DMS-5). In this context, recent opinions conceptualize obesity and overeating as disorders related to addictive-like processes. Indeed, some forms of obesity strongly resemble to drug addiction, both at the behavioural level and in terms of underlying neural processes (Ziauddeen et al. 2012). Indeed, obese individuals are driven by an excessive motivation for food and by a compulsive pattern of eating even though obeses make frequent, yet unsuccessful, efforts to control it. These obese individuals continue to overeat despite knowledge that their behaviour causes negative health consequences that could reduce the individual ability to participate in a full range of social, occupational, and recreational activities. Thus, it seems that the face value of eating addiction construct is strong when it is applied to certain individuals with obesity.

Obesity and addiction has also a strong neurobiological common substrate. Indeed, addiction and obesity share several traits such as habits due to the reinforcing properties of powerful and repetitive

rewards (Volkow & Wise 2005). Indeed, the ST hypofunction that characterizes both obesity and drug addiction has been well documented (Volkow et al. 2011). These parallelisms have generated interest in understanding also the shared vulnerabilities between addiction and obesity. Indeed, some obese and drug-addict patients could be linked to similar genetic polymorphisms. The most widely studied polymorphism has been the Taq1A minor allele of the DA D2R, which is associated with alcoholism (Munafò et al. 2007) cocaine (Noble 1993), opioid dependence (Doehring et al. 2009) and also obesity (Davis et al. 2009).

Although similarities between obesity and addiction have been described, there are also important differences according to clinical evidence. An addiction model of obesity assumes that overeating is the primary cause of obesity, just as compulsive drug use causes addiction. However, although obesity is generally associated with food overconsumption, other factors may also contribute to body weight gain (Blair & Nichaman 2002). Moreover, eating addiction has also been diagnosed in lean patients (Gearhardt et al. 2009). Thus, despite similarities between addicted and obese phenotypes, the overlap between these two disorders is only partial (Ziauddeen et al. 2012).

In summary, obesity is a heterogeneous condition with a complex and incompletely understood etiology and is not considered a mental disorder per se. The hypothesis that overeating and obesity could be understood under the addiction framework has fuelled a series of studies aimed at testing this proposed link, but future

works are still needed to shed light on the role of eating addiction in obesity.

3. THE OPIOID SYSTEM

3.1 Components and physiological role

The endogenous opioid system is integrated by three different families of endogenous opioid peptide precursors, proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN), and three main different opioid receptors, mu (MOR), delta (DOR) and kappa (KOR), widely distributed in the central nervous system and peripheral tissues. Within the central nervous system, opioid receptors are found in many areas, including the cortex, the limbic system and the spinal cord.

The distribution of MOR, DOR and KOR is similar in several aspects. They are located at the same components of the limbic system, such as the NAc and amygdala, explaining the role of these receptors in mood and reward. In addition, MOR is predominantly localized in the VTA, habenula and thalamic nuclei and is also highly present in cortex, brainstem and reticular core nuclei. DOR is prominent in cerebral cortex and HCP, indicating the involvement of this receptor in cognition and memory, and is also highly expressed in the olfactory tubercle and caudate-putamen. The MOR and DOR location at the cortex, periaqueductal gray, 4th ventricle and substantia gelatinosa of the spinal cord reveals the involvement of these receptors in the control of nociceptive stimuli. In contrast, KOR is mainly expressed in hypothalamic and some thalamic

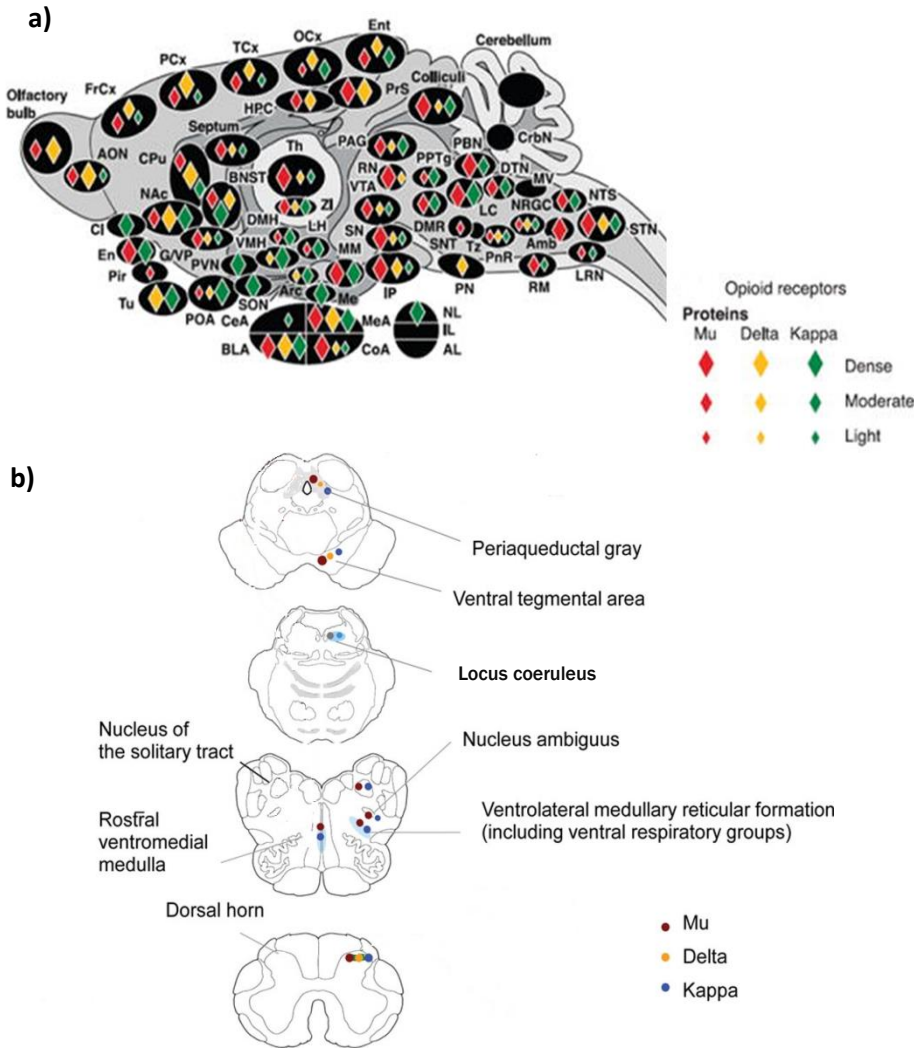


Figure 16. Schematic distribution of opioid receptors in the a) brain (sagittal plane) and b) spinal cord. Densities are represented by symbols of different sizes, from low to high. Localization of opioid receptors is determined by ligand autoradiography in main areas. Amb, nucleus ambiguus; AD, anterodorsal thalamus; AL, anterior lobe, pituitary; AON, anterior olfactory nucleus; Arc, arcuate nucleus, hypothalamus; BLA, basolateral nucleus, amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus, amygdala; Cl, claustrum; CL, centrolateral thalamus; CM, centromedial thalamus; CoA, cortical nucleus, amygdala; CPu, caudate-putamen; CrbN, cerebellar nuclei; DMH, dorsomedial

hypothalamus; DMR, dorsal and medial raphé; DTN, dorsal tegmental nucleus; En, endopiriform cortex; Ent, entorhinal cortex; FrCx, frontal cortex; G, nucleus gelatinosus, thalamus; G/VP, globus pallidus/ventral pallidum; HbL, lateral habenula; HbM, medial habenula; HPC, hippocampus; IL, intermediate lobe, pituitary; IP, interpeduncular nucleus; LC, locus coeruleus; LD, laterodorsal thalamus; LG, lateral geniculate, thalamus; LH, lateral hypothalamus; LRN, lateral reticular nucleus; MD, mediodorsal thalamus; Me, median eminence; MEA, median nucleus, amygdala; MG, medial geniculate; MM, medial mammillary nucleus; MV, medial vestibular nucleus; NAc, nucleus accumbens; NL, neuronal lobe, pituitary; NRGC, nucleus reticularis gigantocellularis; NTS, nucleus tractus solitarius; OCx, occipital cortex; PAG, periaqueductal gray; PCx, parietal cortex; Pir, piriform cortex; PN, pontine nucleus; PnR, pontine reticular; PO, posterior thalamus; POA, preoptic area; PPTg, pedunculopontine nucleus; PrS, presubiculum; PV, paraventricular thalamus; PVN, paraventricular hypothalamus; RE, reuniens thalamus; RN, red nucleus; RM, raphé magnus; SON, supraoptic nucleus; SN, substancia nigra; SNT, sensory trigeminal nucleus; STN, spinal trigeminal nucleus; TCx, temporal cortex; Th, thalamus; Tu, olfactory tubercle; Tz, trapezoid nucleus; VL, ventrolateral thalamus; VM, ventromedial thalamus; VMH, ventromedial hypothalamus; VPL, ventroposterolateral thalamus; VTA, ventral tegmental area; ZI, zona incerta. (adapted from Le Merrer et al. 2009a).

nuclei, important sites in sensory processing and homeostasis. It is also highly present in cortex, caudate-putamen, olfactory tubercle, amygdala and brainstem (George et al. 1994; Mansour et al. 1994; Le Merrer et al. 2009a). In the peripheral nervous system, these receptors are expressed in both the myenteric plexus and submucous plexus of the wall of the gut and are responsible for powerful constipating effects. Opioid receptors belong to the large

family of receptors which possess 7 transmembrane-spanning domains of aminoacids coupled to G-proteins. Once activated, they inhibit the enzyme adenylate cyclase, with a consequent reduction in the production of cyclic AMP and entry of Ca^{2+} ions together with the stimulation of the inwardly rectifying K^{+} channels. These processes cause cellular hyperpolarization and inhibit neural activity. Thus, the activation of opioid receptors at presynaptic level leads to a reduction of neurotransmitter release (Law et al. 2000). Opioid receptors are also expressed at postsynaptic level and their activation usually produces an inhibitory response. Activation of opioid receptors has the potential to produce profound analgesia, mood changes, physical dependence, tolerance and a hedonic ('rewarding') effect. Among all these functions, it is important to highlight that sustained opioid treatment produces tolerance and can potentially lead to physical dependence (Matthes et al. 1996). Desensitization of MOR seems to be the major factor involved in the development of tolerance since the evidence for receptor down-regulation is not consistent (Christie 2008).

The three families of endogenous peptides POMC, PENK and PDYN generate several final active peptides including beta-endorphin, met- and leu-enkephalin, dynorphins and neo-endorphins, respectively, that exhibit different affinities for each opioid receptor (Kieffer & Gavériaux-Ruff 2002). Beta-endorphin binds with higher affinity to MOR than DOR or KOR. The affinity of met- and leu-enkephalin for DOR is 20-fold greater than that for MOR, and dynorphins are the putative endogenous ligands for KOR

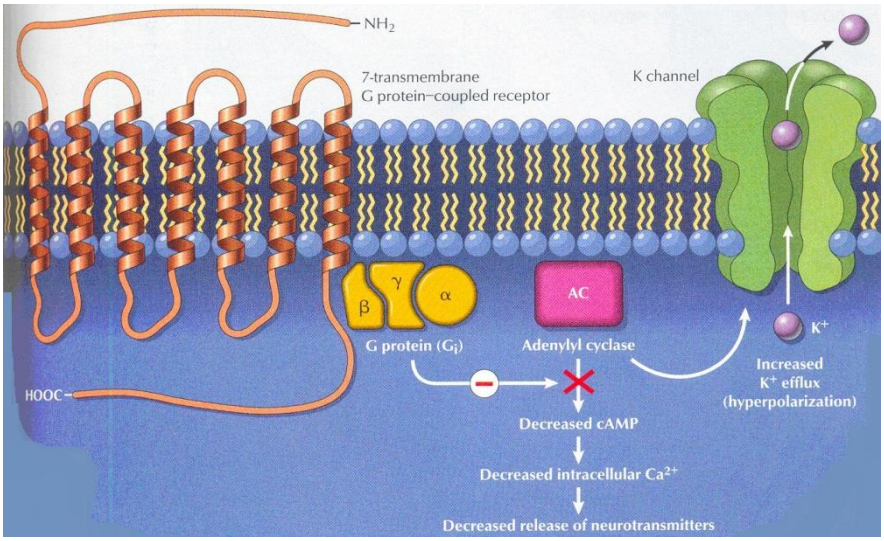


Figure 17. Intracellular signal transduction mechanism mediated by opioid receptors.

(Akil et al. 1996). All these opioid peptides have an N-terminal sequence (Tyr-Gly-Gly-Phe-Met-Leu) indispensable to activate opioid receptors (Akil et al. 1996). Neurons containing enkephalins have been found in many different brain regions, suggesting that these peptides are involved in multiple physiological functions. Indeed, enkephalins control emotional responses by acting in limbic areas, such as the amygdala, NAc and ventral pallidum and also regulate cardiovascular, respiratory, feeding functions by acting on autonomic nuclei in the hypothalamus and brainstem. In addition, enkephalins mediate pain perception by acting in the spinal cord and periaqueductal gray region of the brain. Most enkephalin-containing neurons have short axons, indicating that enkephalins act close to their points of synthesis. The distribution of neurons

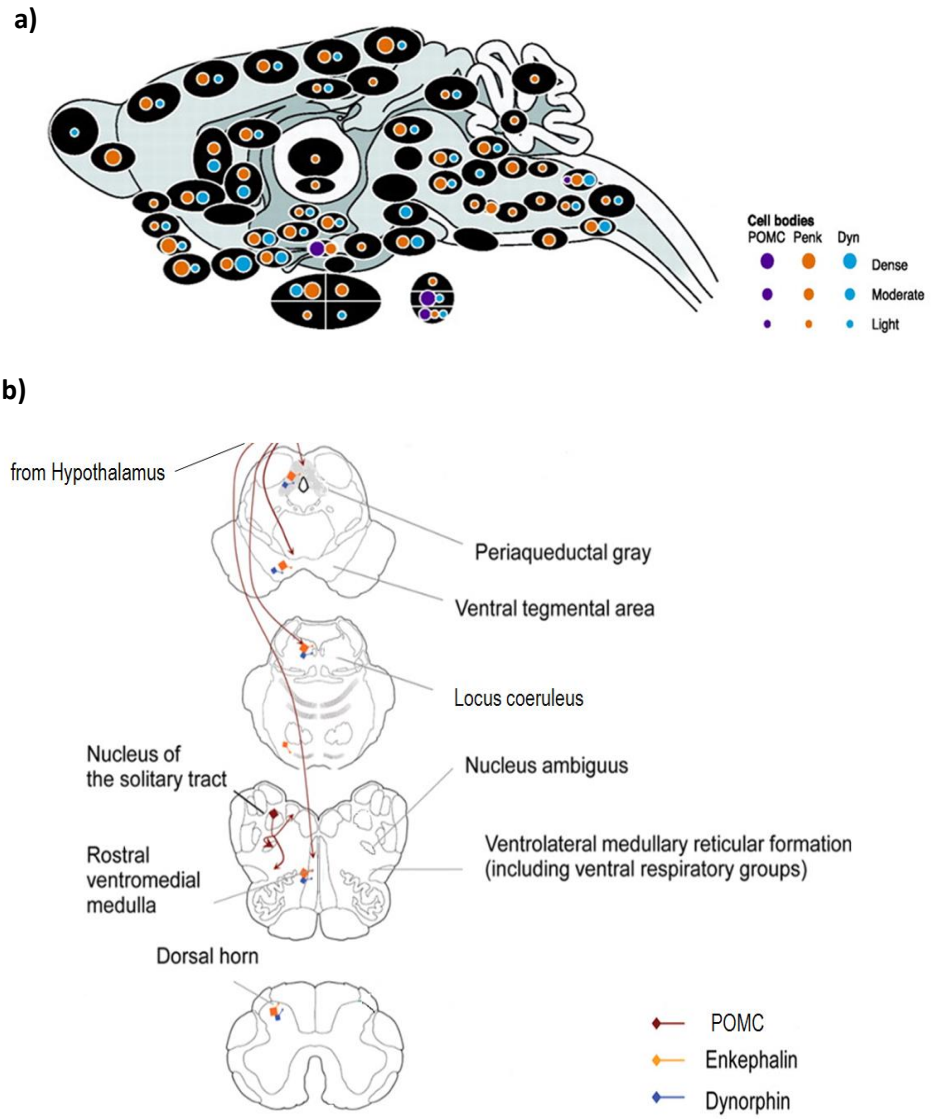


Figure 18. Distribution of opioid peptide neurons in the a) brain (sagittal plane) and b) spinal cord. Densities are represented by symbols of different sizes, from low to high. Map of cell bodies expressing opioid peptides is evaluated by immunohistochemical and in situ hybridization studies (adapted from Le Merrer et al. 2009a).

containing the other two types of opioid peptides, beta-endorphin and dynorphins, is not as diffuse as that of the neurons containing enkephalins. Neurons that contain beta-endorphin are found predominately in the hypothalamus and brainstem. The dynorphin-containing neurons are located primarily in the hypothalamus, NAc and caudate-putamen, and are also present in the PFC. In contrast to the enkephalin-containing neurons, those contain beta-endorphin or dynorphins have long axons that extend to distant brain regions as well as to the pituitary gland, brainstem, and spinal cord, indicating that beta-endorphin and dynorphins act distant from their points of synthesis (Froehlich 1997). Opioid receptors and their endogenous ligands have been demonstrated to play an important role in brain reward processes and to modulate neurochemical effects of multiple drugs of abuse.

3.2 The opioid system in drug addiction

Multiple studies have underlined the important role of opioid receptors and their endogenous ligands in opioid, alcohol, nicotine, cannabinoid and psychostimulant addiction (Conte-Devolx et al. 1981; Ghozland et al, 2002; Lee et al.; Charbogne et al. 2014). Systemic administration of MOR and DOR agonists produce positive reinforcement. These reinforcing effects are due to the indirect increase of ventral striatal DA release in the NAc shell (Di Chiara & Imperato 1988; Fusa et al. 2005), which is suggested to be critical in “drug liking” (Robinson & Berridge 2003; Daglish et al. 2008). Instead, KOR agonists induce aversion and dysphoria (Yoo

et al. 2012) via the suppression of DA release in the NAc (Shippenberg & Elmer 1998; Van Ree et al. 2000). The mechanism by which the endogenous opioid system regulates the reinforcing properties of drugs is related to the anatomical distribution of opioid receptors and neurons containing opioid peptides. MOR and DOR are preferentially located at presynaptic GABAergic neurons of the VTA. Indeed, the activation of MOR and DOR results in the inhibition of GABAergic neurons (Margolis et al. 2008; Johnson & North 1992; Spanagel et al. 1992). The consequence of this inhibition is a reduction of the inhibitory effect on VTA DA neurons which, in turn, is followed by an increased DA release in the NAc. Furthermore, the KOR/dynorphin system is mainly expressed at DA neurons in the NAc (Svingos et al. 2001; Mansour et al. 1994) and the activation of presynaptic KOR inhibits DA release in the NAc (Spanagel et al. 1992; Shippenberg et al. 2007). In agreement, extracellular DA levels in the NAc are elevated in mice lacking KOR (Chefer et al. 2005).

Pharmacological and genetic studies have suggested a role for opioid receptors in the acquisition, maintenance and relapse of drug use (Gerrits et al. 2003). The pharmacological administration of MOR antagonists or MOR genetic deletion attenuates cocaine-, ethanol-, nicotine-, opioid- and THC-induced conditioned place preference (Bechtholt & Cunningham 2005; Ghozland et al. 2002; Soderman & Unterwald 2008; Becker et al. 2002) and reduces cocaine, nicotine, ethanol and amphetamine self-administration (Becker et al. 2000; Liu & Jernigan 2011; Tang et al. 2005; Ward et al. 2003; Mathon et al. 2005; Häggkvist et al. 2009).

Pharmacological and genetic studies have also suggested the involvement of DOR in drug reinforcing effects (Le Merrer et al. 2009a). Naltrindole significantly blocks cocaine- and MDMA-induced place preference (Suzuki et al. 1994; Reid et al. 1995; Belkaï et al. 2009). In addition, DOR antagonists can increase or decrease cocaine self-administration in rats depending on the brain area microinjected (Ward et al. 2003). Indeed, naltrindole decreased cocaine self-administration when injected into the NAc, but it increased this behaviour when administered in the VTA and it had no effect when injected in the amygdala (Ward & Roberts 2007). Different results were obtained in alcohol-seeking behaviour. DOR activation in the VTA robustly decreases ethanol consumption in rats (Margolis et al. 2008), whereas increased ethanol consumption was displayed in constitutive DOR knockout animals (Roberts et al. 2001). DOR-deficient mice also showed increased operant behaviour maintained by nicotine intravenous self-administration, although mainly for lower doses of nicotine (Berrendero et al. 2012). In addition, naltrindole treatment dose-dependently impaired nicotine self-administration (Berrendero et al. 2012), while other pharmacological studies failed to reveal an effect of naltrindole on nicotine self-administration in rats (Liu & Jernigan 2011). DOR does not seem to be involved in THC-induced conditioned place preference (Ghozland et al. 2002).

KOR is also involved in the reinforcing effect of several drugs of abuse such as cocaine, THC, ethanol and nicotine. Several studies have demonstrated that KOR agonists are effective in decreasing cocaine-induced conditioned place preference in rats (Suzuki et al.

1992) and the rate of cocaine self-administration in rats and monkey (Glick et al. 1995; Negus et al. 1997). In addition, the administration of selective KOR agonists decreased amphetamine-induced enhancement of DA extracellular outflow in the ventral ST (Gray et al. 1999). Moreover, KOR has also a critical role in ethanol intake. The selective KOR antagonist norbinaltorphimine selectively decreased ethanol self-administration in alcohol-dependent rats (Walker & Koob 2008) and reduction of KOR mRNA in the VTA and NAc were reported in rats following chronic ethanol exposure (Rosin et al. 1999). In contrast, the role of KOR in the rewarding effects of nicotine is difficult to interpret. Indeed, a high dose of the KOR agonist U50,488 decreased nicotine self-administration, while a low dose tended to increase nicotine self-administration in rats (Ismayilova & Shoaib 2010). Nevertheless, the selective pharmacological antagonism of KOR has no effect on nicotine self-administration (Liu & Jernigan 2012) and nicotine conditioned place preference (Jackson et al. 2010). KOR is critically implicated in the dysphoric effects induced by THC. Indeed, THC-induced conditioned place aversion has been abolished in KOR deficient mice, even though THC-induced conditioned place preference was not modified (Ghozland et al. 2002).

Different drugs of abuse that enhance mesolimbic DA levels also increase dynorphin content (Trigo et al. 2010). Indeed, increased dynorphin in the NAc (Lindholm et al. 2000), and decreased KOR mRNA levels in the VTA and NAc have been reported in rats following chronic ethanol exposure (Rosin et al. 1999). In addition,

the acute administration of MDMA increases PDYN mRNA in the ST and PFC, but not in the NAc (Di Benedetto et al. 2006). Similarly, repeated cocaine administration increases the level of dynorphin, PDYN and preprodynorphin mRNA in the ST (Trifilieff & Martinez 2013). Conversely, a decrease of the dynorphin level was observed in the ST of mice for a protracted time (from 30 min to 72 hours) following discontinuation of chronic administration of nicotine (Isola et al. 2008). The involvement of dynorphin in the dysphoric effects of cannabinoids was revealed in mice lacking the PDYN gene since THC-induced conditioned place aversion was abolished in these mice (Zimmer et al. 2001). Considering these data together, the activation of the KOR/dynorphin system in regions associated with reward might be part of a protective compensatory mechanism to counteract elevated DA level in the NAc due to the action of different drugs of abuse (Yoo et al. 2012).

The regulation of the reinstatement of drug-seeking behaviour by the opioid system confirms the central role of this system in addiction. The relapse induced by cue-stimuli associated with drug use is mainly mediated by MOR and DOR, while stress-induced relapse is generally related to KOR/dynorphin system. Thus, the MOR antagonist naltrexone inhibits cue-induced reinstatement of drugs, such as heroin (Shaham & Stewart 1996), nicotine (Liu et al. 2008), cocaine (Burattini et al. 2008), methamphetamine (Anggadiredja et al. 2004) and alcohol (Ciccocioppo et al. 2002). However, naltrexone does not alter the relapse to these drugs when it is induced by stress (Shaham & Stewart 1996; Ciccocioppo et al. 2002). The DOR antagonist naltrindole inhibits cue-induced

reinstatement of alcohol seeking-behaviour (Ciccocioppo et al. 2002) and abolished the cocaine reinstated effect of a DOR agonist in the conditioned place preference (Kotlinska et al. 2010). Furthermore, KOR blockade by specific antagonists or the deletion of genes encoding both the endogenous opioid peptide dynorphin or KOR inhibits the stress-induced reinstatement of cocaine (Beardsley et al. 2005), alcohol (Funk et al. 2014) and nicotine seeking behaviour (Jackson et al. 2013).

Opioid peptides derived from PENK have been postulated to mediate the reinforcing effects of many drugs of abuse (Berrendero et al. 2005; Marinelli et al. 2005; Shoblock & Maidment 2007). An enhancement of PENK expression has been reported in the ST following acute or chronic nicotine administration in both mice (Dhatt et al. 1995) and rats (Mathieu et al. 1996). Conversely, decreased PENK mRNA levels have been found in the caudate-putamen area in post-mortem brains of humans with a history of cocaine abuse (Hurd & Herkenham 1992). More recent studies have reported an increase of PENK mRNA levels in caudate-putamen after chronic cocaine treatment in rats (Zhang et al. 2012) or no change in this brain area, as well as in the NAc and central nucleus of amygdala in rat after contingent or noncontingent cocaine administration (Ziółkowska et al. 2006). Therefore, changes in PENK mRNA level after chronic cocaine exposure are complex and unclear. In addition, chronic ethanol exposure in rats increases PENK mRNA levels in the central amygdala, but decreases these levels in the NAc (Cowen & Lawrence 2001).

Less consistent changes have been reported for POMC. Thus, no changes in POMC mRNA levels have been observed in the hypothalamus following chronic cocaine exposure and withdrawal (Zhou et al. 2005). In contrast, long-lasting inhibition of POMC gene expression in the mediobasal hypothalamus was reported after chronic nicotine administration (Rasmussen 1998). Interestingly, polymorphisms of the POMC gene might constitute an important risk factor for the development of alcohol dependence in humans (Zhang et al. 2006).

In summary, the opioid system has a key role in drug seeking-behaviour through the modulation of the DA system. In this context, the endogenous opioid system more properly seems to mediate the emotional hedonic/aversive responses to drugs of abuse.

3.3 The opioid system in the control of eating behaviour

3.3.1 The opioid system in the homeostatic control of food intake

Opioid peptides and receptors are located in several brain areas related to the regulation of energy homeostasis and exert a crucial role in the control of food intake. The opioid system modulates feeding mainly in the PVN, LH, ARC, and DMH of the hypothalamus (Bodnar 2004a), as well as in the nucleus tractus solitarius and the mesolimbic system. Activation of MOR in the PVN by local agonist injections produces an increase in food intake. Conversely, blocking MOR or KOR but not DOR in the PVN,

reduces deprivation-induced feeding (Bodnar 2004a). Similarly, the injection of the MOR agonist DAMGO in the nucleus tractus solitarius stimulated food intake, whereas the injection of either the DOR agonist DSLET, or the KOR agonist dynorphins A-(1–17) in the nucleus tractus solitarius had no effect (Kotz et al. 1997). In addition, injections of MOR or DOR, but not KOR agonists in the NAc or VTA, stimulated food intake (Bodnar 2004b), highlighting that the modulation of the reward system by opioid mechanisms participates in feeding behaviour. The precise molecular mechanism by which the opioid system modulates food intake is not clearly understood, although interactions with melanocortin, NPY, AgRP, CART, leptin, PYY, CCK, gherelin, orexin system and insulin among others, have been described. Melanocortins are a family of proteins that reduce appetite, and their precursor, POMC, encodes α-MSH that decreases food intake, and beta-endorphin that influences mood and food intake (Pennock & Hentges 2014). In the PVH, melanocortins act mainly though two receptors to decrease food intake, melanocortin receptor 3 and 4 (Hagan et al. 2001). Interestingly, POMC neurons express melanocortin receptors 3 and 4, but also MOR at pre and post synaptic level. Activation of MOR inhibits the release of endogenous opioids and melanocortins, which underlines the relationships between these two systems (Pennock et al, 2002). The interaction between them has also been supported by the observation that the orexigenic effect of beta-endorphin (MOR ligand) is blunted by an agonist for melanocortin receptors 3 and 4 (Grossman et al. 2003). The opioid system also modulates the effect of other anorexigenic neuropeptides/hormones. Indeed, central

(fourth ventricular) and peripheral injections of naloxone blocked PYY-induced food intake (Hagan & Moss 1993). Previous studies also showed that MOR and DOR, but not KOR agonists, suppress K^+-stimulated release of CCK and substance P from cortical and hypothalamic tissues and this suppression was blocked by naloxone (Micevych et al. 1982; Micevych et al. 1984).

The regulation of the effects of orexigenic peptides/hormones by the opioid system is also been demonstrated. An important central mediator of feeding is NPY. NPY, in the ARC, is a potent orexigen factor and its action is mediated by the opioid system especially by MOR and KOR, as demonstrated by the fact that KOR and MOR antagonists, but not DOR antagonists, blunt NPY-induced feeding (Kotz et al. 1993). Moreover, central and peripheral administration of naloxone decreases NPY-induced feeding behaviour (Rudski et al. 1996; Kotz et al. 1993). It has also been found that long-term morphine treatments increased hypothalamic AgRP gene expression, whereas short-term treatments decreased leptin receptors in the hypothalamus (Anghel et al. 2010). In agreement, combination of MOR and KOR antagonist treatment injected into the third ventricle significantly reduced feeding elicited by AgRP (Brugman et al. 2002). Another central mediator of feeding is the orexin system. Hypothalamic injections of orexin increased enkephalin gene expression in the VTA, PVH and central nucleus of the amygdala. Interestingly, naltrexone also blocked the effects of orexin A when administrated in the NAc of rats (Karatayev et al. 2009). It was also demonstrated that the specific blockade of MOR, DOR and KOR reduced the orexigenic effects of MCH (Lopez et al,

2010) and regulated ghrelin functions (Kawahara et al. 2013). It is well-known that ghrelin stimulates feeding behaviour when injected in the VTA or the NAc (Naleid et al. 2005). Systemic ghrelin administration followed by consumption of regular food increased DA levels in the NAc via preferential activation of MOR, whereas systemic ghrelin administration followed by consumption of palatable food suppressed the increase in DA levels in the same brain area via preferential activation of KOR (Kawahara et al. 2013). However, pre-treatment with naltrexone in the VTA or NAc did not blunt the orexigenic action of ghrelin (Naleid et al. 2005). Pharmacological evidence indicates that the endogenous opioid system also regulates the effects of insulin. Indeed, subcutaneous administration of insulin produced hypoglycemia in rats with a concomitant induction of feeding (Levine & Morley 1981). In this condition, naloxone significantly suppressed eating during the first hour, antagonizing the effects of insulin (Levine & Morley 1981). In addition, the MOR antagonist β-funaltrexamine significantly inhibited insulin-induced feeding during a long time period (6 hours). In contrast, insulin hyperphagia was only transiently (2 hours) inhibited by the selective KOR antagonist nor-binaltorphamine. However, the DOR-antagonistic action of DALCE failed to affect insulin-induced feeding (Beczkowska & Bodnar 1991).

3.3.2 The opioid system in the hedonic control of food intake

The opioid system also plays an important role in the rewarding aspects of eating, modulating the hedonic response promoted by palatable food. The opioid system works together with the DA limbic system to accomplish this physiological role (Olszewski et al. 2011). DA transmission is implicated in the motivation for food, while opioids are possibly involved in the hedonic evaluation of food (Ackroff & Sclafani 2011; Oliveira-Maia et al. 2011). The principal structures involved in these mechanisms are the NAc and the VTA. Indeed, activation of the ventral ST opioid system encodes the positive effect induced by tasty foods and triggers behavioural responses associated with food-seeking. In agreement, the shell of the NAc contains a hedonic hotspot in which the stimulation of opioid receptors increases the "liking" for food reward, measured by the amplification of positive affective orofacial reactions to sucrose in rats (Peciña & Berridge 2005). Indeed, the MOR agonist DAMGO, the DOR agonist DPDPE and the KOR agonist U50488H administered within the rostro-dorsal boundaries of the NAc shell enhanced hedonic reactions to sucrose "liking" responses, whereas all three opioid receptor agonists administered into the caudal half of the medial NAc shell suppressed hedonic reactions to sucrose "liking" responses (Castro & Berridge 2014). In addition, this response is enhanced with DAMGO microinjections in the posterior subregion of the ventral pallidum (Smith 2005), which plays a role in amplifying the rewarding responses (Peciña & Berridge 2005). Other sites were

reciprocally activated in the enhancement of "liking" responses (Smith & Berridge 2007), such as the rostro-dorsal part of the NAc shell projecting to the lateral pre-optic area, anterior and lateral hypothalamus and the lateral septum (Zahm et al. 2013).

Opioids, besides promoting hedonic responses, seem to be also involved in learned-associative appetitive processes that underlie food acceptance and selection (Cottone et al. 2008) through a mechanisms involving orbitofrontal cortex, HCP and amygdala circuits (Volkow & Morales 2015). Indeed, several results support that opioids influence food intake based on flavour preferences (Woolley et al. 2006). A decrease in sucrose preference has been observed after systemic treatments with naltrexone (Parker et al. 1992), whereas morphine injections cause an increase in preferred food intake (Glass et al. 1996a). However, the pattern of effects on preferred versus non-preferred food depends on the site of agonist/antagonist injection: NAc injections of naltrexone decrease intake of the preferred food, whereas injections of naltrexone in the PVH decrease the intake of both, preferred and not preferred foods (Glass et al. 2000). Injections of DAMGO into the NAc preferentially increase intake of food with the preferred flavour (Woolley et al. 2006). In agreement, the blockade of MOR in the shell of the NAc by injection of β-funaltrexamine induced a persistent decrease in the consumption of a palatable glucose solution, with no effect on the intake of standard chow (Le Merrer et al. 2009b). In addition, the administration of the KOR agonist U-50,488H into the NAc of rats decreased the consumption of the

preferred flavour, but increased the intake of the non-preferred flavour food (Le Merrer et al. 2009b).

Several studies also support a role for the opioid system in controlling the intake of specific macronutrients. Thus, morphine increases fat intake and decreases carbohydrate intake, whereas naloxone preferentially decreases fat intake (Marks-Kaufman 1982). In addition, KOR agonists selectively increased the intake of high-fat diet when offered along with a high carbohydrate diet (Romsos et al. 1987).

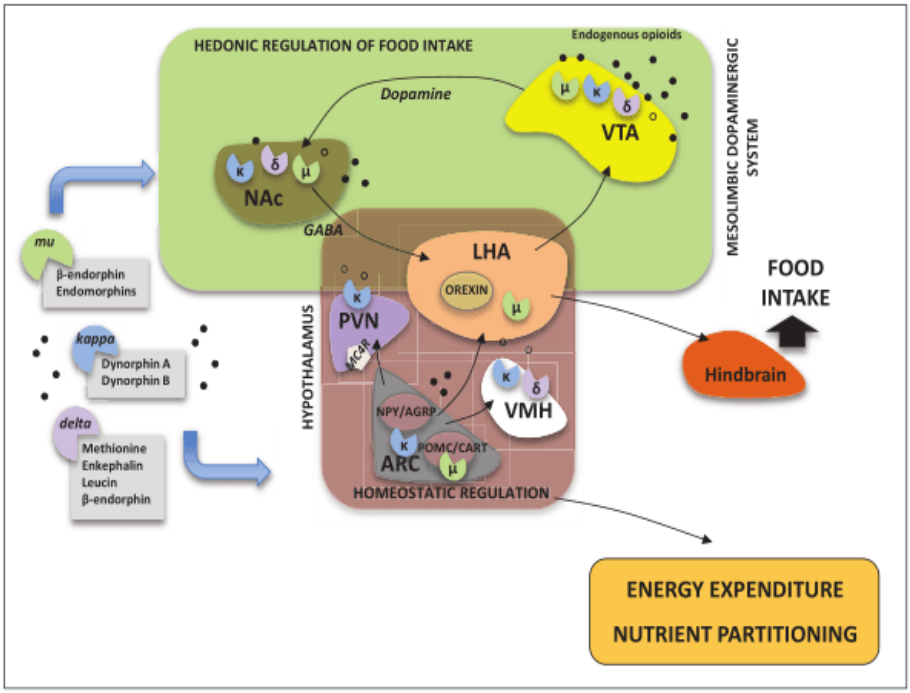


Figure 19. Effects of the opioid system on energy balance. Opioid receptors have been found in the hypothalamus (modulating homeostatic signals) and in extra-hypothalamic areas such as the mesolimbic DA system (regulating hedonic signals) (Nogueiras et al. 2012).

3.3.3 The opioid system in eating disorders

Alterations in the expression of different opioid peptides have been reported in neuropsychiatric conditions associated with eating disorders such as anorexia, bulimia, BED and obesity. Several studies have found that underweight anorexic patients have significantly reduced beta-endorphin levels in the cerebrospinal fluid compared to healthy volunteers. At the same time, beta-endorphin levels remain significantly below normal after short-term weight restoration (Brewerton et al. 1992). Conversely, dynorphin levels have been reported to be normal in the cerebrospinal fluid in all stages of anorexic and in bulimic patients (Swedo et al. 1992).
While there is little evidence of the specific function of the opioid system in anorexia, the involvement of this system in binge-eating episodes has been better described. A reduction in MOR density in the insular cortex has been reported in human patients with bulimia, (Nathan & Bullmore 2009). Moreover, bulimic patients showed a reduction in the size and frequency of binging and purging following naltrexone administration (Kaye et al. 1990; Marrazzi et al. 1995). Neuroadaptations of endogenous opioids and receptors in the medial PFC, such as increased POMC and reduced PDYN gene expression, have been reported in rodents subjected to a binge-eating regime of intermittent access to highly palatable food (Blasio et al. 2014). In addition, the G allele of the OPRM1 (MOR), A118G SNP, related to an exaggerated response after MOR activation, was associated with higher frequency of BED (Davis et al. 2012). Recently, it was also found that obese patients present a down-

regulation of MOR in brain regions relevant for reward processing, including ventral ST, insula, and thalamus associated with unaltered D2R density (Karlsson et al. 2015). The effects of naloxone and naltrexone on feeding behaviour have also been studied in obese patients (Lee & Fujioka 2009; Wolkowitz et al. 1988). Both opioid antagonists suppressed food intake in these patients, and some of the obese patients report decrease in hunger. In spite of these short-term effects, naltrexone fails to produce consistent weight loss in a longer time period (Atkinson et al. 1985; Mitchell et al. 1987). However, combination therapy with naltrexone and bupropion, an antidepressant that selectively binds to the DA transporter, appears to be efficient in obese patients, as previously reported. Taken together, these data indicate that the assessment of the opioid system is an important target in research for eating disorders.

4. THE ENDOCANNABINOID SYSTEM

4.1 Components and physiological role

The ECS is a neuromodulatory system in the mammalian physiology that comprises cannabinoid receptors, their endogenous ligands, mainly anandamide (AEA) and 2-arachidonoylglycerol (2-AG), as well as their synthesis and degradation enzymes (Mechoulam & Parker 2013).

4.1.1 Cannabinoid receptors

Two main subtypes of cannabinoid receptor have been identified: cannabinoid receptor 1 (CB1R) (Matsuda et al. 1990) and cannabinoid receptor 2 (CB2R) (Munro et al. 1993). Both receptors belong to the seven transmembrane domain receptor families associated with G proteins. CB1R is highly expressed in the central nervous system (Herkenham et al. 1991; Tsou et al. 1998) and has been found in the basal ganglia (caudate-putamen, globus pallidus, ectopeduncular nucleus, substantia nigra) and the molecular layer of the cerebellum, explaining the effects of cannabinoids on locomotor activity (Compton et al. 1990). High receptor density has also been found in the HCP and PFC, indicating that cannabinoids are involved in cognition, memory and inhibitory control (Herkenham et al. 1991). The expression of CB1R has also been observed in the limbic system, including the amygdala, hypothalamus, and NAc

(Compton et al. 1990; Tsou et al. 1998), explaining the role of the cannabinoid system in mood and emotional behaviour. In addition, CB1R in the NAc is associated with the brain reward system, and its expression in the hypothalamus correlates with the role of cannabinoids in food intake control and energy homeostasis (Osei-Hyiaman et al. 2006; Solinas et al. 2008). The presence of CB1R has been detected in the thalamus, periaqueductal gray, Rostral ventromedial medulla and dorsal horn of the spinal cord, important sites in pain transmission pathways (Kano et al. 2009; Tsou et al. 1998). In all these areas, CB1R is mainly expressed at the presynaptic level in GABAergic and glutamatergic neurons (Howlett 2002). CB1R has also been found in multiple peripheral locations, such as adipocytes (Cota et al. 2003), liver (Osei-Hyiaman et al. 2006), pancreas (Bermúdez-Silva et al. 2008), lungs, smooth muscle, gastrointestinal tract (Calignano et al. 1997), vascular endothelium (Liu et al. 2000), human eye (Straiker et al. 1999), and other peripheral tissues. Peripheral CB1R plays an important role in the modulation of metabolism. Thus, the activation of CB1R promotes lipogenesis, lipid storage, insulin and glucagon secretion, and adiponectin modulation (Bermúdez-Silva et al. 2008; Cota et al. 2003; Osei-Hyiaman et al. 2006). Moreover, CB1R exerts proinflammatory effects in the cardiovascular system (Slavic et al. 2013) and its presence in endothelial cells of various vascular beds (Golech et al. 2004) contributes to its vasodilatory actions (Wagner et al. 1998). Activation of CB1R in various structures of the human eye (Straiker et al. 1999), such as the ciliary body (Straiker et al. 1999), decreases intraocular pressure

(Oltmanns et al. 2008; Laine et al. 2003). In addition, the presence of CB1R in reproductive organs highlights the involvement of this receptor in male fertility and in several critical stages of pregnancy in female (Maccarrone et al. 2015). CB1R is also involved in other peripheral functions, such as immunosuppression, skin proliferation, differentiation and cell survival as well as intestinal motility and changes in adrenal functions (Maccarrone et al. 2015; Howlett et al. 2004), among others.

The CB2R has been mainly found in immune cells and tissues, namely the tonsils, spleen, thymus and various circulating immune cell populations (Galiègue et al. 1995). The presence of CB2R in brain neurons has been a controversial topic over the last few years. Some studies have reported the expression of CB2R in structures such as PFC, ST, HCP, amygdala, brainstem, dorsal root ganglia and lumbar spinal cord (Van Sickle et al. 2005; Gong et al. 2006a). Moreover, immunohistochemical analysis reveals CB2R immunostaining in apparent neuronal and glial cells in a number of brain areas in rats (Gong et al. 2006b). Indeed, this receptor can modulate neuroinflammatory responses upon microglial activation (Atwood & Mackie 2010). The presence of CB2R has also been demonstrated at the central level on vascular endothelial cells (Golech et al. 2004). The functional role of CB2R in the central nervous system has not been yet clarified, although it has been demonstrated that activation of CB2R decreases locomotion (Gong et al. 2006a), neuropathic and osteoarthritic pain (Racz et al. 2008; La Porta et al. 2013; Elmes et al. 2004) and it may exert a

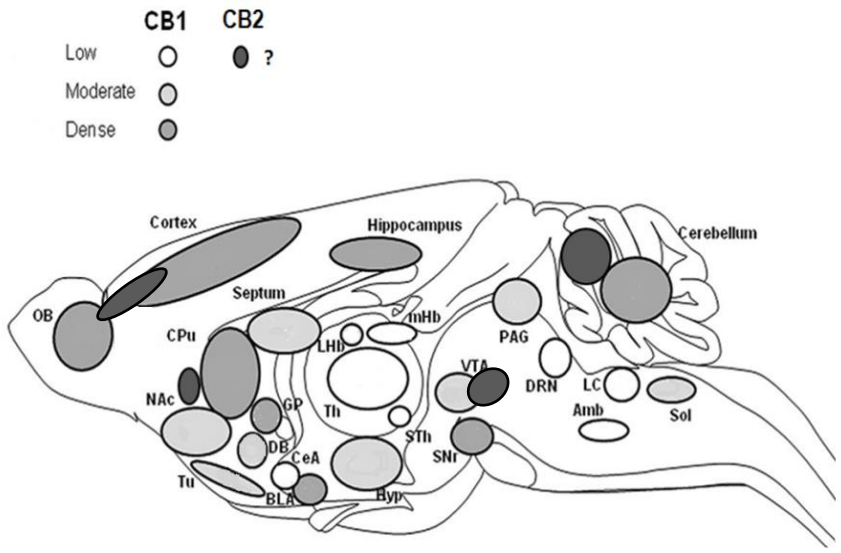


Figure 20. Schematic representation of the distribution of CB1R and CB2R in the brain. CB1R distribution is indicated by circle shapes with low (white), moderate (gray) and high (dark gray) expression. Major localization of CB1R (mRNA and protein) is in cortical areas, hippocampus, amygdala, striatum, and cerebellum. Moderate and low expression levels are observed in thalamic, hypothalamic, and brainstem regions. Interestingly, CB2R is expressed in brain areas overall in glial cells, although quantitative Real Time Polymerase chain reaction experiments also demonstrated the expression of CB2R mRNA in the brainstem, cortex and cerebellars granule cells (Van Sickle et al 2005). Amb, ambiguous nucleus; BLA, basolateral amygdala; CeA, central amygdala; CPu, caudate-putamen; DB, diagonal band; DRN, dorsal raphe nucleus; GP, globus pallidus; Hyp, hypothalamus; LC, locus coeruleus; LHb, lateral habenular nucleus; mHb, medial habenular nucleus; NAc, nucleus accumbens; OB, olfactory bulb; PAG, periaqueductal gray; SNr, substantia nigra pars reticulate; Sol, nucleus of the solitary tract; STh, subthalamic nucleus (ventral thalamus); Th, dorsal thalamus; Tu, olfactory tubercle; VTA, ventral tegmental area (adapted from Befort 2015).

regulatory function in drug rewarding effects (Xi et al. 2011a; Aracil-Fernández et al. 2012). Stimulation of CB1R and CB2R activates a great variety of signal transduction pathways via Gi/o

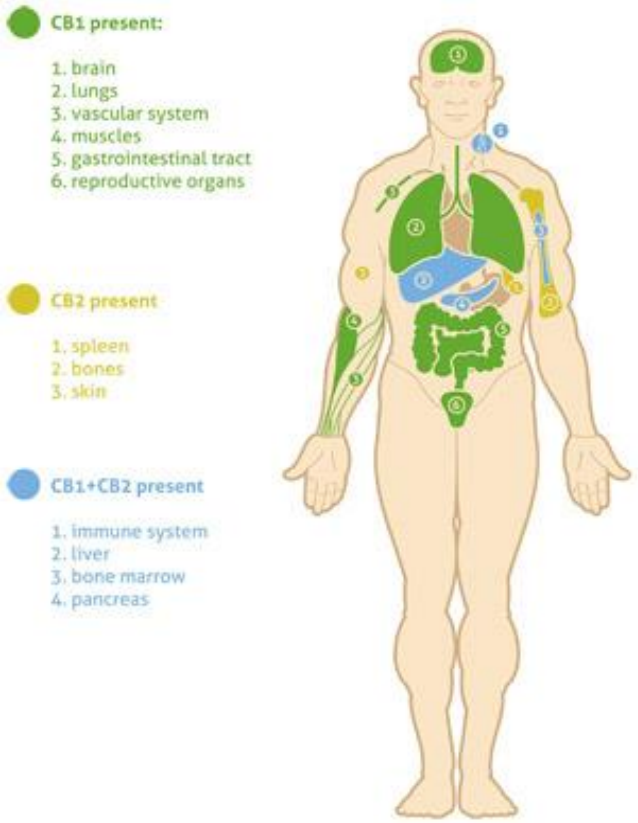


Figure 21. Schematic representation of the peripheral distribution of CB1R and CB2R. The localization of these receptors in different peripheral locations highlights their involvement in several physiological functions.

proteins. When the cannabinoid receptor is activated, the βγ subunit of the G protein dissociates and the α subunit inhibits the enzyme adenylate cyclase (Howlett 2005). This inactivation of adenylate

cyclase leads to a decrease of cAMP production from adenosine-5'-triphosphate, and subsequent PKA activity is inhibited (Bonhaus et al. 1998). Reduction of PKA activity promotes intracellular responses, such as modification of K^+ and Ca^{2+}channel functions and of focal adhesions kinase that is an important factor for integrating cytoskeletal changes with signal transduction events. On the other hand, the βγ subunit activates G protein-gated inwardly rectifying K^+ channels and inhibits voltage gated Ca^{2+} channels. The βγ subunit also activates multiple members of the mitogen-activated protein kinase family, including extracellular signal-regulated kinase 1 and 2, p38 and c-Jun N-terminal

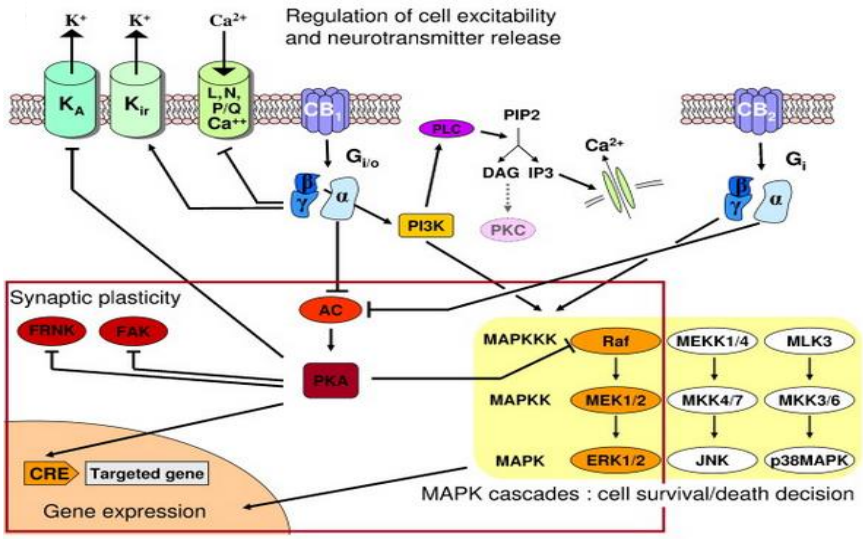


Figure 22. Cannabinoid receptor signalling. Both CB1 and CB2 cannabinoid receptors are associated with Gαi-dependent inhibition of adenylyl cyclase activity and Gβγ-dependent activation of the different mitogen-activated protein kinases cascades (adapted from Bosier et al. 2010).

kinase (JNK) as signalling pathways to regulate nuclear transcription factors (Howlett 2005). Furthermore, cannabinoids could activate protein kinase c signalling in vitro (Hillard & Auchampach 1994). The effects of cannabinoids on these multiple families of kinases indicate the relevance of changes on protein phosphorylation in the mechanism of action of these compounds. These cascades of signalling participate in the inhibition of synaptic transmission mediated by cannabinoid receptors, consisting in the hyperpolarization of the neural membrane and consequent decrease of neurotransmitter release.

Recent evidence suggests the existence of additional cannabinoid-like receptors, distinct from CB1R and CB2R. Indeed, the orphan receptor GPR55 (Sawzdargo et al. 1999) is widely expressed in the central nervous system and peripheral tissues and has been proposed as a new cannabinoid receptor. This receptor has been tentatively classified as a cannabinoid receptor based on its activation by THC and synthetic cannabinoids (Lauckner et al. 2008), although its natural ligand appears to be the lysophospholipid, lysophosphatidylinositol (Oka et al. 2007; Sylantyev et al. 2013). The role of GPR55 has not been thoroughly characterized and it may be involved in pain transmission considering its major expression in dorsal root ganglia neurons (Lauckner et al. 2008). However, several experiments have also demonstrated the role of GPR55 in neuroprotection (Kallendrusch et al. 2013), energy homeostasis (Simcocks et al. 2014), inflammatory processes (Lanuti et al. 2015; Schicho & Storr 2012), and cancer (He et al. 2015).

Moreover, other G-protein coupled receptors (GPR) GPR1 and GPR119, interact with atypical cannabinoids. Besides GPRs, the transient receptor potential vanilloid type 1 (TRPV1) channel has also been considered as a potential cannabinoid-like receptor (Di Marzo & De Petrocellis 2010).

4.1.2 Endogenous cannabinoid ligands

Endogenous cannabinoid receptor ligands derive from arachidonic acid. AEA acts as a partial agonist at CB1R and CB2R and it is an endogenous ligand also for TRPV1. AEA is obtained after the hydrolysis of N-acylphosphatidylethanolamine by NAPE-PLD enzyme. 2-arachidonoylglycerol (2-AG) is the most abundant endocannabinoid in the brain and acts as a full agonist for CB1R and CB2R. 2-AG is synthesized from AA-containing membrane phospholipids through the action of phospholipase C, leading to the formation of diacylglycerol and then through the diacylglycerol lipases. AEA and 2-AG can be degraded by fatty acid amide hydrolase and monoacylglycerol lipase, respectively. AEA and 2-AG might also become substrates for lipoxygenases (van der Stelt et al. 2002), cyclooxygenases-2 (Rouzer & Marnett 2011) and cytochrome P450s (Snyder et al, 2010) when monoacylglycerol lipase or fatty acid amide hydrolase activity is suppressed. Endocannabinoids are not stored and they are generated on demand in response to depolarization-induced increases in intracellular Ca^{2+} (Witting et al. 2004). The principal mechanism by which endocannabinoids regulate synaptic function

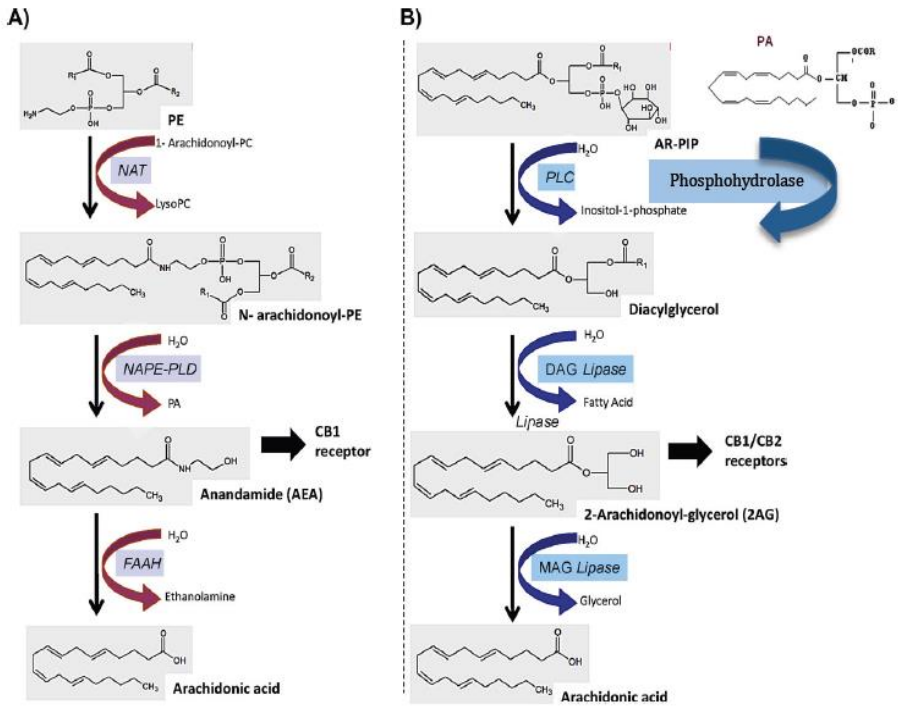


Figure 23. Summary of the major pathways involved in the biosynthesis and degradation of: AEA (A) and 2-AG (B). PE, phosphatidylethanolamine; PC, phosphatidylcholine; NAT, N-acyl-transferase; NAPEselective phospholipase D, N-acylphosphatidylethanolamine hydrolysing phospholipase D; PA, phosphatidic acid; FAAH, fatty acid amide hydrolase; phospholipase C; AR-PIP, arachidonic acid - containing inositol phospholipids; diacylglicerol, diacylglycerol; MAG, monoacylglycerol (Lipina et al. 2012).

is through retrograde signalling (Kano et al. 2009). Postsynaptic activity leads to the production of endocannabinoids that move backwards across the synapse, bind presynaptic cannabinoid receptors, and decrease neurotransmitter release.

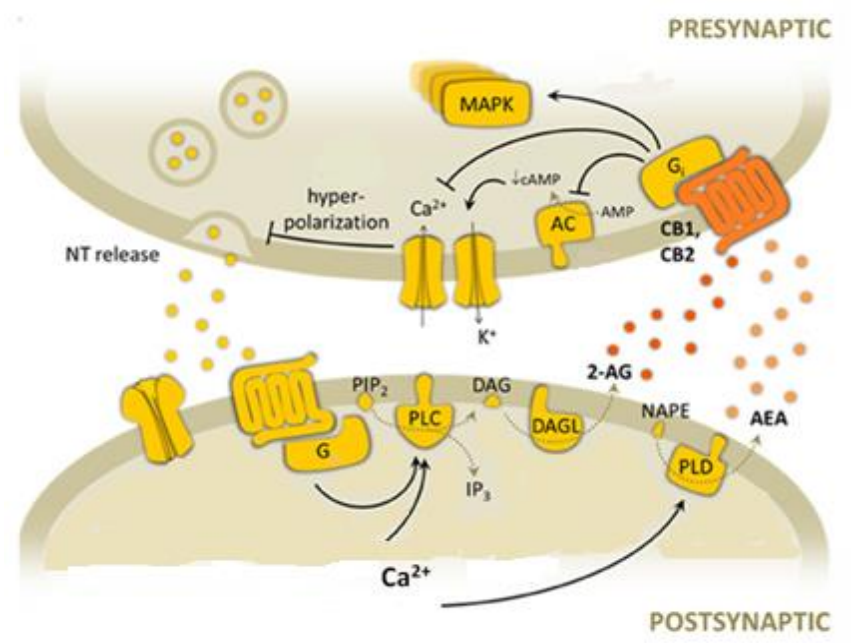


Figure 24. Overview of the retrograde modulation of neurotransmitter release by ECS. The neurotransmitter is released from presynaptic terminals and stimulates ionotropic and/or metabotropic receptors. High Ca^{2+} concentration stimulates endocannabinoid synthesis in the post synaptic terminal through phospholipase C and PLD. 2-AG synthesis could be also mediated by G-protein activation, depending on the receptor. Endocannabinoids are released from the post synaptic terminal to the synaptic cleft and activate presynaptic CB1R and CB2R. Some of the main downstream consequences of CBR activation are: inhibition of AC activity; membrane hyperpolarization after modulation of K^+ and Ca^{2+} channels, and subsequent inhibition of neurotransmitter release; activation of protein kinase cascades, such as mitogen-activated protein kinases pathway (adapted from Flores et al. 2013).

There is evidence suggesting that endocannabinoids could also modulate synaptic functions in a non-retrograde or autocrine manner by engaging TRPV1 and CB1R located within postsynaptic cells (Di Marzo & De Petrocellis 2010). In addition, recent studies

indicate that endocannabinoids could act via astrocytes to indirectly modulate presynaptic or postsynaptic function (Hegyi et al. 2012; Stella 2010).
Dysregulation in the ECS has been linked to both drug addiction and eating disorders (Maldonado et al. 2006; Marco et al. 2012).

4.2 The endocannabinoid system in drug addiction

The ECS is implicated in several phases of the addictive process and this system has been considered an important common neuronal substrate of addiction (Maldonado et al. 2006). Firstly, the ECS is involved in the initial step of addiction, acting as a crucial substrate mediating positive reinforcement (Cheer et al. 2007). The role of the ECS in brain reward processes is not limited to cannabinoid drugs, but also to natural rewards (Sanchis-Segura et al. 2004) and to different classes of drugs of abuse including nicotine, psychostimulants, alcohol and opioids (Maldonado et al. 2006). Genetic deletion of CB1R impairs acquisition of the conditioning place preference and self-administration maintained by different drugs of abuse (Maldonado et al. 2006). The modulatory role of the ECS on the primary rewarding effects of drugs of abuse might mainly depend on the ability of this system to regulate DA transmission in the mesocorticolimbic circuit (Gardner 2005), and in particular the phasic DA release (Cheer et al. 2007). Indeed, CB1R negatively modulates the glutamatergic excitatory and GABAergic inhibitory synaptic inputs into the DA neurons of the VTA, acting as a retrograde feedback mechanism. The final effects

of ECS on the modulation of DA activity depend on the functional balance between the inhibitory GABAergic and excitatory glutamatergic input to the VTA, with the latter being predominant (Maldonado et al. 2006). Thus, the endogenous cannabinoid system seems to be a crucial substrate mediating the positive reinforcement. The ECS is also involved in the motivation to seek the drug by a mechanism independent from release of DA in the NAc. CB1R is present in the PFC, an important brain area involved in the reinforcing value of rewards, in decision making and expectation, as well as in the mediation of 'hedonic experience' (Kringelbach 2005). Endocannabinoids could be involved in the motivation to obtain the drug by linking reward to a 'hedonic experience' (Maldonado et al. 2006). This role is highlighted by a reduction of the breaking point on a PR schedule task mediated by the blockade of CB1R (Solinas et al. 2003) when rodents are trained to self-administer psychostimulants and opioids (Solinas et al. 2003; Soria et al. 2005), which might also be the case for other drugs of abuse. Moreover, the ECS plays a role in the reinstatement of drug consumption, in which inactivation of CB1R in mice strongly reduces drug priming and cue-induced reinstatement of drug-seeking behaviour of almost all drugs of abuse (De Vries & Schoffelmeer 2005). Indeed, endocannabinoids acting as retrograde

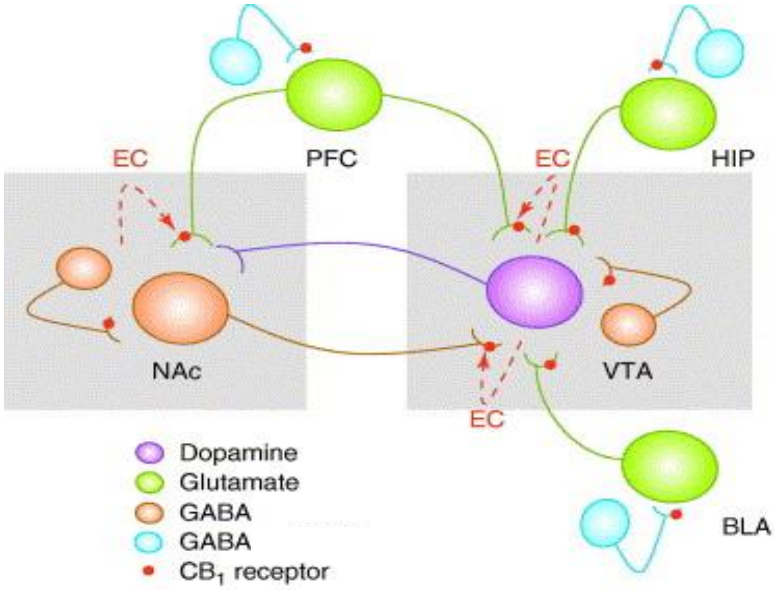


Figure 25. Possible sites of endocannabinoid action in modulation of drug rewarding effects. In the ventral tegmental area (VTA), CB1R is located on presynaptic glutamatergic and GABAergic neurons. Activation of CB1R in the VTA by endocannabinoids (EC; broken red arrows) produces inhibition of GABA release, thus removing the inhibitory effect of these GABAergic cells on DA neurons. In addition, the increase of DA neuron activity induces release from the DA cells of endocannabinoids that, acting in a retrograde manner on presynaptic CB1R, inhibit both inhibitory GABAergic and excitatory glutamatergic inputs to VTA DA neurons. Glutamatergic projections from the basolateral amygdala (BLA) and hippocampus (HIP), which are involved in motivation and memory processes related to drug rewarding effects, are also under the control of CB1R, through a presynaptic inhibitory action. In the nucleus accumbens (NAc), endocannabinoids behave as retrograde modulators acting mainly on CB1R on the axon terminals of glutamatergic neurons. The subsequent inhibition of glutamate release inhibits the GABAergic neurons that originate in the NAc and project to the VTA, thus indirectly activating VTA DA neurons (adapted from Maldonado et al. 2006).

messengers mediate LTP and LTD of synaptic transmission in several reward and memory related brain areas, including the NAc, PFC, amygdala and HCP (De Vries & Schoffelmeer 2005), as later discussed. These effects of endocannabinoids on synaptic plasticity might consolidate the reward-driven behaviour required to establish the addictive processes (Maldonado et al. 2006). Thus, the ECS could represent a crucial neurobiological substrate underlying the long-term behavioural alterations that characterize addiction, such as persistent relapse.

The ECS is also involved in the development of tolerance and physical dependence after chronic cannabinoid consumption. Tolerance is essentially due to adaptative phenomena consisting in pharmacodynamic events such as down-regulation/desensitization of cannabinoid receptors, although evidence exists on additional pharmacokinetic implications (Gonzalez et al. 2005). Tolerance and physical dependence often develop concomitantly and, in some cases, the severity of the physical withdrawal syndrome is directly related to the magnitude of tolerance. Thus, chronic cannabis use leads to adaptative changes in endocannabinoid signalling that could contribute to the development of cannabis physical dependence (Piomelli 2004). In addition, the ECS is also involved in the withdrawal syndrome of several drugs of abuse such as morphine, heroin and ethanol, among others (Maldonado et al. 2006; Naassila et al. 2004). Therefore ECS is an important neurophysiological system underlying drug negative reinforcement.

CB1R and endocannabinoids play a crucial role in the modulation of brain synaptic functions and plasticity (Castillo et al. 2012). Indeed, the ECS induces short-term plasticity by:

1) depolarization-induced suppression of inhibition through modulation of GABA presynaptic release;

2) depolarization-induced suppression of excitation via inhibition of glutamate presynaptic release.

In addition, the ECS is also responsible for the induction of LTD of GABAergic (in the amygdala: Marsicano et al. 2002, and in the HCP: Chevaleyre & Castillo 2004) and glutamatergic (in the ST: Gerdeman et al. 2002, in the NAc: Robbe et al. 2002 and in the cortex: Sjöström et al. 2003) transmission, following the activation of postsynaptic mGluR1. It is tempting to speculate that 2-AG may be the endocannabinoid mediating this process (Kano 2014). The difference between endocannabinnoid-mediated LTD and endocannabinnoid-mediated short-term plasticity relies on the duration of CB1R activity, which engages distinct signalling events in the same target neuron leading to a short or long suppression of neurotransmitter release (Diana & Marty 2004). In addition, the structural plasticity modification mediated by the ECS has been described as a consequence of this synaptic function (Díaz-Alonso et al. 2012).

The potential involvement of CB2R in drug addiction has been suggested in the last years. Indeed, a role of CB2R in the rewarding and motivational effects induced by drugs of abuse has been suggested (Navarrete et al. 2013; Zhang et al. 2014), even though it needs to be better clarified. Accordingly, several contradictory

results have been reported in the scientific literature (Katia 2015; Onaivi et al. 2008). Indeed, genetic deletion or pharmacological blockade of CB2R results in impairment in the acquisition of nicotine self-administration and in reduced nicotine withdrawal syndrome (Navarrete et al. 2013). However, the activation of CB2R does not seem to affect nicotine self-administration and reinstatement of nicotine-seeking behaviour in a previous study (Gamaleddin et al. 2012a). Moreover, activation of CB2R inhibited cocaine self-administration and cocaine-enhanced locomotion (Xi et al. 2011b). In agreement, transgenic mice overexpressing CB2R in the central nervous system showed a reduction of cocaine-induced place preference, self-administration and locomotor sensitization (Aracil-Fernández et al. 2012). On the other hand, activation of CB2R is involved in cocaine priming-induced reinstatement of cocaine-seeking behaviour, but not in cue-induced reinstatement (Adamczyk et al. 2012). Furthermore, CB2R expression seems to be modified by different drugs of abuse. An increase of CB2R gene expression in the whole mouse brain were reported after chronic treatment with heroin or cocaine and decreased CB2R gene expression in ST and ventral midbrain of mice were revealed after chronic ethanol intake (Onaivi et al. 2008).

In conclusion, the ECS participates in the addictive properties of all prototypical drugs of abuse through direct involvement in primary drug rewarding effects, in the motivation to seek the drug and in relapse to drug-seeking behaviour participating in the motivational effects of drug-related environmental stimuli and drug re-exposure,

probably by modulation of the synaptic plasticity underlying memory processes.

4.3. The endocannabinoid system in eating behaviour

4.3.1 The endocannabinoid system in the homeostatic control of food intake

The ECS is a crucial element in the regulation of energy balance and food intake in the central nervous system and peripheral tissues (Di Marzo 2008; Heyman et al. 2012; Pagotto et al. 2006). At the central level, its role in the homeostatic regulation of food intake is due to the expression of the CB1R in hypothalamic circuits (Horvath 2003) and in brainstem structures such as the nucleus tractus solitarius that can sense signals from different peripheral tissues that participate in energy balance (e.g. gastrointestinal tract) (Berthoud 2006). In the nucleus tractus solitarius, CB1R is present in afferent and efferent neurons (Tsou et al. 1998) modulating both glutamatergic and GABAergic transmission in these cells (Roux et al. 2009). CB1R expression in vagal afferent neurons is increased in fasting and decreased after refeeding under the control of CCK (Burdyga et al. 2004).

CB1R activation in the hypothalamus enhances appetite by regulating the response of several orexigenic and anorectic mediators (Di Marzo & Matias 2005; Kirkham et al. 2002). Thus, CB1R activity enhances the release of several orexigenic agents, such as AgRP, orexins and MCH (Matias et al. 2008). The

orexigenic effects of endocannabinoids could also be mediated in part by NPY since stimulation or blockade of hypothalamic CB1R increases or decreases the level of this peptide, respectively (Verty et al. 2004). In addition, CB1R regulates the activation of neuronal populations with anorexigenic properties. Indeed, exogenous cannabinoids inhibit glutamatergic signalling in POMC neurons via CB1R stimulation, thus reducing the release of melanocortins (Hentges et al. 2005). Furthermore, CB1R is expressed in CART and CRH neurons and the endogenous cannabinoid signalling may promote appetite by decreasing these satiety signals (Cota et al. 2003). Functional interactions between the ECS and the satiety system is also supported by the observation that defect in leptin signalling is associated with elevated hypothalamic levels of endocannabinoids (Di Marzo et al. 2001a).

At the peripheral level, the ECS is involved in the reduction of energy expenditure acting on the adipose tissue, liver, skeletal muscle, gastrointestinal tract and pancreas (Matias et al. 2008). CB1R activation in the adipose tissue and liver increases expression of proteins involved in fatty acid synthesis, leading to enhanced lipid levels and fat accumulation (Matias et al, 2008). In the endocrine pancreas, cannabinoid receptors modulate insulin secretion by regulating glucose-induced Ca^{2+} transients (Li et al. 2011). Endocannabinoid signalling in the gastrointestinal tract is involved in the inhibition of gastric emptying and intestinal motility (Davis & Perkins; 2007) as well as the release of enteroendocrine peptides such as CCK (Sykaras et al. 2012). In addition, in the small intestine activation of CB1R serves as an orosensory positive

feedback mechanism that facilitates food intake (DiPatrizio et al. 2011). In skeletal muscle cells, endocannabinoids reduce mitochondrial activity, insulin signalling and glucose uptake via activation of CB1R, possibly through inhibition of IRS1 phosphorylation and insulin-dependent ERK activation (Lipina et al. 2010).

The role of CB2R in the metabolic control and food intake at the central level is still not understood and studies have reported contrasting results concerning this issue. Indeed, previous studies showed that overexpression of brain CB2R could produce a reduction in food intake. This decrease eventually leads to a loss of body weight gain and increase basal glucose level, with transgenic mice showing chronic hyperglycaemia and glucose intolerance (Romero-Zerbo et al. 2012). In the same line, the activation of CB2R increases obesity-associated insulin resistance and the expression of inflammatory markers in adipose tissue, but not in the liver (Deveaux et al. 2009a). On the other hand, the pharmacological CB2R antagonist inhibits food consumption in mice (Emmanuel S. Onaivi et al. 2008). Moreover, CB2R deficient mice fed with a high-fat diet showed a reduction of adipose tissue, hepatic inflammation and insulin resistance when compared to wild-type mice under similar experimental conditions (Agudo et al. 2010a; Deveaux et al. 2009a).

All this evidence demonstrates that central and peripheral CB1R act in a coordinated fashion to regulate energy homeostasis and eating behaviour. More studies are needed to understand the exact implication of the CB2R in these functions.

4.3.2 The endocannabinoid system in the hedonic control of food intake

Multiple studies have demonstrated the role of the ECS in the modulation of hedonic aspects of eating (Cota et al. 2003). Indeed, CB1R is expressed in brain areas directly involved in rewarding processes. Microinfusions of AEA in the NAc shell increase positive responses to sucrose and enhance the intake of sweet solutions (Mahler et al. 2007; Shinohara et al. 2009). In agreement, blockade of CB1R activity in the NAc shell of rodents decreases palatable food intake (Melis et al. 2007). The ECS in the mesocorticolimbic system mediates the motivation to obtain palatable food. Indeed, inactivation of CB1R decreases operant responses in a PR task maintained by palatable food (Hernandez & Cheer 2012). This could be related to the decrease of palatable food-induced DA release in the NAc shell due to the blockade of CB1R activity (Melis et al. 2007). In contrast, cannabinoid agonists increase the hedonic value of food and induce intake in satiated animals where the motivation to eat is minimal (Higgs et al. 2003).
Prolonged consumption of palatable food produces alterations of the endocannabinoid signalling, not only in the NAc (Bello et al. 2012), but also in several regions of the mesocorticolimbic circuit. A down regulation of CB1R expression was found in the HCP (Harrold et al. 2002), cingulate cortex and VMH (Timofeeva et al. 2009) of animals fed a high palatable diet. This effect can be considered to be part of a compensatory mechanism aimed to counteract increased level of endocannabinoids resulting from the consumption of these

palatable foods. Moreover, elevated 2-AG and CB1R levels have been observed in the central amygdala of rats during abstinence from palatable food (Blasio et al. 2013).

The ECS is also involved in the orosensorial aspect of food intake making the substances more palatable (Arnone et al. 1997) due to the CB1R presence in taste buds, and its activation enhances neural responses to sweet foods (Yoshida et al. 2009). Indeed, several studies have reported that THC predominantly increased the consumption of sweet foods rather than less palatable ones (Koch & Matthews 2001). In addition, CB1R is present in the parabrachial nucleus, a hindbrain area that integrates taste information. Local administration of 2-AG in this brain area increases intake of palatable fat-rich diet, but not standard chow, revealing a key role of the ECS in gating neurotransmission inherent to fat and sweet taste (Dipatrizio & Simansky 2008).

The possible involvement of CB2R in the regulation of the reinforcing and motivational properties of food has still not been investigated. However, deletion of CB2R seems to be a protective factor in the development of diet-induced obesity in rodents, although CB2R knockout and wild-type mice showed similar intake of palatable high-fat food (Agudo et al. 2010; Deveaux et al. 2009).

In summary, the ECS participates in the homeostatic, pleasurable and motivational aspects of food intake. The extended role of the ECS in the control of eating behaviour supports the hypothesis that alterations of this system may lead to the development of eating disorders.

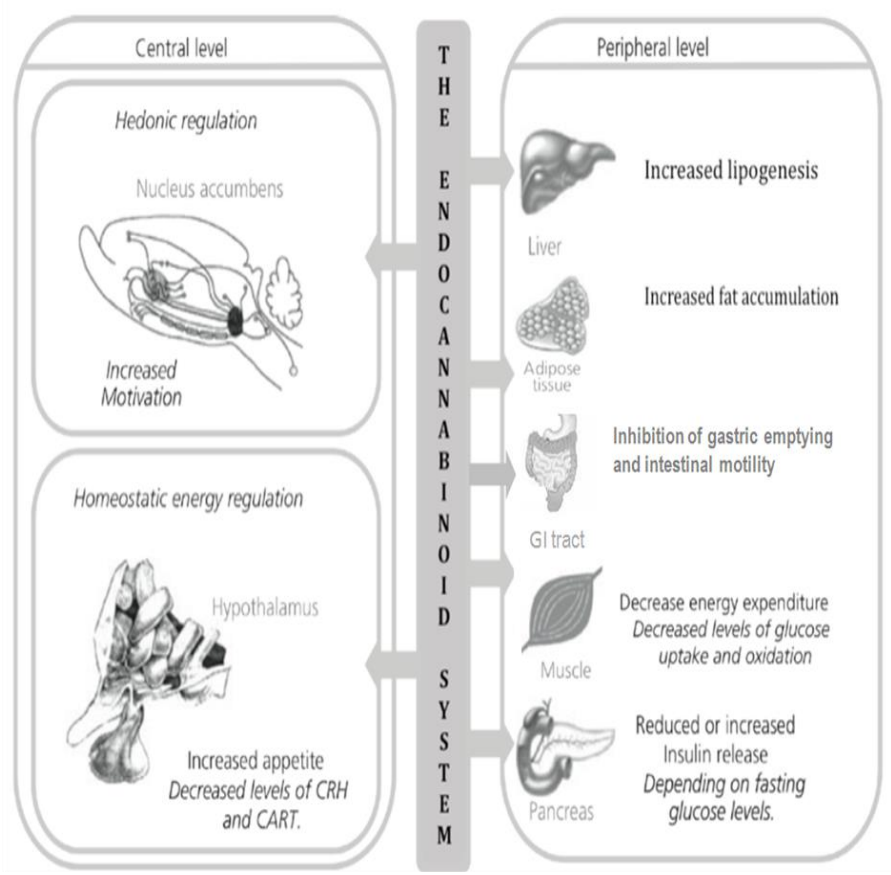


Figure 26. Central and peripheral actions of the endogenous cannabinoid system (ECS). Schematic diagram showing the location and function of the ECS in different central brain structures and peripheral organs involved in the control of food intake and energy metabolism (adapted from Matias et al. 2008).

4.3.3 The endocannabinoid system in eating disorders

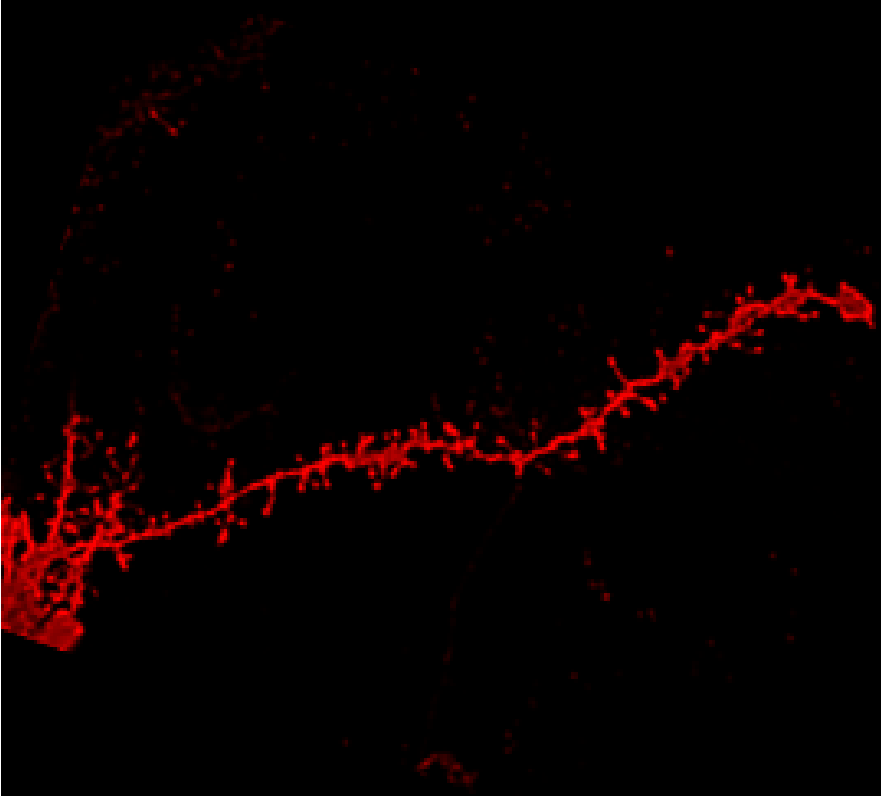
The widespread role of the ECS as a modulator of homeostatic and hedonic aspects of eating has promoted investigations to understand the involvement of this system in eating disorders. A possible

hyperactivation of the ECS is observed in obesity (Matias & Di Marzo) and a significant elevation of AEA plasma levels has been detected in anorexics, binge-eaters and some obese patients, inversely correlated with plasma leptin concentrations (Monteleone et al. 2005). Animal studies using CB1R-deficient mice and human studies also confirm the important role of this receptor in the pathophysiology of obesity and other eating disorders. Indeed, elevated levels of CB1R mRNA have been detected in the blood of women with anorexia or bulimia (Frieling et al. 2009), as well as in the insula, frontal and temporal cortex of these patients (Gérard et al. 2011). Clinical trials with the CB1R antagonist rimonabant and taranabant have already demonstrated efficacy at reducing food intake as well as obesity (Hagmann 2008). However, they produce significant side effects, such as depression and suicidal ideation that have produced the withdrawal from the market two years after the commercialization of rimonabant (Moreira & Crippa 2009).

Less is known about the function of the CB2R in the physiopathological aspect of food intake. No alteration of CB2R mRNA expression has been shown in the blood of patients with anorexia or bulimia when compared to controls (Frieling et al. 2009).

Finally, human genetic studies report a positive association between eating disorders and specific polymorphisms of genes encoding for different components of the ECS system, such as CB1R (Monteleone & Maj 2008), CB2R (Ishiguro et al. 2010) and fatty acid amide hydrolase (Monteleone et al. 2009). Therefore, the ECS

appears to be a modulatory system that integrates different neurotransmitters and hormonal signalling.



OBJECTIVE

Objective 1. Involvement of MOR, DOR, PENK and PDYN in the acquisition and reinstatement of cocaine- and food-seeking behaviour. Our aim was to evaluate the participation of the two main opioid receptors involved in drug reinforcing effects, MOR and DOR, and opioid peptides derived from PENK and PDYN that represent their endogenous ligands, in the reinstatement of cocaine- and palatable food-seeking behaviour. We have also evaluated the impact of the deletion of these opioid components on cue-induced reinstatement using c-Fos expression as a marker of neuronal activity in main brain areas involved in addiction.

Article # 1: Effects of genetic deletion of endogenous opioid system components on the reinstatement of cocaine-seeking behaviour in mice. J. Gutiérrez-Cuesta*, A. Burokas*, S. Mancino[#], S. Kummer[#], E. Martín-García and R. Maldonado; [*,#] Equally contributed to the study*;* Neuropsychopharmacology (2014).
http://www.nature.com/npp/journal/v39/n13/full/npp2014149a.htm

Objective 2. Validation of a mouse model of addictive-like behaviour promoted by palatable food leading to differential epigenetic and protein expression changes in specific brain reward areas. Our aim was to validate an animal model of eating addictive-like behaviour based on the DSM-5 substance use disorder criteria using operant conditioning maintained by chocolate-flavoured pellets in an outbred mouse population. We also evaluated the differential epigenetic and protein expression changes revealed in specific brain areas including HCP, ST, NAc

and PFC of mice showing a compulsive eating behaviour (vulnerable to addiction) and in mice that did not show this behaviour (resistant to addiction). Additionally, we were interested in demonstrating the involvement of CB1R in the development of eating addictive-like behaviour.

Article # 2: Epigenetic and proteomic expression changes promoted by eating addictive-like behaviour. S. Mancino[*], A. Burokas[*], J. Gutiérrez-Cuesta[*], M. Gutiérrez-Martos, E. Martín-García, M. Pucci, A. Falconi, C. D'Addario, M. Maccarrone[#] and R. Maldonado[#]*;* *,[#] Equally contributed to the study*;* Neuropsychopharmacology (2015).
http://www.nature.com/npp/journal/v40/n12/full/npp2015129a.html

Objective 3. Involvement of DOR in the neuroplastic mechanisms underlying food reward and seeking behaviour. Our aim was to investigate the involvement of DOR in the regulation of palatable food rewarding effect by a mechanism that implies structural plasticity changes in three brain areas involved in addictive processes, PFC, HCP and NAc.

Article # 3: Role of DOR in neuronal plasticity changes promoted by food-seeking behaviour. S. Mancino, S. Mendonça Netto, E. Martín-García, R. Maldonado. In preparation.

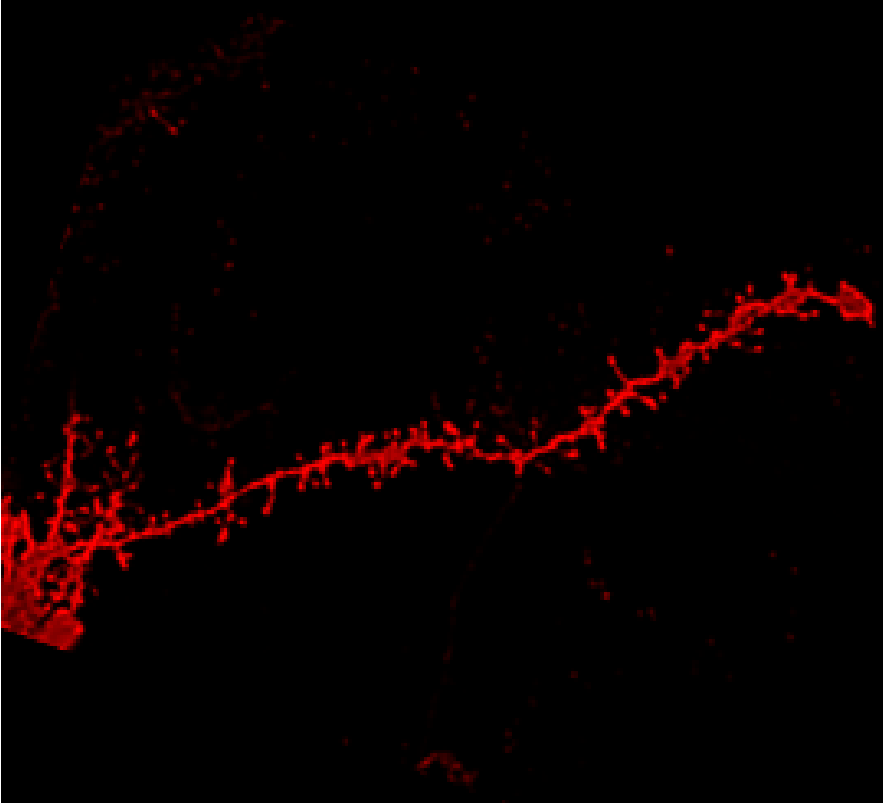
Objective 4. Involvement of CB2R in the reinforcing effects of chocolate flavoured-pellets and eating addictive-like behaviour.

Our aim was to assess the involvement of CB2R in compulsive eating behaviour promoted by highly palatable food using an operant model of eating addictive-like behaviour already validated in our laboratory.

Title: Involvement of CB2 cannabinoid receptor in eating addictive-like behaviour. S. Mancino, E. Martín-García, J. Manzanares and R. Maldonado. In preparation.

.

RESULTS



Objective 1

Involvement of MOR, DOR, PENK and PDYN in the acquisition and reinstatement of cocaine- and food-seeking behaviour.

This article will be also presented in the thesis of Sami Kummer.

Neuropsychopharmacology (2014) 39, 2974–2988

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Effects of Genetic Deletion of Endogenous Opioid System Components on the Reinstatement of Cocaine-Seeking Behavior in Mice

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The repeated cycles of cessation of consumption and relapse remain the major clinical concern in treating drug addiction. The endogenous opioid system is a crucial component of the reward circuit that participates in the adaptive changes leading to relapse in the addictive processes. We have used genetically modified mice to evaluate the involvement of μ-opioid receptor (MOR) and δ-opioid receptor (DOR) and their main endogenous ligands, the enkephalins derived from proenkephalin (PENK) and prodynorphin (PDYN), in the reinstatement of cocaine-seeking behavior. Constitutive knockout mice of MOR, DOR, PENK, and PDYN, and their wild-type littermates were trained to self-administer cocaine or to seek for palatable food, followed by a period of extinction and finally tested on a cue-induced reinstatement of seeking behavior. The four lines of knockout mice acquired operant cocaine self-administration behavior, although DOR and PENK knockout mice showed less motivation for cocaine than wild-type littermates. Moreover, cue-induced relapse was significantly decreased in MOR and DOR knockout mice. In contrast, PDYN knockout mice showed a slower extinction and increased relapse than wild-type littermates. C-Fos expression analysis revealed differential activation in brain areas related with memory and reward in these knockout mice. No differences were found in any of the four genotypes in operant responding to obtain palatable food, indicating that the changes revealed in knockout mice were not due to unspecific deficit in operant performance. Our results indicate that MOR, DOR, and PDYN have a differential role in cue-induced reinstatement of cocaine-seeking behavior.
Neuropsychopharmacology (2014) **39,** 2974–2988; doi:10.1038/npp.2014.149; published online 23 July 2014

INTRODUCTION

Addiction is a chronic brain disease characterized by the compulsive use of drugs in spite of their adverse consequences, loss of control over drug taking, and relapse even after long periods of abstinence according to the 'Diagnostic and Statistical Manual of Mental Disorders (5th edition; DSM-5; American Psychiatric Association, 2013), the most widely accepted nomenclature used by clinicians and researchers for the classification of mental disorders. The repeated cycles of relapse after cessation of consumption remain the major clinical concern in the treatment of drug addiction (Miller and Gold, 1994; Weiss, 2010). In our laboratory, we have recently validated novel models of reinstatement of drug and food-seeking behavior (Martin-Garcia *et al*, 2011; Soria *et al*, 2008) in mice that allow to study the neurobiological mechanisms of relapse through the use of genetically modified mice.

Complex adaptive changes within the brain reward circuits occurring during the addictive processes are responsible of drug relapse. Several neurotransmitters, including the endogenous opioid system are involved in these changes (Bodnar, 2008; Volkow *et al*, 2009). Chronic exposure to the different prototypical drugs of abuse, including opioids, alcohol, nicotine, psychostimulants, and cannabinoids has been reported to produce significant alterations within the endogenous opioid system, which seem to have an important role in the development of the addictive process (Trigo *et al*, 2010). The endogenous opioid system is integrated by different families of endogenous opioid peptides, and three different opioid receptors, μ (MOR), δ (DOR), and κ (KOR), widely distributed in the central nervous system and peripheral tissues. The activation of these opioid receptors leads to different intracellular responses that produce an inhibition of neuronal activity and a reduction of neurotransmitter release (Law *et al*, 2000).

Three families of endogenous peptides derived from either proopiomelanocortin (POMC), proenkephalin (PENK), or prodynorphin (PDYN) generate several final

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active peptides including β-endorphin, met- and leu-enkephalin, dynorphins, and neo-endorphins, respectively, that exhibit different affinities for each opioid receptor (Kieffer and Gaveriaux-Ruff, 2002). The opioid peptides derived from PENK represent the main endogenous ligands that activate MOR and DOR in multiple brain areas, although endogenous enkephalins also derive from PDYN (Kieffer and Gaveriaux-Ruff 2002). These receptors have an important role in regulating mood and reward, and are key components in the control of drug reinforcing effects leading to addictive behavior. Multiple studies have suggested an important role for opioid receptors and their endogenous ligands in cocaine addiction (Charbogne *et al*, 2014). Administration of MOR antagonists attenuates cocaine-induced conditioned place preference and reduces cocaine self-administration and reinstatement in rats (Soderman and Unterwald, 2008; Tang *et al*, 2005; Ward *et al*, 2003), and cocaine reinforcement was reduced in MOR knockout mice (Mathon *et al*, 2005), suggesting the involvement of MOR in cocaine addiction. DOR antagonists can increase or decrease cocaine self-administration in rats depending on the brain area microinjected (Ward *et al*, 2003). The role of endogenous opioid peptides derived from PENK in cocaine responses is still unknown, although these peptides participate in the reinforcing effects of other drugs of abuse (Berrendero *et al*, 2005; Marinelli *et al*, 2005). Several opioid peptides have opposite roles in the control of behavioral responses such as dynorphins and Leu-enkephalin derived from PDYN (Butelman *et al*, 2012), and their specific involvement in cocaine reinforcement remains unclaryfied.

Both drug and food reward has in common the involvement of similar neurochemical pathways within the mesolimbic system (Lutter and Nestler, 2009). Indeed, the endogenous opioid system also has an important role in the mechanisms underlying the behavioral responses directed to obtain food (Kelley, 2004; Shippenberg *et al*, 2007). In agreement, pharmacological agonism or antagonism of MOR and DOR increased or decreased, respectively, food intake (Bodnar, 2004; Zhang *et al*, 1998).

The aim of this study is to investigate the participation of the two main opioid receptors involved in drug reinforcing effects, MOR and DOR, and enkephalins derived from PENK and PDYN that represent their main endogenous ligands, in the reinstatement of cocaine-seeking behavior by using knockout mice deficient in these four components of the endogenous opioid system and their wild-type littermates. We have also evaluated the impact of the deletion of the components of the opioid system on cue-induced reinstatement using c-Fos expression as a marker of neuronal activity in brain areas involved in addiction. The use of knockout mice is an essential tool for understanding the role of the opioid system in drug reinforcement and relapse and complement the information previously obtained on pharmacological studies (Lutz and Kieffer, 2013).

MATERIALS AND METHODS

Animals

Homozygous knockout mice deficient in MOR, DOR, PENK and PDYN on a C57BL/6J background and their respective wild-type littermates were used (Matthes *et al*, 1996; Filliol *et al*, 2000; König *et al*, 1996; Galeote *et al*, 2009). Previous studies have shown that the genetic ablation of a specific opioid receptor did not result in major changes in other opioid receptor sites (Kieffer and Gaveriaux-Ruff, 2002). Mice were housed individually in controlled laboratory conditions with the temperature maintained at $21 \pm 1\,^{\circ}C$ and humidity at $55 \pm 10\%$. Mice were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 08:00 h and on at 20:00 h). Food and water were available *ad libitum* in mice used in the cocaine experiment. For operant behavior maintained by food, mice were food deprived (85% of the initial weight) and water was available *ad libitum*. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Cocaine Self-Administration Apparatus

Cocaine self-administration training was performed in operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive (see Supplementary Information).

Surgery

Mice were anaesthetized with a ketamine/xylazine mixture (20 ml/kg of body weight) and then implanted with indwelling i.v. silastic catheters as previously described (Soria *et al*, 2005) (see Supplementary Information). The success rate for maintaining patency of the catheter (mean duration of 13 days) until the end of the cocaine self-administration training was 88%.

Drugs

Cocaine hydrochloride was obtained from Ministerio de Sanidad y Consumo (Spain) and dissolved in sterile 0.9% physiological saline. Ketamine hydrochloride (100 mg/kg) (Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg/kg) (Sigma, Madrid, Spain) were mixed and dissolved in ethanol (5%) and distilled water (95%). This anesthetic mixture was administered intraperitoneally in an injection volume of 20 ml/kg of body weight. Thiopental sodium (5 mg/ml) (Braun Medical S.A, Barcelona, Spain) was dissolved in distilled water and delivered by infusion of 0.1 ml through the i.v. catheter.

Food-Maintained Operant Behavior Apparatus

Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two retractable levers, one randomly selected as the active lever and the other as the inactive (see Supplementary Information).

Experimental Design

A first group of mice ($n = 101$) was trained for cocaine self-administration experiments, during 2-h daily sessions

to acquire operant responding maintained by cocaine (0.5 mg/kg/infusion, i.v.) under fixed ratio 1 (FR1) (5 consecutive days) and FR3 (7 consecutive days). The criteria for acquisition of operant responding were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of infusions earned in three consecutive sessions, with at least 75% responding on the reinforced nose-poke, and a minimum of 10 reinforcements per session (Martin-Garcia *et al*, 2011; Soria *et al*, 2008). After the 12 FR sessions, animals were tested in a progressive ratio (PR) schedule where the response requirement to earn infusions escalated according to the following series: 1–2–3–5–12–18–27–40–60–90–135–200–300–450–675–1000. The maximum duration of the PR session was 4 h or until mice did not respond on any hole within 1 h, and was performed only once. After PR session, the thiopental test was applied and only mice that showed patency of catheter were moved to the extinction and relapse phases. The first extinction session occurred 48 h after the thiopental tests to avoid any possible influence of thiopental residual effects.

During the extinction phase, the experimental conditions were similar to the acquisition sessions except that cocaine was not available and the cue light was not presented after active responding. Mice were given 2 h daily extinction sessions, during 15 consecutive days until the criteria for extinction was achieved ie, during three consecutive sessions, mice responded on the active lever less than 30% of the responses reached in the three last acquisition days, and made less than 15 active responses per session. After extinction, mice were tested for reinstatement. Cues-induced reinstatement was conducted under the same conditions used in the acquisition phase except that cocaine was not delivered. Each response on the active manipulandum in this phase led to the presentation of the cue light for 2 s. The reinstatement criterion was achieved when responding in the nose-poke doubled with respect to extinction responding.

For food-maintained operant behavior experiments, a second group of mice ($n=63$) was trained during 1 h for 10 consecutive days to lever press for highly caloric and banana-flavored food pellets (14% protein, 60% fat, 26% carbohydrate, with a caloric value of 5.32 kcal/g) (Bio-Serv, Frenchtown, NJ, USA) paired with the presentation of a cue light on a FR1 schedule followed by 10 sessions under FR5, using the same criteria for the acquisition of operant behavior previously described. After the 20 FR sessions, animals were trained in one single PR schedule session where the response requirement to earn pellets escalated according to the following series: 1–5–12–21–33–51–75–90–120–155–180–225–260–300–350–410–465–540–630–730–850–1000–1200–1500–1800–2100–2400–2700–3000–3400–3800–4200–4600–5000–5500. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h. This second group of mice was food deprived during the whole experiment at 85% of their *ad libitum* initial weight adjusted for growth.

Immunohistochemistry Studies

See Supplementary Information for tissue preparation, immunofluorescence and c-Fos quantification.

Statistical Analysis

Analysis of the data obtained during the acquisition and extinction phase was conducted using two-way ANOVA with manipulandum (active/inactive) as within-subjects factor and genotype as between-subjects factor. *Post hoc* analysis (Newman–Keuls) was performed when required. Progressive ratio, day of extinction, cues-induced reinstatement, and immunohistochemistry results were compared using one-way ANOVA.

All results are expressed as mean ± SEM. Differences were considered significant at $p<0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS 19.0 (SPSS, Chicago, USA).

RESULTS

Acquisition and Maintenance of Cocaine Self-Administration

MOR knockout mice ($n=11$) and wild-type littermates ($n=13$) were trained to self-administer cocaine under FR1 (5 days), and FR3 (7 days) schedule of reinforcement. Two-way ANOVA revealed significant main effects of hole during the training period indicating a continuous operant responding for cocaine and discrimination between holes. Significant effects of genotype on day 2, 3 and 4, and a significant interaction between genotype and hole were revealed on day 3 (Table 1A). Subsequent *post hoc* analysis (Newman–Keuls) showed significant differences between genotypes on day 3 ($p<0.05$) (Figure 1a). The acquisition criteria were achieved by 90% of the MOR knockout mice and 77% of wild-type littermates. No significant difference was revealed between the breaking point achieved by MOR knockout and wild-type littermates in the PR session [$F_{(1,22)}=0.652$; NS] (Figure 1e).

For DOR knockout ($n=14$) and wild-type littermates ($n=10$), two-way ANOVA revealed significant main effects of hole during the training period, indicating a continuous operant responding for cocaine and discrimination between holes. No main genotype effects but significant interactions between genotype and hole on days 8, 9, 10, and 11 were revealed (Table 1A). Subsequent *post hoc* analysis (Newman–Keuls) showed significant differences between genotypes on day 8 ($p<0.01$), 9 ($p<0.05$) and 11 ($p<0.05$) (Figure 1b). The acquisition criteria were achieved by 93% of the DOR knockout mice and 100% of wild-type littermates. One-way ANOVA showed a significant decrease of the breaking point achieved by DOR knockout mice when compared with wild-type littermates [$F_{(1,23)}=5.673$; $p<0.05$] (Figure 1f).

For PENK knockout ($n=14$) and wild-type littermates ($n=14$), two-way ANOVA revealed a significant effect of hole during the whole training period, indicating a continuous operant responding for cocaine and discrimination between holes. Significant effects of genotype on day 1, 2, 11, and 12, and a significant interaction between genotype and hole on day 4, 11 and 12 were revealed (Table 1A). Subsequent *post hoc* analysis (Newman–Keuls) showed significant differences between genotypes on day 4 ($p<0.05$), 11 ($p<0.05$) and 12 ($p<0.05$) (Figure 1c). The acquisition criteria were achieved by 76% of the PENK

Table 1A Two-Way ANOVA of the Operant Responses During the Acquisition Phase

	Dependent variables	Two-way ANOVA															
		Cocaine self-administration								Food-maintained operant behaviour							
		MOR		DOR		PENK		PDYN		MOR		DOR		PENK		PDYN	
		F(1.44)	Sig.	F(1.46)	Sig.	F(1.52)	Sig.	F(1.46)	Sig.	F(1.28)	Sig.	F(1.28)	Sig.	F(1.28)	Sig.	F(1.26)	Sig.
Genotype	Day 1	1.55	NS	1.92	NS	5.97	$p<.05$	2.30	NS	2.54	NS	.07	NS	.00	NS	.49	NS
	Day 2	6.47	$p<.05$	.05	NS	4.17	$p<.05$	1.56	NS	.06	NS	1.12	NS	.93	NS	.35	NS
	Day 3	6.71	$p<.05$	.00	NS	1.93	NS	.00	NS	1.34	NS	1.96	NS	1.23	NS	.19	NS
	Day 4	5.5	$p<.05$	.2	NS	3.95	NS	.18	NS	.01	NS	.27	NS	.17	NS	.01	NS
	Day 5	3.51	NS	.1	NS	1.88	NS	.20	NS	.86	NS	.81	NS	.00	NS	1.05	NS
	Day 6	.72	NS	.93	NS	.6	NS	.47	NS	.15	NS	.18	NS	.00	NS	.2	NS
	Day 7	.32	NS	.79	NS	.53	NS	.37	NS	.86	NS	.01	NS	.01	NS	4.82	NS
	Day 8	.29	NS	2.94	NS	1.10	NS	.01	NS	.16	NS	1.18	NS	.04	NS	1.04	NS
	Day 9	.31	NS	1.94	NS	2.96	NS	.00	NS	1.00	NS	2.55	NS	.10	NS	9.7	$p<.01$
	Day 10	.05	NS	.45	NS	3.00	NS	.01	NS	.98	NS	1.74	NS	.16	NS	5.88	$p<.05$
	Day 11	.13	NS	1.57	NS	4.23	$p<.05$	.01	NS	.00	NS	2.29	NS	.27	NS	2.29	NS
	Day 12	.01	NS	.05	NS	5.28	$p<.05$	.07	NS	0.00	NS	3.91	NS	.19	NS	.25	NS
	Day 13									1.16	NS	2.28	NS	.41	NS	.22	NS
	Day 14									.35	NS	1.28	NS	.34	NS	.64	NS
	Day 15									.60	NS	1.24	NS	.01	NS	.84	NS
	Day 16									.02	NS	.59	NS	.05	NS	3.7	NS
	Day 17									.33	NS	.31	NS	.61	NS	1.12	NS
	Day 18									.23	NS	.50	NS	.80	NS	.04	NS
	Day 19									.53	NS	.72	NS	.29	NS	.7	NS
	Day 20									.01	NS	.58	NS	1.2	NS	1.08	NS
Hole / lever	Day 1	1.66	NS	7.21	$p<.05$	7.94	$p<.01$	.37	NS	8.7	$p<.01$	11.95	$p<.01$	2.9	NS	.13	NS
	Day 2	10.51	$p<.01$	10.59	$p<.01$	7.38	$p<.01$	4.91	$p<.05$	13.45	$p<.001$	38.28	$p<.001$	8.82	$p<.01$	7.10	$p<.05$
	Day 3	12.36	$p<.01$	2.21	NS	5.48	$p<.05$	9.19	$p<.01$	41.68	$p<.001$	54.36	$p<.001$	41.59	$p<.001$	31.30	$p<.001$
	Day 4	19.71	$p<.001$	10.55	$p<.01$	19.00	$p<.001$	16.87	$p<.001$	49.72	$p<.001$	55.55	$p<.001$	43.95	$p<.001$	42.40	$p<.001$
	Day 5	25.82	$p<.001$	8.36	$p<.01$	11.15	$p<.01$	13.73	$p<.01$	102.46	$p<.001$	104.11	$p<.001$	41.31	$p<.001$	63.60	$p<.001$
	Day 6	19.19	$p<.001$	41.93	$p<.001$	29.72	$p<.001$	17.75	$p<.001$	35.79	$p<.001$	216.37	$p<.001$	41.08	$p<.001$	106.30	$p<.001$
	Day 7	21.32	$p<.001$	34.00	$p<.001$	36.61	$p<.001$	25.08	$p<.001$	83.16	$p<.001$	399.66	$p<.001$	70.01	$p<.001$	146.80	$p<.001$
	Day 8	27.24	$p<.001$	73.53	$p<.001$	45.4	$p<.001$	24.51	$p<.001$	48.12	$p<.001$	152.71	$p<.001$	39.92	$p<.001$	106.70	$p<.001$
	Day 9	39.72	$p<.001$	107.6	$p<.001$	64.02	$p<.001$	28.69	$p<.001$	68.61	$p<.001$	170.91	$p<.001$	72.28	$p<.001$	227.70	$p<.001$
	Day 10	41.03	$p<.001$	157.13	$p<.001$	75.22	$p<.001$	34.35	$p<.001$	64.01	$p<.001$	301.18	$p<.001$	36.81	$p<.001$	310.00	$p<.001$
	Day 11	62.00	$p<.001$	149.36	$p<.001$	120.44	$p<.001$	35.94	$p<.001$	54.55	$p<.001$	125.64	$p<.001$	48.78	$p<.001$	174.50	$p<.001$
	Day 12	74.35	$p<.001$	152.87	$p<.001$	108.45	$p<.001$	32.39	$p<.001$	45.35	$p<.001$	135.59	$p<.001$	64.97	$p<.001$	247.10	$p<.001$
	Day 13									83.22	$p<.001$	249.98	$p<.001$	44.83	$p<.001$	170.00	$p<.001$

Table 1A (Continued)

	Dependent variables	Two-way ANOVA															
		Cocaine self-administration								Food-maintained operant behaviour							
		MOR		DOR		PENK		PDYN		MOR		DOR		PENK		PDYN	
		F(1.44)	Sig.	F(1.46)	Sig.	F(1.52)	Sig.	F(1.46)	Sig.	F(1. 28)	Sig.	F(1.28)	Sig.	F(1.28)	Sig.	F(1.26)	Sig.
	Day 14									97.59	$p<.001$	325.17	$p<.001$	63.06	$p<.001$	127.20	$p<.001$
	Day 15									79.42	$p<.001$	182.57	$p<.001$	53.31	$p<.001$	81.20	$p<.001$
	Day 16									83.50	$p<.001$	146.48	$p<.001$	40.37	$p<.001$	107.60	$p<.001$
	Day 17									99.72	$p<.001$	134.11	$p<.001$	45.75	$p<.001$	120.30	$p<.001$
	Day 18									108.65	$p<.001$	137.28	$p<.001$	52.95	$p<.001$	86.20	$p<.001$
	Day 19									144.22	$p<.001$	209.91	$p<.001$	58.54	$p<.001$	283.80	$p<.001$
	Day 20									135.97	$p<.001$	249.66	$p<.001$	55.56	$p<.001$	171.20	$p<.001$
Genotype * Hole / lever	Day 1	.23	NS	.03	NS	2.64	NS	.19	NS	3.13	NS	.01	NS	.58	NS	.22	NS
	Day 2	3.6	NS	1.05	NS	1.02	NS	.14	NS	.10	NS	1.12	NS	1.37	NS	.10	NS
	Day 3	5.39	$p<.05$	.50	NS	2.42	NS	.54	NS	1.11	NS	.14	NS	.90	NS	.41	NS
	Day 4	1.82	NS	.25	NS	4.63	$p<.05$	.89	NS	.22	NS	.9	NS	2.11	NS	6.76	$p<.05$
	Day 5	.41	NS	.54	NS	2.38	NS	.28	NS	.31	NS	.10	NS	3.86	NS	5.22	$p<.05$
	Day 6	.71	NS	3.24	NS	.74	NS	.93	NS	.27	NS	.27	NS	.04	NS	1.18	NS
	Day 7	.43	NS	3.51	NS	.63	NS	.26	NS	.00	NS	.47	NS	.07	NS	.27	NS
	Day 8	.00	NS	11.38	$p<.01$	1.51	NS	.00	NS	.27	NS	2.32	NS	1.86	NS	.08	NS
	Day 9	.52	NS	8.84	$p<.01$	3.52	NS	.06	NS	1.00	NS	3.23	NS	1.16	NS	.00	NS
	Day 10	.06	NS	4.94	$p<.05$	2.32	NS	.06	NS	5.95	$p<.05$	1.53	NS	.07	NS	2.46	NS
	Day 11	.02	NS	6.96	$p<.05$	5.49	$p<.05$	.14	NS	.22	NS	.86	NS	.58	NS	3.01	NS
	Day 12	.18	NS	2.86	NS	5.56	$p<.05$	.39	NS	.19	NS	1.71	NS	.11	NS	.13	NS
	Day 13									2.53	NS	1.2	NS	.58	NS	2.22	NS
	Day 14									1.08	NS	.83	NS	.10	NS	.00	NS
	Day 15									.18	NS	.24	NS	.00	NS	.11	NS
	Day 16									.00	NS	.28	NS	.03	NS	2.12	NS
	Day 17									.03	NS	.14	NS	.21	NS	.32	NS
	Day 18									.00	NS	.15	NS	.48	NS	.62	NS
	Day 19									.43	NS	.49	NS	.20	NS	.14	NS
	Day 20									.00	NS	.16	NS	.88	NS	.55	NS

Table 1B Two-Way ANOVA of the Operant Responses During the Extinction Phase

	Dependent variables	Two-way ANOVA							
		Extinction							
		MOR		DOR		PENK		PDYN	
		F(1.38)	Sig.	F(1.32)	Sig.	F(1.40)	Sig.	F(1.36)	Sig.
Genotype	Day 1	.47	NS	.01	NS	.49	NS	6.54	$p<0.05$
	Day 2	.04	NS	.00	NS	.01	NS	8.11	$p<0.05$
	Day 3	.01	NS	.54	NS	.06	NS	3.94	NS
	Day 4	.52	NS	.73	NS	.13	NS	8.49	$p<0.01$
	Day 5	.01	NS	.30	NS	6.47	$p<0.05$	1.73	NS
	Day 6	3.33	NS	.47	NS	.01	NS	11.97	$p<0.01$
	Day 7	5.79	$p<0.05$	.08	NS	.92	NS	3.12	NS
	Day 8	3.43	NS	.04	NS	.11	NS	.95	NS
	Day 9	2.57	NS	.21	NS	.79	NS	3.31	NS
	Day 10	5.85	$p<0.05$	2.13	NS	.98	NS	5.23	$p<0.05$
	Day 11	3.96	NS	.08	NS	2.08	NS	12.11	$p<0.01$
	Day 12	.33	NS	1.75	NS	.66	NS	6.86	$p<0.05$
	Day 13	1.26	NS	3.11	NS	.18	NS	2.94	NS
	Day 14	1.26	NS	.71	NS	.00	NS	.14	NS
	Day 15	4.21	$p<0.05$	1.39	NS	.14	NS	1.10	NS
Hole	Day 1	37.84	$p<0.001$	33.34	$p<0.001$	154.23	$p<0.001$	59.82	$p<0.001$
	Day 2	26.69	$p<0.001$	14.23	$p<0.01$	112.36	$p<0.001$	32.37	$p<0.001$
	Day 3	24.65	$p<0.001$	7.61	$p<0.01$	95.73	$p<0.001$	27.62	$p<0.001$
	Day 4	34.55	$p<0.001$	4.52	$p<0.05$	84.69	$p<0.001$	21.87	$p<0.001$
	Day 5	34.27	$p<0.001$	26.34	$p<0.001$	60.60	$p<0.001$	37.38	$p<0.001$
	Day 6	16.40	$p<0.001$	17.05	$p<0.001$	53.00	$p<0.001$	54.52	$p<0.001$
	Day 7	20.08	$p<0.001$	6.12	$p<0.05$	48.03	$p<0.001$	54.62	$p<0.001$
	Day 8	11.35	$p<0.01$	14.22	$p<0.01$	35.49	$p<0.001$	22.76	$p<0.001$
	Day 9	10.78	$p<0.01$	11.49	$p<0.01$	46.10	$p<0.001$	30.39	$p<0.001$
	Day 10	21.1	$p<0.001$	13.99	$p<0.01$	50.17	$p<0.001$	27.27	$p<0.001$
	Day 11	16.4	$p<0.001$	10.84	$p<0.01$	28.30	$p<0.001$	32.26	$p<0.001$
	Day 12	29.16	$p<0.001$	8.94	$p<0.01$	15.66	$p<0.001$	26.02	$p<0.001$
	Day 13	10.95	$p<0.01$	4.06	NS	33.83	$p<0.001$	35.23	$p<0.001$
	Day 14	6.94	$p<0.05$	7.34	$p<0.05$	31.23	$p<0.001$	12.70	$p<0.01$
	Day 15	8.65	$p<0.01$	3.26	NS	25.51	$p<0.001$	24.46	$p<0.001$
Genotype*	Day 1	.78	NS	2.46	NS	1.93	NS	5.61	$p<0.05$
Hole	Day 2	.65	NS	1.79	NS	1.90	NS	7.80	$p<0.01$
	Day 3	.66	NS	.09	NS	1.84	NS	8.84	$p<0.01$
	Day 4	.92	NS	2.05	NS	2.95	NS	5.24	$p<0.05$
	Day 5	.2	NS	7.76	$p<0.01$	.09	NS	2.69	NS
	Day 6	.23	NS	4.05	NS	.51	NS	8.66	$p<0.01$
	Day 7	.14	NS	2.14	NS	.01	NS	8.52	$p<0.01$
	Day 8	.00	NS	3.81	NS	.67	NS	5.86	$p<0.05$
	Day 9	.45	NS	2.68	NS	.04	NS	4.09	NS
	Day 10	.51	NS	4.94	$p<0.05$	.07	NS	5.29	$p<0.05$
	Day 11	.03	NS	4.1	NS	.85	NS	4.66	$p<0.05$
	Day 12	.05	NS	.14	NS	2.02	NS	7.61	$p<0.01$
	Day 13	.49	NS	.05	NS	1.23	NS	2.48	NS
	Day 14	.00	NS	2.18	NS	1.16	NS	.46	NS
	Day 15	.12	NS	.21	NS	.94	NS	.81	NS

knockout mice and 100% of wild-type littermates. One-way ANOVA showed a significant decrease of the breaking point achieved by PENK knockout mice when compared with wild-type littermates [$F_{(1,26)} = 7.123$; $p < 0.05$] (Figure 1g).

For PDYN knockout ($n = 14$) and wild-type littermates ($n = 11$), two-way ANOVA revealed significant main effects of hole during the whole training period, indicating a continuous operant responding for cocaine and discrimination between holes. Two-way ANOVA did not reveal main effects of genotype nor interaction between genotype and hole through the entire acquisition phase (Table 1A; Figure 1d). All PDYN KO mice and wild-type littermates achieved the acquisition criteria in the last experimental sequence. One-way ANOVA showed no significant differences in the breaking point achieved by PDYN knockout mice and wild-type littermates [$F_{(1,23)} = 0.213$; NS] (Figure 1h).

Extinction and Cues-Induced Reinstatement of Cocaine-Seeking Behavior

The extinction criteria were achieved by all mice. Two-way ANOVA revealed significant main effects in hole during the whole extinction phase in the four experiments (Table 1B).

In MOR experiment, two-way ANOVA showed significant effects of genotypes on day 7, 10, and 15 without interaction between genotype and hole (Table 1B; Figure 2a). One-way ANOVA did not reveal significant differences in the time required to achieve the extinction criteria [$F_{(1,19)} = 0.012$; NS] (Figure 2e). After 15 daily sessions of extinction, one-way ANOVA demonstrated a significant decrease in cues-induced reinstatement of cocaine-seeking behavior in MOR knockout mice compared with wild-type littermates [$F_{(1,19)} = 5.255$; $p < 0.05$] (Figure 2i).

In DOR experiment, no significant main effects of genotype were obtained (two-way ANOVA), although a significant interaction between genotype and hole was revealed on day 5 and 10 (Table 1B). Subsequent *post hoc* analysis (Newman–Keuls) showed significant differences between genotypes on day 5 ($p < 0.05$) and 10 ($p < 0.001$) (Figure 2b). One-way ANOVA did not reveal significant differences in the time required to achieve the extinction criteria [$F_{(1,21)} = 1.492$; NS] (Figure 2f). After extinction, one-way ANOVA showed a significant decrease in cues-induced reinstatement in cocaine-seeking behavior in knockout mice compared with wild-type littermates [$F_{(1,21)} = 17.581$; $p < 0.001$] (Figure 2j).

In PENK experiment, two-way ANOVA revealed significant main effects of genotype on day 5 without significant interaction between genotype and hole (Table 1B; Figure 2c). One-way ANOVA did not show significant differences in the time required to achieve extinction [$F_{(1.23)} = 0.172$; NS] (Figure 2g) nor in cues-induced reinstatement [$F_{(1,23)} = 0.55$; NS] (Figure 2k).

In PDYN experiment, two-way ANOVA revealed significant main effects of genotype on day 1, 2, 4, 6, 10, 11, and 12, as well as a significant interaction between genotype and hole on day 1, 2, 3, 4, 6, 7, 8, 10, 11, and 12 (Table 1B). *Post hoc* analysis (Newman–Keuls) showed significant differences between genotypes on day 1 ($p < 0.01$), 2 ($p < 0.001$), 3 ($p < 0.01$), 4 ($p < 0.001$), 6 ($p < 0.01$), 7 ($p < 0.01$), 8 ($p < 0.05$), 10 ($p < 0.01$) and 12 ($p < 0.001$) (Figure 2d). One-way ANOVA revealed a significant increase in the time required to achieve extinction [$F_{(1,18)} = 10.618$; $p < 0.01$] (Figure 2h) and cues-induced reinstatement of cocaine-seeking behavior in PDYN knockout mice compared with wild-type littermates [$F_{(1,18)} = 12.892$; $p < 0.01$] (Figure 2l).

C-Fos Expression

Fos protein levels were evaluated in brain areas involved in drug operant reinstatement (striatum, nucleus accumbens core, amygdala, prelimbic cortex, and CA1, CA2, CA3 regions of the hippocampus) in different knockout mice and wild-type littermates after the cues-induced reinstatement session. One-way ANOVA showed significant decreases in c-Fos levels in MOR knockout mice compared with wild-type littermates in CA1 [$F_{(1,11)} = 9.48$; $p < 0.05$], CA2 [$F_{(1,11)} = 7.60$; $p < 0.05$] and CA3 [$F_{(1,11)} = 5.42$; $p < 0.05$] regions of the hippocampus. No significant differences were observed in other brain areas (Figures 3a and 4).

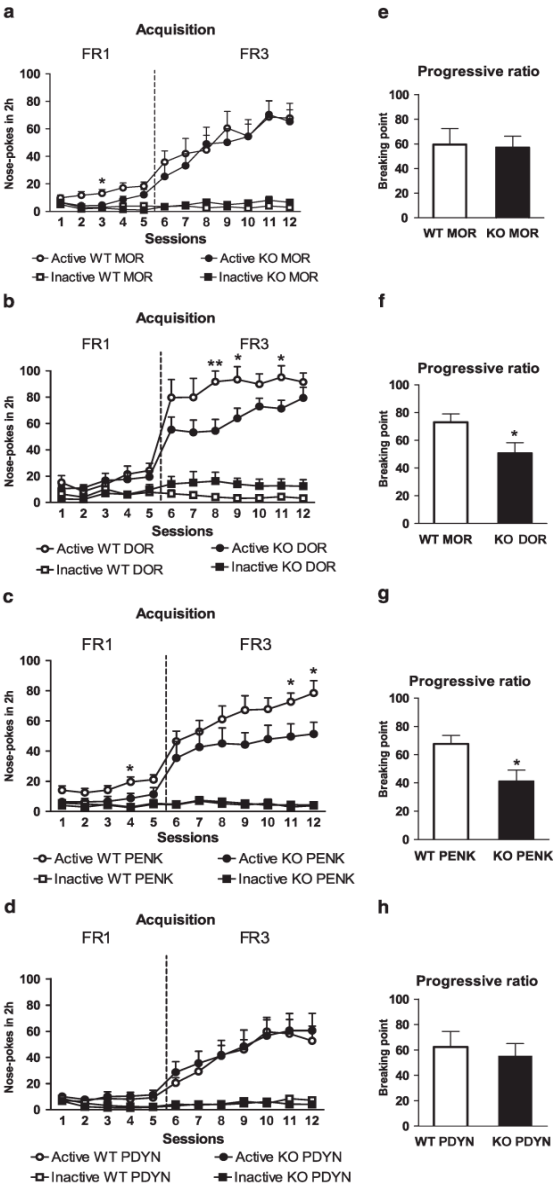
One-way ANOVA showed a significant decrease in c-Fos levels in DOR knockout mice compared with wild-type littermates in the striatum [$F_{(1,11)} = 6.04$; $p < 0.05$] and CA1 region of the hippocampus [$F_{(1,11)} = 57.24$; $p < 0.001$]. No significant differences were revealed in other brain areas (Figures 3b and 4). Significant decreases in c-Fos levels in PENK knockout mice compared with wild-type littermates were shown in the striatum [$F_{(1,11)} = 13.12$; $p < 0.01$], amygdala [$F_{(1,11)} = 7.53$; $p < 0.05$], CA2 [$F_{(1,11)} = 10.45$; $p < 0.01$] and CA3 [$F_{(1,11)} = 37.46$; $p < 0.001$] region of the hippocampus, without significant differences in other brain areas (Figures 3c and 4).

One-way ANOVA showed a significant increase in c-Fos levels in PDYN knockout mice compared with wild-type littermates in the striatum [$F_{(1,16)} = 4.51$; $p < 0.05$], nucleus accumbens core [$F_{(1,16)} = 5,12$; $p < 0.05$] and CA2 region of the hippocampus [$F_{(1,16)} = 5,42$; $p < 0.05$]. No significant differences were revealed in other brain areas (Figures 3d and 4).

Acquisition and Maintenance of Operant Conditioning Maintained by Food

MOR knockout ($n = 8$) and wild-type littermates ($n = 8$) mice were trained to acquire an operant responding

Figure 1 Operant behavior to obtain cocaine in MOR, DOR, PENK, and PDYN knockout mice. Mean number of nose-pokes in the active and inactive hole during the acquisition training in fixed ratio 1 (FR1) and FR3 schedule of reinforcement to obtain cocaine (0.5 mg/kg/infusion) (a) MOR knockout mice ($n = 11$) and wild-type littermates ($n = 13$). (b) DOR knockout mice ($n = 14$) and wild-type littermates ($n = 10$). (c) PENK knockout mice ($n = 14$) and wild-type littermates ($n = 14$). (d) PDYN knockout mice ($n = 14$) and wild-type littermates ($n = 11$). (e) Breaking point achieved in the progressive ratio session in MOR knockout mice and wild-type littermates. (f) Breaking point in DOR knockout mice and wild-type littermates. (g) Breaking point in PENK knockout mice and wild-type littermates. (h) Breaking point in PDYN knockout mice and wild-type littermates. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ *vs* knockout group (Newman–Keuls test (acquisition) or one-way ANOVA (progressive ratio)).


a
Acquisition
FR1
FR3
Nose-pokes in 2h
Sessions
Active WT MOR
Active KO MOR
Inactive WT MOR
Inactive KO MOR
b
Acquisition
FR1
FR3
Nose-pokes in 2h
Sessions
Active WT DOR
Active KO DOR
Inactive WT DOR
Inactive KO DOR
c
Acquisition
FR1
FR3
Nose-pokes in 2h
Sessions
Active WT PENK
Active KO PENK
Inactive WT PENK
Inactive KO PENK
d
Acquisition
FR1
FR3
Nose-pokes in 2h
Sessions
Active WT PDYN
Active KO PDYN
Inactive WT PDYN
Inactive KO PDYN
e
Progressive ratio
Breaking point
WT MOR
KO MOR
f
Progressive ratio
Breaking point
WT MOR
KO DOR
g
Progressive ratio
Breaking point
WT PENK
KO PENK
h
Progressive ratio
Breaking point
WT PDYN
KO PDYN

maintained by high palatable food pellets under FR1 (10 days), and FR5 (10 days) schedule. Two-way ANOVA revealed significant main effects of lever during the whole training period indicating a continuous operant responding for food pellets and discrimination between levers. No significant effects of genotype were revealed (Tables 1A and B). The mean number of active lever presses during the 3 days when the acquisition criteria were achieved was 216.17 ± 11.96 in MOR knockout group and 226.83 ± 21.08 in wild-type littermates (number of inactive lever presses 13.67 ± 3.63 and 16.25 ± 4.43, respectively). The breaking point achieved by MOR knockout mice in the PR session was 248.13 ± 33.57 and 213.13 ± 20.72 in wild-type littermates [$F_{(1,14)} = 0.787$; NS] (data not shown).

In DOR knockout ($n = 8$) and wild-type littermates ($n = 8$), two-way ANOVA revealed significant main effects of lever during the whole training period, indicating a continuous operant responding for food pellets and discrimination between levers. No significant effects of genotype were revealed (Table 1A). The mean number of active lever presses during the 3 days when the acquisition criteria were achieved was 333.5 ± 33.21 in DOR knockout group and 364.13 ± 42.73 in wild-type littermates (number of inactive lever presses 12.42 ± 3.25 and 19.75 ± 6.17, respectively). The breaking point achieved by DOR knockout mice in the PR session was 305 ± 21.46 and 426.88 ± 63.21 in wild-type littermates [$F_{(1,14)} = 3.333$; NS] (data not shown).

In PENK knockout mice ($n = 8$) and wild-type littermates ($n = 8$), two-way ANOVA revealed significant main effects of lever during the whole training period indicating a continuous operant responding for food pellets and discrimination between levers. No significant effects of genotype were revealed (Table 1A). The mean number of active lever presses during the 3 days when the acquisition criteria were achieved was 249.88 ± 43.62 in PENK knockout group and 304.25 ± 39.87 in wild-type littermates (number of inactive lever presses 19.25 ± 5.32 and 24.25 ± 3.97, respectively). The breaking point achieved by PENK knockout mice in the PR session was 237.5 ± 33.74 and 275 ± 37.86 in wild-type littermates [$F_{(1,14)} = 0.547$; NS] (data not shown).

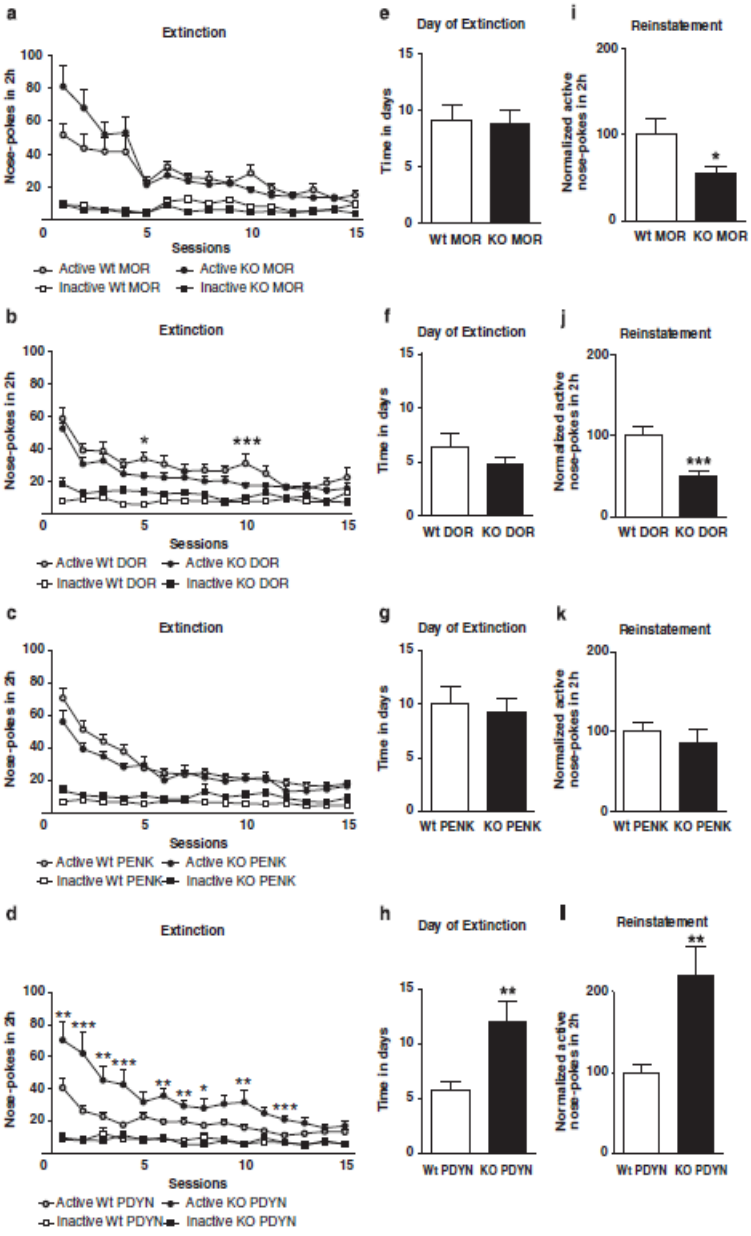
In PDYN knockout mice ($n = 5$) and wild-type littermates ($n = 10$), two-way ANOVA revealed significant main effects of lever during the whole training period indicating a continuous operant responding for food pellets and discrimination between levers. Significant effects of genotype were revealed only on day 9 and 10 (Tables 1A and B). The mean number of active lever presses during the 3 days when the acquisition criteria were achieved was 324.87 ± 37.38 in PDYN knockout group and 334.6 ± 23.63 in wild-type littermates (number of inactive lever presses 16.2 ± 7.22 and 36.63 ± 7.9, respectively). The breaking point achieved by PDYN knockout mice in the PR session was 248 ± 27.14 and 251.5 ± 23.66 in wild-type littermates [$F_{(1,13)} = 0.008$; NS] (data not shown).

DISCUSSION

The present study shows the specific involvement of four components of the endogenous opioid system in the acquisition and relapse of cocaine self-administration in mice. We have used a reliable operant model of reinstatement validated in our laboratory (Martin-Garcia *et al*, 2011; Soria *et al*, 2008) to demonstrate that the constitutive deletion of MOR, DOR, PENK, and PDYN differentially modifies the acquisition and reinstatement of cocaine-seeking behavior, but has no significant consequences in a similar operant training for palatable food. Indeed, a similar performance in operant responding maintained by food was obtained in all knockout mice and wild-type littermates under our experimental conditions, which ruled out a potential learning impairment for operant training in these four lines of knockout mice. This experimental control was mandatory considering that MOR knockout mice displayed learning impairment in the radial-maze task (Jamot *et al*, 2003) and DOR knockout mice showed impaired place conditioning (Le Merrer *et al*, 2011). These spatial memory impairments have no consequences in the performance of an operant training to obtain a rewarding stimulus, such as palatable food.

Previous studies have reported that selective MOR antagonists attenuate cocaine-conditioned place preference (Schroeder *et al*, 2007) and self-administration in rats (Ward *et al*, 2003), while the deletion of MOR in knockout mice reduced oral ethanol self-administration and intravenous cocaine self-administration (Becker *et al*, 2002; Mathon *et al*, 2005). In our experimental conditions, no major differences in the acquisition of cocaine self-administration were revealed in MOR knockout mice, as only a single significant reduction of active nose-poking was observed on day 3, and no differences in cocaine motivation was shown in the PR. This discrepancy could be due to differences in experimental protocol used in terms of cocaine dose, and time of conditioning sessions, as the previous study only found significant differences at high cocaine doses in shorter session times. Our results reveal

Figure 2 Operant behavior in extinction phase, the day of extinction and cues-induced reinstatement of cocaine-seeking behavior in MOR, DOR, PENK, and PDYN knockout mice. Mean number of nose-pokes in the active and inactive hole during the extinction training. (a) MOR knockout mice ($n = 10$) and wild-type littermates ($n = 11$). (b) DOR knockout mice ($n = 13$) and wild-type littermates ($n = 9$). (c) PENK knockout mice ($n = 11$) and wild-type littermates ($n = 14$). (d) PDYN knockout mice ($n = 9$) and wild-type littermates ($n = 11$). (e) Time in days necessary to accomplish extinction criteria in MOR KO mice ($n = 10$) and wild-type littermates ($n = 11$), (f) DOR KO mice ($n = 13$) and wild-type littermates ($n = 9$), (g) PENK KO mice ($n = 11$) and wild-type littermates ($n = 14$), (h) PDYN KO ($n = 9$) mice and wild-type littermates ($n = 11$). (i) Cues-induced reinstatement of cocaine-seeking behavior shown as normalized nose-pokes in the active hole in MOR KO mice ($n = 10$) and wild-type littermates ($n = 11$), (j) DOR KO mice ($n = 13$) and wild-type littermates ($n = 9$), (k) PENK KO mice ($n = 11$) and wild-type littermates ($n = 14$), (l) PDYN KO mice ($n = 9$) and wild-type littermates ($n = 11$). Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* KO group; (Newman–Keuls test (extinction) or one-way ANOVA (day of extinction and cues-induced reinstatement)).


a
Extinction
Nose-pokes in 2h
Sessions
Active Wt MOR
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Inactive Wt MOR
Inactive KO MOR
e
Day of Extinction
Time in days
Wt MOR
KO MOR
i
Reinstatement
Normalized active nose-pokes in 2h
Wt MOR
KO MOR
b
Extinction
Active Wt DOR
Active KO DOR
Inactive Wt DOR
Inactive KO DOR
f
Day of Extinction
Wt DOR
KO DOR
j
Reinstatement
Wt DOR
KO DOR
c
Extinction
Active Wt PENK
Active KO PENK
Inactive Wt PENK
Inactive KO PENK
g
Day of Extinction
Wt PENK
KO PENK
k
Reinstatement
Wt PENK
KO PENK
d
Extinction
Active Wt PDYN
Active KO PDYN
Inactive Wt PDYN
Inactive KO PDYN
h
Day of Extinction
Wt PDYN
KO PDYN
l
Reinstatement
Wt PDYN
KO PDYN

that MOR is involved in cocaine relapse, as reinstatement of cocaine-seeking behavior was attenuated in MOR knockout mice. In agreement, pharmacological studies have shown that MOR antagonists reduced cocaine relapse in rats (Tang *et al*, 2005). Furthermore, the non-selective opioid antagonist naltrexone reduced cue-induced cocaine-seeking behavior (Burattini *et al*, 2008), and had no effects on cocaine priming-induced reinstatement in rats (Comer *et al*, 1993), although repeated naltrexone treatment suppressed this priming-induced reinstatement (Gerrits *et al*, 2005). Accordingly, microinjection of selective MOR agonists into the nucleus accumbens reinstated cocaine-seeking behavior in rats (Simmons and Self, 2009). Interestingly, the number of positive c-Fos-immunostained cells was lower in MOR knockout mice after cue-induced cocaine reinstatement than in wild-type mice in CA1, CA2 and CA3 regions of the hippocampus. This result reflects a decreased neuronal activation in this brain structure closely involved in memory processing after the exposure to the cocaine-associated cues when the activity of MOR is absent. These behavioral and neurochemical results suggest that MOR is involved in cocaine reinstatement by modifying the neuronal activity in brain areas involved in memory.

Cocaine self-administration was significantly attenuated in DOR knockout mice when trained in FR3, but not in FR1, suggesting that the response is impaired only when the effort required to obtain the reward is enhanced. Other studies showed that DOR knockout mice acquired morphine self-administration similarly to wild-type mice (Le Merrer *et al*, 2011), although these mutants showed a decreased operant responding when trained to obtain high doses of intravenous nicotine (Berrendero *et al*, 2012). In agreement, with our acquisition data, the breaking point achieved by DOR knockout mice was significantly lower than wild-type littermates during the PR session. In contrast, DOR knockout were reported to achieve a similar breaking point for morphine than wild-type mice under a PR schedule (Le Merrer *et al*, 2011). The reinstatement of cocaine-seeking behavior was also significantly reduced in DOR knockout mice. In agreement, pharmacological studies have suggested the participation of DOR in particular brain areas in cocaine-reinforcing effects (Le Merrer *et al*, 2009), and microinjection of selective DOR agonists in the nucleus accumbens reinstated cocaine-seeking behavior in rats (Simmons and Self, 2009). Furthermore, the enhancement of positive c-Fos-immunostained cells induced by cocaine reinstatement was attenuated in DOR knockout mice in the striatum, and the CA1 region of the hippocampus. These results revealed a decreased neuronal activation in these brain structures involved in motor and motivation control, and memory processing after the exposure to cocaine-associated cues in the absence of DOR activity. Our findings suggest that DOR modulates the motivation to obtain cocaine and cocaine reinstatement by modifying neuronal activity in brain areas involved in motor, motivation, and memory processing.

Cocaine self-administration was attenuated in PENK knockout mice, mainly when animals were trained in FR3. In agreement, the breaking point achieved by PENK knockout mice was also reduced during the PR session suggesting that opioid peptides derived from PENK have an important role in cocaine-reinforcing properties. PENK has been postulated to mediate the reinforcing effects of other drugs of abuse (Berrendero *et al*, 2005; Marinelli *et al*, 2005; Shoblock and Maidment, 2007), and changes in PENK gene expression have been revealed after long-term cocaine self-administration (Crespo *et al*, 2001). In contrast, cue-induced reinstatement of cocaine-seeking behavior was not modified in PENK knockout mice, which suggests that other opioid peptides different from those derived from PENK must be involved in the reinstatement of cocaine-seeking behavior. However, the number of positive c-Fos-immunostained cells was decreased in PENK knockout in the striatum, amygdala, CA2, and CA3 regions of the hippocampus after cue-induced reinstatement. Therefore, the absence of PENK decreases neuron activation in several brain structures during cue-induced reinstatement session, although these changes were not associated with a significant modification of cues-induced reinstatement of cocaine-seeking behavior.

The acquisition of cocaine self-administration and the motivation to obtain cocaine in the PR schedule were not modified in PDYN knockout mice. However, PDYN knockouts showed slower extinction and increased cues-induced reinstatement of cocaine-seeking behavior when compared with wild-type littermates, the opposite result to that obtained in MOR and DOR knockout mice on cocaine reinstatement. Opioids derived from PDYN include the MOR and DOR agonist leu-enkephalin as well as other opioid peptides with preferential KOR agonist properties, such as dynorphins (Kieffer and Gaveriaux-Ruff 2002). Considering the opposite role on the control of the rewarding pathways of KOR with regards to MOR and DOR (Trigo *et al*, 2010), and the opposite responses on cocaine reinstatement of our lines of knockout mice, we can postulate that the enhanced reinstatement of cocaine seeking revealed in PDYN knockout mice would be related to opioid peptides acting on KOR, such as dynorphins. In agreement, the DYN/KOR system appears to participate in the aversive effects related to cocaine exposure. Thus, KOR reduces the effects of stress on the reinstatement of cocaine-seeking behavior in mice (McLaughlin *et al*, 2006) and rats (Beardsley *et al*, 2005). Furthermore, repeated cocaine administration increases levels of dynorphins and prePDYN mRNA in animals and humans (Trifilieff and Martinez, 2013). In support of our hypothesis, c-fos mapping reveals an opposite result to other lines of opioid knockout in PDYN knockouts after cocaine reinstatement. Indeed, the number of positive c-Fos-immunostained cells induced by cocaine reinstatement was enhanced in PDYN knockout mice in the striatum, the core of nucleus accumbens and CA2 region of the hippocampus, revealing an increased neuronal activation in these brain structures related to motor, motivation, and memory processing. The findings suggest that PDYN modulates cocaine reinstatement by modifying neuronal activity in these brain areas.

Our behavioral and neurochemical results suggest that DOR and PENK are involved in the motivation to obtain cocaine, and the absence of these opioid components reduces cocaine self-administration mainly when the effort to obtain the reward is increased. Moreover, cocaine reinstatement is reduced in MOR and DOR knockout mice, whereas it is not modified in the absence of PENK and

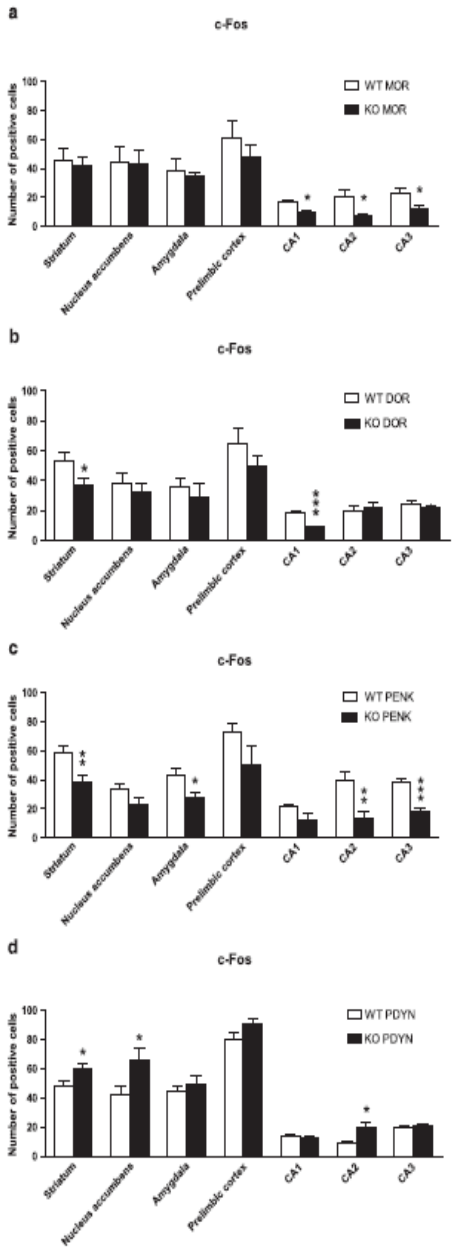

Figure 3 Levels of c-Fos expression in different brain areas after cues-induced reinstatement of cocaine-seeking behavior in MOR, DOR, PENK, and PDYN knockout mice. Number of positive immunostained cells in the striatum, nucleus accumbens core, amygdala, prelimbic cortex, CA1 of the hippocampus, CA2 of the hippocampus, and CA3 of the hippocampus. (a) MOR knockout mice ($n=7$) and wild-type littermates ($n=6$). (b) DOR knockout mice ($n=7$) and wild-type littermates ($n=6$). (c) PENK knockout mice ($n=7$) and wild-type littermates ($n=6$). (d) PDYN knockout mice ($n=8$) and wild-type littermates ($n=10$). Data are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* wild-type (one-way ANOVA).

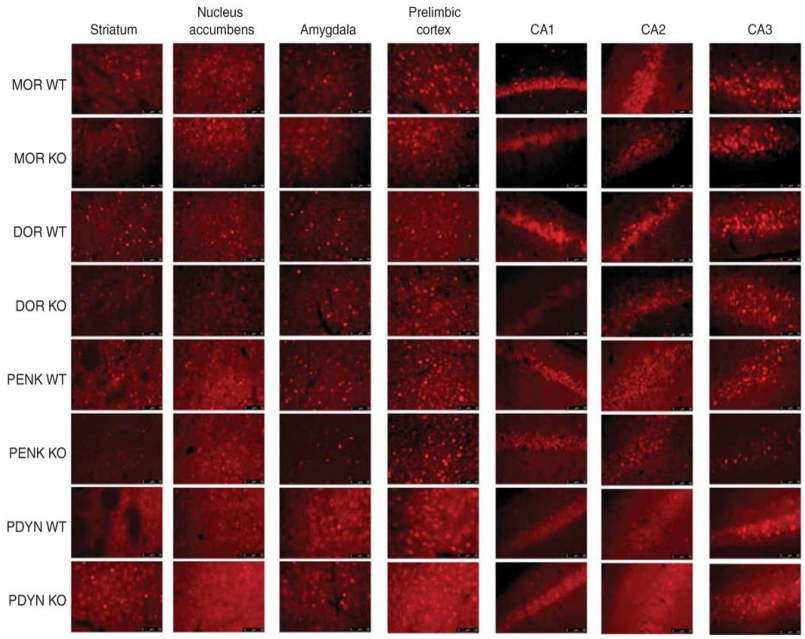

Figure 4 Representative images of c-Fos immunoreactivity in different brain areas after cues-induced reinstatement of cocaine-seeking behavior in MOR, DOR, PENK, and PDYN knockout mice. Striatum, nucleus accumbens core, amygdala, prelimbic cortex, CA1 of the hippocampus, CA2 of the hippocampus, and CA3 of the hippocampus.

results increased in the absence of PDYN. Therefore, the reduced cocaine reinstatement revealed in MOR and DOR was not mediated by the main endogenous ligand of these receptors, enkephalins, as the deletion of the two precursors of these endogenous opioid peptides, PENK and PDYN, did not mimic this behavioral response. In agreement, we have previously demonstrated that another MOR and DOR endogenous ligand, β-endorphin, has a crucial role in the rewarding properties of other drugs of abuse such as nicotine (Trigo *et al*, 2009).

The genetic deletion of MOR, DOR, PENK, and PDYN did not modify the acquisition and motivation to maintain operant responding to obtain palatable food in deprived mice. In agreement, opioid receptor antagonists did not significantly modify food-seeking behavior (Abdoullaye *et al*, 2010) nor preference for high-caloric food in rats (Dela Cruz *et al*, 2012). However, the enhancement of opioid activity by administration of opioid agonists increased preferentially high-caloric food intake (Taha, 2010). In addition, rats with chronic access to highly palatable food increased their mRNA expression of POMC in the medial prefrontal cortex (Blasio *et al*, 2013). Taken together, these results suggest that the absence of the basal tone of MOR, DOR, PENK, and PDYN did not modify food-seeking behavior in agreement with previous pharmacological studies, whereas opioid system activation promotes this behavior.

In conclusion, our results suggest that opioid peptides derived from PENK acting on DOR have an important role in cocaine-reinforcing properties. MOR and DOR, and endogenous opioid peptides different from enkephalins are crucial for cue-induced reinstatement of cocaine-seeking behavior by modulating neuronal activation of brain areas involved in the control of motor, motivation, and memory processes. Opioid peptides derived from PDYN have an opposite role to MOR and DOR in the control of cocaine reinstatement. The elucidation of these neurobiological mechanisms involved in cocaine-reinforcing effects and relapse opens new possibilities for developing new therapeutic strategies targeting the endogenous opioid system.

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The authors declare no conflict of interest.

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Author Contributions

J. G-C., A.B., S.M., S.K., and E. M-G. conducted the behavioral studies and participated in the interpretation and manuscript writing. R.M. participated in the experimental design, interpretation and manuscript writing and funded the project.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Supplementary Materials and Methods

Cocaine self-administration apparatus

Cocaine self-administration training was performed in operant chambers (Model ENV-307A-CT, Med Associates, Inc., Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive. Pump noise and a stimulus-light located above active hole were paired with the delivery of the infusion. Chambers had grid floors and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and ambient noise. When mice responded on the reinforced hole, the stimulus light went on, and a cocaine (0.5 mg/kg) infusion was delivered via a syringe mounted on a microinfusion pump (PHM-100A, Med Associates, Inc., Georgia, VT, USA) connected via Tygon tubing (0.96 mm o.d., Portex Fine Bore Polythene Tubing, Portex Limited, Hythe, Kent, UK) to a single channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA), and to the mouse intravenous (i.v.) catheter.

Surgery

Mice were anaesthetized with a ketamine/xylazine mixture (20 ml/kg of body weight) and then implanted with indwelling i.v. silastic catheters as previously described (Soria et al, 2005). Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic®, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, UK) that was bent at a right angle and then embedded in a cement disk

(Dentalon Plus, Heraeus Kulzer, Wehrheim, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain). After surgery, animals were allowed to recover for 3 days prior to initiation of self-administration sessions. The catheter was flushed daily with heparinised saline (30 USP units/ml). The patency of i.v. catheters was evaluated after the last self-administration session and whenever the behaviour appeared to deviate dramatically from that observed previously by infusion of thiopental through the catheter. If prominent signs of anaesthesia were not apparent within 3s of the infusion, the mouse was removed from the experiment. The success rate for maintaining patency of the catheter (mean duration of 13 days) until the end of the cocaine self-administration training was 88%.

Food maintained operant behaviour apparatus

Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two retractable levers, one randomly selected as the active lever and the other as the inactive. Pressing on the active lever resulted in a pellet delivery together with a stimulus-light located above the active lever. Pressing on the inactive lever had no consequences. The chambers housed in sound- and light-attenuated boxes equipped with fans to provide ventilation

and white noise. A food dispenser equidistant between the two levers permitted delivery of food pellets when required.

Immunohistochemistry studies

Tissue preparation. For c-Fos detection, mice were transcardially perfused with 4% paraformaldehyde immediately after the 2h cue-induced reinstatement session. Subsequently, the brains were extracted and postfixed in the same fixative for 4h and cryoprotected overnight in a solution of 30% sucrose at 4ºC. Coronal frozen sections were made at 30 μm on a freezing microtome and stored in a 5% sucrose solution at 4°C until use.

Immunofluorescence. A previously described protocol (Balerio et al, 2004) was adapted for immunofluorescence. Sections were incubated with the primary antibody in 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB (NGS-T-PB) overnight at 4°C. Next day, sections were incubated with the secondary antibody at room temperature in NGS-T-PB for 2h. After incubation, sections were rinsed and mounted onto glass slides coated with gelatin in Mowiol mounting medium. A rabbit polyclonal antiserum (1:1000) (Ab-5; Calbiochem) was used for c-Fos detection. Cy3-conjugated goat antirabbit (Jackson ImmunoResearch) was used (1:500) as secondary antibody.

c-Fos quantification. The stained sections of the brain were analyzed at 40× objective using a Leica DMR microscope equipped with a digital camera Leica DFC 300FX. ImageJ software was used for the quantification of c-Fos positive nuclei. Three samples of each mouse (3–7) were quantified per group. Circular particles were

analyzed in transformed binary images using a constant region of interest (ROI). The threshold was adjusted to distinguish the particles from the background.

Objective 2

Validation of a mouse model of addictive-like behaviour promoted by palatable food leading to differential epigenetic and protein expression changes in specific brain reward areas.

This article will be also presented in the thesis of Miriam Gutierrez-Martos.

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Epigenetic and Proteomic Expression Changes Promoted by Eating Addictive-Like Behavior

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An increasing perspective conceptualizes obesity and overeating as disorders related to addictive-like processes that could share common neurobiological mechanisms. In the present study, we aimed at validating an animal model of eating addictive-like behavior in mice, based on the DSM-5 substance use disorder criteria, using operant conditioning maintained by highly palatable chocolate-flavored pellets. For this purpose, we evaluated persistence of food-seeking during a period of non-availability of food, motivation for food, and perseverance of responding when the reward was associated with a punishment. This model has allowed identifying extreme subpopulations of mice related to addictive-like behavior. We investigated in these subpopulations the epigenetic and proteomic changes. A significant decrease in DNA methylation of CNR1 gene promoter was revealed in the prefrontal cortex of addict-like mice, which was associated with an upregulation of CB_1 protein expression in the same brain area. The pharmacological blockade (rimonabant 3 mg/kg i.p.) of CB_1 receptor during the late training period reduced the percentage of mice that accomplished addiction criteria, which is in agreement with the reduced performance of CB_1 knockout mice in this operant training. Proteomic studies have identified proteins differentially expressed in mice vulnerable or not to addictive-like behavior in the hippocampus, striatum, and prefrontal cortex. These changes included proteins involved in impulsivity-like behavior, synaptic plasticity, and cannabinoid signaling modulation, such as alpha-synuclein, phosphatase 1-alpha, doublecortin-like kinase 2, and diacylglycerol kinase zeta, and were validated by immunoblotting. This model provides an excellent tool to investigate the neurobiological substrate underlying the vulnerability to develop eating addictive-like behavior.
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INTRODUCTION

The increased incidence of obesity and overeating disorders represent a major health problem in developed countries. Based on the latest estimates, overweight affects >30% and obesity >15% of adults in the European Union (WHO, 2008), while in the USA 35.3% of adults are considered overweight and the obesity rate is 27.7% (Gallup, 2014). Obesity is defined as excessive fat accumulation that results from a caloric imbalance, meaning that the number of calories consumed exceeds the calories expended (Brownell, 2004). Unfortunately, few obese people achieve significant weight reduction mainly owing to the difficult to control food intake. The low rate of success of treatments to prevent or reverse obesity highlights the fact that this condition is not only a metabolic disorder but also a behavioral alteration (Volkow and Wise, 2005). In agreement, obese individuals are led by an excessive motivational drive for food, eat more than intended, and make frequent unsuccessful efforts to control overeating. These behavioral alterations seem to be mediated by changes in the mesolimbic reward circuits that mimic the changes occurring during addictive-like behavior (Volkow *et al*, 2012). In addition, several behavioral modifications occurring during obesity that parallel to those described in DSM-5 for substance use disorder had allowed to hypothesize that overeating may be considered as an addictive-like behavior (Hebebrand *et al*, 2014). Binge eating disorder (BED) seems particularly linked to addiction given specific features of the disorder as compulsive eating, excess consumption despite adverse consequences, and diminished self-control over eating behavior. Indeed, the concept of eating addiction includes the loss of control over eating, the persistent desire for food, the unsuccessful efforts to control

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food use, the high motivation to seek the food, and the continued use despite negative consequences (Hebebrand *et al*, 2014). However, BED does not entirely overlap with drug addiction as the sense of feeling out-of-control occurs in BED when a large quantity of food is consumed during a discrete time period. In contrast, substance dependence is not marked by a specific time course of consumption but rather by drug use occurring over a longer time and in higher frequency. Moreover, addiction diagnosis places a greater emphasis on the contribution of the substance (addictive potential of drug) while BED diagnosis does not consider specific types of food consumed (merely the amount) (Gearhardt *et al*, 2011; Gearhardt *et al*, 2014). Furthermore, the possible development of an addictive-like behavior to food has been accepted (Gearhardt *et al*, 2013; Pretlow *et al*, 2015) and it is thought that it may participate in obesity, although normal-weight people may also be vulnerable to eating addiction. Eating addiction and BED do not entirely overlap; and despite multiple similarities, a diagnosis of food addiction was met by 57% of BED patients (Gearhardt *et al*, 2011). Eating addiction, BED, and the co-occurrence of both could have an implication in obesity due to the excess of food consumption. Although evidence of eating addiction has been found in normal-weight patients, a higher rate has been reported in obese patients (Gearhardt *et al*, 2011). Indeed, eating addiction has been considered as a behavior resembling drug addiction only in a subgroup of obese patients or binge eaters (Curtis and Davis, 2014), and genes involved in drug addiction may also be associated with eating addiction (Heber and Carpenter, 2011).

Epigenetics is the study of heritable changes that affect gene expression without changing the DNA sequence transducing environmental stimuli in stable alterations of DNA or chromatin structure (Jaenisch and Bird, 2003). Animal studies have shown that epigenetic mechanisms produced by repeated exposure to drugs mediate addictive-like behavior (Schroeder *et al*, 2008) and could also participate in the possible development of eating addiction. The endocannabinoid system is a key candidate to explore its involvement in this complex behavior through epigenetic, genetic, and pharmacological approaches due to its crucial role in the reward circuit and the pathogenesis of obesity (D'Addario *et al*, 2014). Epigenetic changes usually promote modifications in the expression of different proteins that can be evaluated by proteomic techniques.

These techniques offer the opportunity for assessing the entire expression, translation, modification of genes, and their expression products (Li and Smit, 2008). In our study, proteomic analyses have been performed to identify proteins differentially expressed in mice vulnerable or not to addictive-like behavior in the hippocampus (HCP), striatum (ST), and prefrontal cortex (PFC). The use of proteomic studies is an essential tool for wide proteomic screening to explore global patterns of protein expression and also to identify markers of risk or protection of addictive-like disorders (Ortega *et al*, 2012).

The aim of the present study was to validate a mouse model of addictive-like behavior promoted by palatable food and to evaluate the differential epigenetic and protein expression changes induced by the development of this behavior in specific brain areas. Four brain regions closely related to addictive processes and overeating have been chosen: PFC, ST, nucleus accumbens (NAc), and HCP. The PFC regulates decision making and emotions and has been related to compulsive food intake disorders (Tomasi and Volkow, 2013). The ST is involved in driving the initial motivation for the different rewarding stimuli and has a crucial role in habit formation (Everitt *et al*, 2008). The NAc is a crucial structure in reward processes, including food- and drug-reinforcing effects (Everitt and Robbins, 2005), and the HCP is essential for memory, reward conditioning, and has a crucial role in food consumption relapse (Haber and Murai, 2006).

MATERIALS AND METHODS

Animals

Male CD1 mice (Charles River, France), weighing 30–32 g at the beginning of the first experiment were used. CB_1KO mice and their wild-type littermates, weighing, respectively, 31–35 and 22–26 g, were used in the second experiment (see Supplementary Information).

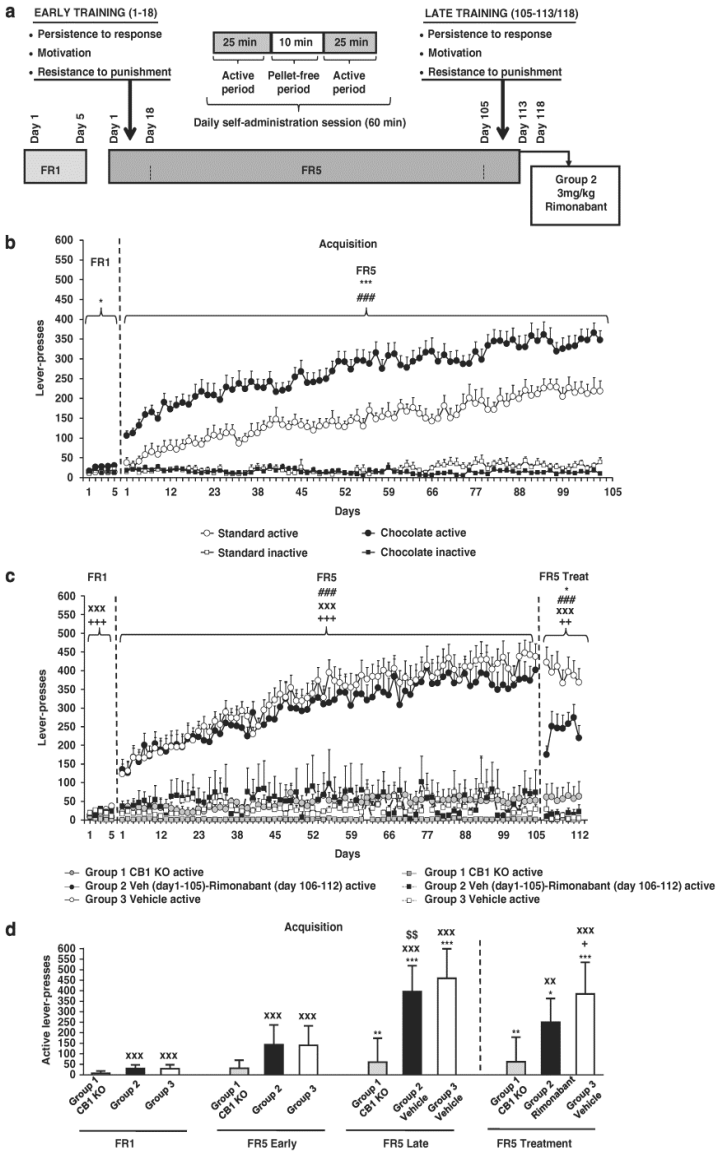
Experimental Design

In a first experiment, WT mice were divided in two groups trained in operant boxes with standard ($n=15$) or chocolate-flavored ($n=30$) pellets. In the second experiment, CB_1KO (Group 1, $n=11$) and WT mice (Group 2, $n=12$ and Group 3, $n=15$) were trained in operant boxes with chocolate-flavored pellets. Animals were trained under a fixed-ratio (FR) 1 schedule of reinforcement in 1-h daily sessions during 5 days, followed by 113 (experiment 1) or 118 (experiment 2) days of training on a FR5 schedule. Every self-administration session was composed by 25 min of normal delivery of pellets (active period), followed by 10 min of non-reinforced active responses (pellets-free period), and 25 additional minutes of active period (Figure 1a). During the pellets-free period, no pellet reinforcer was delivered, signaled by the light that illuminated the entire box. A stimulus light, located above the active lever, was paired contingently with the delivery of the reward during the active periods. A time-out period of 10 s was established after each pellet delivery. During this period, the cue light was off and no reinforcer was provided after responding on the active lever. Responses on the active lever and all the responses performed during the time-out period were recorded. The beginning of each operant responding session was signaled by turning on a house light placed on the ceiling of the box only during the first 3 s of the session. The criteria for acquisition of operant responding were achieved when mice maintained a stable responding with $<20\%$ deviation from the mean of the total number of food pellets earned in three consecutive sessions, with at least 75% responding on the reinforced lever, and a minimum of 10 reinforcers per session (Martin-Garcia *et al*, 2011).

Three addiction-like criteria of eating addiction model were evaluated at two different time points in each mouse, first during the early training sessions (1–18) and then during the late training sessions (days 105–113 experiment 1; days 110–118 experiment 2; Figure 1a). The experimental procedure was the same for experiments 1 and 2 until the day 105. From day 105, WT mice in experiment 2 were treated with vehicle (Group 3, $n=15$) or rimonabant (Group

2, 3 mg/kg, i.p. $n = =12$) and tested for the three criteria of loss of control from day 110 to day 118.

The score of food addiction criteria was attributed considering the responses obtained during the late training sessions using the following three behaviors resembling DSM-5 criteria for addiction: (1) persistence to response or difficulty to cut down of food seeking even if it is not available, measured by the persistence in active responding



during the 10-min periods of signaled no food pellets availability, (2) high motivation for food pellets, measured by a progressive ratio (PR) schedule of reinforcement, and (3) resistance to punishment when food pellets use is maintained despite its negative consequences, measured by resistance to foot shock-induced punishment during operant responding maintained by food. This mouse model of food addiction-like behavior was adapted from that previously described for cocaine addiction in rats (Deroche-Gamonet *et al*, 2004).

Attribution of the three addiction-like criteria. The following behavioral tests were used to measure the criteria of eating addictive-like behavior:

Persistence to response. Responses for active lever-presses during the 10 min of unavailability of pellet delivery (pellets-free period) were measured as a persistence of food-seeking behavior. The active lever responses during the 10 min pellet-free period of the first 3 consecutive days of the early and late training period were evaluated.

Motivation. The PR schedule of reinforcement was used to evaluate the motivation for the food pellet during the early (days 6–13) and late (days 108–110 experiment 1 and days 113–115 experiment 2) period. The response required to earn the pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000, 1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, and 5500. The maximal number of responses that the animal performs to obtain one pellet is the last ratio completed, referred to as the breaking point. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h.

Resistance to punishment. Mice were placed for 1-day session in a self-administration chamber with a different kind of grid in the floor during the early (days 15–18) and late (days 111–113 experiment 1 and days 116–118 experiment 2) period. This environmental change acted as a contextual cue. The schedule was as follows: mice received an electric foot-shock (0.20 mA, 2 s) after four responses and received both, an electric foot-shock (0.20 mA, 2 s) and a pellet, associated with the corresponding conditioned stimulus (cue light), after the fifth response. The schedule was reinitiated at the end of the time-out period, ie, 10 s after the pellet delivery. If mice after the fourth response did not complete the fifth response within a minute, the sequence was reinitiated.

Supplementary Information includes extended experimental procedures with details of the apparatus, the procedure of operant responding maintained by food, and establishment of mice subpopulations. Description about the methods of sample preparation, epigenetic analysis, real-time qPCR (RT-qPCR), proteomic analysis, immunoblotting, and statistical analysis are also provided in the Supplementary Information.

RESULTS

Acquisition of Operant Training Maintained by Food

In experiment 1, body weight was registered in mice trained with standard ($n = 10$, 39.7 ± 1.1 g) or chocolate-flavored pellets ($n = 27$, 40.9 ± 0.5 g) during the entire experiment, and no significant differences between groups were found ($F_{(1,35)} = 0.18$, NS). Both groups of mice underwent a FR1 schedule of reinforcement (5 days) followed by FR5 (113 days) (Figure 1a). Mice that did not achieve the acquisition criteria after day 33 were excluded from the study ($n = 5$ standard group and $n = 3$ chocolate group). Three-way ANOVA revealed a significant interaction between 'group' and 'lever' during the acquisition of operant responding under FR1 ($F_{(1,35)} = 5.44$, $p < 0.05$; Figure 1b; Supplementary Information; Supplementary Table S2A), showing higher number of active responses in the chocolate than in the standard group. The acquisition criteria in FR5 were achieved after an average of 20.2 ± 2.5 sessions by 66.7% of mice trained with standard pellets and of 8.78 ± 1.72 sessions by 90% of mice trained with chocolate pellets. During FR5, three-way ANOVA revealed significant main effects of 'lever' ($F_{(1,35)} = 130.36$, $p < 0.001$), indicating a discrimination in all the groups and an interaction between 'group' and 'lever' ($F_{(1,35)} = 15.69$, $p < 0.001$), indicating higher number of active

Figure 1 (a) Experimental design. Experimental sequence of the eating addiction-like behavior model for the experiments 1 and 2, in which mice were trained for chocolate-flavored pellets or standard pellets (experiment 1) under a fixed-ratio (FR) 1 schedule of reinforcement on 1-h daily sessions during 5 days followed by 113 days (experiment 1) or 118 days (experiment 2) on a FR5 schedule of reinforcement paired with the presentation of a cue light. Each session was composed by 25 min of normal delivery pellets named active period, followed by 10 min of pellets-free period in which the persistence to response was registered, and other 25 min of active period. In the FR5, two time points were considered, early and late period of the training, to measure the three addiction-like criteria: (1) persistence to response, (2) motivation, and (3) resistance to punishment. The experimental procedure was the same for the experiments 1 and 2 until the day 105. From day 105, WT mice in experiment 2 were treated with vehicle (Group 3, $n = 15$) or rimonabant (Group 2; 3 mg/kg, i.p. $n = 12$) and tested for the three criteria of loss of control from day 110 to 118. (b) Acquisition of operant training maintained by food. Mean number of active and inactive lever-presses during the acquisition training in FR1 and FR5 schedule of reinforcement in mice trained with standard pellets ($n = 10$) and mice trained with chocolate-flavored pellets ($n = 27$). Differences are reported as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ (chocolate group vs standard group); $^{\#\#\#}p < 0.001$ (active lever vs inactive lever). (c) Acquisition of operant training maintained by chocolate-flavored pellets. Mean number of active and inactive lever-presses during the acquisition training in FR1 and FR5 schedule of reinforcement in CB_1KO mice (Group 1; $n = 11$), WT mice treated with rimonabant (3 mg/kg, i.p.) (Group 2; $n = 12$) or vehicle (Group 3; $n = 15$) and maintained by chocolate-flavored pellets. Differences are reported as mean ± SEM. $^{xxx}p < 0.05$, (Group 3 vehicle vs Group 1 CB_1KO); $^{++}p < 0.01$, $^{+++}p < 0.001$ (Group 2 rimonabant vs Group 1 CB_1KO); $^{*}p < 0.05$, (Group 1 vehicle vs Group 2 rimonabant); $^{\#\#\#}p < 0.001$ (active lever vs inactive lever). (d) Operant behavior to obtain chocolate-flavored pellets. Mean number of active lever-presses during the acquisition training in FR1 schedule of reinforcement (day 3-5), FR5 early period (day 1-3), FR5 late period (day 103-105), and FR5 during the treatment (days 110–112) in CB_1KO mice (Group 1; $n = 11$), WT mice treated with rimonabant (3 mg/kg, i.p.) (Group 2; $n = 12$,) or vehicle (Group 3; $n = 15$). Differences are reported as mean ± SEM. $^{**}p < 0.05$, $^{***}p < 0.001$ (early period vs late period), $^{xxx}p < 0.01$, $^{xxxx}p < 0.001$ (vs Group 1); $^{+}p < 0.05$ (vs Group 2), and $^{\$\$}p < 0.01$ (Group 2 FR5 late vs Group 2 FR5 treatment).

responses in the chocolate group (Figure 1b; Supplementary Information; Supplementary Table S2A).

In experiment 2, body weight was registered in CB_1KO mice (Group 1; $n=11$, 39.12 ± 1.20 g), WT mice treated after day 105 with rimonabant (3 mg/kg, i.p.) (Group 2; $n=12$, 43.91 ± 1.15 g), or vehicle (Group 3; $n=15$, 47.91 ± 1.03 g). Significant differences between groups were reported ($F_{(2,35)}=15.37$, $p<0.001$) with CB_1KO mice showing decreased body weight compared with vehicle ($p<0.001$) and rimonabant ($p<0.05$) treated mice. All the groups of mice underwent a FR1 schedule of reinforcement (5 days) followed by FR5 (105 days) and by an additional period of FR5 (7 days) in which WT mice were treated with rimonabant (3 mg/kg, i.p.) or vehicle (Figure 1a). Three-way ANOVA revealed a significant interaction between 'day', 'group', and 'lever' during the acquisition of operant responding under FR1 ($F_{(1,8)}=3.07$, $p<0.005$; Supplementary Information; Supplementary Table S2B). Subsequent *post hoc* analysis (Bonferroni) showed differences between groups (Figure 1c and d), indicating that CB_1KO mice performed less active lever-presses than the rest of the groups ($p<0.001$). The acquisition criteria in FR5 were achieved after an average of 3.64 ± 3.44 sessions by 18.2% mice of CB_1KO mice and after 10.4 ± 3.02 or 6.27 ± 2.67 sessions by 100% of the WT groups that will receive vehicle or rimonabant, respectively. During FR5, three-way ANOVA revealed significant main effects of 'day', 'group', and 'lever' ($F_{(1,35)}=2.21$, $p<0.001$), and subsequent *post hoc* analysis (Bonferroni) showed significant interactions between groups ($p<0.001$); Figure 1c and d; (Supplementary Information; Supplementary Table S2B). During the 7 days of pharmacological treatment, three-way ANOVA revealed a significant interaction between 'day', 'group', and 'lever'. Subsequent *post hoc* analysis (Bonferroni) showed significant differences between CB_1KO mice and mice treated with rimonabant (3 mg/kg, i.p., $p<0.05$) or vehicle ($p<0.001$) and between mice treated with vehicle and rimonabant ($p<0.05$), indicating that rimonabant decreased the active lever-presses when compared with the control group and with the previous FR5 training period but to a lower extent than the CB_1KO group (Figure 1c and d) (Supplementary Information Supplementary Table S2B).

Calculation of Addiction Score Based on the Three Addiction-Like Criteria and Distribution of Animals With Different Scores for Addiction-Like Behavior

In experiment 1, the two groups of animals were tested for the three behaviors used to evaluate the addiction-like criteria (persistence to response, motivation, resistance to punishment) during the early (days 1–18) and late (days 105–113) phases of the operant training. A mouse was considered positive for an addiction-like criterion when its score for each behavior was equal or major the 75th percentile of the distribution of chocolate group in the late period (Figure 2a–c). All animals were divided into four subgroups based on the number of criteria for which they scored (Figure 3a). During the early training period, 70% of mice trained with standard pellets presented 0 criteria, 20% reached 1 criteria, 10% showed 2 criteria, and 0% met the 3 criteria, while 40.7% of mice trained with chocolate pellets exhibited 0 criteria, 40.7% reached 1 criteria, 14.8% 2 criteria, and 3.7% obtained the 3 criteria. During the late training period, 70% of mice trained with standard pellets presented 0 criteria, the remaining 30% only reached 1 criteria, and 0% showed 2 or 3 criteria (Figure 3b), whereas 55.6% of mice trained with chocolate pellets exhibited 0 criteria, 22.2% reached 1 criteria, 7.4% got 2 criteria, and 14.8% obtained the 3 criteria (Figure 3c). To confirm that the high score of addiction was reached only by the chocolate group and not by the standard pellet-trained group, the top quartile of this standard group was calculated considering the 75th percentile of the same standard group for the three behaviors resembling addiction. Results showed that none of the mice reached the 3 criteria. Indeed, 40% of mice trained with standard pellets presented 0 criteria in the late period, 50% obtained 1 criteria, 10% reached 2 criteria, and 0% showed the 3 criteria.

In experiment 2, all groups of animals were tested for the three behaviors used to evaluate the addiction-like criteria (persistence to response, motivation, and resistance to punishment) during the early (days 1–18) and late (days 110–118) phases of the operant training, as reported in experiment 1 (behavior was equal or major the 75th percentile of the distribution of vehicle-treated WT mice in the late period), (Figure 2d–f). All animals were divided into four subgroups based on the number of criteria for which they scored (Figure 3d). In all, 91% of CB_1KO mice trained with chocolate pellets presented 0 criteria and 9% reached 1 criteria, and the same distribution was obtained during the late training period (Figure 3e). Moreover, 42% of WT mice trained with chocolate pellets that will receive later rimonabant presented 0 criteria, 42% 1 criteria, and 17% 2 criteria, while 60% of WT mice trained with chocolate pellets that will receive later the vehicle exhibited 0 criteria, 6.7% 1 criteria, 26.7% 2 criteria, and 6.7% 3 criteria. After rimonabant treatment during the late period, 67% of WT mice trained with chocolate reached 0 criteria, 25% 1 criteria, and 8% 2 criteria (Figure 3f), whereas 40% of WT mice treated with vehicle exhibited 0 criteria, 26.7% 1 criteria, 20% 2 criteria, and 13.3% 3 criteria (Figure 3g). Thus vehicle-treated mice increased the percentage of mice reaching 3 criteria in the late period when compared with the early period, whereas rimonabant-treated mice decreased the percentage of animals reaching 1 and 2 criteria in the late period and none reached the 3 criteria in this group.

Differences in Operant Responding Between the 0 and 2–3 Criteria Subgroups and Comparisons in Impulsivity-Like Behavior, Pellets Intake, and Body Weight

In experiment 1, three subgroups were selected for subsequent studies considering the data obtained during the late training period: (1) mice reaching 2–3 criteria ($n=6$), (2) 0 criteria ($n=6$) in the chocolate group, and (3) 0 criteria ($n=6$) in the standard group. In the persistence to response test (pellet-free period), standard pellets-trained mice presenting 0 criteria and chocolate-trained mice reaching the 2–3 criteria increased significantly the number of active lever-presses during the late training ($p<0.01$); (Figure 4a). In the motivation test, only mice trained with chocolate pellets reaching the 2–3 criteria significantly increased the breaking point during the late training ($p<0.05$; Figure 4b). In the resistance to punishment, only mice trained with

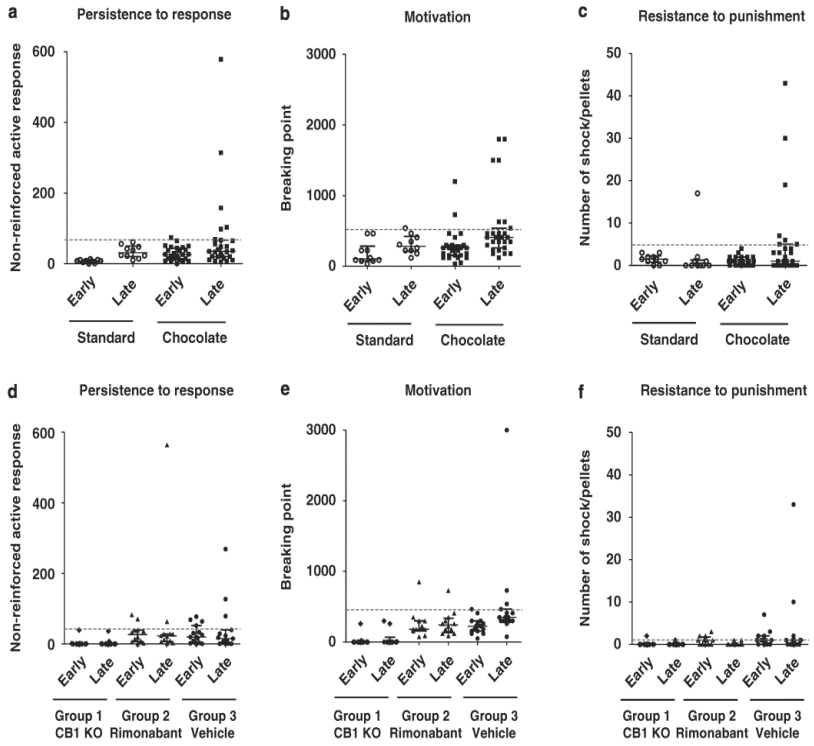


Figure 2 (a–f) Calculation of addiction score based on the three addiction-like criteria for the experiments 1 and 2. Distribution of individual data in the three tests used to measure addiction-like criteria during the early and late training of the (a–c) WT standard and chocolate group; (d–f) CB_1KO mice (Group 1; $n=11$), WT mice treated with rimonabant (3 mg/kg, i.p.) (Group 2; $n=12$), or vehicle (Group 3; $n=15$) chocolate group calculated with median and interquartile range during the test of (a, d) persistence to response, (b, e) motivation, and (c, f) resistance to punishment. The line indicates the 75th percentile of distribution of (a–c) WT chocolate group during the late training period and (d–f) WT chocolate group treated with vehicle during the late training and is used as the criterion to attribute one point of the score to each individual located equal or above this percentile.

chocolate pellets reaching the 2–3 criteria significantly increased the pellets intake during the foot-shock test ($p<0.05$); (Figure 4c). The number of lever-presses during the time-out period was also evaluated as an indirect measure of impulsivity-like behavior. Mice trained with standard (0 criteria) and chocolate pellets reaching the 2–3 criteria significantly increased the number of active lever-presses during the time-out period in the late training ($p<0.01$; Figure 4d). Pearson correlation between time-out and pellets-free period lever-presses in 2–3 criteria chocolate pellets-trained mice revealed the absence of correlation between both variables ($r=0.486$; $p=$NS). This lack of correlation is not surprising considering that the behavioral responses during time-out period, thus after reaching a reward, should be different to those obtained during a signalled pellet-free period. Despite the differences between addiction-like scores groups, mice showing 0 and 2–3 criteria did not significantly differ in the intake of high palatable chocolate pellets during the early and late operant training periods (Figure 4e) and had the same body weight (Figure 4f). Therefore, the differences in these two subgroups were not caused by variation in satiety or energy requirement.

In experiment 2, four subgroups of mice were selected according to the number of criteria attributed during the late training period: (1) 2–3 criteria WT mice treated with vehicle ($n=5$), (2) 0 criteria WT mice treated with vehicle ($n=5$), (3) 0 criteria WT mice treated with rimonabant ($n=5$), and (4) 0 criteria CB_1KO mice ($n=5$). In the persistence to response test (pellet-free period), 2–3 criteria WT mice treated with vehicle increased significantly active lever-presses during the late training when compared with 0 criteria WT mice treated with vehicle or rimonabant and CB_1KO mice ($p<0.01$). Moreover, 0 criteria WT mice treated with vehicle ($p<0.05$) and rimonabant ($p<0.01$) showed significant differences with respect to CB_1KO mice (Figure 4g). In the motivation test, only WT mice treated

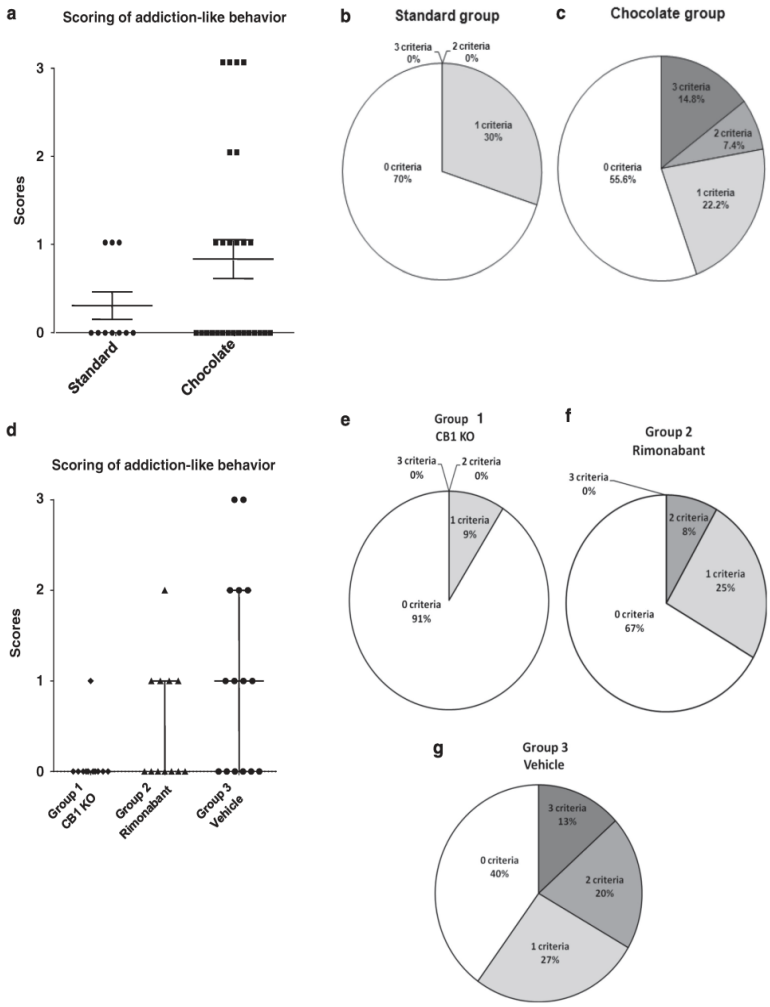


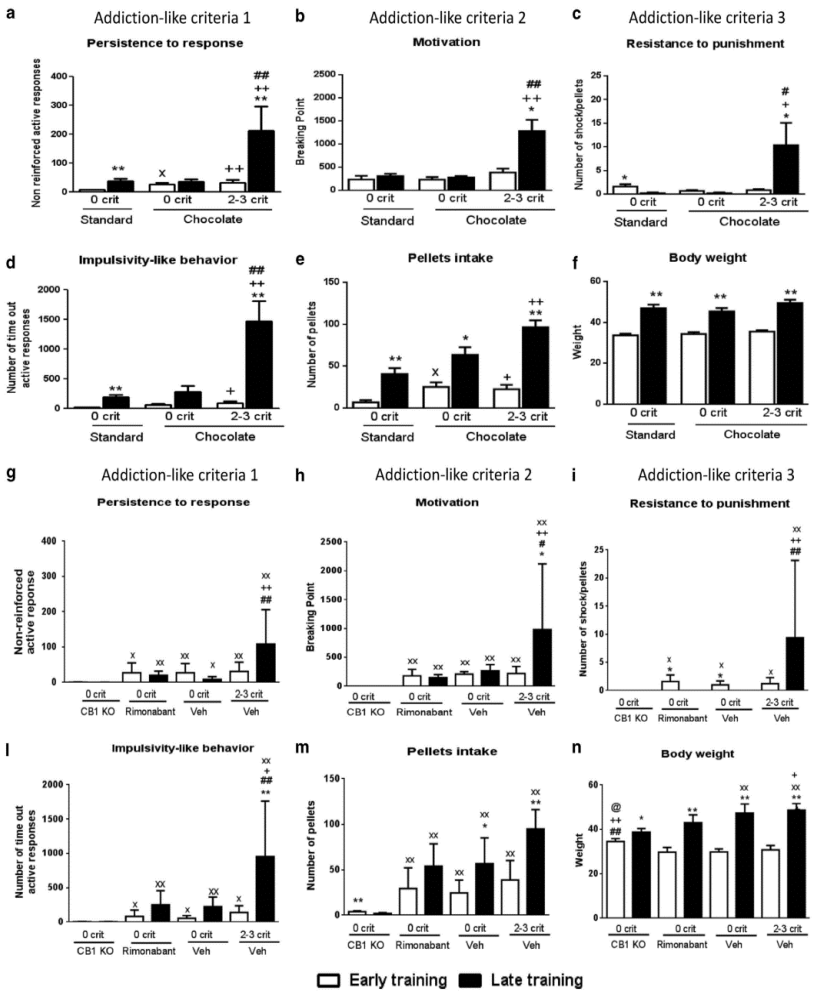
Figure 3 (a–d) Distribution of animals with different scores for addiction-like behavior. It was calculated as the algebraic sum of the scores obtained in each of the three addiction-like criteria. The addiction score was thus distributed along a scale from 0 to 3. Data are expressed with median and interquartile range. (b, c, e–g) Distribution of the different criteria subgroups in percentages. Animals were assigned to a criteria subgroup based on the amount of criteria met for which they scored equal or above the 75th percentile. (b) Percentage of distribution of the WT animals that were exposed to standard pellets, (c) percentage of distribution of WT animals that were exposed to high palatable chocolate-flavored pellets, (e) percentage of distribution of CB_1KO animals exposed to high palatable chocolate-flavored pellets, (f) percentage of distribution of WT animals treated with vehicle or rimonabant (3 mg/kg; i.p.) exposed to high palatable chocolate-flavored pellets, and (g) percentage of distribution of WT animals treated with vehicle exposed to high palatable chocolate-flavored pellets.

with vehicle reaching the 2–3 criteria significantly increased the breaking point during the late training ($p<0.05$) and showed significantly higher breaking point than vehicle ($p<0.05$) or rimonabant ($p<0.01$) WT mice reaching 0 criteria and CB_1KO mice ($p<0.01$; Figure 4h). In the resistance to punishment, only WT vehicle-treated mice reaching 2–3 criteria significantly increased the pellets intake during the foot-shock test when compared with the different

subgroups of 0 criteria ($p<0.01$) (Figure 4i). In addition, mice treated with vehicle reaching 2–3 criteria significantly increased the number of active lever-presses during the time-out period in the late training ($p<0.01$) and showed significant differences with respect to the different 0 criteria subgroups ($p<0.05$ or $p<0.01$; Figure 4l). Finally, a significant increase of pellets intake during the late training period was only observed in 0 and 2–3 criteria vehicle-treated mice ($p<0.05$ and $p<0.01$), whereas CB_1KO mice decreased the pellets intake during this late period (Figure 4m). All groups of mice significantly increased the body weight during the late training ($p<0.05$ and $p<0.01$), although this gain of weight was significantly higher in vehicle-treated WT mice then in WT mice treated with rimonabant and CB_1KO mice (Figure 4n).

Epigenetic Analysis

In the first experiment, specific DNA methylation at CNR1 gene promoter was analyzed by real-time methylation-specific



PCR in PFC, ST, NAc, and HCP. In PFC, DNA methylation of CNR1 gene promoter region was decreased in mice reaching 2–3 criteria trained with chocolate pellets when compared with 0 criteria mice trained with standard pellets ($p<0.05$; Figure 5a). No differences were reported in CB_1 mRNA level, although a significant increase of CB_1 protein expression was revealed in the PFC of mice trained with chocolate pellets reaching 2–3 criteria ($p<0.01$; Figure 5e and i). In HCP, no significant differences were observed in DNA methylation at CNR1 gene promoter and a significant increase in CB_1 gene expression was found in chocolate-trained mice with 2–3 criteria compared with mice trained with standard pellets (Figure 5b, f, and l), although no modification was revealed at the CB_1 protein level. In NAc, no differences between the subgroups were reported in DNA methylation of CNR1 gene promoter, and a significant increase in CB_1 mRNA level was shown when compared with 0 criteria standard pellets-trained mice and 2–3 criteria chocolate-trained mice, although this regulation did not lead to an increase of CB_1 protein expression (Figure 5c, g, and m). In ST, no significant differences were observed in DNA methylation at the CNR1 gene promoter, CB_1 mRNA and protein levels (Figure 5d, h, and n).

Proteomic Analysis

Proteomic profile was analyzed among the subgroups of the first experiment in HCP, ST, and PFC (see Supplementary Information).

Immunoblotting

Immunoblotting was performed to validate the most relevant changes obtained in the proteomic study in HCP, ST, and PFC (see Supplementary Information).

DISCUSSION

We have validated a novel operant model of eating addictive-like behavior in a heterogenic population of mice exposed to long-term operant training to obtain palatable food. This model permits to distinguish different subpopulations of mice vulnerable (22.2%) or resistant (55.6%) to addiction. Several changes in gene and protein expression were identified in the mouse subpopulations in PFC, HCP, NAc, and ST through epigenetic and proteomic studies. Using genetic and pharmacological approaches, we demonstrated the crucial involvement of CB_1 receptor in the development of this addictive-like behavior.

Food palatability strongly promoted operant seeking-behavior in our experimental conditions. Thus mice trained with chocolate-flavored pellets increased their operant active responses during FR1 and FR5 schedule more than animals trained with standard pellets. The percentage of chocolate-trained mice that achieved the acquisition criteria was higher and in a shorter duration time (90% in 8 sessions) than in standard pellets-trained mice (66.7% in 20 sessions). A differential response depending on the kind of pellets was also revealed when testing for the three criteria of loss of behavioral control that mimic addictive-like behavior. Indeed, 14.7% of mice trained with chocolate pellets reached in the late training period the 3 criteria and 7.4% achieved 2 criteria, whereas none of the mice trained with standard pellets achieved these criteria. These mice with high scores also revealed increased operant responses even when no reward can be obtained during the time-out period, suggesting an enhancement of food seeking that can be related to increased impulsivity. The identification of this subgroup of mice losing behavioral control supports the hypothesis that addiction-like behavior represents a pathological continuum from controlled to compulsive use that is only reached by a limited percentage of users (Piazza and Deroche-Gamonet, 2013). Experiments performed in rodents also using the three criteria of loss of control of operant seeking to obtain a drug of abuse (cocaine) revealed a higher percentage of animals reaching these criteria (17.2% 3 criteria and 13.8% 2 criteria); (Deroche-Gamonet *et al*, 2004). This could be due to the different behavioral responses promoted by natural rewards and drugs of abuse, as well as by the different animal species (rats and mice) and experimental conditions required to achieve these distinct experiments. Considering the strict criteria defined in our

Figure 4 (a–c) Differences in operant responding between the 0 and 2–3 criteria subgroups. Mean ± SEM operant responses in the 0 and 2–3 criteria subgroups during the early and late period of training. (a) in the persistence to response. (b) in the motivation, and (c) in the resistance to punishment for standard and chocolate group. (d–f) Comparisons in impulsivity-like behavior, pellets intake, and body weight. (d) Mean of active lever-presses during the time-out period during the 3 consecutive day sessions in the early and late operant training periods for the standard subgroup and chocolate subgroups (0 criteria and 2–3 criteria). (e) Mean number of pellets intake during 3 consecutive sessions in the early and late period of the operant training for the standard subgroup and chocolate subgroups (0 criteria and 2–3 criteria). (f) Mean of body weight during the early and late periods of the operant training for the standard subgroup and chocolate subgroups (0 criteria and 2–3 criteria). Data are expressed as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ (early training vs late training); $^{\#}p<0.05$, $^{\#\#}p<0.01$ (2–3 criteria vs 0 criteria chocolate), $^{+}p<0.05$, $^{++}p<0.01$ (2–3 criteria vs 0 criteria standard); $^{\S}p<0.05$ (0 criteria chocolate vs 0 criteria standard). (g–n) Differences in operant responding between the 0 and 2–3 criteria subgroups. Mean ± SEM operant responses in the 0 and 2–3 criteria subgroups during the early and late period of training. (g) in the persistence to response. (h) in the motivation, and (i) in the resistance to punishment for the CB_1KO group, WT group treated with rimonabant (3 mg/kg; i.p.), and WT group treated with vehicle that were exposed to high palatable chocolate-flavored pellets. (l–n) Comparisons in impulsivity-like behavior, pellets intake, and body weight. (l) Mean of active lever-presses during the time-out period during the 3 consecutive day sessions in the early and late operant training periods for the CB_1KO group, WT group treated with rimonabant (3 mg/kg; i.p.), and WT group treated with vehicle that were exposed to high palatable chocolate-flavored pellets (0 criteria and 2–3 criteria). (m) Mean number of pellets intake during 3 consecutive sessions in the early and late period of the operant training for the CB_1KO group, WT group treated with rimonabant (3 mg/kg; i.p.) and WT group treated with vehicle that were exposed to high palatable chocolate-flavored pellets (0 criteria and 2–3 criteria). (n) Mean of body weight during the early and late periods of the operant training for the CB_1KO group, WT group treated with rimonabant (3 mg/kg; i.p.), and WT group treated with vehicle that were exposed to high palatable chocolate-flavored pellets (0 criteria and 2–3 criteria). Data are expressed as mean ± SEM $^{*}p<0.05$, $^{**}p<0.01$ (early training vs late training); $^{\#}p<0.05$, $^{\#\#}p<0.01$ (vs 0 criteria vehicle); $^{+}p<0.05$, $^{++}p<0.01$ (vs 0 criteria rimonabant); $^{\S}p<0.05$, $^{\S\S}p<0.01$ (0 criteria CB_1KO), $^{\$\$}p<0.05$ (vs 2–3 criteria vehicle).

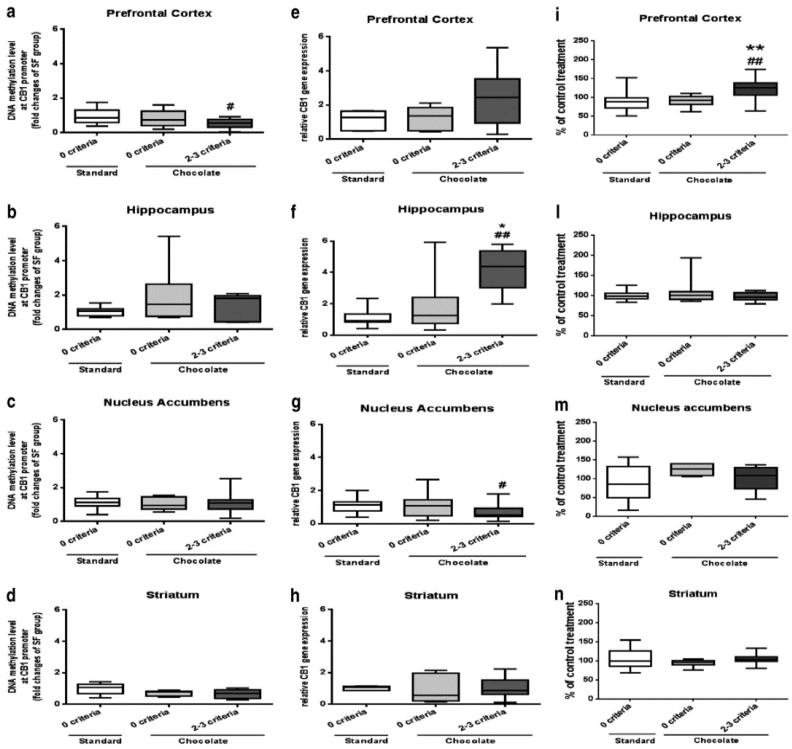


Figure 5 CB_1 epigenetic regulation through DNA methylation at its *CNR1* gene promoter, *CNR1* gene expression, and CB_1 protein levels in eating addiction like-behavior. (a–d) Level of DNA methylation in the (a) prefrontal cortex, (b) hippocampus, (c) striatum, and (d) nucleus accumbens. (e–h) *CNR1* gene expression regulation in the (e) prefrontal cortex, (f) hippocampus, (g) striatum, and (h) nucleus accumbens. (i–n) Quantification of CB_1 protein levels in the (i) prefrontal cortex, (l) hippocampus, (m) striatum, and (n) nucleus accumbens. The optical density was normalized to GAPDH in the same samples. Data are reported as a fold change of standard subgroup; $^{\#}p<0.05$, $^{\#\#}p<0.01$ (vs mice 0 criteria standard pellets); $^{*}p<0.05$, $^{**}p<0.01$ (vs mice 0 criteria chocolate pellets).

protocol to achieve a loss of control of a natural reward seeking in mice and the difficulties to accomplish a complex operant responding in these animal species, we have established that mice achieving 2 and 3 criteria (22.2%) have reached a reliable loss of control of this behavioral response. This percentage is similar to those reported in other studies using operant responding to obtain drugs of abuse and allow obtaining a minimum amount of biological samples from the mouse brains to achieve the subsequent epigenetic and proteomic analysis. Only mice reaching the high and low score were selected for the subsequent studies. None of the mice of the standard pellet group reached the 2–3 criteria. The lack of standard pellets mice reaching these criteria could represent a potential limitation in the interpretation of proteomic and epigenetic data as comparisons between the chocolate 2–3 criteria and standard 2–3 criteria groups could not be performed.

Long-term operant training to obtain highly palatable food produced adaptive changes at the epigenetic level. Mice that reach the 2–3 criteria showed a significant reduction in DNA methylation at CNR1 gene promoter in PFC, which led to an upregulation of CNR1 gene expression and the subsequent increase in CB_1 protein receptor in the same brain area. The increased level of CB_1 protein may be due to multiple factors that are involved in gene transcription and protein synthesis, including methylation of the gene promoter. Differential changes were also observed in the CNR1 gene expression in the NAc and HCP of the addict-like mice, although these regulations could not be functionally relevant as no modification was revealed at the CB_1 protein level, the ending product through which the genetic information determines cellular functions.

CB_1 receptors has a crucial role in the reinforcing and motivational properties of highly palatable food (Maccioni *et al*, 2008). Indeed, CB_1R modulates the glutamatergic excitatory and GABAergic inhibitory synaptic inputs in several brain regions, acting as a regulatory feedback

mechanism to modulate synaptic transmission (D'Addario *et al*, 2014). Previous studies proposed a bimodal regulation of food intake by CB_1R in cortical glutamatergic transmission, responsible for the orexigenic effect, and CB_1R in ventrostriatal GABAergic neurons, mediator of the hypophagic action by reducing local inhibitory transmission (Bellocchio *et al*, 2010). We hypothesized that the CB_1R epigenetic regulation in PFC and the subsequent translation in increased CB_1 protein level found in our study may modulate the primary glutamatergic neuronal output of this region, as already described in other studies (Steketee, 2003). CB_1R on cortical glutamatergic cells are less abundant but produce more pronounced effects than on GABAergic cells (Steindel *et al*, 2013). Specifically, the decrease in DNA methylation at CNR1 gene could promote an upregulation of CB_1R on glutamatergic projection terminals leading to a CB_1-dependent inhibition of excitatory glutamatergic transmission that promotes food intake. This CB1R overexpression on glutamatergic projections could decrease glutamate release in the NAc contributing to a less activation of the NAc GABAergic inputs to VTA, removing the inhibitory modulation on Da neurons, as already reported (Lupica *et al*, 2004; Melis *et al*, 2004). Hence, CB_1R acting indirectly via presynaptic inhibition of neurons and interneurons (Szabo *et al*, 2002) may ultimately affect brain reward processes through their ability to enhance extracellular DA levels in the NAc (Fadda *et al*, 2006; Lecca *et al*, 2006).

We have performed pharmacological studies with a selective CB_1 receptor antagonist and genetic approaches with constitutive CB_1 knockout mice to evaluate our hypothesis about the involvement of CB_1 receptor in the development of this addictive-like behavior produced by palatable food. The pharmacological treatment with the CB_1 receptor antagonist rimonabant (3 mg/kg) at the beginning of the late training period reduced the percentage of mice that reached addiction scores. Thus none of rimonabant-treated mice reached the 3 criteria, whereas 67% of treated mice achieved 0 criteria. In contrast, 13% of vehicle-treated mice reached the 3 criteria and 20% achieved 2 criteria, similarly to the previous experiment although the responses in the three behavioral tests were decreased with respect to the previous experiment as expected considering the stressor event that represents the repeated intraperitoneal injection. These results supports the hypothesis that CB_1 receptors are involved in the achievement of addiction-like behavior, thus in the transition to addiction that evolves from controlled to compulsive intake. In agreement, deletion of CB_1 receptors in constitutive CB_1KO mice reduced operant seeking behavior in our experimental conditions. Thus CB_1KO mice trained with chocolate-flavored pellets significantly reduced operant active responses during FR1 and FR5 compared with WT mice. The percentage of CB_1KO mice that reached addiction criteria in the late period was significantly lower than WT mice, and none of CB_1KO mice reached the 3 or 2 criteria in the late training period. These CB_1KO mice also showed decreased operant responses during the time-out period, which can be related to a lack of impulsivity. Therefore, the pharmacological or genetic disruption of CB_1 receptor activity reduced the reinforcing effect of palatable food and prevented the transition to addiction. Future studies are needed to elucidate the functional and structural interactions between CB_1 receptors and the main neurotransmitter system in the context of eating addiction.

Proteomic studies have revealed changes in the protein expression and in the level of phosphorylation of synaptic proteins depending on the experimental subgroup and the brain area analyzed. These proteomic data were compared with previous studies with drugs of abuse in order to identify similar molecular targets underlying addictive-like behaviors. Protein expression profiles were compared in the three brain regions studied and specific common proteins related to impulsive-like behavior, synaptic plasticity, and cannabinoid signaling that could promote neurobiological changes leading to eating addiction-like behavior were selected and validated by immunoblot technique: α-Syn (impulsive control: Ambermoon *et al*, 2011), PP1α, DCalmK 2, (synaptic plasticity processes: Edelman *et al*, 2005; Hou *et al*, 2013), and DGKζ (regulation of the endocannabinoid activity: Liu *et al*, 2001). An overexpression of α-Syn was revealed in the HCP of mice trained with chocolate pellets reaching the 2–3 criteria and in the PFC of mice trained with chocolate pellets presenting 0 criteria, which could facilitate impulsivity-like behavior. In agreement, a similar increase of α-Syn expression in the HCP was reported in cocaine-addicted rats (Brenz Verca *et al*, 2003). α-Syn is a neuronal protein regulating dopaminergic transmission (Boyer and Dreyer, 2007) involved in synaptic vesicular transport and synaptic plasticity (Murphy *et al*, 2000) and has been implicated in impulsive control disorders, such as drug addiction (Pena-Oliver *et al*, 2012). In contrast, a decreased expression of the same protein was found in the ST of mice trained with standard pellets that leads to speculate impairment in dopaminergic neurotransmission. Indeed, α-Syn-deficient mice exhibited dopaminergic hyperactivity and a reduction in presynaptic striatal DA store (Abeliovich *et al*, 2000; Senior *et al*, 2008).

A downregulation of PP1α expression in HCP and an upregulation in ST was observed in chocolate-trained mice reaching the 2–3 criteria, while no modifications were reported in PFC. This protein localized in excitatory synapses is involved in synaptic plasticity (Hou *et al*, 2013) and seems to be important for triggering long-term depression (LTD) through the dopamine-induced phosphorylation of DARPP-32 (Yan *et al*, 1999). PP1α overexpression in the ST of mice reaching the highest criteria could induce LTD as PP1 is crucial for maintaining NMDAR-dependent LTD (Mulkey *et al*, 1993). The opposite regulation of PP1α expression in HCP and ST could modify synaptic strength leading to the plasticity changes promoted by palatable food training.

Palatable food training increased the expression of DCalmK 2 in mice reaching the 2–3 criteria and 0 criteria in the HCP and ST, respectively, whereas no modifications were reported in the PFC. DCalmK 2 is a protein localized in the distal dendrites that suppresses the maturation of spine structures (Shin *et al*, 2013), covering an important role in the regulation of synaptic plasticity (Edelman *et al*, 2005). The increased expression of the DCalmK 2 protein after long-term exposure to chocolate pellets may therefore regulate the maturation of dendritic spines and synaptic plasticity.

An overexpression of DGKζ protein was selectively observed in the HCP and ST of chocolate-trained mice with

0 criteria. DGKζ modules subcellular levels of diacylglycerol and phosphatidic acid, as well the synthesis of triacylglycerols (Topham and Epand, 2009). Previous research demonstrated that high-fat diet stimulated DGKζ expression in hypothalamus, and this expression was reduced in obese animals being inversely related to body fat mass and serum leptin level (Liu *et al*, 2001). Furthermore, DGKζ seems to modulate the cannabinoid signaling (Gantayet *et al*, 2011) and could lead to hypothesize an inversely expression regulation between DGKζ and CB1R in specific brain areas under our experimental conditions.

In conclusion, our research validated for the first time an operant model of eating addiction in a heterogenic mouse population and identified extreme subpopulations vulnerable or resistant to addiction. We detected in these subpopulations specific epigenetic and proteomic alterations in the HCP, ST, NAc, and PFC. Changes in DNA methylation at *CNR1* gene promoter and its encoding transcript were observed in the PFC. The involvement of these changes in CB_1R in the development of this addictive-like behavior was demonstrated by using genetic and pharmacological approaches. Moreover, we identified proteins expressed in different subpopulations of mice that have allowed formulating novel hypotheses on the molecular mechanisms orchestrating eating addiction. These changes could participate in the biological substrate underlying the behavioral alterations that could eventually lead to eating-related disorders and provide an important advance in understanding the mechanisms engaged in the hedonic aspects of food consumption furthering eating addiction.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Animals

Male wild-type CD1 mice used in the fist experiment were purchased from Charles River (France). Male CB1 knockout mice and their wild-type littermates were used in the second experiment. The generation of mice lacking CB1 cannabinoid receptor was performed as described previously (Soria et al., 2005). Mice were housed individually in controlled laboratory conditions (temperature at 21 ± 1°C and humidity at 55 ± 10%) and were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 8.00 hours and on at 20.00 hours). Food and water were available ad libitum. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Operant behaviour apparatus

Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two retractable levers, one randomly selected as the active lever and the other as the inactive. Pressing on the active lever resulted in a pellet delivery together with a stimulus-light (associated-cue), located above the active lever, while pressing on the inactive lever had no consequences. The chambers were made of aluminium and acrylic, and were

housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise. A food dispenser equidistant between the two levers permitted delivery of food pellets when required.

Food pellets

During the operant experimental sessions, animals received after active responding a 20 mg standard pellet (TestDiet, Richmond, IN, USA) or a 20 mg highly palatable isocaloric pellet (TestDiet, Richmond, IN, USA). The standard pellet formula was similar to the standard maintenance diet provided to mice in their home cage (24.1% protein, 10.4% fat, 65.5% carbohydrate, with a caloric value of 3.30 kcal/g). Highly palatable isocaloric pellets presented similar caloric value of standard pellets (20.5% protein, 12.7% fat, 66.8% carbohydrate, with a caloric value of 3.48 kcal/g) with some slight differences in their composition: addition of chocolate flavour (2% pure unsweetened cocoa) and modification in the sucrose content. Indeed, although the carbohydrate content was similar in standard (65.5%) and highly palatable isocaloric pellets (66.8%), the proportion of sugars within this carbohydrate amount was different: sucrose content in standard pellets was 3.1% of the total carbohydrates and 50.1% in highly palatable isocaloric pellets. These pellets were presented only during the operant behaviour sessions and animals were maintained on standard chow for their daily food intake.

Experimental design

Establishment of mice subpopulations

A mouse was considered positive for a particular addiction-like criterion when the score for this behaviour was equal or above the 75th percentile of the distribution achieved by the group showing the highest rate of responses that in all the cases corresponded to the late training in the chocolate group in the first experiment and in the WT chocolate group treated with vehicle in the second experiment. Animals were scored for each addiction-like behaviour (three) independently and the algebraic sum of scores was calculated. Four subgroups of mice were identified depending on the number of positive criteria met (0crit, 1crit, 2crit and 3crit) (Figure 3). Based on these behavioural results, in the first experiment three different animal subgroups were considered for further analysis (behavioural and proteomic studies) depending on the diet and criteria met, as follows: a) 2-3 criteria chocolate subgroup (n=6), b) 0 criteria chocolate subgroup (n=6) and c) 0 criteria standard subgroup (n=6). Due to the accomplishment of several criteria of loss of control, we considered the chocolate trained mice reaching the 2 criteria as addict-vulnerable phenotypes and therefore were included in the subgroup with the highest score. For the epigenetic and the immunoblot studies, subgroups of mice of a previous study were added to obtaining a representative amount of biological samples from the mouse brains and the total number of animals used are reported as follows: a) 2-3 criteria chocolate subgroup (n=13), b) 0

criteria chocolate subgroup (n=16) and c) 0 criteria standard subgroup (n=14).

In the second experiment, the following subgroups were considered for the behavioural study: a) 2-3 criteria WT mice treated with vehicle (n=5), b) 0 criteria WT mice treated with vehicle (n=5) c) 0 criteria WT mice treated with rimonabant (3mg/kg; ip.) (n=5) and d) 0 criteria CB1KO mice (n=5).

Sample preparation

All animals were decapitated immediately after the last training session. The brains were quickly removed and the following brain areas dissected according to the atlas of stereotaxic coordinates of mouse brain (Paxinos and Franklin, 1997): PFC, ST, NAc and HCP. Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at -80ºC for later proteomic and epigenetic studies. Each brain sample was treated individually. Studies of gene expression at the level of transcription and translation by quantification of DNA, RNA and proteins were made isolating DNA, RNA, and proteins from the selected animals using the Nucleo Spin TriPrep kit (Macherey-Nagel GmbH & Co.KG, Germany). Isolated DNA and RNA were used for epigenetic studies and isolated proteins for proteomic studies and validation with immunoblot techniques.

Epigenetic analysis

Methylation status of CB1R (CNR1) promoter regions was determined using methylation-specific primer real-time PCR. After

the extraction, 0.5 µg of DNA from each sample was treated with bisulfite, using DNA methylation kit (Zymo Research, Orange, CA, USA), in according to the manufacturers protocol. Methylation analysis was performed by fluorescence-based real-time qRT-PCR, using SensiFAST™ SYBR® Kit (Bioline, London, UK), on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). Amplified CNR1 sequence, containing 17 CpG sites, was located within 5' upstream region of the gene. The amplification program was as follow: 95 °C for 2 min; 50 cycles at 95 °C for 10 s, 60 °C for 30s. PCR was also performed for non-CpG-containing region of β-Act that served as control gene. The primers used for amplification (M: methylation specific, U: specific for unmethylated sequence) were the following: M_CNR1 (F: 5'-tttatttggtcggttttttgtc-3', R: 5'-ctcaccctactctactccctaacg-3'), U_CNR1 (F: 5'-tttatttggttggttttttgttgt-3', R: 5'-ctcaccctactctactccctaacac-3') and β-Act (F: 5'-ggtattgttgataggatgtagaagga-3', R: 5'-tctaaatactaaaattccccttaaacc-3')
The DNA methylation level was calculated as follows: DNA methylation level= (1/1+2-ΔCt). ΔCt= CtU-CtM (Lu et al., 2007).The data reported are shown as fold of induction over standard food group.

Real-time qPCR (RT-qPCR)

RT-PCR reactions were performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The relative abundance of each mRNA species was assessed by quantitative real-time RT-PCR (qRTPCR), using

SensiFAST SYBR No-ROX Kit (Bioline) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). All data were normalized to the endogenous reference genes β-actin and GAPDH. The primers used for PCR amplification are the following: CB1R (F: 5'-ccaagaaaagatgacggcag-3', R: 5'-aggatgacacatagcaccag-3'); β-ACT (F: 5'-tgttaccaactgggacga-3', R: 5'-gtctcaaacatgatctgggtc-3'); GAPDH (F: 5'-aacgggaagctcactggcat-3', R: 5'-gcttcaccaccttcttgatg-3'). One μl of the first strand cDNA product was used for amplification in triplicate in 20 μl reaction solution, containing 10 μl of SensiFAST SYBR No-ROX Kit and 10 pmol of each primer. The following PCR program was used: 95°C for 10 min, followed by 45 amplification cycles of 95°C for 10 sec, 57°C for 30 sec and 72 for 30 sec.

Proteomic analysis

Shotgun analysis using high-resolution mass spectrometry together with label-free quantification for the analysis of diet-induced neuropeptide expression in mice brain was performed. Phosphoproteomic and protein analysis were studied in HCP, ST and PFC regions. The enrichment in phosphoprotein or phosphopeptide content could help to identify the activation of particular proteins or pathways. Indeed, a change in phosphorylation status may reflect a modification in protein activity, and can provide new insights to clarify the intricate cellular networks involved in these addiction-like processes (Tannu et al., 2008). Samples from 6 representative animals of each subgroup

were pooled together in 3 pools of 2 animals per pool in equal proportion. Every sample was prepared in triplicate. Precipitated protein samples were dissolved in 4% SDS in 0.1 M HEPES (pH=8.5) followed by concentration measurement using DC-micro plate assay (Bio-Rad, Spain), following manufacturer's instructions. 200 ug of each sample of every pooled protein sample were subjected to trypsin digestion following the FASP protocol as previously described (Wisniewski et al., 2009) for subsequent mass spectrometric analysis of the cellular proteome. In addition, 100 □g of each digested sample was further subjected to phosphopeptide enrichment using TiO2. Briefly, TiO2 microcolumns were prepared in gel loading tips (0.5 mg) and were equilibrated with loading buffer (80 % ACN in water + 6 % TFA). Samples were loaded in loading buffer and the columns were washed with 80 % ACN in water + 0.1 % TFA. Finally, phosphopeptides were eluted with 30 µL of elution buffer (5 % NH3 in water) into an eppendorf containing 30 µL of 20% formic acid in water. 1ug of the non-enriched samples and all the samples enriched in phosphorylated peptides were run on an LTQ-Orbitrap Velos (Thermo Fisher Scientific, United States) fitted with a nanospray source (Thermo Fisher Scientific, United States) previous nanoLC separation in a EasyLC system (Proxeon, Denmark). Peptides were separated in a reverse phase column, 75µm x 150mm (Nikkyo Technos Co., Ltd., Japan) with a gradient of 5 to 35% ACN with 0.1% FA in 60 min at a flow of 0.3 □L/min. The Orbitrap Velos was operated in positive ion mode with nanospray voltage set at 2.2 kV and source temperature at 325 °C. The instrument was externally calibrated

using Ultramark 1621 for the FT mass analyzer. An internal calibration was performed using the background polysiloxane ion signal at m/z 445.120025 as the calibrant. The instrument was operated in data-dependent mode. In all experiments, full MS scans were acquired over a mass range of m/z 350-2000 with detection in the Orbitrap mass analyzer at a resolution setting of 60,000. For each MS scan, the twenty most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation with CID at normalized collision energy of 35% in the LTQ linear ion trap. All data were acquired with Xcalibur 2.2 (Thermo Fisher Scientific, United States). Protein identification was performed by Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific, United States) using MASCOT v2.3 (Matrix Science, United Kingdom) as search engine and a mouse SwissProt database that included the most common contaminants. Carbamidomethylation for cysteines was set as fixed modification whereas acetylation in protein N-terminal, oxidation of methionine and phosphorylation in Ser, Thr and Tyr were set as variable modifications (being the latter only set in samples enriched in phosphorylated peptides). Peptide tolerance was 7 ppm in MS and 0.5Da in MS/MS mode, maximum number of missed cleavages was set at 3. Peptides were filtered based on the FDR (False Discovery rate) lower than 1%. Protein quantification was calculated using the R package MSStats (Clough et al., 2012). Two analyses were performed with different treatment of the missing values. The "strict" analysis considered only features that had at least two values in each group, while the "relaxed" analysis considered also

the features with one or two groups with no values. In this case, a value corresponding to the background noise was imputed.

Immunoblotting

Proteomic results were validated using immunoblot techniques. For immunoblot antibodies anti-CB1R (1:500) (Frontier Institute, Japan), anti- alpha syn (1:800) (Cell Signaling, Merck KGaA, Germany), anti-DCalmK 2 (1:600) (Abcam, UK), anti-PP1 alpha (1:600) and anti-DGKζ (1:500) (Santa Cruz Biology, USA) were used (see Supplementary Information). Shortly, proteins from HCP, ST and PFC of the three different animal subgroups were processed (2-3 criteria chocolate pellets, 0 criteria chocolate pellets, 0 criteria standard pellets subgroup). Bound primary antibodies were detected with horseradish peroxidase-conjugated antibodies to rabbit, or mouse, or goat antibodies (Pierce, diluted 1: 4000) and visualized by enhanced chemiluminescence detection (West-Fetmo-SuperSignaling, Pierce). Western Blotting was performed with BioRad Criterion System (Bio-Rad Laboratories Headquarters, USA), using precast polyacrylamide 4–20% gradient gel, 13.3 × 8.7 cm (W × L), for use with Criterion electrophoresis cells. The optical density of the immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v4.6.3 (Bio-Rad). Representative cropped immunoblots for display were processed with Adobe Photoshop 7.0. For quantitative purposes, the optical density values of the specific antibodies were normalized to the detection of specific antibodies to GAPDH values in the same sample and expressed as a percentage of control treatment.

Statistical analysis

In the acquisition phase, in the experiment 1 the behavioural data of operant responding maintained by food were analysed using three-way repeated measures analysis of variance (ANOVA) with diet (chocolate versus standard food) as between-subject factor and lever (active/inactive) and day as within-subjects factors. In the behavioural tests to evaluate the addictive-like behaviour, comparisons between the subgroups (2-3 criteria chocolate, 0 criteria chocolate, 0 criteria standard) were made through Mann–Whitney U test (non-normally distributed data according to Kolmogorov test) (Supplementary Information Table S1A). In epigenetic study, comparisons among the three subgroups (2-3 criteria chocolate, 0 criteria chocolate and 0 criteria standard pellets) were achieved through Mann–Whitney U test. In the immunoblot, analysis of protein changes validation was made using a parametric Student t-test.

In the acquisition phase, in the experiment 2 the behavioural data of operant responding maintained by food were also analyzed using three-way repeated measures analysis of variance (ANOVA) with group (CB1KO versus WT) as between-subject factor and lever (active/inactive) and day as within-subjects factors. When the pharmacologic treatment started on day 105, data analysis was performed considering the between-subject factor group with three levels (CB1KO, WT rimonabant and WT vehicle). In the behavioural tests to evaluate the addictive-like behaviour, comparisons between the subgroups (2-3 criteria WT mice treated with vehicle, 0 criteria WT mice treated with vehicle, 0 criteria WT

mice treated with rimonabant and 0 criteria CB1KO mice were made through Mann–Whitney U test (non-normally distributed data according to Kolmogorov test) (Supplementary Information Table S1B).

Statistical analysis in proteomic was made to compare the following subgroups: 1) 2-3 criteria vs. 0 criteria chocolate subgroup, 2) 2-3 criteria vs. 0 criteria standard subgroup and 3) 0 criteria chocolate vs. 0 criteria standard subgroup. Correction for multiple testing was performed by adjusting the p-value with a false discovery rate (FDR) of 1%. As the features with no values detected were considered when comparing the three groups, a value corresponding to the background noise was imputed and the results were represented in terms of "expression/no expression" for each protein (see Supplementary Information Table S3). Differences in the significance of some peptides forming part of the same protein were also observed. The relationships within the three comparisons were analyzed and the following issues were considered: (a) protein expression changes in mice trained with chocolate pellets reaching 0 criteria (resistance to addiction-like behaviour) (b) protein expressed only in mice trained with chocolate pellets reaching the 2-3 criteria (presence in addiction-like subgroup) (c) proteins expressed in mice trained with chocolate and standard pellets with 0 criteria, but not in chocolate trained mice reaching the 2-3 criteria (absence in addiction-like subgroup) (d) protein expression changed only in mice trained with chocolate pellets (2-3 criteria or 0 criteria) but not in mice trained with standard pellets (palatable food training), (Figure S2A). A subsequent second proteomic analysis

was performed and only features that had at least two of three values detected in each group were considered (see Supplementary Information Table S4). Protein identification was processed by a search against a mouse UniProt database. Canonical pathway enrichment analyses were performed using Kyoto encyclopaedia of genes and genome (KEGG) pathways as well as Gene Ontology (GO) enrichment analyses.

The data are expressed as mean ± SEM, distribution of individual data with median and interquartile range or fold change of control group. $P<0.05$ was the criterion for statistical significance for behavioural, epigenetic and immunoblot analysis, while $p<0.01$ was used for the proteomic analysis. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 15.0 (SPSS Inc, Chicago, USA).

SUPPLEMENTARY RESULTS

Proteomic analysis

The NAc was excluded due to the reduced amount of protein obtained in this brain area, which impedes the proteomic studies in this animal species. In HCP, changes in the expression of 13 proteins were revealed in the chocolate trained mice presenting 0 criteria (resistance to addiction-like behaviour). Moreover, 21 proteins and 1 phosphoprotein were significantly identified only in the chocolate trained mice reaching the 2-3 criteria (presence in addiction-like subgroup). Also, 19 proteins and 2 phosphoproteins were absent in the chocolate trained mice reaching the 2-3 criteria

(absence in addiction-like subgroup) (Figure S2B, C). In ST, changes in the expression of 16 proteins and 31 phosphoproteins were reported in the chocolate trained mice presenting 0 criteria. Moreover, 40 proteins and 6 phosphoproteins were modified in the chocolate trained mice reaching the 2-3 criteria, whereas 9 proteins and 25 phosphoproteins were absent in this group (Figure S2D, E). In PFC, changes in the expression of 37 proteins and 26 phosphoproteins were reported in the chocolate trained mice obtaining 0 criteria. Furthermore, 13 proteins and 32 phosphoproteins were significantly identified in the chocolate trained mice reaching the 2-3 criteria, whereas 16 proteins and 14 phosphoproteins were absent in this subgroup (Figure S2F, G). All the proteins and phosphoproteins resulted from the comparisons are listed in Table S3 and S4.

We compared protein expression profiles through analysis of canonical pathways enrichment and we validated common proteins expressed in the three brain regions related to neurological processes. More specifically, due to our results obtained on impulsivity like-behaviour (time-out period), we selected alfa-SYN, a protein reported to be specially implicated in this behaviour (Pena-Oliver et al., 2012). Moreover, we were interested in the possible alteration in the expression of proteins involved in synaptic plasticity during addictive-like behaviour. Particularly in the phenomenon of polarization/depolarization which directly involves the PP1alpha protein. In addition, we have selected DCAMKL2 as a protein closely related to the possible morphological changes that could be associated to these plasticity changes. Finally DGK is a

protein directly connected to the endocannabinoid system that together with CB1R could be of particular relevance for the behavioural alterations reported. As reported in the analysis of canonical pathways enrichment, alpha-synuclein (alfa-Syn) peptide involved in the "regulation of DA uptake implicated in synaptic transmission" pathway was identified in HCP and ST areas of chocolate trained mice reaching the 2-3 criteria and in PFC of the chocolate trained mice presenting 0 criteria. Protein phosphatase 1 alpha (PP1alfa) involved in the "long term potentiation" pathway was detected in HCP of chocolate and standard subgroup presenting 0 criteria and in ST of chocolate trained mice reaching 2-3 criteria. Moreover, two additional proteins were considered in HCP: doublecortin-like kinase 2 (DCalmK 2) involved in the "nervous system development" pathway expressed in chocolate trained mice reaching the 2-3 criteria and diacylglycerol kinase zeta (DGKζ) involved in the "protein kinase C-activating G-protein coupled receptor signaling" pathway expressed in the chocolate trained mice presenting 0 criteria.

Immunoblotting

In HCP, changes in the expression of PP1alfa protein were validated in the chocolate and standard subgroup presenting 0 criteria ($p<0.05$) and the presence of alfa-Syn and DCalmK proteins were identified in chocolate trained mice reaching the 2-3 criteria ($p<0.01$). An increased expression of DGKζ protein was also revealed in the chocolate trained mice with 0 criteria ($p<0.05$) (Figure S3A).

In ST, change in the expression of PP1alfa protein was validated in the chocolate trained mice reaching the 2-3 criteria ($p<0.01$). Decrease of alfa-Syn expression was found in the standard trained mice presenting 0 criteria ($p<0.05$). Interestingly, significant expression changes in DCalmK 2 and DGKζ protein were additionally revealed in the chocolate subgroup presenting 0 criteria ($p<0.05$) (Figure S3B).

In PFC, significant increase in alfa-Syn expression was revealed in the chocolate group presenting 0 criteria ($p<0.05$). No changes were observed in DCalmK 2, PP1 alfa and DGKζ proteins expression, in agreement to the proteomic study (Figure S3C).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Effects of CB1 epigenetic regulation by highly palatable food. Immunoblot of CB1 in (A) PFC, (B) HCP, (C) NAc and (D) striatum homogenates from mice of 0 criteria standard (n = 14), 0 criteria chocolate (n = 16) and 2-3 criteria chocolate (n = 13). The optical density was normalized to GAPDH in the same samples.

Figure S2. Protein expression changes caused by operant responding maintained by food: expression / no expression. (A) Venn Diagrams of differentially expressed proteins for each comparison in the three brain areas. Questions considered: (a) protein expression changes in mice trained with chocolate pellets reaching 0 criteria (resistance to addiction-like behaviour) (b)

protein expressed only in mice trained with chocolate reaching the 2-3 criteria (presence in addiction-like subgroup) (c) proteins expressed in mice trained with chocolate and standard pellets with 0 criteria, but not in chocolate trained mice reaching the 2-3 criteria (absence in addiction-like subgroup) (d) protein expression changed only in mice trained with chocolate pellets (2-3 criteria or 0 criteria) but not in mice trained with standard pellets (palatable food exposure). (B-G) Total number of proteins differentially expressed for each comparison in (B) HCP, (D) striatum and (F) PFC. Total number of phosphoproteins expressed in (C) HCP, (E) striatum and (G) PFC.

Figure S3. Immunoblot and quantification of alpha-synuclein, protein phosphatase 1 alpha, doublecortin-like kinase 2 and diacylglycerol kinase zeta in (A) HCP, (B) striatum and (C) PFC homogenates from mice of 0 criteria standard, 0 criteria chocolate and 2-3 criteria chocolate (n =6 mice per group). The optical density was normalized to GAPDH in the same samples. Data are expressed as mean±SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (2-3 criteria chocolate vs. 0 criteria standard), # $p<0.05$, ## $p<0.01$, ### $p<0.001$ (2-3 criteria chocolate vs. 0 criteria chocolate), + $p<0.05$ (0 criteria chocolate vs. 0 criteria standard).

Table S3 (3.1) Spectral counts of mouse (A) HCP proteins, (B) ST proteins, (C) PFC proteins, with 1 or more peptide identifications subjected to label-free quantification. Proteins are grouped according to their abundance in the four issues considered ($p<0.01$).

(3.2) Spectral counts of mouse (A) HCP phosphoproteins, (B) ST phosphoproteins, (C) PFC phosphoproteins, with 1 or more peptide identifications subjected to label-free quantification. Proteins are grouped according to their abundance in the four issues considered ($p<0.01$).

Table S4 (4.1) Spectral counts of mouse (A) HCP proteins, (B) ST proteins, (C) PFC proteins with 1 or more peptide identifications subjected to label-free quantification. Peptides that were significantly differentially expressed ($p<0.05$) are shown. **(4.2)** Spectral counts of mouse (A) HCP phosphoproteins, (B) ST phosphoproteins, (C) PFC phosphoproteins with 1 or more peptide identifications were subjected to label-free quantification. Phosphopeptides that were significantly differentially expressed ($p<0.05$) are shown.

SUPPLEMENTARY TABLES

Table S1A Normality test

		Kolmogorov-Smirnov					
		Persistance to response		Motivation		Resistance to punishment	
		F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Early	0 criteria standard	$F_{(1,6)} = 0.32$	*n.s*	$F_{(1,6)} = 0.28$	*n.s*	$F_{(1,6)} = 0.21$	*n.s*
	0 criteria chocolate	$F_{(1,6)} = 0.23$	*n.s*	$F_{(1,6)} = 0.24$	*n.s*	$F_{(1,6)} = 0.41$	$P < 0.01$
	2-3 criteria chocolate	$F_{(1,6)} = 0.23$	*n.s*	$F_{(1,6)} = 0.18$	*n.s*	$F_{(1,6)} = 0.48$	$P < 0.001$
Late	0 criteria standard	$F_{(1,6)} = 0.23$	*n.s*	$F_{(1,6)} = 0.14$	*n.s*	$F_{(1,6)} = 0.49$	$P < 0.001$
	0 criteria chocolate	$F_{(1,6)} = 0.23$	*n.s*	$F_{(1,6)} = 0.32$	*n.s*	$F_{(1,6)} = 0.49$	$P < 0.001$
	2-3 criteria chocolate	$F_{(1,6)} = 0.27$	*n.s*	$F_{(1,6)} = 0.31$	*n.s*	$F_{(1,6)} = 0.31$	*n.s*

Table S1B Normality test

		Kolmogorov-Smirnov					
		Persistance to response		Motivation		Resistance to punishment	
		F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Early	0 criteria CB1 KO	$F_{(1,10)} = 0.28$	$P < 0.05$	$F_{(1,10)} = 0.52$	$P < 0.001$	$F_{(1,10)} = 0.52$	$P < 0.001$
	0 criteria Rimonabant	$F_{(1,5)} = 0.26$	*n.s*	$F_{(1,5)} = 0.47$	$P < 0.001$	$F_{(1,5)} = 0.37$	$P < 0.05$
	0 criteria vehicle	$F_{(1,9)} = 0.16$	*n.s*	$F_{(1,9)} = 0.15$	*n.s*	$F_{(1,9)} = 0.41$	$P < 0.001$
	2-3 criteria vehicle	$F_{(1,5)} = 0.30$	*n.s*	$F_{(1,5)} = 0.26$	*n.s*	$F_{(1,5)} = 0.27$	*n.s*
Late	0 criteria CB1 KO	$F_{(1,10)} = 0.47$	$P < 0.001$	$F_{(1,10)} = 0.51$	$P < 0.001$	$F_{(1,10)} = 0.52$	$P < 0.001$
	0 criteria Rimonabant	$F_{(1,8)} = 0.26$	*n.s*	$F_{(1,8)} = 0.17$	*n.s*	$F_{(1,8)} = 0.51$	$P < 0.001$
	0 criteria vehicle	$F_{(1,6)} = 0.24$	*n.s*	$F_{(1,6)} = 0.37$	$P < 0.05$	$F_{(1,6)} = 0.49$	$P < 0.001$
	2-3 criteria vehicle	$F_{(1,5)} = 0.23$	*n.s*	$F_{(1,5)} = 0.39$	$P < 0.05$	$F_{(1,5)} = 0.31$	*n.s*

Table S2A Experiment 1: operant responding maintained by food during acquisition

Three-way ANOVA				
	Acquisition FR1		Acquisition FR5	
	F-value	*P*-value	*F*-value	*P*-value
Group	$F_{(1,35)} = 2.33$	*n.s*	$F_{(1,35)} = 11.28$	$P < 0.01$
Lever	$F_{(1,35)} = 2.89$	*n.s*	$F_{(1,35)} = 130.36$	$P < 0.001$
Day	$F_{(4,140)} = 2.02$	*n.s*	$F_{(78,2730)} = 14.09$	$P < 0.001$
Day × Lever	$F_{(4,140)} = 1.02$	*n.s*	$F_{(78,2730)} = 13.28$	$P < 0.001$
Group × Lever	$F_{(1,35)} = 5.44$	$P < 0.05$	$F_{(1,35)} = 15.69$	$P < 0.001$
Group × Day	$F_{(4,140)} = 0.33$	*n.s*	$F_{(78,2730)} = 0.63$	*n.s.*
Group × Lever × Day	$F_{(4,140)} = 2.28$	*n.s*	$F_{(78,2730)} = 0.97$	*n.s.*

Three-way ANOVA between-subjects factor and repeated measures in the factors day and lever (active/inactive). See materials and methods for details. *n.s.:* non significant

Table S2B Experiment 2: operant responding maintained by food during acquisition

Three-way ANOVA						
	Acquisition FR1		Acquisition FR5		Acquisition FR5 Treatment	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Group	$F_{(1,35)} = 12.90$	*P<0.001*	$F_{(1,35)} = 20.51$	*P<0.001*	$F_{(1,35)} = 21.43$	*P < 0.001*
Lever	$F_{(1,35)} = 1.02$	*n.s*	$F_{(1,35)} = 101.90$	*P<0.001*	$F_{(1,35)} = 68.87$	*P < 0.001*
Day	$F_{(4,140)} = 6.38$	*P<0.001*	$F_{(74,2590)} = 12.17$	*P<0.001*	$F_{(6,210)} = 102.02$	*P < 0.001*
Day × Lever	$F_{(4,140)} = 5.54$	*P<0.001*	$F_{(74,2590)} = 11.01$	*P<0.001*	$F_{(6,210)} = 40.73$	*P < 0.001*
Group × Lever	$F_{(2,35)} = 2.34$	*n.s*	$F_{(2,35)} = 15.21$	*P<0.001*	$F_{(2,35)} = 25.85$	*P < 0.001*
Group × Day	$F_{(8,140)} = 2.91$	$P < 0.005$	$F_{(74,2590)} = 2.60$	*P<0.001*	$F_{(12,210)} = 19.60$	*P < 0.001*
Group × Lever × Day	$F_{(8,140)} = 3.07$	$P < 0.003$	$F_{(148,2590)} = 2.20$	*P<0.001*	$F_{(12,210)} = 6.66$	*P < 0.001*

Three-way ANOVA between-subjects factor and repeated measures in the factors day and lever (active/inactive). See materials and methods for details. *n.s.:* non significant

Figure S1

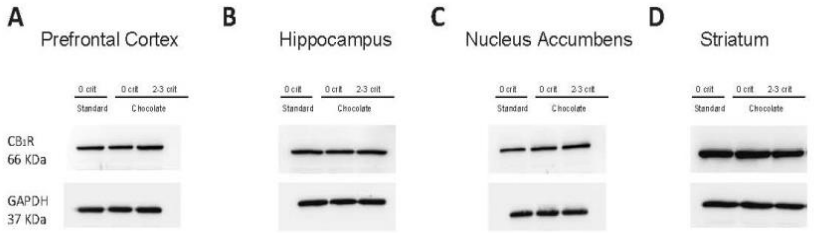

Figure S2

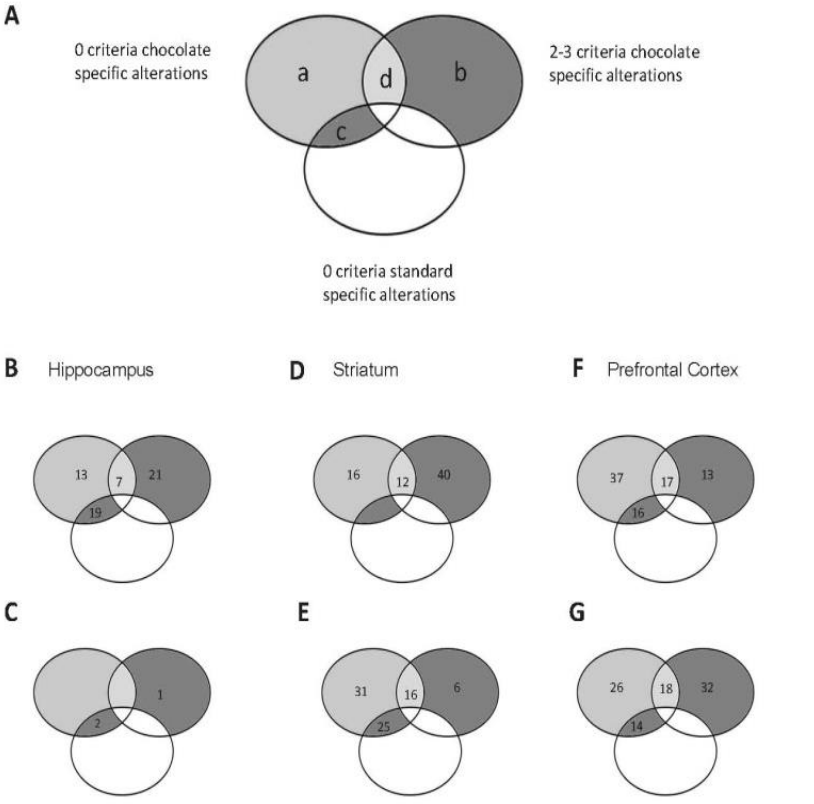

Figure S3

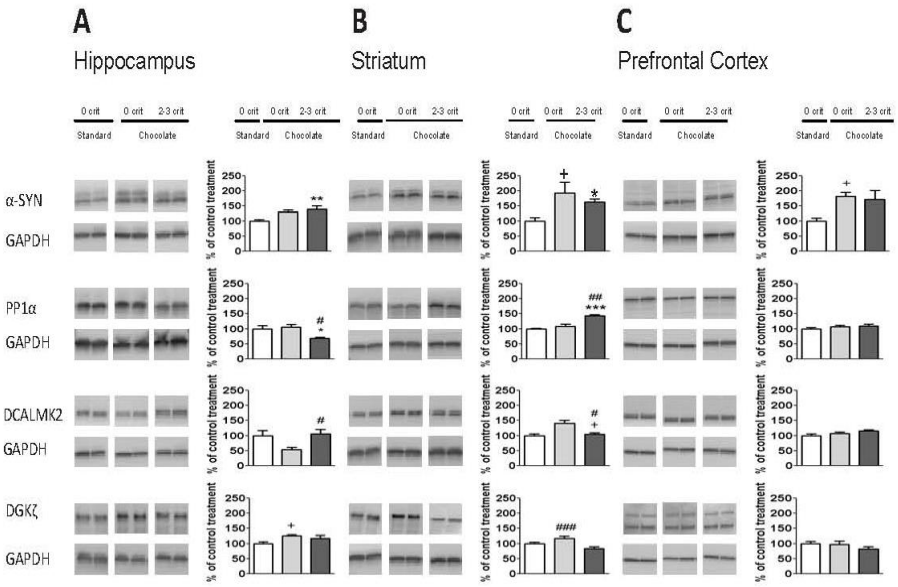
A
Hippocampus
B
Striatum
C
Prefrontal Cortex
0 crit
2-3 crit
Standard
Chocolate
α-SYN
GAPDH
PP1α
DCALMK2
DGKζ
% of control treatment

Table S3

3.1 Proteins showing differential abundance among the subgroups.

A Hippocampus

Expressed in	UniProt accesion	Protein name
GROUP2-3criteria	Q62318	Transcription intermediary factor 1-beta
	Q64105	Sepiapterin reductase
	Q6PGN3-6	Serine/threonine-protein kinase DCLK2
	Q8K0S0	Phytanoyl-CoA hydroxylase-interacting protein
	Q8R5M8-4	Cell adhesion molecule 1
	O55042-2; Q91ZZ3	Alpha-synuclein, Beta-synuclein
	O70172	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha
	O88643	Serine/threonine-protein kinase PAK 1
	P62823	Ras-related protein Rab-3C
	P70336	Rho-associated protein kinase 2
	Q91VR5	ATP-dependent RNA helicase DDX1
	Q9ES97-2	Reticulon-3
	P35762	CD81 antigen
	P47791-2	Glutathione reductase, mitochondrial
	P99027	60S acidic ribosomal protein P2
	Q61838	Alpha-2-macroglobulin
	Q6ZPJ8	Ubiquitin-conjugating enzyme E2 O
	Q9CR16	Peptidil-prolil cis-trans isomerasa D
	Q9CYG7	Mitochondrial import receptor subunit TOM34
	Q9JKW0	ADP-ribosylation factor-like protein 6-interacting protein 1
	Q9R0P3	S-formylglutathione hydrolase
GROUP2-3criteria and GROUP0criteria	O35682 P62702	Myeloid-associated differentiation marker 40S ribosomal protein S4, X isoform
	Q62417-5	Sorbin and SH3 domain-containing protein 1
	P14869	60S acidic ribosomal protein P0
	Q4KML4	Costars family protein ABRACL
	B2RSH2;P08752	Guanine nucleotide-binding protein G(i) subunit alpha-1; Guanine nucleotide-binding protein G(i) subunit alpha-2
	Q640R3	Hepatocyte cell adhesion molecule
GROUP0criteria and control	O08788	Dynactin subunit 1
	P61294	Ras-related protein Rab-6B
	P62137 Q5SRX1-2	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit TOM1-like protein 2
	Q64674	Spermidine synthase
	Q9Z268	RasGAP-activating-like protein 1
	Q8R1Q8	Cytoplasmic dynein 1 light intermediate chain 1
	P45376	Aldose reductase
	P49722	Proteasome subunit alpha type-2
	P59325	Eukaryotic translation initiation factor 5
	P61148	Fibroblast growth factor 1
	P84099	60S ribosomal protein L19
	Q6P1B1	Xaa-Pro aminopeptidase 1
	Q8VDD5;Q61879	Myosin-9; Myosin-10
	Q99JG2	Endothelin B receptor-like protein 2
	Q99KJ8	Dynactin subunit 2
	Q99LP6	GrpE protein homolog 1, mitochondrial

	Q9D173	Mitochondrial import receptor subunit TOM7 homolog
	Q9JMA1	Ubiquitin carboxyl-terminal hydrolase 14
GROUP0criteria	Q60598	Src substrate cortactin
	P31648	Sodium- and chloride-dependent GABA transporter 1
	P34022	Ran-specific GTPase-activating protein
	P63141	Potassium voltage-gated channel subfamily A member 2
	P68181-2;P05132-2	cAMP-dependent protein kinase catalytic subunit beta; cAMP-dependent protein kinase catalytic subunit alpha
	Q80UP3	Diacylglycerol kinase zeta
	Q8CGC7	Bifunctional glutamate/proline--tRNA ligase
	Q9D0F9	Phosphoglucomutase-1
	Q60625	Intercellular adhesion molecule 5
	Q8VCM7	Fibrinogen gamma chain
	Q91ZP9-2	N-terminal EF-hand calcium-binding protein 2
	Q9D898	Actin-related protein 2/3 complex subunit 5-like protein
	Q9R0Q6	Actin-related protein 2/3 complex subunit 1A
GROUP2-3criteria and control	Q80TE7	Leucine-rich repeat-containing protein 7
	P62331	ADP-ribosylation factor 6
	Q8VHI6	Wiskott-Aldrich syndrome protein family member 3
	P70195	Proteasome subunit beta type-7
	Q64310	Surfeit locus protein 4
	Q9CXS4	Centromere protein V
	Q9CY58-3	Plasminogen activator inhibitor 1 RNA-binding protein

B Striatum

Expressed in	UniProt accession	Protein name
GROUP2-3criteria	O35633-2;O35295;P42669;Q8R4E6-2	Vesicular inhibitory amino acid transporter; Transcriptional activator protein Pur-beta;Transcriptional activator protein Pur-alpha; Purine-rich element-binding protein gamma
	P21836	Acetylcolinesterase
	P22005	Proencefalin-A
	P28661-6	Septin-4
	P62137;P62141	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit; Serine/threonine-protein phosphatase PP1-beta catalytic subunit
	Q69ZK9	Neuroligin-2
	Q6PH08-2	ERC protein 2
	Q80Z24	Neuronal growth regulator 1
	Q8BRU6	Synaptic vesicular amine transporter
	Q8K406	Leucine-rich repeat LGI family member 3
	O55042-2; Q91ZZ3	Alpha-synuclein; Beta-synuclein
	Q99LX0	Protein DJ-1
	Q9ES97-2	Reticulon-3
	Q9R0N7	Synaptotagmin-7
	O88487	Cytoplasmic dynein 1 intermediate chain 2
	P13595-2	Neural cell adhesion molecule 1
	P57776-2	Elongation factor 1-delta
	P63001;P60766;P84096	Ras-related C3 botulinum toxin substrate 1; Cell division control protein 42 homolog; Rho-related GTP-binding protein RhoG
	Q64010-2	Adapter molecule crk
	Q6PB44-2	Tyrosine-protein phosphatase non-receptor type 23E
	Q8BGQ7	Alanine--tRNA ligase, cytoplasmic
	Q8CI32	BAG family molecular chaperone regulator 5
	Q8VHI6	Wiskott-Aldrich syndrome protein family member 3

	Q91VH6	Protein MEMO1
	Q91XL9-3	Oxysterol-binding protein-related protein 1
	Q9D1A2	Cytosolic non-specific dipeptidase
	Q9QZQ8-2	Core histone macro-H2A.1
	Q9R0Q7	Prostaglandin E synthase 3
	A2ARS0	Ankyrin repetir dominio que contienen proteinas 63
	O54901	OX-2 membrane glycoprotein
	P61957	Small ubiquitin-related modifier 2
	Q00623	Apolipoprotein A-I
	Q8BP67	60S proteina ribosomal L24
	Q8CI71-2	Coiled-coil domain-containing protein 132
	Q8K1Z0	Ubiquinone biosynthesis protein COQ9, mitochondrial
	Q8R464	Cell adhesion molecule 4
	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial
	Q9CQJ8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9
	Q9D1A2	Cytosolic non-specific dipeptidase
	Q9DBF1-2	Alpha-aminoadipic semialdehyde dehydrogenase
GROUP2-3criteria and GROUP0criteria	Q8R071	Inositol-trisphosphate 3-kinase A
	P62821;P61027;Q6PHN9;Q9D1G1	Ras-related protein Rab-1A; Ras-related protein Rab-10; Ras-related protein Rab-35; Ras-related protein Rab-1B
	Q5SQX6;Q7TMB8-2	Cytoplasmic FMR1-interacting protein 2; Cytoplasmic FMR1-interacting protein 1
	Q6IRU5-3	Clathrin light chain B
	Q99JY8	Lipid phosphate phosphohydrolase 3
	P51410	60S ribosomal proteina L9
	P62702	40S ribosomal S4 proteinas, X isoforma
	Q05186	Reticulocalbin-1
	Q60625	Intercellular adhesion molecule 5
	Q6NS60	F-box only protein 41
	Q91YQ5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
	Q9R1P3	Proteasome subunit beta type-2
GROUP0criteria and control	Q9CTY5	Calcium uptake protein 3, mitochondrial
	Q80TZ3-2	Putative tyrosine-protein phosphatase auxilin
	Q63912	Oligodendrocyte-myelin glycoprotein
	Q91Z69	SLIT-ROBO Rho GTPase-activating protein 1
	P16460	Argininosuccinate synthase
	P56379	6.8 kDa mitochondrial proteolipid
	P62334	26S protease regulatory subunit 10B
	P97355	Spermine synthase
	Q9JHI5	Isovaleryl-CoA dehydrogenase, mitochondrial
GROUP0criteria	P42859-2	Huntingtin
	P63213	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2
	Q60629	Ephrin tipo-A receptor 5
	Q61207	Sulfated glycoprotein 1
	Q64331	Unconventional miosina VI
	Q6P5F9	Exportin-1
	Q9CY58-4	Plasminogen activator inhibitor 1 RNA-binding protein
	Q9WV69	Dematin
	O88343	Electrogenic sodium bicarbonate cotransporter
	P24369	Peptidyl-prolyl cis-trans isomerase
	P63073	Eukaryotic translation initiation factor 4E
	Q99LY9	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5
	Q9CZM2	60S ribosomal protein L15

	Q9D0L7-2	Armadillo repeat-containing protein 10
	Q9D0S9	Histidine triad nucleotide-binding protein 2, mitochondrial
	Q9DC07	LIM zinc-binding domain-containing Nebulette
GROUP2-3criteria and control	O70161-2	Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma
	Q05816	Fatty acid-binding protein, epidermal
	Q8C419	Probable G-protein coupled receptor 158
	Q924D0	Reticulon-4-interacting protein 1, mitochondrial
	Q99JF8-2	PC4 and SFRS1-interacting protein
	Q99KB8-2	Hydroxyacylglutathione hydrolase, mitochondrial
	Q9CPR4	60S ribosomal protein L17
	Q9CQJ6	Density-regulated protein
	Q9Z130;Q60668-4	Heterogeneous nuclear ribonucleoprotein D-like; Heterogeneous nuclear ribonucleoprotein D0

C Prefrontal cortex

Expressed in	UniProt accesion	Protein name
GROUP2-3criteria	Q99JG2	Endothelin B receptor-like protein 2
	Q9CPW4	Actin-related protein 2/3 complex subunit 5
	Q9CQV7	Mitochondrial import inner membrane translocase subunit TIM14
	Q9CR16	Peptidyl-prolyl cis-trans isomerase D
	Q9WTU3-2	Sodium channel protein type 8 subunit alpha
	P61087	Ubiquitin-conjugating enzyme E2 K
	P21278	Guanine nucleotide-binding protein subunit alpha-11
	O88342	WD repeat-containing protein 1
	O35685	Nuclear migration protein nudC
	O08585	Clathrin light chain A
	O70133-3	ATP-dependent RNA helicase A
	Q00PI9	Heterogeneous nuclear ribonucleoprotein U-like protein 2
	Q9JKR6	Hypoxia up-regulated protein 1
GROUP2-3criteria and GROUP0criteria	P15209-2	BDNF/NT-3 growth factors receptor
	P35803-3	Neuronal membrane glycoprotein M6-b
	Q9WV92-6	Band 4.1-like protein 3
	P62827	GTP-binding nuclear protein Ran
	O54774	AP-3 complex subunit delta-1
	P02104	Hemoglobin subunit epsilon-Y2
	P06795	Multidrug resistance protein 1B
	P17665	Cytochrome c oxidase subunit 7C, mitochondrial
	P51410	60S ribosomal protein L9
	P52503	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial
	P84096	Rho-related GTP-binding protein RhoG
	Q5SVL6	Rap1 GTPase-activating protein 2
	Q6ZWN5	40S ribosomal protein S9
	Q8CCT4	Transcription elongation factor A protein-like 5
	Q99MN9	Propionyl-CoA carboxylase beta chain, mitochondrial
	Q9JMH9-5	Unconventional myosin-XVIIIa
	Q9R0P3	S-formylglutathione hydrolase
GROUP0criteria and control	P48453-2	Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform
	P61957	Small ubiquitin-related modifier 2
	Q810U4-2	Neuronal cell adhesion molecule

Q68FF6	ARF GTPase-activating protein GIT1
P27048	Small nuclear ribonucleoprotein-associated protein B
P47955	60S acidic ribosomal protein P1
Q9CR62	Mitochondrial 2-oxoglutarate/malate carrier protein
Q8CCB4	Vacuolar protein sorting-associated protein 53 homolog
Q8R164	Valacyclovir hydrolase
Q9CRB8	Mitochondrial fission process protein 1
Q9R1P3	Proteasome subunit beta type-2

3.2 Phosphoproteins showing differential abundance among the subgroups

A Hippocampus

Expressed in	peptide	UniProt access	phosphoRS.Site.Probabilities	protein name
GROUP2-3 criteria	DMSPSAETEAPLAK	P27546	S(3): 99.6; S(5): 0.4; T(8): 0.0	Microtubule-associated protein 4
GROUP0criteria and control	ESSPLYSPGFSDSTSAAK	P14873	S(2): 0.5; S(3): 99.5; Y(6): 0.5; S(7): 99.5; S(11): 0.0; S(13): 0.0; T(14): 0.0; S(15): 0.0	Microtubule-associated protein 1B
	YLATASTMDHAR	P04370	Y(1): 0.0; T(4): 0.0; S(6): 96.5; T(7): 3.5	Myelin basic protein
GROUP2-3 criteria and control	SEAEDDEEDEVDDLPSSR	Q61097	S(1): 100.0; S(16): 0.0; S(17): 0.0	Kinase suppressor of Ras 1
	SPFEIISPPASPPEMTGQR	Q9QYR6	S(1): 0.0; S(7): 100.0; S(11): 100.0; T(16): 0.0	Microtubule-associated protein 1A

B Striatum

Expressed in	peptide	UniProt access	phosphoRS.Site.Probabilities	protein name
GROUP2-3criteria	DFQEYVEPGEDFPASPQR	Q8R1Q8	Y(5): 0.0; S(15): 100.0	Cytoplasmic dynein 1 light intermediate chain 1
	GGSSEELHDSPR	Q3UMU9	S(3): 0.0; S(4): 0.0; S(10): 100.0	Hepatoma-derived growth factor-related protein 2
	MESEAGADDSAEEGDLLDD DDNEDRGDDQLELK	Q9Z204	S(3): 0.0; S(10): 100.0	Heterogeneous nuclear ribonucleoproteins C1/C2
	RFSNVGLVHTSER	Q9QWI6	S(3): 100.0; T(10): 0.0; S(11): 0.0	SRC kinase signaling inhibitor 1
	SASQDCIETTPGAQEGK	P98084	S(1): 9.2; S(3): 90.8; T(9): 0.0; T(10): 0.0	Amyloid beta A4 precursor protein-binding family A member 2
	SYHSSLR	Q61290	S(1): 0.0; Y(2): 0.0; S(4): 0.3; S(5): 99.7	Voltage-dependent R-type calcium channel subunit alpha-1E
GROUP2-3 criteria and 0criteria	AFYGSEEDEAK	Q9JME5	Y(3): 0.0; S(5): 100.0	AP-3 complex subunit beta-2

	AGGGRPSSPSPSVVSEK	Q80TJ1	S(7): 0.0; S(8): 0.0; S(10): 0.0; S(12): 0.0; S(15): 100.0	Calcium-dependent secretion activator 1
	AGSVADSDAVVK	Q8VED9	S(3): 0.0; S(7): 100.0	Galectin-related protein
	DDDDIDLFGSDDEEESEEAK	O70251	S(10): 100.0; S(16): 0.0	Elongation factor 1-beta
	ELASPVSPELR	A2AN08	S(4): 100.0; S(7): 0.0	E3 ubiquitin-protein ligase UBR4
	GAASGPSAAEEAGSEEAGPAGEPR	O88703	S(4): 0.0; S(7): 0.0; S(14): 100.0	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2
	INVYYNEATGGNYVPR	Q9D6F9	Y(4): 0.0; Y(5): 0.0; T(9): 100.0; Y(13): 0.0	Tubulin beta-4A chain
	LHQVYFDAPSCVK	Q62277	Y(5): 100.0; S(10): 0.0	Synaptophysin
	SGTSTPTTPGSTAITPGTPPSYSSR	P20357	S(1): 0.0; T(3): 0.0; S(4): 0.0; T(5): 0.0; T(7): 0.0; T(8): 0.0; S(11): 0.0; T(12): 0.0; T(15): 0.0; T(18): 100.0; S(21): 0.0; Y(22): 0.0; S(23): 0.0; S(24): 0.0	Microtubule-associated protein 2
	SGYSSPGSPGTPGSR	P10637	S(1): 0.0; Y(3): 0.0; S(4): 0.0; S(5): 0.0; S(8): 0.0; T(11): 100.0; S(14): 0.0	Microtubule-associated protein tau
	SHSLDSLSR	Q3UHB8	S(1): 0.0; S(3): 100.0; S(6): 0.0; S(8): 0.0	Coiled-coil domain-containing protein 177
	SSSTGNLLDK	Q8BKX1	S(1): 0.0; S(2): 0.0; S(3): 4.2; T(4): 95.8	Brain-specific angiogenesis inhibitor 1-associated protein 2
	SYSSPDITQALQEEEK	Q80U87	S(1): 5.9; Y(2): 5.9; S(3): 87.7; S(4): 0.4; T(8): 0.0	Ubiquitin carboxyl-terminal hydrolase 8
	TISDGTISAAK	Q8K012	T(1): 0.4; S(3): 99.6; T(6): 0.0; S(8): 0.0	Formin-binding protein 1-like
	VDSTTCLFPVEEK	P47856	S(3): 93.9; T(4): 5.7; T(5): 0.4	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1
	VRTESSEALK	Q9QYR6	T(3): 0.0; S(5): 95.3; S(6): 4.7	Microtubule-associated protein 1A
GROUP0criteria and control	AEGEPQEESPLK	Q8K019	S(9): 100.0	Bcl-2-associated transcription factor 1
	ALYLEPSDGVSPTQETGEAQSQDDER	Q6ZPE2	Y(3): 0.0; S(7): 0.1; S(11): 99.9; T(13): 0.0; T(16): 0.0; S(21): 0.0	Myotubularin-related protein 5
	APSWDAGAPPPR	Q3UNH4	S(3): 100.0	G protein-regulated inducer of neurite outgrowth 1
	DDYLETLSSPK	Q8VD37	Y(3): 0.0; T(6): 0.0; S(8): 0.1; S(9): 99.9	SH3-containing GRB2-like protein 3-interacting protein 1

EESLATDPAPGTQK	Q8BR92	S(3): 92.4; T(6): 7.6; T(12): 0.0	Paralemmin-2
GPVAPAESK	Q8R4A3	S(8): 100.0	Dapper homolog 1
GSPPLLDAPASPPQAPR	Q3UHE1	S(2): 100.0; S(11): 0.0	Membrane-associated phosphatidylinositol transfer protein 3
HNSVEDAEQGK	Q9D7X1	S(3): 100.0	BTB/POZ domain-containing protein KCTD4
RDSEEESESTAL	Q5SSM3	S(3): 100.0; S(7): 0.0; S(9): 0.0; T(10): 0.0	Rho GTPase-activating protein 44
RHSSPSSPTSPK	O35495	S(3): 92.3; S(4): 0.6; S(6): 7.1; S(7): 0.1; T(9): 0.0; S(10): 0.0	Cyclin-dependent kinase 14
SALSSSLR	Q8R0S2	S(1): 0.0; S(4): 0.0; S(5): 0.1; S(6): 99.9	IQ motif and SEC7 domain-containing protein 1
SESDASSLDAK	Q8C8R3	S(1): 99.5; S(3): 0.5; S(6): 0.0; S(7): 0.0	Ankyrin-2
SGTSQEELR	P33173	S(1): 0.0; T(3): 0.2; S(4): 99.8	Kinesin-like protein KIF1A
SLSAPLHPEFEEVYR	Q8VDN4	S(1): 50.0; S(3): 50.0; Y(14): 0.0	Coiled-coil domain-containing protein 92
SLSSPTDNLELSAR	Q68FF6	S(1): 0.0; S(3): 0.3; S(4): 99.6; T(6): 0.0; S(12): 0.0	ARF GTPase-activating protein GIT1
SSSAPNVHINTIEPVNIDEK	P28028	S(1): 33.3; S(2): 33.3; S(3): 33.3; T(11): 0.0	Serine/threonine-protein kinase B-raf
SVSTSPSILPAYLK	Q8BWS5	S(1): 0.0; S(3): 0.0; T(4): 50.0; S(5): 50.0; S(7): 0.0; Y(12): 0.0	G protein-regulated inducer of neurite outgrowth 3
TQVLSPDSLFTAK	Q9QZQ1	T(1): 0.0; S(5): 100.0; S(8): 0.0; T(11): 0.0	Afadin
TSCGSPNYAAPEVISGR	Q8BRK8	T(1): 94.7; S(2): 5.3; S(5): 0.0; Y(8): 0.0; S(15): 0.0	5'-AMP-activated protein kinase catalytic subunit alpha-2
TSLADQEEVR	P70704	T(1): 50.0; S(2): 50.0	Probable phospholipid-transporting ATPase IA
VALAAGSPTRPPPAR	A2AJA9	S(7): 93.2; T(9): 6.8	Uncharacterized protein C9orf172 homolog
VASEEEEVPLVVYLK	Q8CC35	S(3): 100.0; Y(13): 0.0	Synaptopodin
VPTEEEDGSSLEADMECPN QRPTESSDVDK	Q61699	T(3): 100.0; S(9): 0.0; S(10): 0.0; T(23): 0.0; S(25): 0.0; S(26): 0.0	Heat shock protein 105 kDa
VSLPTANPDVSSGITQIK	Q8C419	S(2): 100.0; T(5): 0.0; S(11): 0.0; S(12): 0.0; T(15): 0.0	Probable G-protein coupled receptor 158
YVESDDEKPTDENVNEK	Q61687	Y(1): 100.0; S(4): 0.0; T(10): 0.0	Transcriptional regulator ATRX

	ASSFSGISILTR	Q80TM6	S(2): 5.9; S(3): 94.0; S(5): 0.0; S(8): 0.0; T(11): 0.0	R3H domain-containing protein 2
GROUP0criteria	ATGPAPGPSVDR	Q7TSJ2	T(2): 0.0; S(9): 100.0	Microtubule-associated protein 6
	DTTEDSITEDDK	P0C913	T(2): 0.0; T(3): 0.0; S(6): 99.7; T(8): 0.3	Overexpressed in colon carcinoma 1 protein homolog
	EIASPSSPVK	Q8C8R3	S(4): 0.0; S(6): 99.7; S(7): 0.3	Ankyrin-2
	ELVGDTGSQEGDNEQPSG SETEEDPSASPQK	E9Q942	T(6): 0.0; S(8): 0.0; S(17): 99.1; S(19): 50.4; T(21): 50.4; S(26): 0.0; S(28): 0.0	Small integral membrane protein 13
	EPAISSQNSPEAR	P10711	S(5): 0.0; S(6): 0.1; S(9): 99.9	Transcription elongation factor A protein 1
	EPSSPGTDDVFTPGSSDSP SSQR	Q61165	S(3): 0.0; S(4): 0.0; T(7): 0.0; T(12): 0.0; S(15): 0.0; S(16): 0.0; S(18): 8.5; S(20): 83.1; S(21): 8.5	Sodium/hydrogen exchanger 1
	FSHSYLSDSDTEAK	Q9JMH9	S(2): 0.0; S(4): 0.0; Y(5): 0.0; S(7): 100.0; S(9): 100.0; T(11): 0.0	Unconventional myosin-XVIIIa
	GAGDEVSELPAPARSPPR	Q66L44	S(4): 0.0; S(7): 0.0; S(14): 100.0	Protein Dos
	GALGEPSAPSAR	Q8QZT2	S(7): 0.1; S(10): 99.9	Centriole, cilia and spindle-associated protein
	GFSQYGVSGSPTK	Q8VE19	S(3): 0.0; Y(5): 0.0; S(8): 0.0; S(10): 94.2; T(12): 5.8	WD repeat-containing protein mio
	GWSPPPEVR	Q9QXG4	S(3): 100.0	Acetyl-coenzyme A synthetase, cytoplasmic
	HASEPQPGPR	Q6PFD5	S(3): 100.0	Disks large-associated protein 3
	HGAPAAPSPPPR	Q8BHL3	S(8): 100.0	TBC1 domain family member 10B
	KLSPTEPR	O35609	S(3): 94.9; T(5): 5.1	Secretory carrier-associated membrane protein 3
	LSPPHSPR	Q60875	S(2): 100.0; S(6): 0.0	Rho guanine nucleotide exchange factor 2
	LSPPLPPK	Q2M3X8	S(2): 100.0	Phosphatase and actin regulator 1
	LSPVSPSAK	Q8C8R3	S(2): 100.0; S(5): 99.6; S(7): 0.4	Ankyrin-2
	LTSIGSDEDEETETYQEK	Q924N4	T(2): 0.2; S(3): 0.2; S(6): 99.6; T(12): 0.0; T(14): 0.0; Y(15): 0.0	Solute carrier family 12 member 6
	NLTSSSLNDISDKPEK	O88343	T(3): 0.0; S(4): 0.3; S(5): 47.9; S(6): 47.9; S(11): 3.9	Electrogenic sodium bicarbonate cotransporter 1

	NVPGLAAPSSPTQK	Q8R3Q2	S(9): 6.9; S(10): 92.6; T(12): 0.6	Serine/threonine-protein phosphatase 6 regulatory subunit 2
	REGPIGGESDSEEVR	Q6NZL0	S(9): 100.0; S(11): 0.0	Protein SOGA3
	RQSESIAAPPVASEDMDK	Q8BPN8	S(3): 100.0; S(5): 0.0; S(13): 0.0	DmX-like protein 2
	RSSVSGISLEDNR	P70271	S(2): 0.4; S(3): 99.6; S(5): 0.0; S(8): 0.0	PDZ and LIM domain protein 4
	RSVSHGSNHAQNAEEQR	Q3TLH4	S(2): 87.8; S(4): 10.7; S(7): 1.4	Protein PRRC2C
	SETSSNPSSPEICPNK	Q5SVL6	S(1): 0.0; T(3): 0.0; S(4): 6.2; S(5): 93.8; S(8): 6.2; S(9): 93.8	Rap1 GTPase-activating protein 2
	SPSPPPR	Q3UTJ2	S(1): 0.0; S(3): 100.0	Sorbin and SH3 domain-containing protein 2
	TASLTSAASIDGSR	Q9QYG0	T(1): 0.9; S(3): 99.0; T(5): 0.1; S(6): 0.1; S(9): 99.9; S(13): 0.0	Protein NDRG2
	TGSSTNNNEEEKSR	Q9WV18	T(1): 6.3; S(3): 64.6; S(4): 64.6; T(5): 64.6; S(13): 0.0	Gamma-aminobutyric acid type B receptor subunit 1
	TPALSPQRPLTTQQPQSGT LK	Q64332	T(1): 100.0; S(5): 100.0; T(11): 0.0; T(12): 0.0; S(17): 0.0; T(19): 0.0	Synapsin-2
	TPGTPGTPSYPR	P20357	T(1): 0.0; T(4): 100.0; T(7): 0.0; S(9): 0.0; Y(10): 0.0	Microtubule-associated protein 2
	AISPTSATSSGR	Q99104	S(3): 100.0; T(5): 0.0; S(6): 0.0; T(8): 0.0; S(9): 0.0; S(10): 0.0	Unconventional myosin-Va
GROUP2-3criteria and control	ASQGLATAPASPPVLQR	A2AJA9	S(2): 0.0; T(7): 0.0; S(11): 100.0	Uncharacterized protein C9orf172 homolog
	GPGGSPSGLQK	Q91VC7	S(5): 93.2; S(7): 6.8	Protein phosphatase 1 regulatory subunit 14A
	GPGLGSTQGQTIALPAQGLI EFR	P42669	S(6): 50.0; T(7): 50.0; T(11): 0.0	Transcriptional activator protein Pur-alpha
	KLSEASDER	Q3UVG3	S(3): 100.0; S(6): 0.0	Protein FAM91A1
	LIHGEDSDSEGDDDGR	Q6ZPJ3	S(7): 100.0; S(9): 100.0	Ubiquitin-conjugating enzyme E2 O
	SADVSPTTEGVK	Q9ESN6	S(1): 0.0; S(5): 3.4; T(7): 48.3; T(8): 48.3	Tripartite motif-containing protein 2
	SASSGAEGDVSSER	Q9DD18	S(1): 0.0; S(3): 0.4; S(4): 99.6; S(11): 0.0; S(12): 0.0	D-tyrosyl-tRNA(Tyr) deacylase 1
	SHEAEVLK	P54227	S(1): 100.0	Stathmin
	SPQGLELPLPNR	Q8C8R3	S(1): 100.0	Ankyrin-2

	SYSPDGKESPSDK	Q8K310	S(1): 5.1; Y(2): 5.1; S(3): 89.9; S(9): 100.0; S(11): 0.0	Matrin-3
	YDDDISPSEDK	O88447	Y(1): 0.0; S(6): 100.0; S(8): 0.0	Kinesin light chain 1

C Prefrontal cortex

Expressed in	peptide	UniProt access	phosphoRS.Site.Probabilities	protein name
GROUP2-3 criteria	AEDGAAPSPSSETPKK	P26645	S(8): 0.0; S(10): 0.0; S(11): 0.0; T(13): 100.0	Myristoylated alanine-rich C-kinase substrate.
	ASPSPSPSEAR	E9PUL5	S(2): 99.1; S(4): 0.9; S(6): 0.0; S(8): 0.0	Proline-rich transmembrane protein 2.
	DSADLLPLDSLK	Q9JHR9	S(2): 100.0; S(10): 0.0	Nuclear receptor-interacting protein 2
	DSDQVAQSDGEESPAAEEQLLGER	Q3UPL0	S(2): 0.0; S(8): 100.0; S(13): 100.0	Protein transport protein Sec31A
	EAAASNTNTLNRK	Q8VHW2	S(5): 0.1; T(7): 7.1; T(9): 92.8	Voltage-dependent calcium channel gamma-8 subunit.
	EIHDVAGDGDSLGSPGPTR	Q80U49	S(11): 0.0; S(14): 100.0; T(18): 0.0	Centrosomal protein of 170 kDa protein B
	EIQSITDESRGSIR	Q91V14	S(4): 0.0; T(6): 0.0; S(9): 0.0; S(12): 100.0	Solute carrier family 12 member 5
	FSPTQDRPESSTVLK	Q8BTI8	S(2): 91.4; T(4): 8.6; S(10): 0.0; S(11): 0.0; T(12): 0.0	Serine/arginine repetitive matrix protein 2.
	GSVSEDELIAAIK	Q8K0T0	S(2): 100.0; S(4): 100.0	Reticulon-1
	GTGGVDTAATGSVFDISNLDR	P30275	T(2): 0.0; T(7): 0.0; T(10): 0.7; S(12): 99.3; S(17): 0.0	Creatine kinase U-type, mitochondrial
	GTPIPDSSSTLASGEFTGVK	Q60902	T(2): 100.0; S(7): 0.0; S(8): 0.0; S(9): 0.0; T(10): 0.0; S(13): 0.0; T(17): 0.0	Epidermal growth factor receptor substrate 15-like 1
	HHSVEAAPGPPR	Q8C8T7	S(3): 100.0	Protein ELFN1
	IDIGRLSPEAK	Q61037	S(7): 100.0	Tuberin
	LAAGAESPQPASGNSPSEDDR	P16054	S(7): 100.0; S(12): 0.0; S(15): 99.9; S(17): 0.1	Protein kinase C epsilon
	LLDPEDVDVSSPDEK	Q91ZU6	S(10): 0.0; S(11): 100.0	Dystonin Cytoskeletal linker

			protein.
LNQSDSIEDPNSPAG R	Q8BUK6	S(4): 0.0; S(6): 0.0; S(12): 100.0	Protein Hook homolog 3
LPAPQEDTASEAGT PQGEVQTR	Q64012	T(8): 12.5; S(10): 87.5; T(14): 0.0; T(21): 0.0	RNA-binding protein Raly
LSPSPTSQR	P48678	S(2): 0.0; S(4): 33.3; T(6): 33.3; S(7): 33.3	Prelamin-A/C
LSSSESPAPDTGSSA ASGEADTSRPGTQQ K	Q9QXL2	S(2): 7.1; S(3): 42.8; S(4): 42.8; S(6): 7.1; T(11): 0.2; S(13): 0.0; S(14): 0.0; S(17): 0.0; T(22): 0.0; S(23): 0.0; T(27): 0.0	Kinesin-like protein KIF21A
MNTAPSRPSPTR	Q91W39	T(3): 0.1; S(6): 92.3; S(9): 7.5; T(11): 0.1	Nuclear receptor coactivator 5
MQAESQSPTNVDLE DK	P58871	S(5): 0.0; S(7): 94.8; T(9): 5.2	182 kDa tankyrase-1-binding protein
RRTEEGPTLSYGR	Q8K310	T(3): 100.0; T(8): 0.0; S(10): 0.0; Y(11): 0.0	Matrin-3
SEESLTSLHAVDGDS K	Q811P8	S(1): 100.0; S(4): 0.0; T(6): 0.0; S(7): 0.0; S(15): 0.0	Rho GTPase-activating protein 32GTPase
SLEAIPEK	Q8K394	S(1): 100.0	Inactive phospholipase C-like protein 2
SPDLGHSTQIPR	Q8K212	S(1): 100.0; S(7): 0.0; T(8): 0.0	Phosphofurin acidic cluster sorting protein 1
SPSDSSTASTPIAEQI ER	Q9QXS6	S(1): 0.6; S(3): 99.4; S(9): 0.0; T(13): 0.0	Drebrin
SQSELDDQHDYDSV ASDEDTDQEPLPSA GATR	Q68FF6	S(1): 0.0; S(3): 0.0; Y(11): 0.0; S(13): 0.1; S(16): 99.9; T(20): 0.1; S(27): 0.0; T(31): 0.0	ARF GTPase-activating protein GIT1 GTPase-activating protein for the ADP ribosylation factor family.
SRTASLTSAASIDGS R	Q9QYG0	S(1): 0.6; T(3): 99.4; S(5): 99.3; T(7): 0.1; S(8): 0.6; S(11): 0.0; S(15): 0.0	Protein NDRG2
SSSMAAGLER	Q8BKX1	S(1): 0.0; S(2): 0.3; S(3): 99.6	Brain-specific angiogenesis inhibitor 1-associated protein 2
STVSIEEAVAK	P20357	S(1): 0.0; T(2): 0.0; S(4): 100.0	Microtubule-associated protein 2
TASLTSAASIDGSR	Q9QYG0	T(1): 9.5; S(3): 90.4; T(5): 0.2; S(6): 0.2; S(9): 99.8; S(13): 0.0	Protein NDRG2

	VLEKPPSPK	Q9ET80	S(3): 0.0; T(5): 99.7; S(8): 50.1; S(9): 50.1	Junctophilin-1
GROUP2-3 criteria and 0criteria	AVSVEAAER	Q9QWI6	S(3): 100.0	SRC kinase signaling inhibitor 1
	HEGLAETPETSPESLSFSPK	Q8C8R3	T(7): 100.0; T(10): 0.0; S(11): 0.0; S(14): 0.0; S(16): 0.0; S(18): 0.0	Ankyrin-2
	HRDTGILDSIGR	P04370	T(4): 100.0; S(9): 0.0	Myelin basic protein
	LPSISDLDSIFGPVLSPK	Q8VD37	S(3): 0.0; S(5): 0.0; S(9): 0.0; S(16): 100.0	SH3-containing GRB2-like protein 3-interacting protein 1
	LSGFSFK	P26645	S(2): 0.0; S(5): 100.0	Myristoylated alanine-rich C-kinase substrate
	RSSFLNAK	Q8CHG7	S(2): 0.2; S(3): 99.8	Rap guanine nucleotide exchange factor 2
	SADVSPTTEGVK	Q9ESN6	S(1): 100.0; S(5): 99.6; T(7): 0.4; T(8): 0.0	Tripartite motif-containing protein 2
	SALSSSLR	Q8R0S2	S(1): 0.0; S(4): 0.0; S(5): 3.5; S(6): 96.5	IQ motif and SEC7 domain-containing protein 1
	SAPSSAPSTPLSTDAPEFLSIPK	Q8K4R4	S(1): 0.1; S(4): 8.8; S(5): 91.1; S(8): 0.0; T(9): 0.0; S(12): 0.0; T(13): 0.0; S(20): 0.0	Cytoplasmic phosphatidylinositol transfer protein 1
	SHLVNEVPVLASPDLLSEVSEMK	Q8C8R3	S(1): 0.0; S(12): 100.0; S(17): 0.0; S(20): 0.0	Ankyrin-2
	SLNIADQEGTLLGK	Q8CH09	S(1): 100.0; T(10): 0.0	SURP and G-patch domain-containing protein 2
	SPNTAILIK	Q9QWI6	S(1): 100.0; T(4): 0.0	SRC kinase signaling inhibitor 1
	SSDDIDYR	Q8BPN8	S(1): 0.3; S(2): 99.7; Y(7): 0.0	DmX-like protein 2
	TLSQSSESGTLPSGPPGHTMEVSC	Q9QYG0	T(1): 76.7; S(3): 10.8; S(5): 10.8; S(6): 1.6; S(8): 0.0; T(10): 0.0; S(13): 0.0; T(19): 0.0; S(23): 0.0	Protein NDRG2
	VASEEEEVPLVVYLK	Q8CC35	S(3): 100.0; Y(13): 0.0	Synaptopodin
	VASVSLEGLNLAR	Q8BSS9	S(3): 0.0; S(5): 100.0	Liprin-alpha-2
	VGSLTPPSSPK	Q3UHJ0	S(3): 0.0; T(5): 99.7; S(8): 50.1; S(9): 50.1	AP2-associated protein kinase 1
	YGSGYLLASTPESEL	Q8BJI1	Y(1): 0.0; S(3): 0.0; Y(5): 0.0; S(9): 0.3; T(10): 99.7; S(13): 0.0	Sodium-dependent neutral amino acid transporter

GROUP0criteria and control	AGSVADSDAVVK	Q8VED9	S(3): 100.0; S(7): 100.0	Galectin-related protein
	ASQGLATAPASPPVLQR	A2AJA9	S(2): 0.0; T(7): 0.0; S(11): 100.0	Uncharacterized protein C9orf172 homolog
	ESPEGSYTDDANQEVR	Q8K012	S(2): 100.0; S(6): 0.0; Y(7): 0.0; T(8): 0.0	Formin-binding protein 1-like
	ESPGGCTSPGSQEK	Q3TRR0	S(2): 0.0; T(7): 50.0; S(8): 50.0; S(11): 0.0	Microtubule-associated protein 9
	GCSQDDECVSLR	Q9D415	S(3): 100.0; S(10): 0.0	Disks large-associated protein 1
	GPVAASPQK	D3YVF0	S(6): 100.0	A-kinase anchor protein 5
	HNSVEDAEQGK	Q9D7X1	S(3): 100.0	BTB/POZ domain-containing protein KCTD4
	IGELEVGQMENISPGQIIDEPIRPVNIPR	Q99104	S(13): 100.0	Unconventional myosin-Va
	NFSVGR	P28652	S(3): 100.0	Calcium/calmodulin-dependent protein kinase type II subunit beta
	RESPESEGPIYEGLIL	Q9EQH3	S(3): 100.0; S(6): 0.0; Y(11): 0.0	Vacuolar protein sorting-associated protein 35
	SAYQDYDSDSDVPEELK	Q8CI71	S(1): 0.0; Y(3): 0.0; Y(6): 0.0; S(8): 6.5; S(10): 93.5	Coiled-coil domain-containing protein 132
	SGTSTPTTPGSTAITPGTPPSYSSR	P20357	S(1): 0.0; T(3): 0.0; S(4): 0.0; T(5): 0.0; T(7): 0.0; T(8): 0.0; S(11): 0.0; T(12): 0.0; T(15): 0.0; T(18): 99.8; S(21): 0.1; Y(22): 0.0; S(23): 0.0; S(24): 0.0	Microtubule-associated protein 2
	SPPESLNDLGAFESLR	Q8CI71	S(1): 100.0; S(5): 0.0; S(14): 0.0	Coiled-coil domain-containing protein 132
	VPGGSPR	Q8R1Q8	S(5): 100.0	Cytoplasmic dynein 1 light intermediate chain 1
GROUP0criteria	AESFYQK	Q9QYR6	S(3): 100.0; Y(5): 0.0	Microtubule-associated protein 1[a].
	DTTEDSITEDDK	P0C913	T(2): 0.0; T(3): 0.0; S(6): 99.7; T(8): 0.3	Overexpressed in colon carcinoma 1 protein homolog
	EASPPTSADK	P20357	S(3): 100.0; T(6): 0.0; S(7): 0.0	Microtubule-associated protein 2.

EEVASEPEEAASPTTPK	Q9D6Z1	S(5): 100.0; S(12): 99.0; T(14): 0.5; T(15): 0.5	Nucleolar protein 56
EGSQGELTPANSQSR	Q99LD4	S(3): 100.0; T(8): 0.0; S(12): 0.0; S(14): 0.0	COP9 signalosome complex subunit 1
EHLLSPPR	Q8C3Q5	S(5): 100.0	Protein shisa-7
GHTEENLSPVSK	Q5XG69	T(3): 0.0; S(8): 100.0; S(11): 0.0	Soluble lamin-associated protein of 75 kDa
GIEFPMADLDALSPIHTPQR	Q5SRX1	S(13): 100.0; T(17): 0.0	TOM1-like protein 2
HMQMSSQEALNK	Q61290	S(5): 4.6; S(6): 95.4	Voltage-dependent R-type calcium channel subunit alpha-1E
HRPSSPATPPPK	Q52KI8	S(4): 0.3; S(5): 99.7; T(8): 100.0	Serine/arginine repetitive matrix protein 1
IVDEQGDMDFQISPDR	Q8C8R3	S(13): 100.0	Ankyrin-2
LDPAQSASRENLLEEQGSIALR	P16546	S(7): 100.0; S(12): 0.0; S(15): 99.9; S(17): 0.1	Spectrin alpha chain, non-erythrocytic 1
NSLETVGTPDSGR	Q9R0N7	S(2): 0.0; T(5): 0.1; T(8): 99.9; S(11): 100.0	Synaptotagmin-7
RFSNVGLVHTSER	Q9QWI6	S(3): 100.0; T(10): 0.0; S(11): 0.0	SRC kinase signaling inhibitor 1
RTSEDTSSGSPPK	O35841	T(2): 0.0; S(3): 0.0; T(6): 0.1; S(7): 0.1; S(8): 0.1; S(10): 99.8	Apoptosis inhibitor 5
RVSLTHR	Q923M0	S(3): 0.2; T(5): 99.8	Protein phosphatase 1
SHSLPNSLDYAQASER	Q3UJU9	S(1): 5.0; S(3): 95.0; S(7): 0.0; Y(10): 0.0; S(14): 0.0	Regulator of microtubule dynamics protein 3
SLTNSHLEK	Q8K2P7	S(1): 99.4; T(3): 0.6; S(5): 0.0	Sodium-coupled neutral amino acid transporter 1
SMSHQAAIASQR	Q8C0E2	S(1): 6.4; S(3): 93.6; S(10): 0.0	Vacuolar protein sorting-associated protein 26B
SPSPPPR	Q3UTJ2	S(1): 0.3; S(3): 99.7	Sorbin and SH3 domain-containing protein 2
SSSPGAGGGHSTSTSTSPATTLQR	Q5DU25	S(1): 33.3; S(2): 33.3; S(3): 33.3; S(11): 0.0; T(12): 0.0; S(13): 0.0; T(14): 0.0; S(15): 0.0; T(16): 0.0; S(17): 0.0; T(20): 0.0; T(21): 0.0	IQ motif and SEC7 domain-containing protein 2

	TGVIQTSTEQSFSK	P20357	T(1): 0.0; T(6): 0.0; S(7): 0.4; T(8): 93.5; S(11): 6.1; S(13): 0.0	Microtubule-associated protein 2
	TSSPTSLPPLAR	Q80XI3	T(1): 0.7; S(2): 0.7; S(3): 98.6; T(5): 0.0; S(6): 0.0	Eukaryotic translation initiation factor 4 gamma 3
	TYSDEASQLR	Q8R361	T(1): 0.5; Y(2): 0.5; S(3): 99.0; S(7): 0.0	Rab11 family-interacting protein 5
	VMHTQCHSTPDSAEDVR	P29699	T(4): 0.0; S(8): 0.0; T(9): 0.0; S(12): 100.0	Alpha-2-HS-glycoprotein
	VTSEPEAVSER	P20357	T(2): 0.4; S(3): 99.6; S(9): 0.0	Microtubule-associated protein 2
GROUP2-3criteria and control				
	APSPVVSPTELSK	Q80Z38	S(3): 0.0; S(7): 95.9; T(9): 4.1; S(12): 0.0	SH3 and multiple ankyrin repeat domains protein 2
	DMDEPSPVPNVEEVTLPK	Q6PDG5	S(6): 100.0; T(15): 0.0	SWI/SNF complex subunit SMARCC2.
	DYDDMSPR	P61979	Y(2): 0.0; S(6): 100.0	Heterogeneous nuclear ribonucleoprotein K.
	GASSPDMEPSYGGGLFDMVK	Q80TZ3	S(3): 93.4; S(4): 6.6; S(10): 0.0; Y(11): 0.0	Putative tyrosine-protein phosphatase auxilin
	GDIYCSDPALYCPDER	Q68EF6	Y(4): 0.0; S(6): 100.0; Y(11): 0.0	Brain-enriched guanylate kinase-associated protein
	GSNYHLSDNDASDVE	Q3UHK1	S(2): 0.7; Y(4): 0.1; S(7): 99.2; S(12): 100.0	Proton myo-inositol cotransporter H+-myo-inositol cotransporter.
	KGSSEESVDEDR	Q8C8R3	S(3): 100.0; S(4): 100.0; S(7): 0.0	Ankyrin-2
	MVEAVDRTIEK	Q8BG89	T(8): 100.0	Protein ZNF365 Homodimer.
	RAPSPVVSPTELSK	Q80Z38	S(4): 0.0; S(8): 99.5; T(10): 0.5; S(13): 0.0	SH3 and multiple ankyrin repeat domains protein 2
	RGFSEEQLR	Q9R1Q8	S(4): 100.0	Transgelin-3
	RGSALGPDEAGGELER	P60469	S(3): 100.0	Liprin-alpha-3 May regulate the disassembly of focal adhesions.
	RLSGGAVPSASMTR	Q8BTG7	S(3): 100.0; S(9): 9.4; S(11): 89.6; T(13): 1.1	Protein NDRG4
	RTSMGGTQQQFVEGVR	Q02248	T(2): 6.8; S(3): 93.2; T(7): 0.0	Catenin beta-1
	SASAPASPR	O35927	S(1): 0.0; S(3): 0.0; S(7): 100.0	Catenin delta-2

SDEELDDGVDDLK	Q6PNC0	S(1): 100.0	DmX-like protein 1
SESGYGFNVR	Q3UHD6	S(1): 0.4; S(3): 99.6; Y(5): 0.0	Sorting nexin-27
SLDLPDR	Q8BJ42	S(1): 100.0	Disks large-associated protein 2
SPDCTHDNPLETR	Q6PIC6	S(1): 100.0; T(5): 0.0; T(12): 0.0	Sodium/potassium-transporting ATPase subunit alpha-3
SPFEIISPPASPPEMTGQR	Q9QYR6	S(1): 0.0; S(7): 100.0; S(11): 100.0; T(16): 0.0	Microtubule-associated protein 1A
SPLAQMEEER	O55131	S(1): 100.0	Septin-7Filament-forming cytoskeletal GTPase.
SPSPPPDGSPAATPEIR	O08539	S(1): 0.6; S(3): 99.4; S(9): 0.0; T(13): 0.0	Myc box-dependent-interacting protein 1
SSGREEDEEELLR	Q8K4G5	S(1): 50.0; S(2): 50.0	Actin-binding LIM protein 1
STSPPPSPEVWAESR	Q9WV69	S(1): 33.3; T(2): 33.3; S(3): 33.3; S(7): 0.0; S(14): 0.0	Dematin Actin-bundling protein.
VAIIRTPPK	P20357	T(6): 100.0	
VDSPTVTTTLK	O88447	S(3): 100.0; T(5): 0.0; T(7): 0.0; T(8): 0.0; T(9): 0.0	Kinesin light chain 1
VQVAPLQGSPPLSHDDR	Q9Z0U1	S(9): 100.0; S(13): 0.0	Tight junction protein ZO-2
YEPAAVSEHGDK	Q8VDN2	Y(1): 0.0; S(7): 100.0	Sodium/potassium-transporting ATPase subunit alpha-1
YHGHSMSDPGVSYR	P35486	Y(1): 0.0; S(5): 92.8; S(7): 7.2; S(12): 92.8; Y(13): 7.2	Pyruvate dehydrogenase E1 component subunit alpha
YLATASTMDHAR	P04370	Y(1): 0.0; T(4): 0.0; S(6): 3.4; T(7): 96.6	Myelin basic protein

Table S4

4.1 Proteins showing differential abundance among the subgroups

A Hippocampus

UniProt accession	comparison	p.value	fold change	protein name
B2RSH2;P08752	GROUP0criteria - GROUPControl	0.0082	0.57	Guanine nucleotide-binding protein G(i) subunit alpha-1 or alpha 2
	GROUP2-3criteria - GROUPControl	0.0386	0.72	
	GROUP2-3criteria - GROUP0criteria	0.1406	1.26	
Q9CR68	GROUP2-3criteria - GROUPControl	0.0253	0.75	Cytochrome b-c1 complex subunit Rieske, mitochondrial
	GROUP0criteria - GROUPControl	0.0432	0.78	
	GROUP2-3criteria - GROUP0criteria	0.6906	0.96	
P20152	GROUP0criteria - GROUPControl	0.0200	0.65	Vimentin
	GROUP2-3criteria - GROUP0criteria	0.0488	1.40	
	GROUP2-3criteria - GROUPControl	0.5241	0.91	
Q640R3	GROUP0criteria - GROUPControl	0.0035	0.77	Hepatocyte cell adhesion molecule
	GROUP2-3criteria - GROUP0criteria	0.0183	1.19	
	GROUP2-3criteria - GROUPControl	0.1096	0.92	
P57776-2	GROUP2-3criteria - GROUP0criteria	0.0184	0.64	Elongation factor 1-delta
	GROUP2-3criteria - GROUPControl	0.0357	0.71	
	GROUP0criteria - GROUPControl	0.3728	1.10	
Q9ESW4	GROUP2-3criteria - GROUP0criteria	0.0085	0.69	Acylglycerol kinase, mitochondrial
	GROUP2-3criteria - GROUPControl	0.0224	0.75	
	GROUP0criteria - GROUPControl	0.3453	1.08	
Q91YR1	GROUP2-3criteria - GROUPControl	0.0101	1.40	Twinfilin-1
	GROUP2-3criteria - GROUP0criteria	0.0324	1.28	
	GROUP0criteria - GROUPControl	0.3887	1.09	
Q9D8Y0	GROUP2-3criteria - GROUPControl	0.0260	1.26	EF-hand domain-containing protein D2
	GROUP2-3criteria - GROUP0criteria	0.0396	1.22	
	GROUP0criteria - GROUPControl	0.7004	1.03	

B Striatum

UniProt accession	comparison	p value	fold change	protein name
O08788-2	GROUP2-3criteria - GROUPControl	0.0125	0.62	Dynactin subunit 1
	GROUP2-3criteria - GROUP0criteria	0.0230	0.69	
	GROUP0criteria - GROUPControl	0.4048	0.89	
O54984	GROUP2-3criteria - GROUP0criteria	0.0365	0.65	ATPase Asna1
	GROUP2-3criteria - GROUPControl	0.0468	0.68	
	GROUP0criteria - GROUPControl	0.8305	1.04	
P97355	GROUP2-3criteria - GROUP0criteria	0.0007	0.68	Spermine synthase
	GROUP2-3criteria - GROUPControl	0.0010	0.66	
	GROUP0criteria - GROUPControl	0.7140	0.98	
Q9Z0P5	GROUP2-3criteria - GROUP0criteria	0.0063	0.73	Twinfilin-2
	GROUP2-3criteria - GROUPControl	0.0379	0.82	
	GROUP0criteria - GROUPControl	0.1968	1.12	
O88533	GROUP2-3criteria - GROUP0criteria	0.0170	1.17	Aromatic-L-amino-acid decarboxylase
	GROUP2-3criteria - GROUPControl	0.0212	1.14	
	GROUP0criteria - GROUPControl	0.5637	0.98	
P63005	GROUP2-3criteria - GROUP0criteria	0.0206	1.20	Platelet-activating factor acetylhydrolase IB subunit alpha
	GROUP2-3criteria - GROUPControl	0.0238	1.19	
	GROUP0criteria - GROUPControl	0.9151	0.99	
P84096	GROUP2-3criteria - GROUP0criteria	0.0056	1.30	Rho relacionada GTP RhoG proteína
	GROUP2-3criteria - GROUPControl	0.0192	1.24	
	GROUP0criteria - GROUPControl	0.4798	0.95	
P97300;P97300-3	GROUP2-3criteria - GROUPControl	0.0063	1.46	Neuroplastin
	GROUP2-3criteria - GROUP0criteria	0.0452	1.25	
	GROUP0criteria - GROUPControl	0.0909	1.17	
Q91VR2	GROUP2-3criteria - GROUP0criteria	0.0103	1.39	ATP synthase subunit gamma, mitochondrial
	GROUP2-3criteria - GROUPControl	0.0263	1.29	
	GROUP0criteria - GROUPControl	0.4360	0.93	

P17809	GROUP2-3criteria - GROUP0criteria	0.0221	0.83	Solute carrier family 2, facilitated glucose transporter member 1
	GROUP0criteria - GROUPControl	0.0379	1.18	
	GROUP2-3criteria - GROUPControl	0.6762	0.98	
Q9CQM5	GROUP0criteria - GROUPControl	0.0179	1.48	Thioredoxin domain-containing protein 17
	GROUP2-3criteria - GROUP0criteria	0.0369	0.72	
	GROUP2-3criteria - GROUPControl	0.5978	1.07	
Q9D9M2-2	GROUP2-3criteria - GROUP0criteria	0.0079	0.80	Ubiquitin carboxilo-terminal hidrolasa 12
	GROUP0criteria - GROUPControl	0.0255	1.17	
	GROUP2-3criteria - GROUPControl	0.2568	0.94	
Q9WV92-6	GROUP2-3criteria - GROUP0criteria	0.0406	1.24	Band 4.1-like protein 3
	GROUP2-3criteria - GROUPControl	0.0470	1.24	
	GROUP0criteria - GROUPControl	0.9408	0.99	
Q9Z1B3-3	GROUP0criteria - GROUPControl	0.0267	1.17	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1
	GROUP2-3criteria - GROUPControl	0.0336	1.15	
	GROUP2-3criteria - GROUP0criteria	0.7702	0.98	
P12970	GROUP2-3criteria - GROUPControl	0.0040	1.70	60S ribosomal protein L7a
	GROUP2-3criteria - GROUP0criteria	0.0460	1.29	
	GROUP0criteria - GROUPControl	0.0474	1.32	
P18572-2	GROUP2-3criteria - GROUPControl	0.0011	1.22	Basigin
	GROUP0criteria - GROUPControl	0.0217	1.11	
	GROUP2-3criteria - GROUP0criteria	0.0302	1.10	

C Prefrontal cortex

UniProt accession	comparison	p.2value	fold change	protein name
B2RSH2;P08752	GROUP0criteria - GROUPControl	0.0105	1.54	Guanine nucleotide-binding protein G(i) subunit alpha-1 or alpha 2
	GROUP2-3criteria - GROUPControl	0.0225	1.43	
	GROUP2-3criteria - GROUP0criteria	0.5617	0.93	
P12023-2	GROUP2-3criteria - GROUPControl	0.0317	1.41	Amyloid beta A4 protein
	GROUP0criteria - GROUPControl	0.0485	1.36	

	GROUP2-3criteria - GROUP0criteria	0.7618	1.04	
P14152	GROUP2-3criteria - GROUPControl	0.0115	1.21	Malate dehydrogenase, cytoplasmic
	GROUP0criteria - GROUPControl	0.0139	1.21	
	GROUP2-3criteria - GROUP0criteria	0.9891	1.00	
P35803-3	GROUP0criteria - GROUPControl	0.0038	1.37	Neuronal membrane glycoprotein M6-b
	GROUP2-3criteria - GROUPControl	0.0073	1.31	
	GROUP2-3criteria - GROUP0criteria	0.5786	0.96	
P61255	GROUP0criteria - GROUPControl	0.0303	1.50	60S ribosomal protein L26
	GROUP2-3criteria - GROUPControl	0.0355	1.47	
	GROUP2-3criteria - GROUP0criteria	0.8296	0.98	
Q63810-2	GROUP0criteria - GROUPControl	0.0066	1.35	Calcineurin subunit B type 1
	GROUP2-3criteria - GROUPControl	0.0225	1.25	
	GROUP2-3criteria - GROUP0criteria	0.3400	0.93	
Q7TSJ2	GROUP2-3criteria - GROUP0criteria	0.0228	0.88	Microtubule-associated protein 6
	GROUP0criteria - GROUPControl	0.0307	1.12	
	GROUP2-3criteria - GROUPControl	0.8678	0.99	
Q8BLF1	GROUP0criteria - GROUPControl	0.0279	1.29	Neutral cholesterol ester hydrolase 1
	GROUP2-3criteria - GROUPControl	0.0421	1.25	
	GROUP2-3criteria - GROUP0criteria	0.7160	0.97	
Q91V14-2	GROUP0criteria - GROUPControl	0.0042	1.40	Solute carrier family 12 member 5
	GROUP2-3criteria - GROUPControl	0.0240	1.26	
	GROUP2-3criteria - GROUP0criteria	0.2143	0.90	
Q99PJ0	GROUP0criteria - GROUPControl	0.0082	1.69	Neurotrimin
	GROUP2-3criteria - GROUPControl	0.0384	1.35	
	GROUP2-3criteria - GROUP0criteria	0.0723	0.80	
Q9R0K7	GROUP0criteria - GROUPControl	0.0114	1.31	Plasma membrane calcium-transporting ATPase 2
	GROUP2-3criteria - GROUPControl	0.0484	1.20	
	GROUP2-3criteria - GROUP0criteria	0.2919	0.92	
P06151;P16125	GROUP0criteria - GROUPControl	0.0009	1.65	L-lactate dehydrogenase A chain

	GROUP2-3criteria - GROUPControl	0.0188	1.30	
	GROUP2-3criteria - GROUP0criteria	0.0273	0.79	
O35633-2	GROUP0criteria - GROUPControl	0.0085	1.16	Vesicular inhibitory amino acid transporter
	GROUP2-3criteria - GROUP0criteria	0.0157	0.88	
	GROUP2-3criteria - GROUPControl	0.6292	1.02	
P0DI97	GROUP0criteria - GROUPControl	0.0096	1.32	Neurexin-1-beta
	GROUP2-3criteria - GROUP0criteria	0.0303	0.81	
	GROUP2-3criteria - GROUPControl	0.3950	1.07	
P31650	GROUP0criteria - GROUPControl	0.0010	1.58	Sodium- and chloride-dependent GABA transporter 3
	GROUP2-3criteria - GROUP0criteria	0.0112	0.76	
	GROUP2-3criteria - GROUPControl	0.0529	1.20	
P49815	GROUP2-3criteria - GROUP0criteria	0.0171	0.84	Vesicular inhibitory amino acid transporter
	GROUP0criteria - GROUPControl	0.0355	1.15	
	GROUP2-3criteria - GROUPControl	0.5002	0.97	
P55200-2	GROUP0criteria - GROUPControl	0.0071	1.51	Histone-lysine N-methyltransferase MLL
	GROUP2-3criteria - GROUP0criteria	0.0383	0.78	
	GROUP2-3criteria - GROUPControl	0.1382	1.18	
Q6PHN9	GROUP0criteria - GROUPControl	0.0027	1.66	Ras-related protein Rab-35
	GROUP2-3criteria - GROUP0criteria	0.0101	0.72	
	GROUP2-3criteria - GROUPControl	0.0570	1.20	
Q9CXW4	GROUP0criteria - GROUPControl	0.0326	1.23	60S ribosomal protein L11
	GROUP2-3criteria - GROUP0criteria	0.0449	0.83	
	GROUP2-3criteria - GROUPControl	0.7745	1.02	
Q9DB05	GROUP0criteria - GROUPControl	0.0179	1.11	Alpha-soluble NSF attachment protein
	GROUP2-3criteria - GROUP0criteria	0.0190	0.91	
	GROUP2-3criteria - GROUPControl	0.9238	1.00	
Q9QYC0	GROUP0criteria - GROUPControl	0.0114	1.22	Alpha-adducin
	GROUP2-3criteria - GROUP0criteria	0.0492	0.88	
	GROUP2-3criteria - GROUPControl	0.2645	1.07	

Q9QYR6	GROUP0criteria - GROUPControl	0.0453	1.26	Microtubule-associated protein 1A
	GROUP2-3criteria - GROUP0criteria	0.0460	0.80	
	GROUP2-3criteria - GROUPControl	0.9752	1.00	
P84086	GROUP2-3criteria - GROUPControl	0.0058	2.29	Complexin-2
	GROUP2-3criteria - GROUP0criteria	0.0285	1.76	
	GROUP0criteria - GROUPControl	0.2353	1.30	
O35381	GROUP2-3criteria - GROUPControl	0.0102	1.63	Acidic leucine-rich nuclear phosphoprotein 32 family member A
	GROUP2-3criteria - GROUP0criteria	0.0120	1.53	
	GROUP0criteria - GROUPControl	0.5505	1.07	
Q9CR62	GROUP2-3criteria - GROUP0criteria	0.0048	2.00	Mitochondrial 2-oxoglutarate/malate carrier protein
	GROUP0criteria - GROUPControl	0.0088	0.54	
	GROUP2-3criteria - GROUPControl	0.6070	1.09	
Q8BHI4	GROUP2-3criteria - GROUPControl	0.0066	1.72	Kelch repeat and BTB domain-containing protein 3
	GROUP0criteria - GROUPControl	0.0453	1.40	
	GROUP2-3criteria - GROUP0criteria	0.1739	1.23	

4.2 Phosphoproteins showing differential abundance among the subgroups

A Hippocampus

Peptide	UniPro accesion	phosphoRS Site Probabilities	Comparison	p.value	fold change	protein name
AEDGAAPSPSSETPK	P26645	S(8): 0.0; S(10): 0.4; S(11): 5.8; T(13): 93.8	GROUP2-3criteria - GROUPControl	0.0090	12.00	Myristoylated alanine-rich C-kinase substrate
			GROUP0criteria - GROUPControl	0.0378	5.39	
			GROUP2-3criteria - GROUP0criteria	0.1971	2.22	
AISLEGEPR	Q91XM9	S(3): 100.0	GROUP0criteria - GROUPControl	0.0148	1.38	Disks large homolog 2
			GROUP2-3criteria - GROUPControl	0.0238	1.31	
			GROUP2-3criteria - GROUP0criteria	0.4724	0.95	
LDHELSLDR	Q8BPN8	S(6): 100.0	GROUP0criteria - GROUPControl	0.0222	1.42	Dim1-like protein 2
			GROUP2-3criteria - GROUPControl	0.0324	1.33	
			GROUP2-3criteria -GROUP0criteria	0.5472	0.93	
LSGFSFK	P26645	S(2): 0.0; S(5): 100.0	GROUP0criteria - GROUPControl	0.0079	2.53	Myristoylated alanine-rich C-kinase substrate
			GROUP2-3criteria - GROUPControl	0.0098	2.46	
			GROUP2-3criteria - GROUP0criteria	0.8994	0.97	
LSSPVLHR	Q9QXS6	S(2): 0.1; S(3): 99.9	GROUP0criteria - GROUPControl	0.0024	2.17	Drebrin
			GROUP2-3criteria - GROUPControl	0.0147	1.89	
			GROUP2-3criteria - GROUP0criteria	0.1578	0.78	
RDSVLAASR	P11881	S(3): 100.0; S(8): 0.0	GROUP2-3criteria - GROUPControl	0.0288	2.87	Inositol 1,4,5-trisphosphate receptor type 1
			GROUP0criteria - GROUPControl	0.0373	2.98	
			GROUP2-3criteria - GROUP0criteria	0.9256	0.96	
SGDETPGSEAPGDK	Q00PI9	S(1): 100.0; T(5): 0.0; S(8): 0.0	GROUP2-3criteria - GROUPControl	0.0159	1.56	Heterogeneous nuclear ribonucleoprotein U-like protein 2
			GROUP0criteria - GROUPControl	0.0277	1.41	
			GROUP2-3criteria - GROUP0criteria	0.3650	1.11	
SHTGEAAAVR	P59017	S(1): 94.8; T(3): 5.2	GROUP0criteria - GROUPControl	0.0213	2.53	Bcl-2-like protein 13
			GROUP2-3criteria - GROUPControl	0.0229	2.26	
			GROUP2-3criteria - GROUP0criteria	0.7006	0.89	
SLDEIHPTR	Q99NE5	S(1): 100.0; T(6): 0.0	GROUP2-3criteria - GROUPControl	0.0229	1.63	Regulating synaptic membrane exocytosis protein 1
			GROUP0criteria - GROUPControl	0.0493	1.44	
			GROUP2-3criteria - GROUP0criteria	0.3561	1.13	
SSASFSTTAVSAR	O08599	S(1): 0.0; S(2): 0.0; S(4): 0.0; S(6): 0.0; T(7): 0.0; T(8): 0.0; S(11): 100.0	GROUP2-3criteria - GROUPControl	0.0093	1.80	Syntaxin-binding protein 1
			GROUP2-3criteria - GROUP0criteria	0.0197	1.54	
			GROUP0criteria - GROUPControl	0.7810	1.04	

B Striatum

Peptid	UniProt accesion	phosphoRS Site Probabilities	Comparison	p value	fold change	Protein name
AIEQADLLQEEDESPR	P61967	S(14): 100.0	GROUP2-3criteria - GROUP0criteria	0.0195	0.68	AP-1 complex subunit sigma-1A
			GROUP2-3criteria - GROUPControl	0.0239	0.70	
			GROUP0criteria - GROUPControl	0.7591	1.03	
EPELEMESLTGSPEDR	O88737	S(8): 0.0; T(10): 0.0; S(12): 100.0	GROUP0criteria - GROUPControl	0.0133	1.81	Protein bassoon
			GROUP2-3criteria - GROUPControl	0.0468	1.44	
			GROUP2-3criteria - GROUP0criteria	0.1391	0.80	
HEDGTGSDSEDPLAK	O80U49	T(5): 0.0; S(7): 99.5; S(9): 0.5	GROUP2-3criteria - GROUP0criteria	0.0146	0.60	Centrosomal protein of 170 kDa protein B
			GROUP2-3criteria - GROUPControl	0.0283	0.67	
			GROUP0criteria - GROUPControl	0.3462	1.12	
MQFSFEGPEK	Q8R0S2	S(4): 100.0	GROUP2-3criteria - GROUP0criteria	0.0020	0.57	IQ motif and SEC7 domain-containing protein 1
			GROUP0criteria - GROUPControl	0.0131	1.47	
			GROUP2-3criteria - GROUPControl	0.1379	0.83	
RGGGSGGGDESEGEEVDED	O35295	S(5): 0.0; S(11): 100.0	GROUP2-3criteria - GROUP0criteria	0.0324	0.59	Transcriptional activator protein Pur-beta
			GROUP0criteria - GROUPControl	0.0430	1.62	
			GROUP2-3criteria - GROUPControl	0.7983	0.96	
RLEDEGPPTLSPK	Q09460	T(9): 0.0; S(11): 100.0	GROUP0criteria - GROUPControl	0.0104	1.26	Calcium-activated potassium channel subunit alpha-1
			GROUP2-3criteria - GROUP0criteria	0.0106	0.80	
			GROUP2-3criteria - GROUPControl	0.9740	1.00	
RSYSPDGK	Q8K310	S(2): 0.0; Y(3): 0.0; S(4): 100.0	GROUP0criteria - GROUPControl	0.0050	0.47	Matrin-3
			GROUP2-3criteria - GROUP0criteria	0.0452	1.47	
			GROUP2-3criteria - GROUPControl	0.0671	0.69	
SDISPLTPR	P14873	S(1): 0.0; S(4): 100.0; T(7): 0.0	GROUP2-3criteria - GROUPControl	0.0084	0.74	Microtubule-associated protein 1B
			GROUP2-3criteria - GROUP0criteria	0.0535	0.86	
			GROUP0criteria - GROUPControl	0.0610	0.86	
SFDDPIVQTER	P48722	S(1): 100.0; T(8): 0.0	GROUP2-3criteria - GROUPControl	0.0193	1.18	Heat shock 70 kDa protein 4L
			GROUP2-3criteria - GROUP0criteria	0.0492	1.14	
			GROUP0criteria - GROUPControl	0.5043	1.04	
SGTSQEELR	P33173	S(1): 0.0; T(3): 0.2; S(4): 99.8	GROUP2-3criteria - GROUPControl	0.0060	0.71	Kinesin-like protein KIF1A
			GROUP2-3criteria - GROUP0criteria	0.0063	0.74	
			GROUP0criteria - GROUPControl	0.8159	0.96	

C Prefrontal cortex

Peptide	UniPro accesion	Phospho_RS	Comparison	p.value	fold change	protein name
AATDLERVSNAEPEPR	Q8CHH9	T(3): 0.0; S(9): 100.0	GROUP2-3criteria - GROUPControl	0.0102	0.27	Septin-8
			GROUP2-3criteria - GROUP0criteria	0.0426	0.46	
			GROUP0criteria - GROUPControl	0.1065	0.58	
AGSISTLDSLDFAR	Q148V7	S(3): 100.0; S(5): 0.0; T(6): 0.0; S(9): 0.0	GROUP2-3criteria - GROUPControl	0.0092	1.27	LisH domain and HEAT repeat-containing protein KIAA1468
			GROUP0criteria - GROUPControl	0.0199	1.19	
			GROUP2-3criteria - GROUP0criteria	0.3200	1.07	
ALGLEESPEEEGK	Q9QYR6	S(7): 100.0	GROUP0criteria - GROUPControl	0.0331	1.33	Microtubule-associated protein 1A
			GROUP2-3criteria - GROUP0criteria	0.0430	0.75	
			GROUP2-3criteria - GROUPControl	0.9908	1.00	
ASSLNFLNK	O88448	S(2): 0.2; S(3): 99.8	GROUP0criteria - GROUPControl	0.0095	0.82	Kinesin light chain 2
			GROUP2-3criteria - GROUP0criteria	0.0240	1.18	
			GROUP2-3criteria - GROUPControl	0.4736	0.97	
DYLSDSELNQLR	O88737	Y(2): 0.0; S(4): 100.0; S(6): 0.0	GROUP2-3criteria - GROUP0criteria	0.0274	0.71	Protein bassoon
			GROUP2-3criteria - GROUPControl	0.0376	0.74	
			GROUP0criteria - GROUPControl	0.6735	1.04	
EGDGSATTDAAPATSPK	P06837	S(5): 0.0; T(7): 0.0; T(8): 0.0; T(14): 0.7; S(15): 99.3	GROUP0criteria - GROUPControl	0.0201	1.61	Neuromodulin
			GROUP2-3criteria - GROUPControl	0.0435	1.41	
			GROUP2-3criteria - GROUP0criteria	0.3812	0.87	
FYYSSGSSSPTHAK	Q60771	Y(2): 0.0; Y(3): 0.0; S(4): 0.0; S(5): 0.0; S(7): 0.0; S(8): 5.3; S(9): 94.7; T(11): 0.0	GROUP2-3criteria - GROUPControl	0.0048	0.61	Claudin-11
			GROUP2-3criteria - GROUP0criteria	0.0295	0.73	
			GROUP0criteria -	0.1791	0.84	

			GROUPControl			
GLYDGPVCEVSVTPK	O08553	Y(3): 0.0; S(11): 0.0; T(13): 100.0	GROUP0criteria - GROUPControl	0.0123	5.03	Dihydropyrimidinase-related protein 2
			GROUP2-3criteria - GROUP0criteria	0.0421	0.29	
			GROUP2-3criteria - GROUPControl	0.4764	1.44	
HSWDSPAFNNDVVQR	Q5DU25	S(2): 0.0; S(5): 100.0	GROUP2-3criteria - GROUPControl	0.0204	0.60	IQ motif and SEC7 domain-containing protein 2
			GROUP0criteria - GROUPControl	0.0496	0.69	
			GROUP2-3criteria - GROUP0criteria	0.2808	0.86	
LDSVDMLLPSK	B1AZP2	S(3): 100.0; S(10): 0.0	GROUP0criteria - GROUPControl	0.0022	2.15	Disks large-associated protein 4
			GROUP2-3criteria - GROUP0criteria	0.0165	0.62	
			GROUP2-3criteria - GROUPControl	0.0566	1.34	
RGSQPDAELDGAGTSLLR	Q6PE13	S(3): 100.0; T(14): 0.0; S(15): 0.0	GROUP2-3criteria - GROUPControl	0.0100	0.41	Proline-rich transmembrane protein 3
			GROUP2-3criteria - GROUP0criteria	0.0283	0.50	
			GROUP0criteria - GROUPControl	0.4384	0.82	
RPVVTTHDLEAPSR	Q8C052	T(5): 0.0; T(6): 0.0; S(13): 100.0	GROUP0criteria - GROUPControl	0.0059	1.39	Microtubule-associated protein 1S
			GROUP2-3criteria - GROUP0criteria	0.0223	0.79	
			GROUP2-3criteria - GROUPControl	0.2011	1.10	
SDFDEFER	P26369	S(1): 100.0	GROUP2-3criteria - GROUPControl	0.0237	1.75	Splicing factor U2AF 65 kDa subunit
			GROUP0criteria - GROUPControl	0.0289	1.69	
			GROUP2-3criteria - GROUP0criteria	0.8539	1.03	
SGGDTHSPPR	Q812A2	S(1): 0.0; T(5): 0.0; S(7): 100.0	GROUP0criteria - GROUPControl	0.0113	0.19	SLIT-ROBO Rho GTPase-activating protein 3
			GROUP2-3criteria - GROUP0criteria	0.0171	4.54	
			GROUP2-3criteria - GROUPControl	0.6749	0.84	

SIPHITSDR	P97427	S(1): 100.0; T(6): 0.0; S(7): 0.0	GROUP0criteria - GROUPControl	0.0046	1.43	Dihydropyrimidinase-related protein 1
			GROUP2-3criteria - GROUP0criteria	0.0361	0.80	
			GROUP2-3criteria - GROUPControl	0.1405	1.15	
SLDEIHPTR	Q99NE5	S(1): 100.0; T(8): 0.0	GROUP0criteria - GROUPControl	0.0053	1.58	Regulating synaptic membrane exocytosis protein 1
			GROUP2-3criteria - GROUPControl	0.0176	1.36	
			GROUP2-3criteria - GROUP0criteria	0.1741	0.86	
VAIIRTPPK	P20357	T(6): 100.0	GROUP0criteria - GROUPControl	0.0136	0.92	Microtubule-associated protein 2
			GROUP2-3criteria - GROUP0criteria	0.0366	1.07	
			GROUP2-3criteria - GROUPControl	0.4548	0.98	
YGGITSFENTAIEVDR	Q6PGE7	Y(1): 0.0; T(5): 0.0; S(6): 99.9; T(10): 0.0	GROUP2-3criteria - GROUP0criteria	0.0241	1.51	Sodium-dependent proline transporter
			GROUP2-3criteria - GROUPControl	0.0302	1.47	
			GROUP0criteria - GROUPControl	0.8326	0.97	
YLATASTMDHAR	P04370	Y(1): 0.0; T(4): 0.0; S(6): 96.5; T(7): 3.5	GROUP2-3criteria - GROUP0criteria	0.0003	0.38	Myelin basic protein
			GROUP2-3criteria - GROUPControl	0.0018	0.51	
			GROUP0criteria - GROUPControl	0.0662	1.33	

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Objective 3

Involvement of DOR in the neuroplastic mechanisms underlying food reward and seeking behaviour.

Role of DOR in neuronal plasticity changes promoted by food-seeking behaviour.

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Abstract

Several lines of evidence support that food overconsumption may be related to the role of the endogenous opioid system in the control of food palatability. The opioid system, and particularly the delta opioid receptor (DOR), plays a crucial role in the regulation of food rewarding properties. In our study, we used operant conditioning maintained by chocolate-flavoured pellets to investigate the role of DOR in the motivation for palatable food and the structural plasticity changes promoted by this behaviour. For this purpose, we evaluated the specific role of this receptor in the behavioural and neuroplastic changes induced by palatable food in the prefrontal cortex (PFC), hippocampus (HCP) and nucleus accumbens (NAc) in constitutive knockout (KO) mice deficient in DOR. Mutant mice and their wild-type littermates were trained to obtain chocolate-flavoured pellets on fixed ratio 1 (FR1), FR5 and progressive ratio (PR) schedule of reinforcement. No significant differences between genotypes were revealed on operant behaviour acquisition in FR1. DOR knockout mice displayed lower number of active lever-presses than wild-type mice on FR5, and a similar decrease was revealed in DOR KO mice in the breaking point during the PR. This operant training to obtain palatable food increased dendritic spine density in the PFC, HCP and NAc shell of wild-type, but these plasticity changes were abolished in DOR KO mice. Our results support the hypothesis that DOR regulates the reinforcing effects and motivation for palatable food through neuroplastic changes in specific brain reward areas.

Key Words: compulsive eating, motivation, operant behaviour, neuroplasticity.

Introduction

Obesity and overeating are among the most important health issues affecting the developed countries (Hill 2006). Genetic predisposition and environmental influences are two prominent risk factors related to these disorders. Obesogenic environment and the high availability of palatable foods contribute to the present high rate of obesity. It is estimated that about 13% of the world's adult population (11% of men and 15% of women) was obese in 2014 and this percentage will tend to increase in the next years (World Health Organization WHO; 2014).

Palatability is an important determinant of the reward value of food that can promote the overconsumption leading to overweight and obesity. The pleasant experience obtained from palatable food contributes to repeat the experience and can result in the development of loss of control over intake (Kenny 2011). Food, like drugs, activates the mesocorticolimbic system involved in the hedonic effect promoted by different rewards and may produce neuroadaptive responses in these circuitries by mechanisms that resemble drugs of abuse (Volkow et al. 2013).

The involvement of the opioid system in food hedonic effects has been known for several decades (Yeomans & Gray 2002). The opioid system is integrated by three different opioid receptors, mu receptor (MOR), delta receptor (DOR) and kappa receptor (KOR), widely distributed in the central nervous system and located in brain areas mediating food intake and reward (Mansour et al. 1995). Rewarding effects are mainly mediated by MOR and DOR, which

activation increases dopamine (DA) release in the nucleus accumbens (NAc) shell (Hirose et al. 2005; Murakawa et al. 2004; Okutsu et al. 2006) by decreasing GABA-inhibition in the ventral tegmental area (VTA) (Bonci & Williams 1997). MOR and DOR are also involved in the inhibitory control process in cortical areas, whose dysregulation is associated with addiction and eating disorders (Mitchell et al., 2007; Selleck et al., 2015). Although the involvement of MOR in eating disorders (Ziauddeen et al. 2012) and obesity (Tabarin et al. 2005) is largely demonstrated, little is known about the potential implication of DOR in these behaviours.

Behavioural alterations induced by rewarding stimuli might be triggered by plasticity adaptations in neuronal circuitries that seem to be mediated by the opioid system (Mazei-Robison & Nestler 2012). Indeed, this system is implicated in cellular and synaptic plasticity through the regulation of glutamatergic and GABAergic transmission (Lüscher & Malenka 2011), and structural plasticity through long-lasting changes of neuronal morphology (Russo et al. 2010). Specifically, DOR seems directly involved in long term depression (LTD) in hippocampus (HCP) (Piskorowski & Chevaleyre 2013) and it is also required to induce long term potentiation (LTP) in the lateral perforant path (Bramham et al. 1991). However, the specific implication of DOR in structural plasticity changes has not been clarified. Neuronal and dendritic spine morphogenesis underlie experience-dependent brain development and information storage (Holtmaat & Svoboda 2009). Dendritic spines are highly dynamic structures, and their stabilization and morphology are influenced by synaptic activity

(Lai & Ip 2013). An increase in synaptic strength by induction of LTP is generally associated with the enlargement of pre-existing spines, the stabilization of newly-formed spines, and the formation of new spines increasing the connectivity of neurons (De Roo et al., 2008). In contrast, a reduction of synaptic strength during the LTD is correlated with spine shrinkage and retraction (Lai & Ip 2013). A high correlation exists between the physiology of synaptic transmission and the shape of the dendritic spine and both phenomena could play complementary functions in neuronal plasticity (Bosch & Hayashi 2012).

In this study, we examine the role of DOR in the motivational properties and morphological plasticity changes induced by palatable food in prefrontal cortex (PFC), HCP and NAc core and shell. These regions are crucial for regulating food hedonic aspects and dysfunctions of these areas have been related to eating disorders (Kelley & Berridge 2002; Volkow et al. 2008). The NAc shell participates in the hedonic impact for rewards (Bodnar et al. 2005), the NAc core in response-reinforcement learning (Di Ciano & Everitt 2001), the PFC in decision-making and inhibitory control processes (Volkow et al, 2008) and the HCP in memory processes that regulate motivational salient events (Kahn & Shohamy 2013).

Materials and methods

Animals

Homozygous knockout (KO) mice deficient in DOR on a C57BL/6J background and their respective wild-type littermates were used (Filliol et al. 2000). A group of C57BL/6J naïve mice of the same age and under the same housing conditions not trained with chocolate pellets was added as a control for the synaptic plasticity experiment. Mice weighted 31 ± 1.5 g at the beginning of the experiment. Mice were housed individually in controlled laboratory conditions with the temperature maintained at 21 ± 1ºC and humidity at 55 ± 10%, and were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 8.00 a.m. and on at 8:00 p.m.). Food and water were available ad libitum to avoid confounding factors linked to deprivation experience (Piazza & Le Moal 1998). Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Operant behaviour apparatus

Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two retractable levers, one randomly selected as the active lever and the other as the inactive, as previously described (Martín-García et al. 2011). Pressing on the

active lever resulted in a pellet delivery together with a stimulus-light located above the active lever. Pressing on the inactive lever had no consequences. The chambers were housed in sound and light-attenuated boxes equipped with fans to provide ventilation and white noise. A food dispenser equidistant between the two levers permitted the delivery of food pellets when required.

Food pellets

During the operant experimental sessions, animals received after each active responding a 20 mg highly palatable isocaloric pellet (20.5% proteins, 12.7% fats and 66.8% carbohydrates, with a caloric value of 3.48 kcal/g; TestDiet, Richmond, IN, USA) with similar caloric value than the maintenance diet provided for mice in their home cage (24.1% proteins, 10.4% fats and 65.5% carbohydrates, with a caloric value of 3.30 kcal/g; Diets Services, Witham, Essex UK) with some slight modifications in its composition: addition of chocolate flavour (2% pure unsweetened cocoa) and modification in the sucrose content. Indeed, although the carbohydrate content was similar in maintenance diet (65.5%) and highly palatable isocaloric pellets (66.8%), the sucrose content in standard chow was 3.1% of the total carbohydrates and 50.1% in highly palatable isocaloric pellets. These pellets were presented only during the 1 h daily operant session and animals were maintained on standard chow for their daily food intake.

Experimental design

DOR KO mice and their wild-type littermates were trained in operant boxes during 1 h daily sessions to acquire operant responding maintained by chocolate-flavoured pellets under FR1 during 16 consecutive days and FR5 during 95 consecutive days. A stimulus light, located above the active lever, was paired contingently with the delivery of the reward during the active periods. The beginning of each operant responding session was signalled by turning on a house light placed on the ceiling of the box only during the first 3 sec of the session. A time-out period of 10 sec was established after each pellet delivery. During this period, the cue light was off and no reinforcer was provided after responding on the active lever. Responses on the active lever and all the responses performed during the time-out period were recorded. The criteria for acquisition of operant responding were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of food pellets earned in three consecutive sessions, with at least 75% responding on the reinforced lever, and a minimum of 10 reinforcers per session (Martín-García et al. 2011). Motivation for food was evaluated in each mouse at day 70 of the operant training using the progressive ratio (PR) schedule where the response required to earn the pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000, 1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, 5500. The maximal number of responses that the animal performs to obtain one pellet is the last ratio completed,

referred to the breaking point. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h.

Sample preparation

Immediately after the last training session, mice were deeply anesthetized by intraperitoneal injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intracardiac perfusion, delivered with a peristaltic pump with 10 ml of Na2HPO4/NaH2PO4/NaCl buffer (PBS) 0.1M, pH 7.5, and followed by perfusion with 40 ml of 4% paraformaldehyde (PFA) in PBS 0.1M, pH 7.5. Brains were quickly removed from the skull and post fixed in 4% PFA for 10 min. Brain coronal sections (100 μm) containing the NAc (from bregma, AP: from 1.54 to 0.98 mm; ML: ± 0.5 mm; DV: - 4 mm), medial PFC (mPFC) (from bregma, AP: from 2.10 to 1.70 mm; ML: ± 0 mm; DV: - 3.5 mm) and HCP (from bregma, AP: from 1.58 to 2.06 mm; ML: ± 0 mm; DV: -2.8 mm) (Paxinos and Franklin, 1997) were obtained by using a vibratome (Leica VT 1000 S, Nussloch, Germany) and kept in PBS 0.1 M until they were processed for fluorescent labelling.

Dendritic spine analysis

Brain slices were labelled by ballistic delivery of fluorescent dye DiI (Molecular Probes, Eugene, OR, USA) using a gene gun apparatus (Helios Gene Gun System, Bio-Rad, Deutschland), as already reported (Grutzendler et al. 2003), and post fixed with PFA overnight at room temperature to further preserve structures and to allow the diffusion of the dye DiI. Brain sections were placed on

microscope gelatine coated slides and cover slipped with mounting medium (Mowiol). Then, images were acquired with confocal microscope (Zeiss LSM 510, Germany) with an oil immersion lens (63x) to analyze dendritic spine density and structure.

Individual pyramidal neurons from the mPFC and HCP, and medium spiny neurons in the NAc were chosen for spine analysis based on several criteria, as described previously (Lee et al. 2006): (i) there was minimal or no overlap with other labelled cells, (ii) at least three primary dendrites needed to be visible for cells to be used for analysis, and (iii) distal dendrites (from secondary dendrites to terminal dendrites) were examined. Dendrites from medium spiny neurons in the core and shell of the NAc, basilar dendrites of pyramidal neurons taken predominantly from the prelimbic and infralimbic areas of the mPFC, usually CA1 and CA3 dendrites of pyramidal neurons of HCP were analyzed. A total of 5 to 8 mice per experimental group were used and 4 to 11 dendrites per brain area and mouse were analyzed (max two dendrites/neuron).

To calculate spine density, a length of dendrite (at least 21 μm long) was traced. All images of dendrites were taken at different z levels (0.3 μm depth intervals) to examine the morphology of dendritic spines. Before the analysis, all images were processed with the deconvolution software (Huygens Essential, The Netherlands) that reduces the optical noise generated by the contribution of out-of-focus fluorescent points and by the distortion due to the optical instrument. All measurements were made using IMARIS 7 analysis software (Bitplane scientific software, Swiss) with the aim of

automating dendritic spine analysis. Protrusions from dendrites were classified into four types based on their morphology pursuant to a logarithm program that sorts each spine according to parameters as the length spine, the width neck and the width head. After visual identification of stubby and headed spines, parameters defined by the program identify and distinguish the spine length against spine head size, and spine minimum diameter against spine head size. Stubby spines stand out from headed spines by the ratio spine minimum diameter/spine head diameter, which was equal to 1 μm for a majority of stubby spines (94%), and less than 1 μm for spines with an identifiable neck. After finding an efficient way of sorting stubby spines, the program defines parameters to sort out large spines. Head size was the first obvious parameter. Accordingly, mushroom spines were classified as having width head greater than width neck multiplied by 2. The remaining spines could be separated on their neck length to head size ratio as thin, if the length spine was > 3 μm, and the width head was greater than the width neck, or filopodia spines, if the length was > 3 μm and the width head was equal to the width neck.

Statistical analysis

Data obtained during the operant acquisition phase were analyzed using three-way repeated measures analysis of variance (ANOVA) with manipulandum (active/inactive) and day as within-subjects factors and genotype as between-subjects factor. PR and body weight data were analyzed using one-way ANOVA between subjects (genotype). Time-out period was analyzed using Mann–

Whitney U due to the non-normally distributed data according to Kolmogorov test (Supporting Information Table S1A). Structural plasticity data were analyzed using one-way ANOVA between subjects (genotype). Post-hoc analysis (Newman-Keuls or Dunnet's) was performed when required. All the results are expressed as mean ± S.E.M. Differences were considered significant at $p < 0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 19.0 (SPSS Inc, Chicago, USA).

Results

Acquisition of operant behaviour to obtain chocolate flavoured-pellets in DOR and wild-type littermate mice.

DOR KO and wild-type littermate mice were trained to acquire an operant responding maintained by chocolate flavoured-pellets under FR1 and FR5 schedule. The acquisition criteria in FR1 were achieved after an average of 11.3 ± 1.03 sessions by 28.6% of the entire DOR KO group and of 12.6 ± 1.03 sessions by 66.7% of wild-type littermates. Chi square test revealed significant differences in the percentage of acquisition criteria ($\chi 2 = 9.14$, $p < 0.05$). Three-way ANOVA revealed a significant main effects of genotype, lever and day indicating a continuous operant responding for chocolate pellets during the whole training period and discrimination between levers [$F(1,24) = 44.52$, $p < 0.001$] (Figure 1). No significant genotype differences were revealed (Table 1).

The acquisition criteria in FR5 were achieved after an average of 8.55 ± 5.06 sessions by 78.6% of the entire DOR KO group and of 6.08 ± 0.71 sessions by 100% of wild-type littermates. Three-way ANOVA revealed significant main effects of lever [$F(1,24) = 125.85$, $p < 0.001$] indicating discrimination in the two groups, and an interaction between genotype and lever [$F(1,24) = 16.95$, $p < 0.001$], indicating higher number of active lever-presses for wild-type littermates (Figure 1) (Table 1). Motivation for food measured at day 70 on the PR schedule showed a significant decrease in DOR KO mice when compared with wild-type littermates [one-way

Table 1. Operant responding maintained by food during acquisition

	Three-way ANOVA				
	Acquisition FR1			Acquisition FR5	
	F-value	*P*-value		*F*-value	*P*-value
Group	$F_{(1,24)} = 0.40$	n.s		$F_{(1,24)} = 12.44$	$P < 0.01$
Lever	$F_{(1,24)} = 44.52$	$P < 0.001$		$F_{(1,24)} = 125.85$	$P < 0.001$
Day	$F_{(15,360)} = 13.40$	$P < 0.001$		$F_{(82,1968)} = 1.97$	$P < 0.001$
Day × Lever	$F_{(15,360)} = 9.47$	$P < 0.001$		$F_{(82,1968)} = 1.87$	$P < 0.001$
Group × Lever	$F_{(1,24)} = 7.44$	$P < 0.05$		$F_{(1,24)} = 16.95$	$P < 0.001$
Group × Day	$F_{(15,360)} = 1.08$	n.s		$F_{(82,1968)} = 0.66$	n.s.
Group × Lever × Day	$F_{(15,360)} = 1.81$	$P < 0.05$		$F_{(82,1968)} = 1.04$	n.s.

Three-way ANOVA between-subjects factor and repeated measures in the factors day and lever (active/inactive). See materials and methods for details. *n.s.:* non-significant

ANOVA, $F(1,24) = 1.03$, $p < 0.05$] (Figure 2A). The number of lever-presses during the time-out period was also evaluated as a measurement of impulsivity like-behaviour and decreased active lever-presses during FR1 and FR5 training sessions were observed in DOR KO when compared with wild-type mice [Mann-Whitney test, $p < 0.05$ and $p < 0.01$, respectively] (Figure 2B).

Figure 1

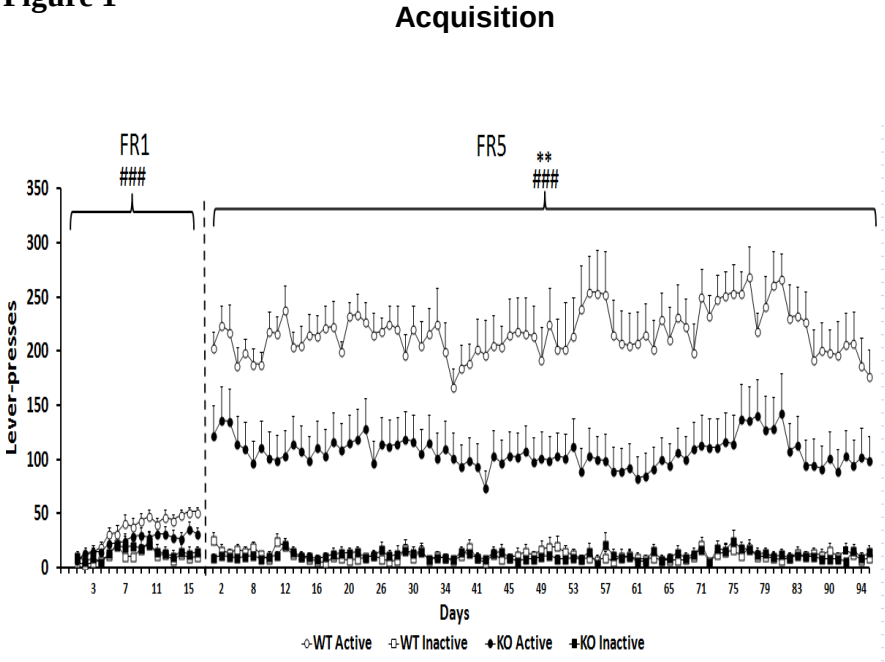

Figure 1. Acquisition of operant training maintained by chocolate-flavoured pellets. Mean number of active and inactive lever-presses during the acquisition training in FR1 (day 1-16) and FR5 (day 1-95) schedule of reinforcement in DOR knockout mice (DOR KO) (n=14) and wild-type littermates (WT) (n=12) trained with chocolate-flavoured pellets. Differences are reported as mean ± SEM. ** $p < 0.01$ (vs. KO group); ### $p < 0.001$ (active lever vs. inactive lever) (three-way ANOVA).

Body weight

No differences in body weight were found between genotypes during the FR1 training. As expected, a significant increase of body weight was reported during the FR5 training period when compared to the previous FR1 period in DOR KO mice and their wild-type

littermates [one-way ANOVA, $F(1,26) = 25.87$, $p < 0.001$; $F(1,22) = 18.32$, $p < 0.001$]. In contrast to the FR1 period, significant differences were revealed between DOR KO mice and their wild-type littermates during FR5 training [one-way ANOVA, $F(1,24) = 4.30$, $p < 0.05$] (Figure 2C).

Figure 2

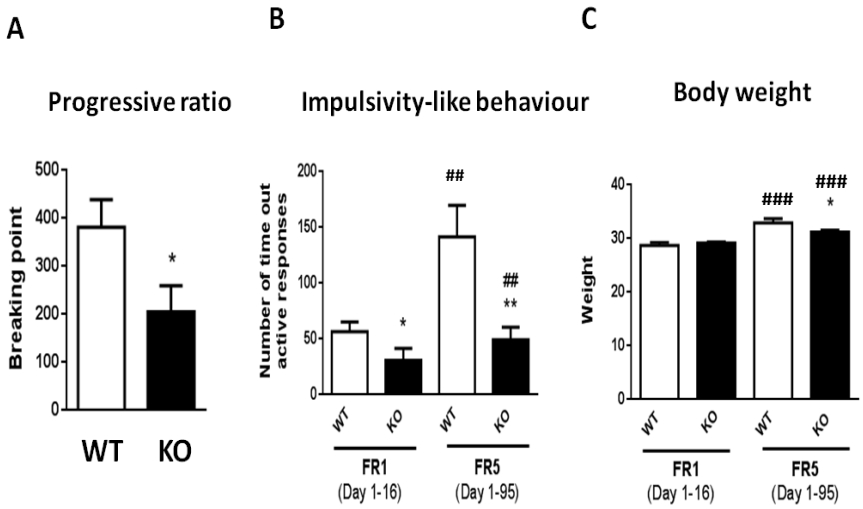

Figure 2. Motivation, impulsivity-like behaviour and body weight in DOR KO and wild-type littermates (A) Breaking point achieved during the progressive ratio (PR) schedule of reinforcement in DOR knockout mice (DOR KO) and wild-type littermates (WT). **(B)** Mean of active lever-presses in the time-out period throughout FR1 and FR5 operant training sessions for DOR KO and wild-type littermates. **(C)** Mean of body weight during the entire experimental period on FR1 and FR5 schedule for DOR KO and wild-type littermates. Data are expressed as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ (DOR KO vs. WT); ## $p < 0.01$ and ### $p < 0.001$ (FR1 vs. FR5 schedule) (one-way ANOVA, PR and body weight, or Mann-Whitney U, impulsive-like behaviour)

Figure 3

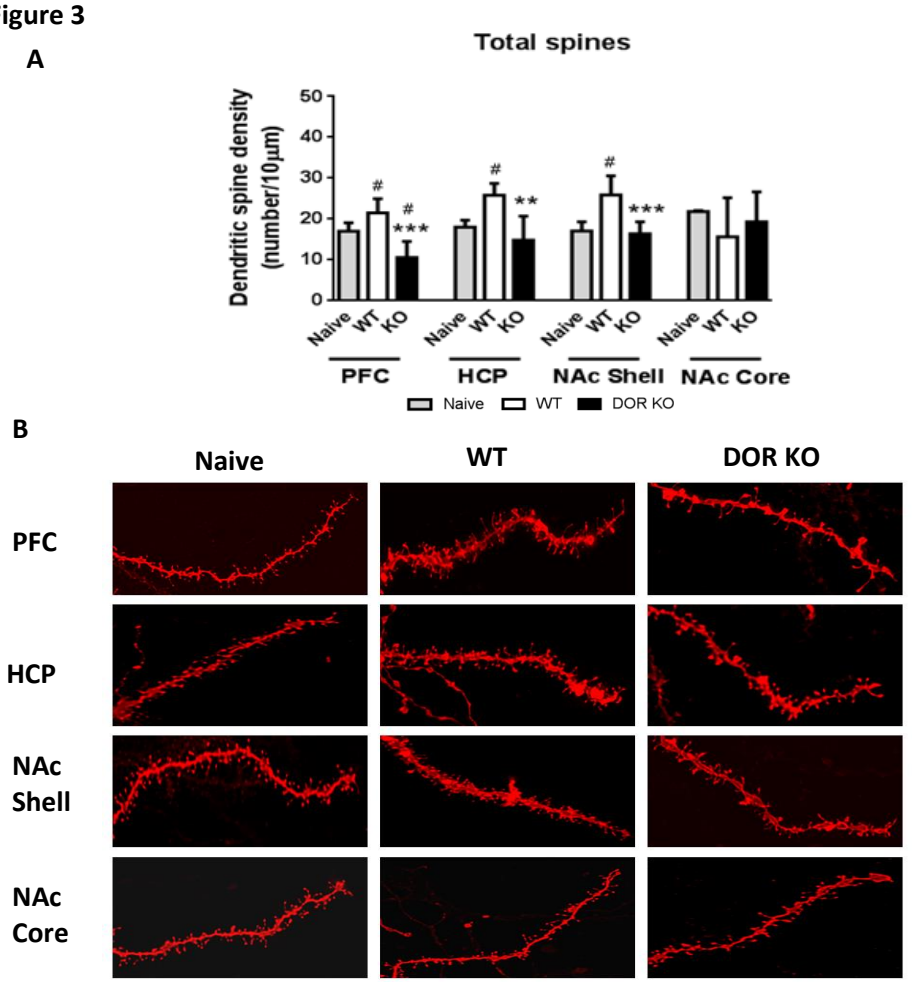

Figure 3. Neuronal morphological changes induced by chocolate-flavoured pellets in DOR and wild-type littermate mice. (A) Total dendritic spine density in neurons from the PFC, HCP, NAc shell and NAc core of DOR knockout mice (DOR KO) and wild-type littermates (WT) after the operant training to obtain chocolate-flavoured pellets respect to naïve mice. **(B)** Illustration of Dil-labelled dendrites of pyramidal neurons from the PFC and HCP and medium spiny neurons from the NAc shell and core of naïve wild-type mice and DOR KO and wild-type littermate mice trained to obtain chocolate pellets. Data are expressed as mean ± SEM. ** $p < 0.01$ and *** $p < 0.001$ (vs. wild-type); # $p < 0.05$ (vs. naïve mice) (one-way ANOVA).

Operant behaviour to obtain chocolate flavoured-pellets modifies structural plasticity in DOR KO and wild-type littermates.

Changes in dendritic spine density were analyzed with ballistic labelling with the fluorescent dye Dil in the PFC, HCP, NAc shell and NAc core. Significant differences in total dendritic spine density in neurons from the PFC, HCP and NAc shell were reported between naïve wild-type mice and wild-type mice trained with chocolate flavoured pellets [one-way ANOVA, $F(1,10) = 5.59$, $p < 0.05$; $F(1,7) = 11.44$, $p < 0.05$; $F(1,10) = 6.10$, $p < 0.05$] and between wild-type and DOR KO mice trained with these pellets [one-way ANOVA, $F(1,14) = 33.72$, $p < 0.001$; $F(1,8) = 11.69$, $p < 0.01$; $F(1,12) = 20.62$, $p < 0.001$]. Moreover, a significant difference in total dendritic spine density in neurons from the PFC was also reported between naïve wild-type mice and DOR KO mice trained with chocolate pellets [one-way ANOVA, $F(1,12) = 9.09$, $p < 0.05$]. However, no differences were found between these two groups in the HCP, NAc shell and NAc core (Figure 3A).

Changes in specific types of dendritic spines were also analyzed in each brain area. In the PFC, significant differences in thin, mushroom and filopodia density were reported between naïve wild-type mice and wild-type mice trained with chocolate pellets [one-way ANOVA, $F(1,9) = 12.01$, $p < 0.01$; $F(1,9) = 14.36$, $p < 0.01$; $F(1,9) = 29.86$, $p < 0.05$] and in thin, mushroom, filopodia and stubby density between wild-type and DOR KO mice trained with these pellets [one-way ANOVA, $F(1,14) = 17.02$, $p < 0.01$; $F(1,14) = 4.68$, $p < 0.05$; $F(1,14) = 36.21$, $p < 0.001$; $F(1,14) = 6.54$, $p <$

Figure 4

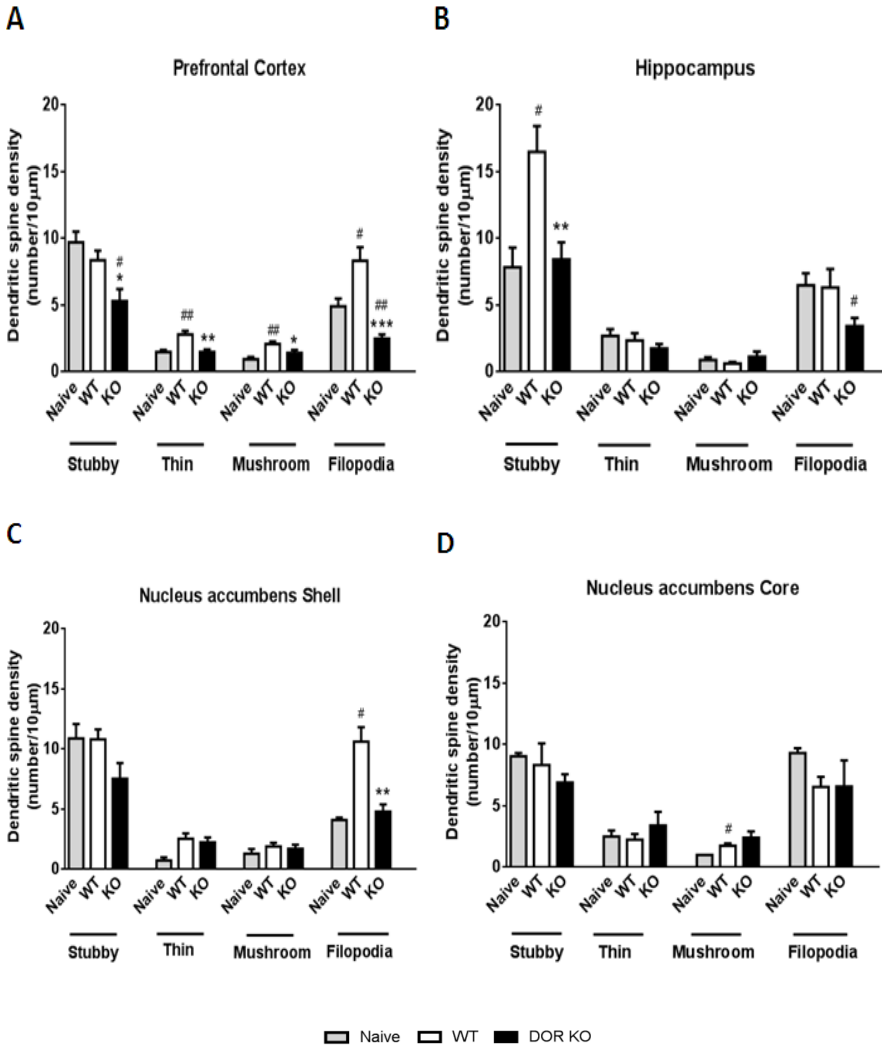

Figure 4. Dendritic density of the different spine types in neurons of (A) PFC (B) HCP (C) NAc shell and (D) NAc core of DOR knockout mice (DOR KO) and wild-type littermate mice (WT) after the operant training to obtain chocolate-flavoured pellets respect to naïve mice. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (vs. wild-type); # $p < 0.05$ and ## $p < 0.01$ (vs. naïve mice) (one-way ANOVA).

0.05]. In addition, decreased stubby and filopodia spine density was reported in DOR KO when compared with naïve wild-type mice [one-way ANOVA, $F(1,11) = 8.85$, $p < 0.05$; $F(1,11) = 15.09$, $p < 0.01$] (Figure 4A).

In the HCP, an increase in the density of stubby spines was shown in wild-type mice trained with chocolate pellets when compared with naïve wild-type mice [one-way ANOVA, $F(1,6) = 8.21$, $p < 0.05$] and DOR KO mice trained with these pellets [one-way ANOVA, $F(1,8) = 13.42$, $p < 0.01$]. Decreased filopodia density was only reported in DOR KO trained with chocolate pellets when compared with naïve wild-type mice [one-way ANOVA, $F(1,6) = 6.54$, $p < 0.05$] (Figure 4B).

In the NAc shell, an increase of filopodia density was observed in wild-type mice trained with chocolate pellets with respect to the naïve wild-type group [one-way ANOVA, $F(1,7) = 7.50$, $p < 0.05$] and DOR KO group trained with these pellets [one-way ANOVA, $F(1,12) = 18.47$, $p < 0.01$]. No significant modifications in the different types of spine density were observed between naïve wild-type mice and DOR KO mice trained with chocolate pellets (Figure 4C).

No major modifications were reported among groups in the NAc core, although increased mushroom spine density was revealed in wild-type mice trained with chocolate pellets when compared with the naïve wild-type group [one-way ANOVA, $F(1,6) = 6.06$, $p < 0.05$] (Figure 4D).

Discussion

The present study reveals a novel role of DOR in mediating the reinforcing effects, motivation and impulsive-like behaviour induced by operant training maintained with chocolate flavoured-pellets. In addition, prolonged operant training to obtain palatable food differently modifies structural plasticity in the PFC, HCP and NAc shell of DOR KO mice and their wild-type littermates, which suggests a specific involvement of DOR in the plasticity changes promoted by this repeated palatable food operant training.

Our results reveal a critical role of DOR in mediating the reinforcing aspects of food-seeking behaviour. During the FR1 schedule of reinforcement, only 28% of DOR KO mice achieved the acquisition criteria in contrast to 66% of wild-type mice. These results provide an initial evidence of the role of DOR in the acquisition of this operant behaviour. The two genotypes showed similar increases in food operant responding across the FR1 period suggesting preserved cognitive/learning abilities to acquire the food-maintained operant task in DOR-deficient mice. A similar performance in operant-responding maintained by high-fat food was already previously reported in food-deprived mice, which facilitates operant responding (Gutiérrez et al. 2014). These findings ruled out a potential learning impairment for operant training in these knockout mice. To avoid possible confounding factors, the role of DOR in the reinforcing and motivational properties of food consumption was examined in the present experimental conditions

in mice trained with chocolate pellets and fed *ad libitum* in their home cage, since restrict access to food induces major changes in the brain opioid system (Wolinsky et al. 1994). During the FR5 schedule, DOR KO mice showed less operant responding than wild-type mice as revealed by the decreased number of active lever-presses during the whole training period. Similar reduction of the operant responding was observed for DOR KO mice during the PR schedule. The breaking point is a sensible and validated measure of the motivational state of the animal (Arnold & Roberts 1997). Therefore, the present result suggests that disruption of DOR reduced the motivation for chocolate-flavoured pellets consumption. Previous studies have also suggested that DOR participates in the rewarding aspects associated with preferred foods (Gosnell & Majchrzak 1989), according with the decreased motivation toward chocolate pellets of DOR KO animals in our experimental conditions. In agreement, central administration of DOR agonists increased saccharin solution intake in rats and this increase was selective for saccharin, since water intake was minimal (Gosnell & Majchrzak 1989). Moreover, non-selective opioid antagonists, such as naltrexone and naloxone suppress consumption of preferred food, but have smaller effects on non-preferred foods further underlying that opioid signalling is involved in determining food preference (Leventhal et al. 1995; Cooper 1983; Le Merrer et al. 2009c).

DOR KO animals also decreased active operant responses during the time-out period, which is an indirect measure of impulsive-like behaviours. Interestingly, opiate addicts tend to display impulsivity

and behavioural disinhibition particularly in the decision-making domain (Clark et al. 2006; Verdejo-Garcia et al. 2007). MOR activity may be positively correlated with impulsivity traits in humans and mice (Love et al. 2009; Olmstead et al. 2009), whereas the opposite implication of DOR in impulsivity and inhibitory control has been reported (Olmstead et al., 2009). Thus, mice lacking DOR appeared more impulsive, in terms of premature responses, than wild-type controls when trained to respond for sucrose in a signalled nose-poke task (Olmstead et al., 2009). In contrast, our results suggest that DOR-deficient mice decrease food-seeking behaviour and impulsive behaviour promoted by the repeated operant training to obtain palatable food. This discrepancy could be due to the different experimental conditions used in terms of rewards, time of sessions and water restriction. Indeed, the previous study found increased impulsivity in response for sucrose solution in session lasting 40 min and when the access to water was restricted for 2 h per day. These conditions could probably alter inhibitory mechanisms, making these mice incapable of refraining from doing an anticipated response. Conversely, a more recent study suggests that the pharmacological activation of DOR increased locomotor activity, although did not increase the rate of non-reinforced lever pressing for sucrose (Befort et al. 2011), which would suggest that the effect of DOR in impulsive-like behaviour in an operant paradigm could be independent of changes in locomotion.

In our study, we observed that mice lacking DOR gained less weigh at the end of the operant training when compared with wild-type

animals. In agreement, mice deficient in DOR resist to weight gain after prolonged exposure to high-energy diet (Czyzyk et al. 2012). This was attributed to the ability of this receptor to increase the energy expenditure and thermogenic activity in the brown adipose tissue (Czyzyk et al. 2012).

Repeated operant training to obtain palatable food changes the morphology of dendritic spines in the mesocorticolimbic system (Guegan et al. 2013) and leads to a reorganization of synaptic connectivity between neurons (Chklovskii 2004). Palatable food-induced structural plasticity changes in the reward circuit appear to be a longer time-dependent process than drug-induced structural plasticity changes (Sarti et al. 2007). In our study, structural spine density alterations induced by operant training with chocolate-flavoured pellets were revealed in several brain areas of the reward circuit. These morphological spine modifications represent a neuronal substrate underlying food experience-induced behavioural changes (Butz et al. 2009). These adaptations include changes in spine number and shape induced by the synaptic activity. Indeed, the density of dendritic spines usually indicates the prevalence of excitatory synaptic inputs into a particular neuron (Lai & Ip 2013). In this line, an increase of total spine density was found in main rewarding brain areas, such as PFC, HCP and NAc shell of wild-type trained to obtain chocolate pellets when compared with naïve and DOR KO mice. In agreement with previous studies (Guegan et al. 2013), this increased spine density might be responsible for the abnormal behavioural responses observed in wild-type mice trained to obtain palatable pellets, including enhanced seeking-behaviour

and related impulsivity. In contrast, reduced spine density was observed in the PFC of DOR KO mice trained to obtain chocolate pellets with respect to naïve wild-type mice. These differences could be attributed to the effect of the operant training in the absence of DOR since the two genotypes revealed opposite modifications in spine density when compared with the non-trained naïve group. Changes in the total spine density were not observed in the NAc core of DOR KO and wild-type mice, underlying that NAc shell and core are involved in different aspects of food seeking behaviour, as previously reported (Parkinson et al. 2002; Peciña & Berridge 2005). Thus, NAc shell encodes pleasurable sensation derived from food consumption (Baldo & Kelley 2007), while the NAc core is associated with the elaboration of habit learning promoted mainly by conditioned association of natural reward-related stimuli (Everitt & Robbins 2005). Accordingly, a previous study reported that operant training to obtain palatable food does not significantly alter dendritic spine density in the NAc core of wild-type mice (Guegan et al. 2013).

Changes in the density of specific dendritic spines were also found in the PFC, HCP and NAc shell between genotypes. Operant training to obtain palatable pellets increased stubby spine density in the HCP and filopodia density in the PFC and NAc shell of wild-type mice in comparison to the naive group. Interestingly, opposite structural changes were observed in DOR KO mice, in which a reduced level of stubby spines was observed in the PFC and HCP and a decreased filopodia spine density was found in the PFC and NAc shell of these mice with respect to their wild-type littermates

trained to obtain chocolate pellets. Stubby and filopodia spines are recognized as immature spines with low stability, easily turned into other spines or eliminated (Kasai et al. 2002). However, filopodia spines seem to be implicated in synaptogenesis mechanisms due to their elongated morphology that can facilitate axodendritic synaptic contacts (García-López et al. 2010). The decreased number of stubby spines in the PFC and HCP of DOR KO mice could be associated to lower spinogenesis or retraction of the existent spines in the memory/learning and inhibitory control circuits and decreased levels of filopodia spines could be associated to a reduction of synaptic connections in the NAc shell of DOR KO mice after this operant training. In this line, spine shrinkages, retractions and turnover are generally correlated with LTD activity (Lai & Ip 2013). Therefore, we can hypothesized that the lower spinogenesis and synaptogenesis in DOR KO mice could be related to possible LTD alterations in these brain areas, leading to decreased motivation for food and reduced operant response and impulsive behaviour.

Operant training with palatable food also enhanced the density of mushroom spines in the PFC and NAc core, as well as thin spines in the PFC in wild-type mice trained to obtain chocolate pellets with respect to the naive group. In contrast, a decrease of thin and mushroom large spines was found in the PFC of DOR KO animals. Mushroom spines hold a decreased motility and consequently display structural and functional stability (Kasai et al. 2004) and are ascribed to mature spines (Nimchinsky et al. 2002) able to maintain pre-existing connections and are considered a structural basis for

long-term memory (Kasai et al, 2004). Thin spines maintain structural flexibility to enlarge and stabilize into mushroom spines making them candidate for 'learning spines'(Bourne & Harris 2007b). The decreased density of these large spines observed after operant training in DOR KO mice could wane the strength of specific synapses in the PFC of these animals. As LTP is generally associated with the enlargement and stability of dendritic spines (De Roo et al. 2008), the present finding may suggest a LTP alteration in the cortical circuit of the mutant mice leading to impairment of food-seeking behaviour.

In conclusion, the present study highlights the role of DOR in the reinforcing and motivational properties of food, as well as in impulsive responses associated to food-seeking behaviour by a mechanism that implies structural plasticity changes in the brain reward circuits. This study provides further advances in understanding neuroadaptations related to food-seeking behaviours that may promote eating disorders and highlights the relevance of DOR as a potential therapeutic target in these diseases.

Funding and Disclosure

The authors have no conflicts of interest to declare.

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Authors contribution

S.M., S.N conducted the behavioural studies. S.M. participated in the experimental design, neuroplasticity studies, interpretation and manuscript writing. E.M-G. participated in the revision of the manuscript. R.M. participated in the experimental design, supervision of experimental studies, interpretation, manuscript writing and funding of the project.

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SUPPLEMENTARY TABLES

Table S1A Normality test

Kolmogorov-Smirnov					
		Progressive ratio		Time-out	
		F-value	*P*-value	*F*-value	*P*-value
WT	FR1 schedule	-	-	$F_{(1,12)} = 0.16$	n.s
	FR5 schedule	$F_{(1,12)} = 0.20$	n.s	$F_{(1,12)} = 0.20$	n.s
DOR KO	FR1 schedule	-	-	$F_{(1,14)} = 0.06$	n.s
	FR5 schedule	$F_{(1,14)} = 0.13$	n.s	$F_{(1,14)} = 0.04$	$P < 0.05$

Objective 4

Involvement of CB2 cannabinoid receptor in the reinforcing effects of chocolate flavoured-pellets and eating addictive-like behaviour.

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Abstract

This study was aimed to evaluate the involvement of CB2 cannabinoid receptor (CB2R) in the reinforcing effects and eating addictive-like behaviour promoted by chocolate-flavoured pellets. We used a recently validated operant model of eating addictive-like behaviour in mice deficient or overexpressing CB2R and wild-type littermates (WT). Three hallmarks of addiction were evaluated at two different time points during the early and late training period in this model: persistence of food seeking during a period of non-availability of food, motivation for food and perseverance of responding when the reward was associated with a punishment. Each mouse was classified as resistant (0 criteria) or vulnerable (2-3 criteria) to this addictive-like behaviour considering these hallmark criteria. Our results revealed a significant difference in the percentage of mice reaching 0 criteria when compared CB2R deficient mice (0%) with control mice (60%) during the early period, although no major differences were reported between mice overexpressing the CB2 protein (33.3%) and control mice. During the late period, a reduced but not significant percentage of CB2R knockout mice resistant to addiction was shown, suggesting that CB2R seems involved in the predisposition to addiction. Thus, CB2R may constitute an interesting potential mechanism involved in eating addictive-like behaviours.

Introduction

Rewarding foods that usually combine palatability and high energy density have become the main driving force promoting overeating and body weight gain in the modern society (Alsiö et al. 2012). Certain forms of overeating may be mediated by an addictive-like process, and individuals may become dependent to these kind of highly rewarding foods in some particular conditions (C. Davis et al. 2011). Indeed, overeating results of eating behaviour disturbances that share neurobiological and psychological similarities with substance use disorder (D'Addario et al. 2014). Among all the neurotransmission systems orchestrating this behaviour, the endocannabinoid system through its two main receptors cannabinoid receptor 1 (CB1R) and 2 (CB2R), results particularly involved due to its important role in the regulation of rewarding responses (Harrold & Williams 2003; Lupica et al. 2004; Zhang et al. 2014). CB1R appears to be an important component of the neural substrate that mediates the reinforcing properties of drugs and palatable foods (Maccioni et al. 2008). The pharmacological antagonism or genetic disruption of CB1R reduced the reinforcing effects of palatable food and prevented the transition from controlled to loss of control over intake (Mancino et al. 2015).

Several studies suggest the potential involvement of CB2R in reward mechanisms by the modulation of brain dopamine (DA) related behaviours (Zhang et al. 2014). CB2R seems to be expressed in DA neurons of the VTA and functionally modulates DA neuronal excitability and DA release (Zhang et al. 2014), although the

mechanisms underlying these actions are still unclear (Morales & Bonci 2012). Accordingly, the role of this receptor in mediating the neurobiological responses of drugs of abuse is not completely understood and several contradictory results were reported during the last years (Katia 2015; Onaivi et al. 2008). Initial results showed increased and decreased CB2R gene expression in mouse striatum (ST) and ventral midbrain after chronic heroin treatment and ethanol intake, respectively (Onaivi et al. 2008). In addition, recent results suggest that the genetic deletion and the pharmacological antagonism of CB2R reduce nicotine self-administration and nicotine withdrawal syndrome (Navarrete et al. 2013). Conversely, it was also shown that neuronal CB2R did not seem to be involved in the reinforcing effects of nicotine (Gamaleddin et al. 2012a). These discrepant results may be due to the different experimental animal species (mice and rats) and differences in the protocol used in terms of food restriction regime and schedules of reinforcement during the operant training. In addition, cocaine self-administration was attenuated in mice overexpressing the CB2R (Aracil-Fernández et al. 2012) and after the pharmacological activation of CB2R (Xi et al. 2011b; Adamczyk et al. 2012).

The possible involvement of CB2R in the regulation of the reinforcing and motivational properties of food has not been yet investigated. However, CB2R seems directly implicated in the homeostatic control of food due to its presence in the main peripheral tissues responsible for the metabolic control, including the liver, adipose tissue, skeletal muscle and pancreatic islets (de Kloet & Woods 2009; Silvestri & Di Marzo 2013). In this context,

CB2R may be a key component in the development of obesity-associated metabolic disorders. Indeed, evidence in mice shows the involvement of CB2R in the regulation of body weight gain, obesity-associated liver and adipose tissue inflammation, and insulin resistance (Agudo et al. 2010a; Deveaux et al. 2009a).

The aim of our study was to understand the involvement of CB2R in the compulsive eating behaviour promoted by palatable food using an operant model of eating addictive-like behaviour already validated in our laboratory (Mancino et al. 2015). For this purpose, we evaluated the persistence of food seeking during a period of non-availability of food, the motivation for food and the perseverance of responding when the reward was associated with a punishment in constitutive transgenic mice overexpressing the CB2R (CB2R Tg), CB2R knockout mice (CB2R KO) and wild-type littermates.

MATERIALS AND METHODS

Animals

CD1 wild-type (WT) male mice (n=15) (Charles River, France), homozygote CB2R Tg (n=12) and CB2R KO (n=11) mice were used. Male CB2R KO mice were initially generated on a C57BL/6J congenic background (provided by Nancy E. Buckley, Cal State Polytechnic University, Pomona, CA, USA), and the CB2R KO founders were crossed with outbred CD1 (Charles River, France) background (Buckley et al. 2000) for eight generation. Male mice overexpressing CB2R (CB2R Tg) were on a CD1 congenic background. These mice were prepared as described elsewhere (Racz et al. 2008). Mice weighed 31 ± 5 g at the beginning of the experiment. Mice were housed individually in controlled laboratory conditions (temperature at 21 ± 1°C and humidity at 55 ± 10%) and they were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 8.00 am and on at 8.00 pm). Food and water were available ad libitum in the home cage. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Operant behaviour apparatus

Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two retractable levers, one randomly selected as the active lever and the other as the inactive, as previously reported (Martín-García et al. 2011). Pressing on the active lever resulted in a pellet delivery together with a stimulus-light (associated-cue), located above the active lever, while pressing on the inactive lever had no consequences. The chambers were made of aluminium and acrylic, and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise. A food dispenser equidistant between the two levers permitted delivery of food pellets when required.

Food pellets

During the operant experimental sessions, animals received a 20 mg highly palatable isocaloric pellet (20.5% proteins, 12.7% fats and 66.8% carbohydrates, with a caloric value of 3.48 kcal/g, TestDiet, Richmond, IN, USA) after each active response. Highly palatable isocaloric pellets presented similar caloric value to the diet provided to mice in their home cage (24.1% proteins, 10.4% fats and 65.5% carbohydrates, with a caloric value of 3.30 kcal/g, Special Diets Services, Witham, Essex UK) with some slight differences in their composition: addition of chocolate flavour (2% pure unsweetened cocoa) and modification in the sucrose content. Indeed, although the

carbohydrate content was similar in chow diet (65.5%) and highly palatable isocaloric pellets (66.8%), the sucrose content was different: in standard chow was 3.1% of the total carbohydrates and 50.1% in highly palatable isocaloric pellets. These pellets were presented only during the operant behaviour sessions and animals were maintained on standard chow for their daily food intake.

Experimental design

WT (*n*=15), CB2R Tg (*n*=12) and CB2R KO (*n*=11) mice were trained in operant boxes to respond for obtaining chocolate-flavoured pellets. Animals were trained under a FR 1 schedule of reinforcement in 1 h daily session during 5 days, followed by 113 days of training on a FR 5 schedule. Every self-administration session was composed by 25 min of normal delivery of pellets (active period), followed by 10 min of non-reinforced active responses (pellets-free period), and 25 additional min of active period. During the pellets-free period, no pellet reinforcer was delivered signalled by the light that illuminated the entire box. A stimulus light, located above the active lever, was paired contingently with the delivery of the reward during the active periods. A time-out period of 10 sec was established after each pellet delivery. During this period, the cue light was off and no reinforcer was provided after responding on the active lever. Responses on the active lever and all the responses performed during the time-out period were recorded. The beginning of each operant responding session was signalled by turning on a house

light placed on the ceiling of the box only during the first three sec of the session. The criteria for acquisition of operant responding were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of food pellets earned in three consecutive sessions, with at least 75% responding on the reinforced lever, and a minimum of 10 reinforcers per session (Martín-García et al. 2011).

Three addiction-like criteria for food-seeking were evaluated as previously described (Mancino et al. 2015), at two different time points in each mouse, first during the early training sessions (1-24) and then during the late training sessions (105-113). The score of addiction criteria was attributed considering the responses obtained during the late training sessions using the following three behaviours resembling DSM-5 criteria for addiction:

1) *Persistence to response:* persistence of food seeking behaviour even if the food reward is not available. It is measured by the number of responses for active lever-presses during the 10 min of unavailability of pellets delivery (pellet-free period). The active lever responses during the 10 min pellet-free period of the first 3 consecutive days of the early and late training period were evaluated.

2) *Motivation:* high motivation for food pellets measured by the PR schedule of reinforcement. It was used to evaluate the motivation for the food pellet during the early (days 13-15) and late (days 108-110) period. The response required to earn the pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000,

1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, 5500. The maximal number of responses that the animal performs to obtain one pellet is the last ratio completed, referred to the breaking point. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h.

3) *Resistance to punishment:* resistance to punishment when food pellets intake is maintained despite its negative consequences. Mice were placed for one day session in a self-administration chamber with a different kind of grid in the floor during the early (days 20-24) and late (days 111-113) period. This environmental change acted as a contextual cue. Mice received an electric foot-shock (0.20 mA, 2 sec) after 4 responses and received both, an electric foot-shock (0.20 mA, 2 sec) and a pellet, associated with the corresponding conditioned stimulus (cue light), after the 5th response. The schedule was reinitiated at the end of the time-out period, i.e. 10 sec after the pellet delivery. If mice after the 4th response did not complete the 5th response within a min, the sequence was reinitiated.

Establishment of mice subpopulations

A mouse was considered positive for a particular addiction-like criterion when the score for this behaviour was equal or above the 75th percentile of the distribution achieved by the control group. Animals were scored for each of addictive-like behaviours (three) independently and the algebraic sum of scores was calculated. Four subgroups of mice were identified depending on the number of

positive criteria met 0 criteria, 1 criteria, 2 criteria and 3 criteria (Figure 5). Due to the accomplishment of several criteria of loss of control, we considered mice reaching 2 or 3 criteria as addict-vulnerable phenotypes and therefore were included in the subgroup with the highest score, as previously reported (Mancino et al. 2015).

Sample preparation

All animals were decapitated immediately after the last training session. The brains were quickly removed and the following brain areas dissected according to the atlas of stereotaxic coordinates of mouse brain (Paxinos and Franklin, 1997): ST, NAc, prefrontal cortex (PFC) and hippocampus (HCP). Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at -80ºC.

Statistical analysis

Data obtained during the operant-acquisition phase were analyzed using three-way repeated measures analysis of variance (ANOVA) with genotype (WT, CB2R Tg and CB2R KO mice) as between-subject factor and lever (active/inactive) and day as within-subjects factors. Post-hoc analysis (Newman-Keuls) was performed when required. Body weight, pellets intake, impulsivity-like behaviour, persistence to response, motivation and resistance to punishment data were analyzed using Mann–Whitney U test due to the non-normally distributed data according to Kolmogorov test.

The data are expressed as mean ± SEM (normally distributed data) and median and interquartile range (non-normally distributed data). Differences were considered significant at $p < 0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 15.0 (SPSS Inc, Chicago, USA).

RESULTS

Acquisition of operant training maintained by food

WT (*n*=15), CB2R Tg (*n*=12) and CB2R KO (*n*=11) mice were trained to acquire an operant responding maintained by chocolate flavoured-pellets under FR1 and FR5 schedule of reinforcement. The acquisition criteria on FR1 were achieved after 5 sessions by 0%, 25% and 36.6% of WT, CB2R Tg and CB2R KO mice respectively. Chi square test revealed significant differences in the percentage of acquisition criteria between the CB2R Tg and WT group [χ^2 = 112495.50, p < 0.001] and between CB2R KO and WT mice [χ^2 = 218176.72, p < 0.001]. On FR1, three-way ANOVA revealed a significant interaction between "genotype", "lever" and "day" [$F_{(18,140)}$ = 2.31, p < 0.05], suggesting a progressive discrimination between levers (Figure 2). Subsequent post hoc analysis (Newman-Keuls) did not show significant differences between genotypes.

The acquisition criteria on FR5 were achieved by the totality of the WT, CB2R Tg and CB2R KO mice after an average of 6.27 ± 2.67, 7.17 ± 2.21 and 5.18 ± 0.52 sessions respectively. During FR5, three-way ANOVA revealed significant main effects of "day", "genotype" and "lever" [$F_{(158,2765)}$ = 1.40, p < 0.01], but not significant interactions between these factors (Figure 2) (Table 1).

Table 1 Operant responding maintained by food during the acquisition.

Three-way ANOVA				
	Acquisition FR1		Acquisition FR5	
	F-value	*P*-value	*F*-value	*P*-value
Group	$F_{(1,35)} = 1.05$	*n.s*	$F_{(1,35)} = 2.68$	*n.s.*
Lever	$F_{(1,35)} = 45.58$	$P < 0.001$	$F_{(1,35)} = 0.23$	*n.s.*
Day	$F_{(4,140)} = 23.12$	$P < 0.001$	$F_{(79,2765)} = 142.36$	$P < 0.001$
Day × Lever	$F_{(4,140)} = 33.53$	$P < 0.001$	$F_{(79,2765)} = 1.11$	*n.s.*
Group × Lever	$F_{(2,35)} = 8.66$	$P < 0.001$	$F_{(2,35)} = 3.15$	*n.s.*
Group × Day	$F_{(8,140)} = 2.78$	$P < 0.01$	$F_{(158,2765)} = 3.15$	$P < 0.001$
Group × Lever × Day	$F_{(8140)} = 2.31$	$P < 0.05$	$F_{(158,2765)} = 1.40$	$P < 0.001$.

Three-way ANOVA between-subjects factor and repeated measures in the factors day and lever (active/inactive). See materials and methods for details. *n.s.:* non significant

Figure 1

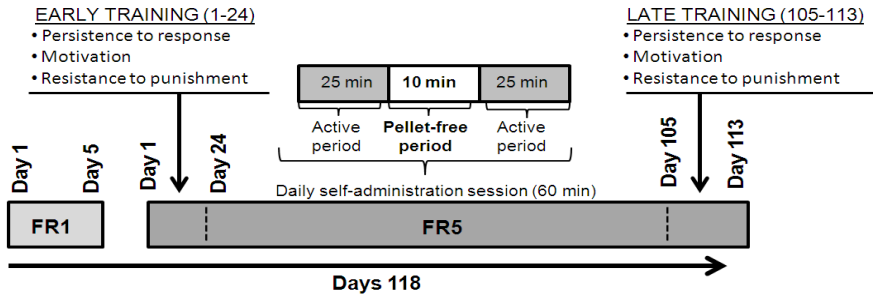

Figure 1. Experimental design. Experimental sequence of the eating addictive-like behaviour model. WT (*n*=15), CB2R Tg (*n*=12) and CB2R KO (*n*=11) mice were trained for chocolate-flavoured pellets with the presentation of a cue light under a fixed-ratio (FR) 1 schedule of reinforcement on 60 min daily sessions during 5 days followed by 113 days on a FR5 schedule of reinforcement. Each session was composed by 25 min of normal delivery pellets named active period, followed by 10 min of pellet-free period in which the persistence to response was registered, and other 25 min of active period. In the FR5 two time points were considered, early (from day 1 to 24) and late period of training (from day 105 to 113) to measure the three addictive-like behaviours: 1) persistence to response, 2) motivation and 3) resistance to punishment.

Figure 2

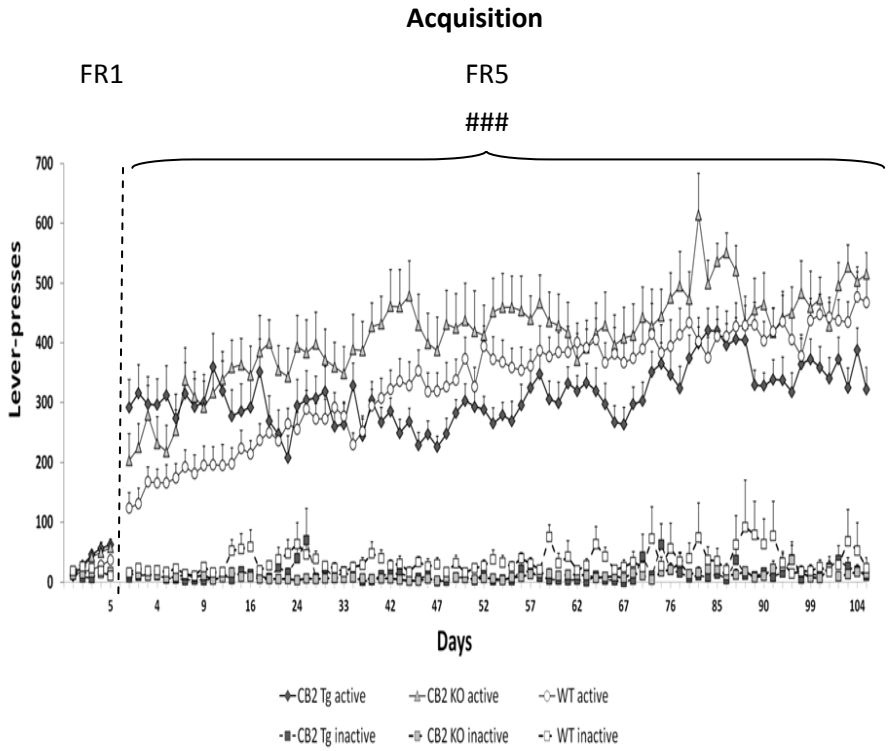

Figure 2. Acquisition of operant training maintained by food. Mean number of active and inactive lever-presses during the acquisition training in the FR1 and FR5 schedule of reinforcement in WT (*n*=15), CB2R Tg (*n*=12) and CB2R KO (*n*=11) mice. Data are expressed as mean ± SEM. ### $p < 0.001$ (active lever vs. inactive lever) (three-way ANOVA).

Comparisons in body weight, pellets intake and impulsivity-like behaviour

Significant differences in body weight were reported only during the late training period (days 105-113) with CB2R Tg mice showing lower body weight than WT ($p < 0.001$) and CB2R KO ($p < 0.05$) mice. A decreased body weight was also observed during the late period for CB2R KO mice with respect to the WT group ($p < 0.05$).

Figure 3

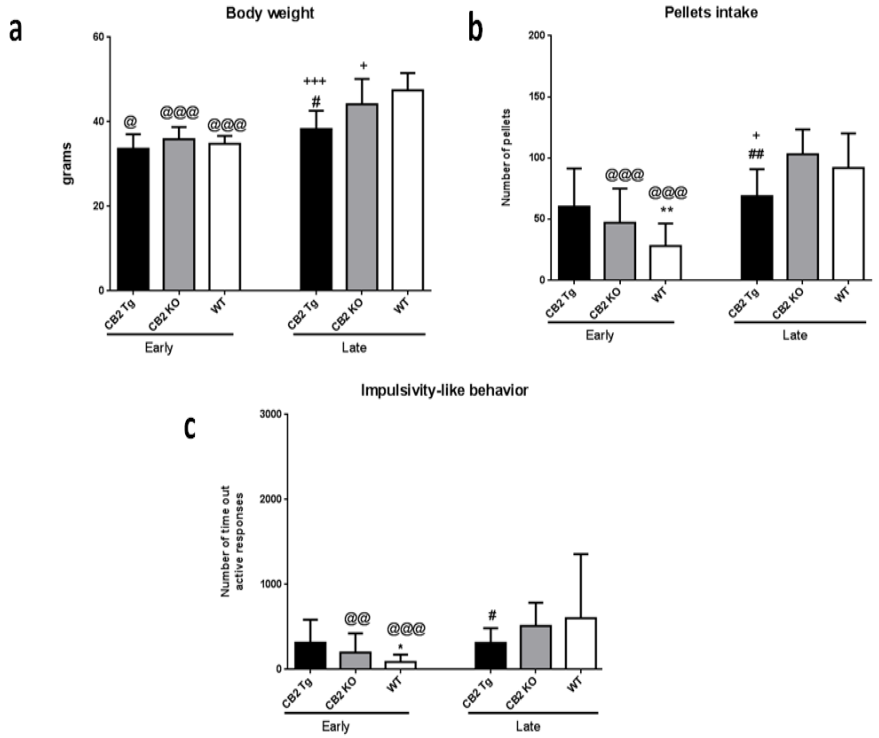

Figure 3. (A-C) Comparisons in body weight, pellets intake and impulsivity-like behaviour. (A) Mean of body weight during the early and late period of the operant training for CB2R Tg, CB2R KO and WT groups that were trained to obtain high palatable chocolate-flavoured pellets. (B) Mean number of pellets intake during three consecutive sessions in the early and late period of the operant training for CB2R Tg, CB2R KO and WT groups. (C) Mean of active lever-presses during the time-out period during three consecutive days sessions in the early and late operant training period for CB2R Tg, CB2R KO and WT groups. Data are expressed as mean ± SEM @ $p < 0.05$, @@ $p < 0.01$, @@@ $p < 0.001$ (early training vs. late training); * $p < 0.05$, ** $p < 0.01$ (vs. CB2R Tg), # $p < 0.05$, ## $p < 0.01$ (vs. CB2R KO); + $p < 0.05$, +++ $p < 0.001$ (vs. WT) (Mann-Whitney U test).

All groups of mice significantly gained weight during the late period when compared to the early period of training (days 1-24) ($p < 0.05$) (Figure 3A).

Pellets intake was significantly increased in CB2R Tg mice when compared to WT mice during the early training period ($p < 0.01$), although during the late training period, CB2R Tg mice decreased pellets intake with respect to the WT and CB2R KO group ($p < 0.05$). Moreover, WT and CB2R KO mice, but not CB2R Tg mice significantly increased the amount of pellets intake during the late period when compared to the early period ($p < 0.001$) (Figure 3B).

Impulsivity-like behaviour was measured by the number of active lever-presses during the time-out period. In the early period, CB2R Tg mice significantly increased the number of active responses when compared with WT mice ($p < 0.05$). Only WT and CB2R KO mice increased the number of time-out active lever-presses during the late period when compared with the early period of training ($p < 0.001$ and $p < 0.01$, respectively). During the late period, a significant decreased in CB2R Tg mice responses were reported with respect to CB2R KO mice ($p < 0.05$) (Figure 3C).

Differences in operant responding between genotypes in the three addiction-like criteria

All groups of animals were tested for the three behaviours used to evaluate the loss of control during the early (days 1-24) and late (days 105-113) periods of the operant training. In the persistence to response test (pellet-free period), only CB2R Tg mice significantly

increased active lever-presses during the early period when compared with WT mice ($p < 0.05$).

During the late period, a significant decrease in operant responding was observed for CB2R Tg when compared with CB2R KO mice ($p < 0.05$), albeit not significant differences were reported with respect to the control group. Furthermore, decreased responses were observed for the CB2R Tg group during the late period when compared to the early one ($p < 0.01$) (Figure 4A).

In the motivation test, CB2R Tg and CB2R KO showed higher breaking point during the early period than WT mice ($p < 0.05$), although no significant differences were reported during the late period between genotypes. Only WT mice increased the breaking point in the late period when compared to the early one ($p < 0.01$) (Figure 4C).

In the resistance to punishment, only CBR2 KO mice increased significantly the amount of pellets intake in the foot-shock test during the early period with respect to the WT group ($p < 0.001$). No significant differences between groups were reported during the late period and a significant decrease in pellets intake was observed in CB2R KO mice during the late period with respect to the early period ($p < 0.001$) (Figure 4E).

Calculation of addiction score based on the three addiction-like criteria

All groups of mice were tested for addiction-like criteria during the early and late period of training. A mouse was considered positive for an addiction-like criterion when its score for the corresponding

Figure 4

MEAN ± SEM MEDIAN ± INTERQUARTILES

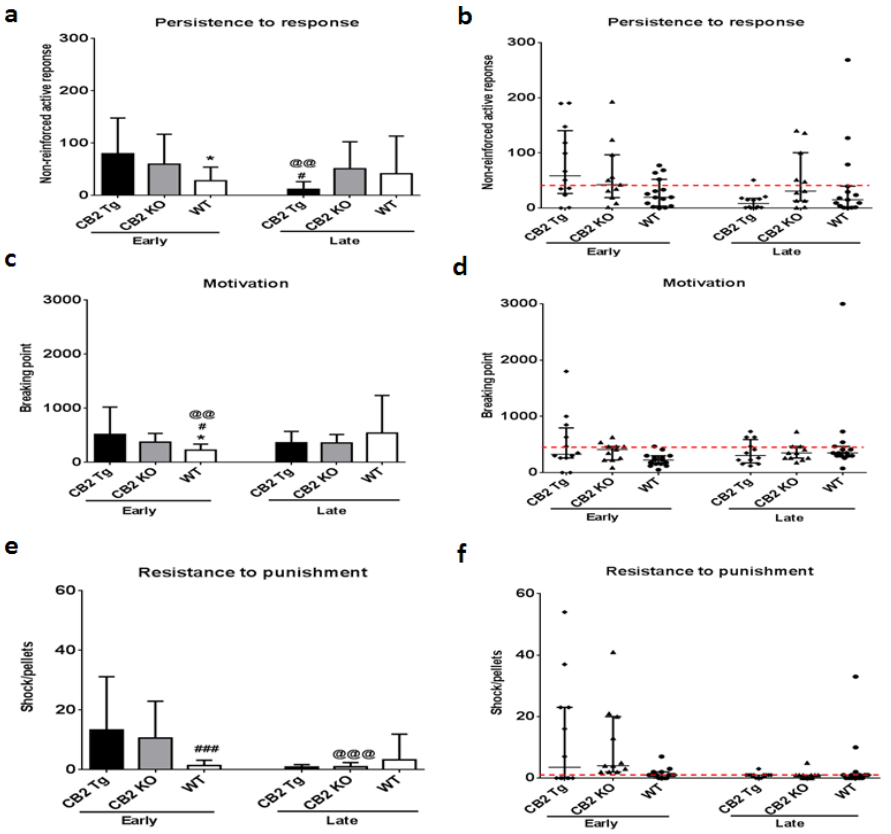

Figure 4. (A-F) Differences in operant responding between groups during the early and late period of training. (A) in the persistence to response, **(C)** motivation and **(E)** resistance to punishment for CB2R Tg, CB2R KO and WT groups exposed to high palatable chocolate-flavoured pellets expressed as mean ± SEM. **(B, D, F)** Distribution of individual data in the three tests used to measure addiction-like criteria during the early and late training of CB2R Tg, CB2R KO and WT groups calculated with median and interquartile range. The line indicates the 75th percentile of distribution of WT group during the late training period and is used as the criterion to attribute one point of the score to each individual located equal or above this percentile. @@ $p < 0.01$, @@@ $p < 0.001$, @@@ $p < 0.001$ (early training vs. late training); * $p < 0.05$, (vs. CB2R Tg), # $p < 0.05$, ### $p < 0.001$ (vs. CB2R KO) (Mann-Whitney U test).

behaviour was equal or major to the 75th percentile of the distribution of the WT group (Figure 4B, D, F). All animals were divided into 4 subgroups based on the number of criteria for which they were scored (Figure 5A). During the early training period 33.3% of CB2R Tg mice exhibited 0 criteria, 0% reached 1 criteria, 33.3% got 2 criteria and 33.3% obtained the 3 criteria, while 0% of CB2R KO mice reached 0 criteria, 36.4% 1 criteria, 27.8% 2 criteria and 36.7 % 3 criteria and finally, 60% of WT mice presented 0 criteria, 6.7% 1 criteria, 26.7% 2 criteria and 6.7% 3 criteria (Figure 5B, D, F). Chi square test revealed significant differences in the percentage of mice subpopulations reaching the high score (2-3 criteria) between the CB2R Tg and the control group [$\chi^2 = 6.00$, $p < 0.05$] and between the CB2R KO and the control group [$\chi^2 = 4.55$, $p < 0.05$]. Interestingly, a significant difference was also shown in the percentage of mice subpopulations reaching the 0 criteria between the CB2R KO and WT group [$\chi^2 = 7.33$, $p < 0.01$].

During the late training period 16.7% of CB2R Tg mice exhibited 0 criteria, 58.3% 1 criteria, 16.7% 2 criteria and 8.3% 3 criteria while, 18.2% of CB2R KO group reached 0 criteria, 45.5% 1 criteria, 18.2% 2 criteria and 18.2% 3 criteria and finally, 40% of WT mice presented 0 criteria, 26.7% 1 criteria, 20% 2 criteria and 13.3% 3 criteria (Figure 5C, E, G). Chi square test did not reveal significant differences in the percentage of mice subpopulations between genotypes during the late training period.

Figure 5

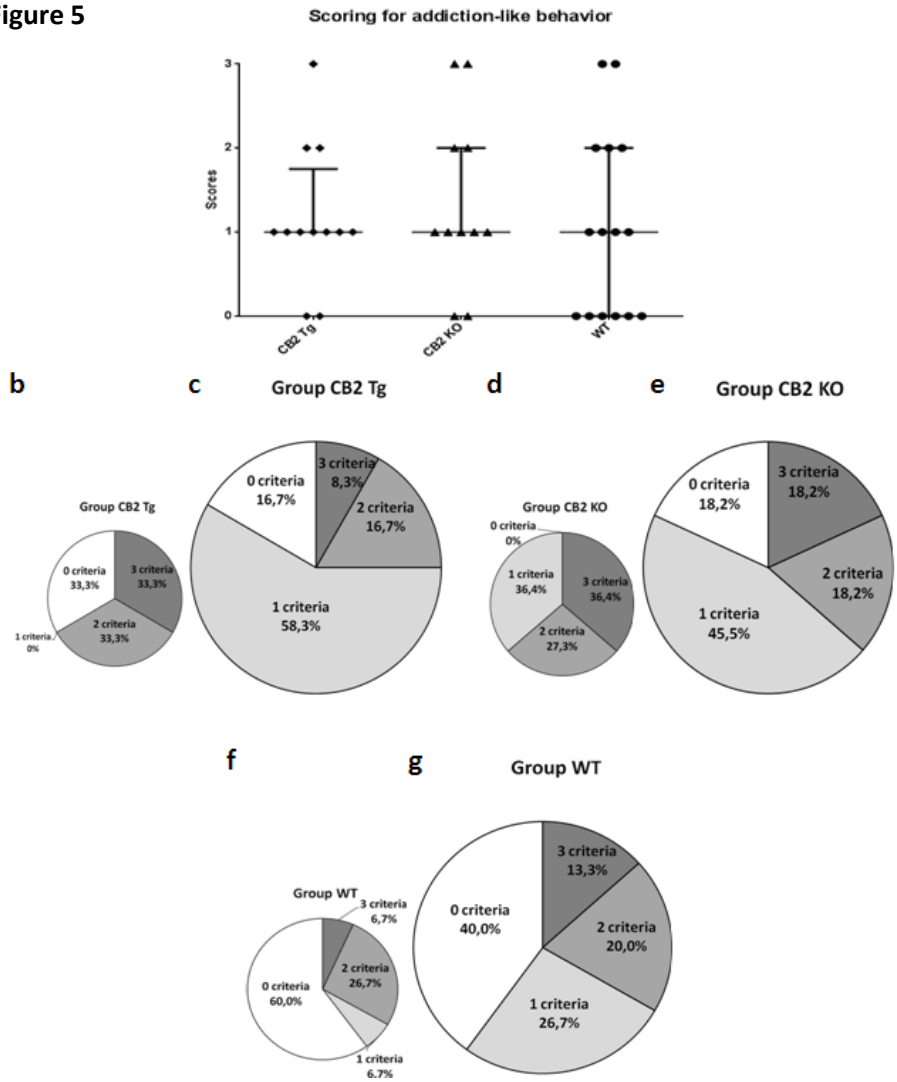

Figure 5. **(A) Distribution of animals with different scores for addictive-like behaviour**. It was calculated as the algebraic sum of the scores obtained in each of the three addiction-like criteria during the late training period. The addiction score was distributed along a scale from 0 to 3. Data are expressed with median and interquartile range. **(B-G) Distribution of the different criteria subgroups in percentage.** Animals were assigned to a criteria subgroup based on the amount of criteria met for which they scored equal or above the 75th percentile. Percentage of distribution of CB2R Tg animals during the early (B) and late (C) period, percentage of distribution of CB2R KO animals during the early (D) and late (E) period and percentage of distribution of WT animals during the early (F) and late (G) period.

DISCUSSION

In the present study, we used a reliable model of eating addictive-like behaviour recently validated in our laboratory (Mancino et al, 2015) to evaluate the involvement of CB2R in the loss of control promoted by long-term operant training maintained by palatable food. Extreme subpopulations of mice related to addictive-like behaviour were identified among the different genotypes studied. The deletion of CB2R leads to a reduction in the percentage of mice resistant to develop this addictive-like behaviour, and this effect was more evident during the early training period.

A previous study of DSM-based animal model of eating addiction revealed a crucial involvement of the CB1R in the addictive-like behaviour promoted by palatable food (Mancino et al, 2015). In this line, the present study showed a possible opposite involvement of CB2R in compulsive food seeking. The genetic disruption or overexpression of CB2R has not major consequences in the operant behaviour maintained by chocolate-flavoured pellets during the FR1 and FR5 schedule of reinforcement, although a trend to increase active responses was revealed in CB2R KO mice. Previous studies reported a different involvement of this receptor in drug operant behaviour depending on the drug of abuse used. Indeed, an impairment in the acquisition of operant behaviour maintained by nicotine was revealed in mice lacking CB2R (Navarrete et al. 2013), although nicotine self-administration was not modified by selective CB2R agonists in rats (Gamaleddin et al. 2012b). On the other

hand, reduction of cocaine-induced place preference and self-administration were reported in transgenic mice overexpressing CB2R (Aracil-Fernández et al. 2012). Moreover, intra-NAc local administration of JWH133, a selective CB2R agonist, dose-dependently inhibited intravenous cocaine self-administration, cocaine-enhanced locomotion, and cocaine-enhanced NAc extracellular DA in WT mice, whereas intra-NAc administration of AM630, a selective CB2R antagonist, elevated extracellular DA, locomotion and blocked the reduction in cocaine self-administration produced by systemic administration of JWH133 (Xi et al. 2011b). These effects could be related to a possible location of CB2R on DA terminals in the NAc (Morales & Bonci 2012) that could mediate a decrease in DA release.

In our experimental conditions, CB2R Tg mice increased the pellet consumption during operant training and showed a higher impulsive-like behaviour during the early period of training. However, an opposite result was observed during the late training in which CB2R Tg mice decreased pellets intake and body weight when compared to the control group. Previous studies reported that CB2R gene expression is increased during obesity (Deveaux et al. 2009b), although pharmacological CB2R agonism did not affect weight gain in obese rats (Jenkin et al. 2014).

A decreased body weight was observed during the late period in CB2R KO with respect to WT mice, even though these two genotypes consumed the same amount of food. It was reported that CB2R deficient mice, fed with high-fat diet for 15 weeks, attenuated the progression of obesity with respect to WT mice by a

mechanism that may involve lipid oxidation (Deveaux et al. 2009b). In addition, young (2-month-old) CB2R KO mice fed with high-fat diet for 8 weeks became obese, but they showed reduced body weight gain compared with WT mice under similar feeding conditions (Agudo et al. 2010b). In contrast, CB2R KO mice fed with standard diet displayed increased body weight gain with age (older than 6 months), which was associated with increased food intake (Agudo et al. 2010b). Nevertheless, the long-term role of CB2R modulation in the control of body weight has not been yet fully clarified (Agudo et al, 2010b).

The increased pellets intake during the late training was also associated to an increased impulsivity-like behaviour in CB2R KO mice with respect to CB2R Tg mice. CB2R has already been proposed to regulate impulsive behaviour, as the treatment with selective CB2R agonists reduced cognitive and motor impulsivity, accompanied by CB2R down-regulation. In addition, CB2R antagonists reduced novelty seeking behaviour in mice (Navarrete et al 2012).

To further explore the role of CB2R in addictive-like behaviour, we tested our genetically modified mice for the three criteria of loss of behavioural control after palatable food operant training (Mancino et al, 2015). During the early period, CB2R Tg mice appear more persistent in the response even when the reward was not available and more motivated for palatable food with respect to control mice. CB2R KO also showed an increased motivation when compared to WT mice and seemed to be more perseverant in food-seeking when the reward was associated with a punishment. These results may

indicate an initial role of CB2R in mediating the loss of control promoted by palatable food during the early phase of the operant training. The involvement of this receptor in this behaviour is highlighted by the higher percentage of CB2R KO and CB2R Tg reaching the 2-3 criteria (64% and 67% respectively) than WT mice (34%). In addition, significant differences between CB2R KO (0%) and control group (60%) in the percentage of mice subpopulations reaching 0 criteria were also reported. This result suggests that the deletion of CB2R may predispose to develop addictive-like behaviour during the early training period, decreasing the percentage of mice resistant to this behaviour.

During the late training, no significant differences were revealed in motivation and resistance to punishment, although CB2R Tg mice decreased active-lever-presses during the pellets free period when compared to WT and CB2R KO mice. In contrast to the early period, no major differences between groups were reported in the percentage of mice achieving the 2-3 criteria (25% CB2R Tg, 35% CB2R KO and 33% WT). Although the percentage of WT and CB2R KO mice reaching the 2-3 criteria is mostly similar, a trend to decrease the percentage of CB2R KO obtaining 0 criteria (18.2%) was revealed when compared to WT mice (40%). These results suggest that the deletion of CB2R may produce adaptive mechanisms underlying the shift from controlled to loss of control over food intake. A possible explanation could be that under our experimental conditions a long-term operant training, compensatory mechanisms could be activated in mice with genetic manipulations of CB2R which would minimize the differences between genotypes.

In conclusion, the present findings support that CB2R could modulate the loss of control promoted by palatable food. The possible involvement of this receptor in the vulnerability to develop palatable food addictive-like behaviour is suggested by the reduced percentage of CB2R KO mice resistant to develop this addictive-like behaviour, an effect mainly observed during the early training period. More studies are needed to clarify the exact involvement of CB2R in eating addictive-like behaviour after long-term operant training. The use of pharmacological tools targeting CB2R or conditional mutants with selective CB2R deletion in particular neuronal subpopulations and brain areas of the reward system could facilitate the understanding of the implication of this receptor in eating disorders.

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DISCUSSION

A growing body of evidence suggests that some forms of overeating disorders should be considered as addictive-like behaviours. Indeed, the pattern of food intake observed in certain obese subjects and in patients with certain eating disorders, resembles behaviours typically endorsed by individuals with substance use disorders (Volkow et al. 2008). This similarity may reflect the involvement of similar neural systems, including those implicated in regulatory self-control and reward (Volkow et al. 2013a). Both, natural reinforcers and drugs of abuse activate the mesocorticolimbic DA system (Volkow et al. 2008). Indeed, the pleasant experience obtained from palatable food and drug consumption positively correlates with the amount of DA released in the ventral ST (Alsiö et al. 2012). However, the magnitude of the DA response to natural reinforcers is usually lower than to drugs of abuse. Nevertheless, both stimuli could produce repeated and excessive release of DA in the mesocorticolimbic system that, in turn, may induce alterations in the reward brain circuits and could trigger complex and long-lasting neurobiological adaptations (Avena et al. 2009; Volkow et al. 2008). Such adaptations may include, at least in part, structural modifications in synaptic connections (Kasai et al. 2002; 2004) that can make the behaviour increasing compulsive and may lead to further loss of control toward drug or food intake (Guegan et al. 2013; Johnson & Kenny 2010) promoting vulnerability to relapse (Volkow & Wise 2005; Martín-García et al. 2011).

Several neurochemical systems seem to play a critical role in these adaptive changes produced by rewarding stimuli. Two of these systems are the endogenous cannabinoid and opioid system, both

involved in the modulation of the rewarding effects mediated by DA (Maldonado & Berrendero 2010). These systems have been proposed to play a common role as neurobiological substrates in addiction. The aim of the present thesis is to study the involvement of the opioid and cannabinoid systems in the compulsive seeking-behaviour promoted by drugs and palatable food and underlying neuroadaptative modifications induced by this behaviour.

The opioid system as a target for cocaine-relapse and food-seeking behaviour (article 1 and 3)

Chronic exposure to prototypical drugs of abuse, including opioids, alcohol, nicotine, psychostimulants and cannabinoids has been reported to produce time-dependent and region-specific alterations in opioid receptor function and expression (Shippenberg et al. 2008). In a similar way, palatable food produces changes in specific opioid components (Chang et al. 2007) that promote food rewarding effects (Avena et al. 2008).

First, we investigated the involvement of several main components of the endogenous opioid system (MOR, DOR, PENK and PDYN) in the acquisition and reinstatement of cocaine-seeking behaviour (article 1). We also studied the role of these opioid components in the regulation of the reinforcing effects of high-fat food and reinstatement of food-seeking behaviour (article 1). Furthermore, we evaluated the specific involvement of DOR in the motivation for seeking chocolate-flavoured pellets and structural plasticity changes promoted by this behaviour (article 3).

Several studies have underlined the important role of opioid receptors and their endogenous ligands in cocaine addiction (Charbogne et al. 2014). Thus, acute and chronic administration of psychostimulants produce adaptive changes in opioid peptide contents and receptor densities depending on the phase of the addiction process (Gorelick et al. 2008). Accordingly, acute cocaine administration increased MOR mRNA levels in PFC, NAc and amygdala (Yuferov et al. 1999). In addition, MOR mRNA levels were also increased in the PFC of rats during early withdrawal from chronic cocaine administration (Bailey et al. 2005) and enhanced beta-endorphin extracellular levels in the NAc were shown during cue-induced reinstatement of cocaine-seeking after a short phase of abstinence (Dikshtein et al. 2013). Our results showed that various components of the opioid system differently mediate cocaine motivational effects during the acquisition, maintenance and reinstatement of drug-seeking behaviour.

Multiple studies reveal that MOR plays an important role in the reinforcing effects induced by natural rewards and several drugs of abuse including cocaine (Le Merrer et al. 2009b). Indeed, selective MOR antagonists attenuated cocaine-conditioned place preference (Schroeder et al. 2007) and cocaine self-administration in rats (Ward et al. 2003). Furthermore, studies with MOR knockout mice demonstrated the role of MOR in the reinforcing effects of nicotine (Berrendero et al. 2002), THC (Ghozland et al. 2002) and alcohol (Becker et al. 2002). However, contradictory results were reported in cocaine reinforcement using these genetically modified mice. Indeed, cocaine place preference was unchanged (Contarino et al.

2002), increased (Becker et al. 2002) or decreased (Hall et al. 2004) and cocaine self-administration reduced (Mathon et al. 2005) in MOR knockout mice. These findings provide an unclear picture of the role of MOR in cocaine reinforcement and they are not in agreement with previous studies using pharmacological manipulations. The discrepancies reported in these studies may be due to differences in the genetic background, gender (Anker & Carroll 2011) and/or the experimental protocol used. However, possible compensatory changes in other neurotransmission systems in constitutive knockout mice could also influence the effects of cocaine on DA release in the NAc. Despite these inherent limitations, we support the hypothesis that the genetic deletion is an essential tool for understanding the role of the opioid system in drug reward and addictive processes (Lutz & Kieffer 2013b). Indeed, pharmacological data in long- term longitudinal studies cannot fully reproduce the effects of the deletion of key components of the opioid system and cannot provide information about the source of endogenous opioid ligands that mediate the behavioural responses associated with cocaine rewarding effects.

In our experimental conditions, no major differences in the acquisition of cocaine self-administration were revealed in MOR knockout mice, and a tendency to reduce the motivation for cocaine was shown in the PR schedule of reinforcement. The discrepancy with studies using MOR pharmacological antagonism may support the hypothesis of a possible compensatory mechanism after the constitutive MOR deletion (Ward et al. 2003).

DOR also seems to play an important role in the reinforcing and motivational effects of cocaine. In fact, several studies showed a diminished release of DA extracellular levels in the NAc of DOR knockout mice in response to acute cocaine (Chefer et al. 2004). In agreement, studies conducted with pharmacological tools suggested that cocaine-induced release of DA in the NAc is partly mediated by a DOR-dependent mechanism (Yoo et al. 2012). However, the mechanism underlying the role of DOR in mediating cocaine-induced DA release is still not clear and may depend on the brain area analyzed (Ward et al. 2003). Several evidence supported an involvement of glutamatergic dependent mechanisms (Fusa et al. 2005) and a possible MOR–DOR heteromeric interaction (Yoo et al. 2012). Our results showed that cocaine self-administration was significantly attenuated in DOR knockout mice when trained in FR3, but not in FR1, suggesting that the response is impaired only when the effort required to obtain the reward is enhanced. A similar result was obtained in the operant acquisition maintained by chocolate flavoured-pellets (Mancino et al, 2015; in preparation), in which DOR-deficient mice decreased active-responses only during the FR5 schedule of the operant training. Interestingly, this last result could be related to the kind of reinforcer, the palatability and/or the caloric content, since no significant differences were obtained in DOR knockout mice trained with high-fat food in our first study. Other studies showed a decreased operant responding of DOR knockout mice when trained to obtain high doses of intravenous nicotine in FR1 (Berrendero et al. 2012). In addition, DOR knockout mice displayed a decreased breaking point to obtain

cocaine during the PR schedule in our experimental conditions, and a similar result was obtained when testing DOR-deficient mice in the motivation for chocolate-flavoured pellets (Mancino et al, 2015; in preparation). The breaking point is a sensible and validated measure of the motivational state of the animal (Arnold & Roberts 1997) indicating that disruption of DOR reduced the motivational properties of cocaine and chocolate-flavoured pellets. All these findings highlight the involvement of DOR in the hedonic value and the motivational effects of cocaine and palatable food.

The role of the different families of endogenous opioid peptides in cocaine responses is not well understood, although endogenous opioid peptides derived from PENK seem to participate in the rewarding effects of cocaine and other drugs of abuse, such as nicotine (Berrendero et al. 2005; Marinelli et al. 2005). Indeed, it was described that DA extracellular levels evaluated by in vivo microdialysis in the NAc were reduced in PENK-deficient mice after nicotine administration (Berrendero et al. 2005). Moreover, long-term cocaine self-administration produced an increased in PENK mRNA levels in the ST, NAc, piriform cortex and olfactory tubercle and a decrease of these levels in the central amygdala (Crespo et al. 2001). In addition, polymorphisms of PENK could be involved in the vulnerability of cocaine use disorder (Moeller et al. 2015). Our results showed an important role of opioid peptides derived from PENK in the reinforcing and motivational properties of cocaine as revealed by the decreased response of PENK-deficient mice for cocaine during the operant acquisition and PR schedule.

The dynorphin system emerged as a powerful regulator of the neurobehavioural consequences of acute and prolonged exposure to several illicit drugs, including cocaine (Butelman et al. 2012). Indeed, activation of the dynorphin/KOR system produces place aversion, social avoidance, and antagonizes the reinforcing/rewarding effects of drugs, mainly cocaine, alcohol and cannabinoids (Wee & Koob 2010; Mendizábal et al. 2006). Animal studies showed that repeated cocaine administration increases levels of dynorphin and preprodynorphin mRNA in ST and caudate-putamen areas (Trifilieff & Martinez 2013). Moreover, selective KOR agonists blocked cocaine-induced place preference in rodents (Y. Zhang et al. 2004) and decreased cocaine self-administration in rats (Schenk et al. 1999) and monkeys (Mello & Negus 1998). In contrast, the acquisition of cocaine self-administration and the motivation to obtain this drug were not modified in PDYN knockout mice in our experimental conditions. In agreement, a recent study showed no involvement of the dynorphin/KOR system in modulating cocaine self-administration in non-human primates (Hutsell et al. 2015).

A crucial hallmark of addiction is the enduring vulnerability to relapse even after long periods of abstinence. The dorsomedial PFC, HCP and amygdala and interactions of these regions with the NAc core are implicated in the reinstatement of drug seeking produced by exposure to stimuli that have previously signalled drug administration. In this context, the HCP is important to generate a long lasting memory that associates good feelings of drug-intake with the circumstances and environment in which it occurs (Lisman

& Grace 2005). These memories, called conditioned associations, often lead to drug craving when the addict re-encounters familiar environmental circumstances. Different components of the opioid system also contribute to reinstatement of cocaine-seeking behaviour produced by a cue-stimulus. Previous studies reported that the non-selective opioid receptor antagonist naltrexone reduced cue-induced cocaine-seeking behaviour in rats, without affecting cue-induced sucrose-seeking behaviour (Burattini et al. 2008). Evidence that opioid system regulates the reinstatement of cocaine-seeking behaviour induced by priming or stress was also reported. Indeed, repeated treatment with the opioid antagonist naltrexone progressively suppressed reinstatement of priming-induced cocaine-seeking behaviour in rats (Gerrits et al. 2005) and the intra NAc injection of selective MOR or DOR agonists reinstated this behaviour (Simmons & Self 2009). Moreover, stress causes dynorphin release activating KOR in monoamine circuits, which results in both potentiation and reinstatement of cocaine and nicotine conditioned place preference (Graziane et al. 2013). Our results reveal that the reinstatement of cocaine-seeking was attenuated in MOR knockout mice. In agreement, pharmacological studies showed that the selective MOR antagonist CTAP reduced cocaine reinstatement in rats (Tang et al. 2005) and MOR up-regulation is positively correlated to cocaine craving intensity (Zubieta et al. 1996).

Similarly, in our experimental conditions the reinstatement of cocaine-seeking behaviour was significantly reduced in DOR-deficient mice. This result could support the previously reported

deficient ability of DOR knockout mice to form drug-context associations rather than deficient reward processes (Le Merrer et al. 2011). However, pharmacological studies have previously described that microinjection of selective DOR agonists in the NAc reinstated cocaine-seeking behaviour in rats (Simmons & Self 2009).

We evaluated the impact of the deletion of the opioid system components on neuronal activity in keys brain areas involved in addiction during cue-induced reinstatement using c-Fos expression. This technique does not provide information about the brain pathways involved, but it allows a general simultaneous screening of neuronal activity in several brain areas in response to specific behaviour. Our results showed that the number of positive c-Fos immunostained cells was lower in MOR knockout mice after cue-induced cocaine reinstatement than in wild-type mice in CA1, CA2 and CA3 regions of the HCP. This result reflects a decreased neuronal activation in brain structures closely involved in memory processing after the exposure to the cocaine associated cues when the activity of MOR is absent. It could suggest a possible implication of MOR in cocaine reinstatement by modifying activity of brain areas involved in memory. Furthermore, our study demonstrated that cocaine reinstatement decreased the activation of positive c-Fos immunostained cells in DOR knockout mice in the ST, and in the CA1 region of the HCP. These findings suggest that DOR modulates the motivation to obtain cocaine and cocaine reinstatement by modifying neuronal activity in brain areas involved in motor, motivation and memory processing.

In contrast, cue-induced reinstatement of cocaine-seeking behaviour was not modified in PENK knockout mice, which suggests that other opioid peptides different from those derived from PENK must be involved in the reinstatement of cocaine-seeking behaviour. However, the number of positive c-Fos immunostained cells was decreased in PENK knockout in the ST, amygdala, CA2 and CA3 regions of the HCP after cue-induced reinstatement. These results suggest that the absence of PENK produces changes in several brain structures that cause a decrease of neuron activation during the cue-induced reinstatement session, although these changes may not be related with the reinstatement of cocaine-seeking behaviour.

The reinstatement of cocaine-seeking behaviour was significantly increased in PDYN knockout mice, the opposite result to that obtained in MOR and DOR knockout mice. In agreement, animal studies of cocaine-seeking behaviour reported that the blockade of KOR decreased the effects of stress on cocaine-seeking behaviour in mice (McLaughlin et al. 2006). Indeed, selective KOR antagonists reduced the ability of a footshock stressor to reinstate cocaine self-administration in rats (Beardsley et al. 2005), while these antagonists had no effects on cue-induced reinstatement of nicotine-seeking (Grella et al. 2014). Furthermore, c-Fos mapping revealed an opposite result to other lines of opioid knockouts in PDYN-deficient mice after cocaine reinstatement. Indeed, the number of positive c-Fos immunostained cells induced by cocaine reinstatement was enhanced in PDYN knockout mice in the ST, NAc core and CA2 region of the HCP, revealing an increased

neuronal activation in these brain structures related to motor, motivation and memory processing.

In summary, our behavioural and neurochemical results suggest that DOR and PENK are involved in the motivation to obtain cocaine, and the absence of these opioid components reduces cocaine self-administration when the effort to obtain the reward is increased. Moreover, cocaine reinstatement is reduced in DOR and MOR knockout mice, whereas is not modified in the absence of PENK and results increased in the absence of dynorphin. Therefore, the reduced cocaine reinstatement revealed in MOR and DOR was not mediated by the main endogenous ligand of these receptors, enkephalins, as the deletion of the two precursors of these endogenous opioid peptides, PENK and PDYN, did not mimic this behavioural response. In agreement, it was previously demonstrated that another MOR and DOR endogenous ligand, beta-endorphin, has a crucial role in the rewarding properties of other drugs of abuse such as nicotine (Trigo et al. 2009). Thus, further studies are needed to investigate the role of this peptide in cocaine addiction and reinstatement.

Previous studies demonstrated the involvement of the opioid system in the modulation of the rewarding properties of palatable food (Gosnell & Levine 2009; Spangler et al. 2004). Indeed, it is currently accepted that the opioid system encodes the palatable/hedonic ("liking") effects of drug and food intake (Zhang et al. 2003; Yeomans & Gray 1997), while the motivational ("wanting") aspect is generally exerted by the DA system. In agremeent, administration of specific MOR and DOR agonists in

the NAc increased sucrose intake in a dose-dependent manner (Zhang & Kelley 2002) and POMC mRNA expression was increased in the mPFC of rats with chronic access to highly palatable food (Blasio et al. 2013). In contrast, the opioid receptor antagonist naltrexone did not significantly modify food-seeking behaviour (Abdoullaye et al. 2010), nor preference for palatable food in rats (Dela Cruz et al. 2012; Baker et al. 2004). However, several studies suggested that opioid antagonists preferentially alter fat consumption depending on the dose. Indeed, low doses of naloxone more specifically reduce fat intake than higher doses (Glass et al. 1996a). In our study, the absence of MOR, DOR, PENK and PDYN in knockout mice did not modify high fat food-seeking behaviour, even though these mice were maintained in food-deprived conditions. Although restricted access to food may facilitate operant responding increasing reinforcer effectiveness and the motivation to consume the reward (Raynor & Epstein 2003), this condition induces major changes in the central opioid system (Wolinsky et al. 1994) making difficult to elucidate opioid system functions in food acquisition and reinstatement. Further studies are needed to clarify the involvement of this system in high-fat food reinforcing effects.

Our next study investigated the specific involvement of DOR in the reinforcing effects, motivation and impulsive-like behaviour induced by palatable chocolate food (article 3). In agreement to our previous results, several studies did not reveal major implications of DOR in the regulation of the rewarding effects promoted by high-fat food in rats with ad libitum access to food (Katsuura & Taha

2010). Indeed, the regulation of high-fat food consumption through a DOR-dependent mechanism was shown only by the injection of high central doses of the DOR antagonist ICI 174,864 that also produces motor dysfunction (Islam & Bodnar 1990).

In our experimental conditions, DOR-deficient mice were trained for chocolate-flavoured pellets as a reward and fed ad libitum in their home cages with standard diet. As we have previously mentioned, our main results are: 1) reduced sensitivity to the primary reinforcing effects of chocolate-flavoured pellets, 2) low motivation for this reinforcer and 3) reduced impulsive-like behaviour displayed by DOR-deficient mice. Impulsivity is a predisposing factor associated with the risk of developing addictive-like behaviour (Jupp et al. 2013). Thus, it can be hypothesized that the phenotype of these mice could be potentially associated to a protection against developing compulsive-like behaviour over palatable food intake.

A reinforcer is a stimulus that increases the probability of strengthening a response and the reinforcer value of that stimulus can be defined in terms of the number of responses made to obtain it (Epstein et al. 2007). Here, we found that the reinforcing value of chocolate pellets was attenuated in DOR-deficient mice during the FR5 schedule. Contradictory results were previously reported with regards to the involvement of DOR in the reinforcing effects of natural rewards. Indeed, chronic administration of the DOR agonist SNC80 produced greater reductions in food-maintained responding (banana-flavoured food pellets) in monkeys, although cocaine self-administration only tended to decrease in the same experimental

conditions (Do Carmo et al. 2006). When a higher dose of SNC80 was tested, it eliminated both cocaine- and food-maintained responding. These results suggest a non-selective suppression of responding for food at the dose in which DOR agonist-induced decreases in cocaine self-administration (Do Carmo et al. 2006). Similarly, SNC80 decreased response rates maintained by food-reinforcement in a dose- and time-dependent manner in monkeys (Negus et al. 1998; Brandt et al. 2001). On the other hand, region-specific pharmacological agonisms revealed opposite effects to those described. Indeed, selective DOR agonist administration in the NAc stimulates palatable feeding (Zhang & Kelley 1997). In addition, the intracerebroventricular infusions of DOR agonist, DPDPE, enhanced intake at higher (2.5%, 10%), but not lower (0.5%), concentrations of sucrose while deltorphin II, increased sucrose intake at lower (0.5%, 2.5%), but not higher (10%), solution concentrations (Ruegg et al. 1997). Although these findings showed a role of DOR in the rewarding aspects of palatable food intake, this role still remains unclear since the administration of DOR antagonists in the NAc also causes an increase in consumption in other studies (Kelley et al. 1996). Moreover, naltrindole, a selective DOR antagonist, injected in the ventral pallidum, significantly increased the intake of saccharin, but not water (Inui & Shimura 2014). Conversely, the selective DOR antagonist naltriben reduced saccharin responses only with high dose (June et al. 1999). In accordance, the constitutive genetic DOR deletion attenuates the palatable food intake, as demonstrated in our study.

A differential involvement of DOR in mediating the rewarding effects of specific food cannot be discarded to explain the previous contradictory results. In fact, the DOR implication in the reinforcing properties of chocolate food in our study, but not of high-fat food (Gutiérrez-Cuesta et al. 2014; Islam & Bodnar 1990), could suggest a possible role for this receptor in mediating this particular food preference. Palatability is an important determinant of food reward value and it is directly correlated with taste responsiveness that promotes food preferences and consumption (Berthoud & Zheng 2012). In this context, the endogenous opioid system plays an important role in the neuronal processing related to palatability. Indeed the opioid system seems implicated in increasing the consumption of palatable foods, but it has little effect on consumption of less pleasant alternative foods (Taha 2010). In this line, opioids have been proposed to promote intake of preferred foods when choosing between alternatives (Glass et al. 2000). In agreement, systemic administration of naltrexone decreased preferred food consumption (Glass et al. 1996b) increasing the intake of the non-preferred one (Cooper & Turkish 1989). Opioid agonists showed a macronutrient-specific effect, increasing food intake through preferential increases in fat consumption (Marks-Kaufman 1982; Zhang et al. 1998; Naleid et al. 2007). Thus, systemically administration of morphine preferentially increases fat intake (Welch et al. 1994). Although several studies explored the effect of opioids on macronutrient preference (fats, carbohydrates and proteins) (Marks-Kaufman et al. 1985; Corwin & Wojnicki 2009), it is still unclear whether opioids effects on choice intake are

primarily related to the taste or the caloric contenent of these food options.

The potential connection between opioid system and preference for chocolate was previously investigated (Drewnowski et al. 1992). A previous study reported that enkephalins in the anteromedial quadrant of the dorsal neostriatum, but not dynorphins, contributes to generating consumption of chocolate. Indeed, rats beginning to consume palatable chocolate showed increased extracellular enkephalin levels in this brain area (DiFeliceantonio et al. 2012). However, the chronic exposure (two weeks) to chocolate liquid food reduced enkephalin gene expression in several striatal regions of rats (Kelley et al. 2003), as a possible compensatory mechanism after chronic chocolate exposure. In addition, epicatechin, a flavonoid present in the dark chocolate, seems to have opioid receptor binding capacity, specifically with DOR (Panneerselvam et al. 2010). In this line, it could be interesting to know which specific substances of the chocolate are able to produce rewarding effects. Besides the chocolate macronutrient composition, sensory properties or psychoactive ingredients such as caffeine and theobromine could also contribute to the uncontrolled consumption (Bruinsma & Taren 1999).

In our experiment, DOR knockout mice have also shown decreased motivation for chocolate-flavoured pellets, in agreement with the role of DOR in mediating palatability (Gosnell & Majchrzak 1989). In the PR schedule, where the work required to obtain each successive reinforcer is progressively increased, the breaking point

is defined as the last ratio completed. The breaking point is a validated measure of the strength of the reinforcer and the motivational state of the animal (Arnold & Roberts 1997). Thus, our results clearly indicate that the deletion of DOR reduced the motivation to obtain palatable food reward. Accordingly, a recent study has also reported that the deletion of DOR in mouse forebrain GABAergic neurons, mainly in olfactory bulb and ST but not in cortex and basolateral amygdala, leads to lower motivation for chocolate pellets in an operant paradigm (Chu Sin Chung et al. 2015). Therefore, these studies provide initial evidence in favour of an important role for DOR in processes underlying the motivational properties of palatable food intake.

A decreased body weight was found in mice deficient in DOR at the end of palatable food operant training in our experimental conditions. Accordingly, a previous study showed that DOR knockout mice gained less body weight and presented lower fat mass than wild-type mice during prolonged exposure to high-fat diet, although they were hyperphagic (Czyzyk et al. 2012). This reduction of fat mass could be related to the higher energy expenditure reported by DOR knockout mice, which was the result of an increased activation of thermogenic markers in brown adipose tissue (Czyzyk et al. 2012). Taken together these findings highlight a role of DOR in the regulation of homeostatic and hedonic responses and suggest that DOR inactivation might be effective in the treatment of obesity and related disorders.

An involvement of DOR in impulsive-like behaviour induced by palatable food was also revealed in our study. Impulsivity has been defined as the predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others (Moeller et al. 2001). A growing number of studies support a strong association between impulsivity and drug (Perry & Carroll 2008) and food-seeking behaviour (Velázquez-Sánchez et al. 2014). Contradictory results have been obtained during the last years about the involvement of DOR in impulsive and inhibitory control. Indeed, a previous study reported that DOR-deficient mice appeared more impulsive, in term of motor responses, than wild-type controls when trained to respond for sucrose in a signalled nose-poke task (Olmstead et al. 2009). In contrast, our results suggest that DOR-deficient mice decrease impulsive behaviour promoted by the repeated operant training to obtain palatable food. This discrepancy may be due to the different reward and experimental protocol used. Conversely, another study showed that activation of DOR increased locomotor activity, although did not increase the rate of non-reinforced lever pressing for sucrose (Befort et al. 2011), which could suggest that the effect of DOR in impulsive-like behaviour in an operant paradigm could be independent of changes in locomotion. Moreover, although all these studies focused only on one type of impulsivity, the response inhibition or motor impulsivity, it is well-known that the construct of impulsivity is multidimensional and consists of several different and possibly independent features (Derefinko et al., 2011). Indeed, evidence

shows that the opioid system also contributes to other types of impulsive behaviours. Thus, morphine increases impulsive decision-making in different rodent tasks, such as delay discounting processes, five-choice serial reaction time task and stop-signal task that provide measures of the sensitivity to delayed rewards, impulsive choices and response inhibition (Harvey-Lewis & Franklin 2015; Pattij et al. 2009; Pitts & McKinney 2005). It should not be surprising that different and contrasting results could be obtained considering the multiple neural systems involved and the different processes participating in impulsive-like behaviours (Befort et al. 2011).

In a next step, we have evaluated whether the persistent alterations in the reinforcing effects, motivation and impulsive-like behaviour revealed in DOR knockout mice and their wild-type littermates include structural plasticity changes in the mesocorticolimbic system. Several studies reported the involvement of the opioid system in synaptic and structural plasticity (Dacher & Nugent 2011; Pitchers et al. 2014). Indeed, repeated exposure to exogenous opiates caused morphological changes in the VTA (Mazei-Robison & Nestler 2012), reduced soma size of DA VTA neurons (Sklair-Tavron et al. 1996; Spiga et al. 2003; Russo et al. 2007; Mazei-Robison et al. 2011) and decreased levels of neurofilament proteins of the neuronal cytoskeleton in this brain structure (Beitner-Johnson et al. 1992). In our study, we observed that repeated operant training maintained by chocolate pellets differentially modified spine density in key areas of the mesocorticolimbic circuit involved in

decision-making, learning/memory and motivational processes, in DOR-deficient mice and their wild-type littermates when compared to non-trained naïve wild-type mice. Indeed, increased total spine density was reported in the PFC, HCP and NAc shell of wild-type mice trained with palatable food with respect to the non-trained naïve wild-type mice and the DOR-deficient group trained with palatable food. In contrast, reduced spine density was observed in the PFC of DOR knockout mice trained to obtain chocolate pellets with respect to non-trained naïve wild-type mice. On the other hand, no significant modifications in total spine density were observed in the NAc core of all mouse groups. Similar results were obtained in a previous study, in which no alteration of dendritic spine density in the NAc core area was reported after the training with palatable food in wild-type mice (Guegan et al. 2013). These results support even more the hypothesis that core and shell structures are involved in different aspects of feeding behaviour (Parkinson et al. 2002; Peciña & Berridge 2005). In this sense, the NAc core plays a more prominent role in the elaboration of habit learning promoted by conditioned association of natural reward-related stimuli (Everitt & Robbins 2005), while the NAc shell encodes pleasurable sensation derived from food consumption, and contributes to motivational aspect of food seeking behaviour (Baldo & Kelley 2007). In agreement, a recent study reports that during cued food reward delivery, DA release increased significantly in the NAc core, but not in the shell subregion of rats while, during the extinction period (24 h), DA significantly decreased in the NAc core, but not in the NAc shell (Biesdorf et al. 2015). These findings demonstrated that

DA release increased in the NAc core only during signalled reward and this increase could be independent of the positive or negative hedonic valence of the taste stimulus and probably linked to associative learning mechanisms (Day & Carelli 2007). In our study increased density of "memory" or mushroom spines is the only modification reported in the NAc core between wild-type mice trained to obtain palatable food and non-trained naive wild-type mice and, although not significant changes were revealed between DOR-deficient mice trained with palatable food and non-trained naive wild-type mice. These changes are likely due to the training effect, although modification in total spine density was not reported in this brain area, as previously described.

Our results highlight the notion that spine formation, turnover and morphology are a consequence of the synaptic activity induced by the training with palatable food, which activity is central to memory formation, learning and motivation and it seems to be mediated by DOR. Similar structural plasticity changes in the reward circuit induced by chronic exposure to psychostimulants or nicotine were hypothesized to participate in the development of the addictive-like behaviour (Russo et al. 2010). However, drugs of abuse also extensively modify dendritic spine density in the NAc core (Russo et al. 2010), suggesting that morphological alterations in this region might play a more prominent role in mediating the effects of drugs of abuse than palatable food (McFarland et al. 2003). The present findings are of relevance to further support the hypothesis that drugs and natural rewards, such as palatable food, can influence similar neurobiological mechanisms and that prolonged consumption of

palatable food, like drugs of abuse, can potentially alter the mechanisms involved in the rewarding processes.

In summary, our results showed a critical role of the opioid system in cocaine-reinforcing effects and reinstatement. These results suggest that dysregulation of this system may contribute to differences in vulnerability to cocaine addiction and relapse. Our data also highlighted the role of DOR in the reinforcing effects and motivation for palatable food. However, these effects seem to depend on the kind of food consumed. In addition, DOR is involved in mediating neuroadaptations in principal rewarding areas such as PFC, HCP and NAc shell after prolonged operant training maintained by chocolate food. To conclude, our results provide some advances in the understanding of the common links between eating disorders and drug addiction, and highlight the relevance of the endogenous opioid system as potential therapeutic targets to treat different addictive-like disorders.

Involvement of the CB1R and CB2R in addictive-like behaviour promoted by palatable food (article 2 and 4)

Behavioural and neurobiological overlaps have been observed between eating and addictive disorders. However, controversial arguments about applying the addiction framework to problematic eating behaviour are still reported. Although food is necessary for survival, the highly palatable processed foods associated with eating addictive-like behaviour may represent an important health concern

(Schulte et al. 2015). In this context, the identification of molecular factors that could be involved in the loss of control over food intake is an important step for further interventions. In this second part of the thesis, we first validated an animal model of eating addictive-like behaviour based on the DSM-5 substance use disorder criteria, and we evaluated protein expression changes in the main brain regions of the reward system of mice showing a compulsive eating behaviour (vulnerable to addiction) and in mice that did not show this behaviour (resistant to addiction) (article 2). We also evaluated the implication of the CB1R in eating addictive-like behaviour through epigenetic, genetic and pharmacological approaches (article 2). In addition, we investigated the possible involvement of CB2R in the vulnerability to develop this addictive-like behaviour (article 4).

We assessed addictive-like behaviour using 3 criteria resembling 5 of the 11 DSM-5 hallmarks of addiction referred to loss of control: 1) persistence of food-seeking during a period of non-availability of food, 2) motivation for food and 3) perseverance of responding when the reward was associated with a punishment. These are the core components of drug addiction across highly reinforcing behaviours and the overconsumption of highly palatable foods can result in the same behavioural outcome (Gearhardt et al. 2009a).

Palatability represents a key factor that influences motivation to seek and consume chocolate-flavoured pellets. Despite the increased effort required to obtain food (FR5) in our experimental conditions, mice trained with palatable food displayed higher levels of operant responding than mice trained with standard pellets. In

agreement, a previous study showed that palatable pellets improve the performance of operant training when compared with standard diet (Barbano et al. 2009). Indeed, mice and rats readily acquired the operant task reinforced by palatable food and were highly motivated to obtain such tastants as measured by the maximum number of responses required to earn rewards on fixed and PR operant behavioural schedules (Alsiö et al. 2009; Salamone et al. 2001). These results suggest that palatable food could potentially alter the functionality of the brain reward circuit generating a pathologic motivational state towards hedonic foods that could facilitate compulsive eating in certain patterns of consumption (Johnson & Kenny 2010). It is important to emphasize that in our study mice were not subjected to a food deprivation regimen, an experimental procedure currently used to facilitate operant conditioning maintained by food. Therefore, the loss of control over palatable food seeking shown in our experiment was not influenced by this factor. In our experimental conditions, repeated and long-term exposure to palatable food produced compulsive seeking behaviour in a consistent mouse subpopulation, as evaluated by the three criteria of addiction previously mentioned.

A possible misconception is that all individuals exposed to hedonic substances lose control over behaviour. Indeed, highly addictive drugs, like cocaine, are used on a regular basis by certain individuals, but not all involved individuals become addicted (Tossmann et al. 2001; Reboussin & Anthony 2006). Similarly, the entire western population is exposed to an energy-dense food environment, but only a subgroup of individual loses the control

over food intake and presents overeating and/or eating disorders. Genetic vulnerability could play an important role in the development of this complex disease. In our experimental conditions, only 14.7% of mice trained with chocolate pellets reached the 3 criteria of addiction and 7.4% achieved 2 criteria, whereas none of mice trained with standard pellets achieved these 2-3 criteria. This percentage of mice reaching the 2-3 criteria for palatable food reflects the high percentage of population with vulnerability to develop addiction-like behaviour despite identical opportunity to seek for chocolate-flavoured pellets in the whole genetically heterogeneous mouse population exposed to this experimental model. As expected, mice reaching this high score showed highest responses in the three addiction like-criteria tests when compared with mice reaching the low score for standard and chocolate trained mice. In addition, differences between these subpopulations were also revealed in impulsive like-behaviour measured by the number of active lever-presses during the time-out period. In this line, long-term operant training with palatable pellets appears to alter inhibitory control processes and produce an unadapted response only in mice reaching the high score. These mice showed enhanced operant responses even when no reward can be obtained and are incapable of withhold an anticipated response. In accordance to our results, a previous work also described the emergence of elevated impulsive behaviour with extended period of operant training with palatable food (Ghitza et al. 2006; Diergaarde et al. 2009). Together, these data suggest that prolonged access to

palatable food can promote the development of impulsive-like behaviours and potentially lead to compulsive food intake.

The identification of a subgroup of mice losing behavioural control supports the hypothesis that addictive-like behaviour represents a pathologic continuum from controlled to compulsive use that is only reached by a limited percentage of users (Piazza & Deroche-Gamonet 2013). Therefore, inter-individual differences in reaction to food could certainly account for the transition from controlled to compulsive food intake, similarly to those that facilitate the shift from drug use to addiction (Le Foll et al. 2009). However, like other common complex disease, addiction has a multifactorial and polygenic component that does not conform to a simple Mendelian transmission pattern. These complex disorders are likely associated with the effects of multiple genes in combination with lifestyle and environmental factors (Hamer 2002; Nestler 2014). The persistence of maladaptive behaviours suggests that long-lasting changes in gene expression occurred within particular regions of the brain in these complex diseases. Epigenetics can be viewed as the vehicle through which environment interacts with an individual genome to determine life-long molecular and behavioural modifications (Nestler 2014). Epigenetic mechanisms produced by repeated exposure to drugs have been reported to participate in addictive-like behaviour (Schroeder et al. 2008), and could also be involved in the development of eating addiction. In our study, operant conditioning maintained by highly palatable chocolate food produced adaptive changes at epigenetic level that were different depending on the

addiction-like criteria reached by mice. Specifically, mice that accomplished the 2-3 criteria showed a significant reduction in DNA methylation at CNR1 gene promoter in PFC, which led to an up-regulation of CNR1 gene expression and the subsequent increase of CB1R protein in the same brain area. Differential changes were also observed in CNR1 gene expression in the NAc and HCP of the addict-like mice, although they were not functionally relevant since no modification was revealed at the CB1 protein level, the ending product of this genetic information. Growing evidence supports the notion that CB1R plays a crucial role in the reinforcing and motivational properties of highly palatable food (Maccioni et al. 2008) and converging studies have led to hypothesized a link between alterations in the ECS and eating disorders. At the peripheral level, elevated levels of CB1R mRNA were detected in the blood of women suffering from anorexia and bulimia nervosa (Frieling et al. 2009) and increased CB1R expression at the central level were shown in the insula and frontal and temporal cortex of anorexic and bulimic patients (Gérard et al. 2011). A large body of evidence also reported that the ECS becomes over activated in obesity and that diet-induced obesity elevates HCP levels of endocannabinoids and CB1R binding (Thanos et al. 2008; Massa et al. 2010). One possible explication to this phenomenon is that dietary conditions can influence central and peripheral ECS regulation, which could contribute to promote obesity and eating disorders (Carr et al. 2008). Indeed, diet-induced changes in the ECS affect not only tissues directly involved in the metabolic regulation, but also brain regions mediating hedonic aspects of

eating and influencing cognitive processes, such as the HCP (Massa et al. 2010). Notably, rats exposed to a sweet palatable food diet for 10 weeks to induce obesity showed a decreased CB1R density in the HCP, cortex, NAc and entopeduncular nucleus, and this decrease was inversely correlated with the intake of palatable food (Harrold et al. 2002). This decrease in CB1R density could be interpreted as the resulting effect of an increased activity of these receptors by endogenous cannabinoids. In fact, chronic treatment with cannabinoid agonists is associated with parallel differentially compensatory decreases in CB1R density, CB1R mRNA expression and G-protein subunit expression in the forebrain, cerebellum and mesencephalon (Rubino et al. 1997; Hoffman & Lupica 2013; Breivogel et al. 1999; Fan et al. 1996; Zhuang et al. 1998). As already described, the opposite CB1R regulation was found in our study, in which CB1R protein level was increased in the PFC of addicted animals. This increase could be interpreted as the result, or the determinant, of the loss of control over palatable food intake presented by this addicted vulnerable subpopulation. Moreover, CB1R mRNA levels were also found to be differentially regulated depending on the schedule of access to high palatable food. Thus, CB1R mRNA levels increased in the nucleus tractus solitarius of rats with continuous access to highly palatable sweet for 6 week, and decreased in the cingulate cortex of rats with intermittent feeding schedule of this palatable food (Bello et al. 2012).

Our results suggest that long-term daily exposure to palatable food leads to alterations of food-seeking behaviour through a CB1R dependent mechanism. These alterations could be due to

dysregulations of the glutamatergic excitatory and GABAergic inhibitory synaptic inputs in several brain regions where CB1R acts as a regulatory feedback mechanism to modulate synaptic transmission (D'Addario et al. 2014). Previous studies proposed a bimodal regulation of food intake by CB1R in cortical glutamatergic transmission, responsible for the orexigenic effect and by CB1R in ventrostriatal GABAergic neurons mediator of the hypophagic action by reducing local inhibitory transmission (Bellocchio et al. 2010). The cortex contains high levels of CB1R in all of its subfields (Glass et al. 1997; Tsou et al. 1998). However, the expression pattern of CB1R in the different neuronal populations within the cortical subregions is a topic of continuous investigation. Almost all cortical neurons expressing CB1R at high or moderate levels constitute a subpopulation of GABAergic interneurons (Marsicano & Lutz 1999). However, glutamatergic neurons in cortical regions contain CB1R at low levels (Monory et al. 2006). Although CB1R on cortical glutamatergic cells are less abundant, they produce more pronounced effects than on GABAergic cells (Steindel et al. 2013). Moreover, anatomical data indicate that the predominant localization of CB1R is on axonal terminals in all cortical regions examined (Egertová & Elphick 2000). Considering this evidence, we hypothesized that the CB1R epigenetic regulation in PFC and the subsequent translation in increased CB1R protein levels found in our study may inhibit the primary glutamatergic neuronal output of this region, as already described in other studies (Steketee 2003). This CB1R overexpression on glutamatergic projections could decrease

glutamate release in the NAc and reduce the activation of NAc GABAergic inputs to VTA, removing the inhibitory modulation on DA neurons, as already reported (Lupica et al. 2004; Melis et al. 2004). Hence, CB1R acting indirectly via pre-synaptic neuronal inhibition (Szabo et al. 2002), may ultimately affect brain reward processes through their ability to enhance extracellular DA levels in the NAc (Figure 27) (Fadda et al. 2006; Lecca et al. 2006). This hypothesis was supported by data showing that the alterated glutamate transmission in the PFC responsible of behavioural inhibition (López-Moreno et al. 2008) mediates behavioural modifications associate to addiction (Kalivas 2004). Indeed, the decreased glutamatergic activation and the functional impairment of cortical areas seem to contribute to the impulsive drug intake seen in addicted individuals (Volkow & Fowler 2000). This is in line with the increased impulsive behaviour observed in mice that achieved the high addiction score in our study.

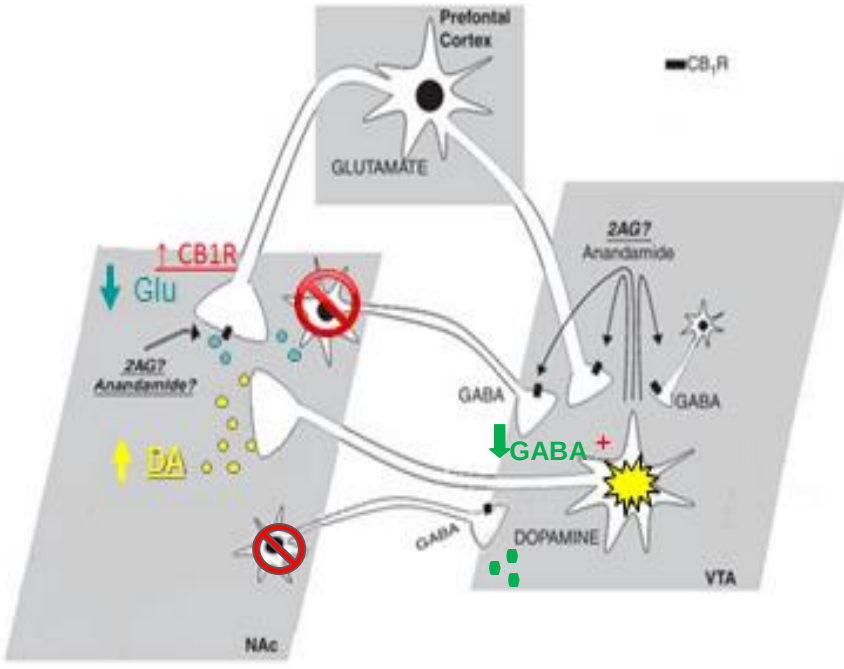


Figure 27. Schematic representation of the first possible mechanism involved in eating addictive-like behaviour mediated by the CB1R. Post-synaptic GABAergic medium spiny neurons in the NAc receive glutamatergic, DA and GABAergic inputs. Both glutamatergic and GABAergic presynaptic terminals contain CB1R, and are thus regulated by endocannabinoids (eCBs). Under physiological conditions, CB1R exerts a regulatory modulation of glutamate and GABA release in the NAc, and subsequently modulates the activation of the GABAergic medium spiny neurons. Long-term training with highly palatable pellets promotes an enhanced DA release in the NAc, together with a possible unbalance between GABA and glutamate release. This unbalance may be due to the increased CB1R expression at the glutamatergic PFC projections toward the NAc able to inhibit glutamate release, and thus producing the decrease of the GABAergic tone. This lower activation of NAc GABAergic inputs to VTA is followed by an increase in the VTA DA neuronal firing responsible of the food rewarding effects. Although the system is more complex, this figure would represent a simplified theoretical and hypothetical tool to understand the mechanism proposed (adapted from Maldonado et al. 2013).

On the other hand, we can also hypothesize a different scenario in the neurotransmitter systems regulated by CB1R. Indeed, the projections from PFC to the NAc and the VTA are both glutamatergic (Kalivas et al. 2005). Glutamate transmission exerts a potent excitatory effect on DA neurons of the VTA, influencing both DA neuron activity and the regulation of the firing properties of these neurons (Geisler & Wise 2008). In this context, it was reported that acute cocaine sensitizes the glutamatergic input from the PFC and enhances the induction of long-term potentiation in DA cells (Almodóvar-Fabregas et al. 2002). However, as previously described, CB1R in the PFC is more abundant on GABAergic interneurons (Steindel et al. 2013).

On this background, we could hypothesize that the CB1R overexpression found in our study is at the GABAergic interneuron level of the PFC. This could lead to a reduced GABA release from these neuronal subpopulations with the following increase of the firing rate of glutamatergic cortical principal neurons in the PFC (Figure 28). This increased excitatory transmission projected to the VTA, could directly stimulate VTA DA neurons, resulting in a major release of DA in the NAc. In support to this second hypothesis, several studies report that acute administration of WIN 55,212-2 into the PFC of rats causes a dose-dependent inhibition of the extracellular levels of GABA (Ferraro et al. 2001). At the same time, we hypothesized that the over activated cortical glutamatergic transmission could stimulate the GABAergic neurons of the NAc as a possible mechanism to compensate the excessive DA release. In any case, the final effect on VTA DA activity will depend upon the

relative level of activation of these inputs under distinct behavioural circumstances (Maldonado & Berrendero 2010). In this context, several studies also reported that activation of PFC regions could be associated with the enhanced desire and craving for the drug (Volkow & Baler 2014; Volkow et al. 1991; Volkow & Fowler 2000). Indeed, the anterior cingulate cortex, ventral orbital cortex and amygdala activities are increased during craving for a variety of addictive drugs (Goldstein & Volkow 2002). The imbalance in glutamatergic transmission is commonly observed in addicted individuals and alteration of this excitatory signalling could lead to the relapse of drug use (Volkow et al. 2008; Addolorato et al. 2005; Volkow & Baler 2014). However, a clear explanation of the glutamatergic neurotransmission regulation mediated by CB1R in different reward areas within stages of addiction is not yet available. Our results together with previous studies suggest that the CB1R plays an important role in mediating palatability-induced rewarding effects and that its modulation in the PFC could lead to the development of an addictive-like behaviour.

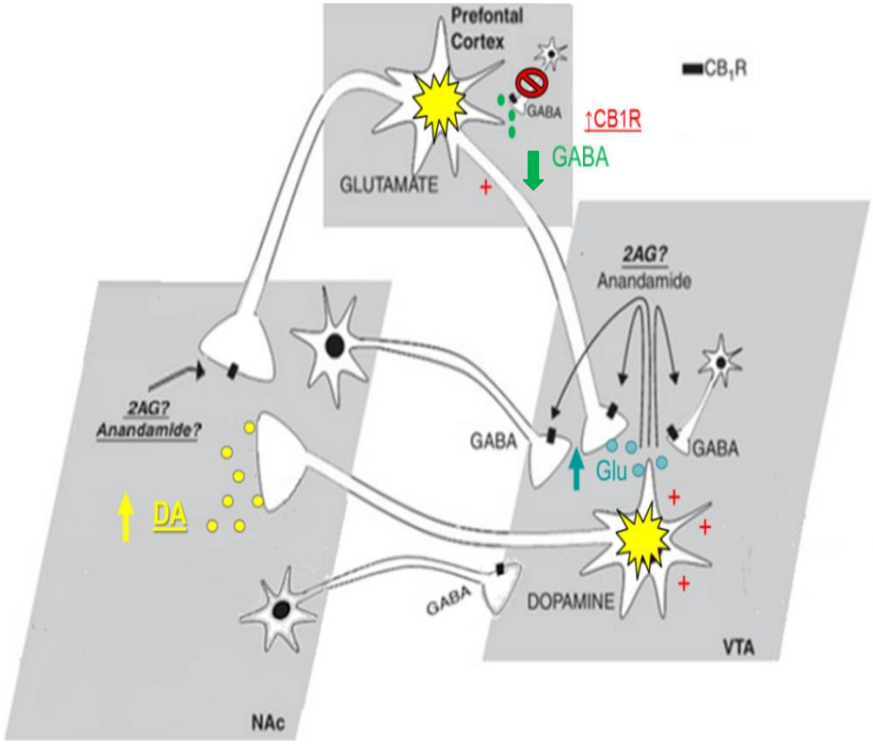


Figure 28 Schematic representation of the second possible mechanism involved in eating addictive-like behaviour mediated by the CB1R. Cortical neurons express CB1R at GABAergic and glutamatergic interneurons. However, low levels of CB1R were found in these glutamatergic terminals, whereas high levels were revealed at GABAergic interneurons (Marsicano and Luz, 1999). Under physiological conditions, CB1R exerts a regulatory modulation of glutamate and GABA release at both levels, and subsequently modulates the activation of the glutamatergic principal neurons. Long-term operant training with palatable food could promote an increase of CB1R level at cortical GABArgic interneurons producing a decrease of GABA release, followed by an increase in the activation of glutamatergic principal neurons. This increased excitatory transmission towards the VTA could directly stimulate the firing rate of VTA DA neurons with the consequent release of DA in the NAc. At the same time, glutamatergic projections stimulate GABAergic neurons in the NAc to compensate the excessive DA release (adapted from Maldonado et al. 2013).

Pharmacological procedures with a selective CB1R antagonist and genetic approaches with constitutive CB1R knockout mice were also performed in our study to clarify the involvement of CB1R in the development of this eating addictive-like behaviour. The pharmacological treatment with the CB1R antagonist rimonabant (3 mg/kg) reduced food-seeking behaviour, as shown by the lower active lever-presses during the FR5 schedule. Moreover, rimonabant treatment in the late training period reduced the percentage of mice that reached high addiction scores. Thus, none of rimonabant treated mice reached the 3 criteria, whereas 67% achieved 0 criteria. In contrast, 13% of vehicle treated mice reached the 3 criteria and 20% 2 criteria, similarly to the previous experiment (14.7% and 7.4%). However, the responses in the three behavioural tests were decreased with respect to the previous experiment, as expected considering the stressor event that represents the repeated intraperitoneal injection. Moreover, mice treated with rimonabant reaching the 0 criteria showed decreased responses in the three criteria tests, consumed significantly less palatable pellets and showed less impulsive-like behaviour when compared with mice treated with saline. In support to our results, the pharmacological blockade of CB1R reduces the enhanced motivation for cocaine developed by rats that have previously escalated their cocaine intake (Orio et al. 2009). Several studies also showed that blockade of CB1R by rimonabant (3 mg/kg) reduces operant responses for palatable food in both FR and PR schedule in animals fed ad libitum, while administration of the CB1R agonist CP-55940 produces opposite effects (Ward & Dykstra 2005).

Moreover, previous studies have also demonstrated that rimonabant administration in food-deprived animals decreased operant responses and intake of both palatable and non-palatable foods (Freedland et al. 2000; Pério et al. 2001), in accordance with the well-described role of endocannabinoids in the regulation of appetite and orexigenic signalling (Di Marzo & Matias 2005). Thus, our results showed that CB1R mediates the reinforcing effects and the motivation for palatable food. Similarly, CB1R knockout mice trained with chocolate-flavoured pellets significantly reduced operant seeking-behaviour during the FR1 and FR5 in our experimental conditions compared to wild-type mice. The decreased operant responses obtained in these knockout mice do not seem to be a consequence of a learning/memory impairment as reported in a previous study conducted in our laboratory demonstrating that the self-administration of natural rewarding stimuli, such as water and standard food, was not altered in CB1R knockout mice (Soria et al. 2005). Moreover, the percentage of CB1R knockout mice that reached addiction criteria was significantly lower than WT mice, and none of CB1R knockout mice reached the 3 or 2 criteria in the late training period. These CB1R knockout mice also showed decreased operant responses during the time-out period, which can be related to reduced impulsive behaviour with respect to wild-type mice. In agreement, the administration of CB1R agonists and antagonists enhanced and decreased, respectively, certain forms of impulsive-like behaviours (McDonald et al. 2003; Pattij et al. 2007). In summary, the pharmacological or genetic disruption of CB1R activity reduced the reinforcing effects of palatable food and

prevented the transition to addiction that evolves from controlled to compulsive intake.

Hedonic food consumption does not only depend on the functional expression and activity of the CB1R in the reward mesocorticolombic pathway, but also it depends on interactions between CB1R and homeostatic signals involved in the modulation of the appetite (Cristino et al. 2014). Indeed, homeostatic and hedonic neural circuits are closely interlinked and the CB1R, at central and peripheral level, is involved in the regulation of appropriate responses in terms of food intake and energy homeostasis. Thus, the endocannabinoid signalling in the hypothalamus seemed to be inversely correlated with leptin plasma levels (Di Marzo et al. 2001b). Such a tight cross-talk between the ECS and leptin also affects the activity of the brain reward system. Indeed, obese rats with defective leptin signalling showed increased CB1R expression in frontal, limbic and striatal brain structures (Thanos et al. 2008). Moreover, the endocannabinoid signalling in the small intestine serves as an orosensory positive feedback mechanism that facilitates reward intake (DiPatrizio et al. 2011). Therefore, it is possible that the deletion of this receptor leads to a decrease of the incentive value and appetite for chocolate food in mice reaching the low score under our experimental conditions. This relationship between the network governing energy and rewarding homeostasis has profound implications for the prevention and treatment of various eating disorders, including some forms of obesity, and perhaps also for addiction (Volkow et al. 2013b).

Epigenetic studies can help to clarify gene regulation involved in the development of behaviours, but cannot provide insight of post–translational modifications in the protein product. Therefore it is necessary to evaluate the proteome in order to understand the intricate neuro-adaptive machinery involved in addictive processes and to know whether palatable food, a reinforcer experienced daily, causes molecular adaptations in terms of protein expression modification. Palatable diets in the modern environment may be harmful not only because of ensuing weight gain and the associated health risks, but also because adaptations have occurred in the neurobiology of the individual, driving the overeating of palatable food away from voluntary control into compulsivity (Alsiö et al. 2009). Proteomic studies contribute greatly to understand gene functions and offer the potential to provide a global view of the neurobiological changes underlying addiction and to identify key proteins involved in compulsive drug and food overintake (Lull et al. 2010). However, technical limitations of proteomic studies were reported in terms of sensitivity and lagging technological capabilities in this field (Lull et al. 2010). Addiction science typically focuses on neuronal populations, although these cells comprise only 20-30% of the total cellular population in the brain tissue (Singh et al. 2003) and the neuronal spatial distribution makes difficult to ensure that the entirety of cell population is subjected to proteomic analysis (Lull et al. 2010), which represent a limitation in the use of proteomic tools in neuroscience. Additionally, rarer proteins are often masked by more abundant proteins, and significant expression changes may not be detected.

Moreover, the proteomic technique used in the present study did not allow the entire detection of the membrane proteins since the technique is focused principally in metabolic cell processes. Nonetheless, proteomic studies have revealed in our experimental conditions differential changes in protein expression and in the level of phosphorylation of synaptic proteins depending on the experimental subgroup and the brain area analyzed (Table S3 and S4 of the article 2). We distinguished between proteins and phosphoproteins because the enrichment in phosphoprotein or phosphopeptide content could help to identify the activation of particular proteins or pathways. Indeed, phosphorylation is a key reversible modification occurring mainly on serine, threonine or tyrosine residues as a post-translational modification that can regulate enzymatic activity, subcellular localization, complex formation and degradation of proteins (Delom & Chevet 2006). A change in phosphorylation status may reflect a modification in protein activity, and can provide new insights to clarify the intricate cellular network involved in these addictive-like processes.

Protein expression profiles were compared in the three brain regions studied and several changes in the expression of proteins involved in structural, transport, motor, signal transducer, catalytic processes were identified within subgroups as result from the list of proteins (Table S3, S4 of the article 2). Changes in the expression of proteins related to intracellular trafficking and cell organelles, Golgi apparatus and endoplasmic reticulum, allow proposing effects on the development of neuronal growth cones, axonal positioning and growth and maturation of dendritic spines (Matus Ortega et al.

2012). Modifications in the expression of proteins involved in the cell adhesion and structural and motor activity of the cytoskeleton microfilaments allow to hypothesize regulation of the spine growth, synapse morphology and formation of new synapses (Matus Ortega et al. 2012.; Gu & Zheng 2009). Expression changes in proteins involved in signal transducer could suggest an alteration in the synaptic transmission. Modifications in the expression of proteins related to lipid and cholesterol metabolisms suggest changes in neuronal functions, plasticity and central nervous system myelination, similar to changes described after the administration of drugs of abuse (Samaha et al. 2004). In this line, all the proteomic data were compared to previous studies with drugs of abuse in order to identify similar molecular targets underlying addictive-like behaviours.

Specific common proteins in the three brain areas selected, related to impulsive-like behaviour, synaptic plasticity and cannabinoid signalling were validated by immunoblot techniques: α-synuclein (α-Syn) (impulsive control: Ambermoon et al. 2011), protein phosphatase 1α and doublecortin-like kinase 2 (PP1α, DCalmK 2) (synaptic plasticity processes: Edelman et al. 2005; Hou et al. 2013) and diacylglycerol kinase zeta (DGKζ) (regulation of the endocannabinoid activity: Liu et al. 2001). Immunoblot remains the staple for confirming and validating proteomic results and it is a useful tool for measuring total levels of a specific protein (Lull et al. 2010). However, the major limitations to antibody-based immunoblot confirmations are the relatively low throughput nature and reliance on the availability of antibodies. Nevertheless,

increased α-Syn level was revealed in the HCP of mice reaching the high score and in the PFC of mice with low score trained with chocolate pellets, while a decreased expression of the same protein was found in the ST of mice trained with standard pellets. α-Syn is an important regulator in DA transmission. It interacts with the DA transporter, and regulates DA neurotransmission and synaptic strength of DA neurons (Boyer & Dreyer 2007). Our data and the literature support the idea that a positive correlation exists between drug and palatable food over intake and increased α-Syn levels. Indeed, α-Syn seems implicated in impulsive control disorder, such as drug addiction (Pena-Oliver et al. 2012), and it is expressed in axons and presynaptic terminals of neurons located in brain areas responsible for emotions and memory, mainly in the HCP (Taguchi et al. 2014). Mice deficient in this protein showed impaired spatial learning and working memory (Kokhan et al. 2012). In agreement, our results showed increased α-Syn protein expression in the HCP of the addict-like group, which it leads to speculate not only enhanced impulsivity in these mice, but also a greater learning ability. Nevertheless, future studies are needed to determine whether the increased α-Syn protein levels induced by drug and palatable food is one of the adaptive mechanisms to mediate addiction.

Protein expression modifications associated with synaptic and structural plasticity changes are also reported in our study. Synaptic plasticity and structural neuronal reorganization underlie learning and memory functions. In this line, the characterization of addiction as a maladaptive learning-related phenomenon has prompted to

evaluate the effects of drugs on the cellular events, cascade signalling and proteins related to synaptic plasticity (Thomas et al. 2008). The molecular mechanisms involved in the structural adaptations induced by the synaptic activity are still not completely understood (Lamprecht & LeDoux 2004). Here, we focus only on two proteins that could contribute to mediate some of the behavioural changes that define addiction. Indeed, we found increased DCalmK 2 expression in HCP and ST of mice reaching the 2-3 criteria and 0 criteria trained with chocolate, respectively. This protein is generally involved in the maturation of dendritic spines and this process could underlie eating addiction.

It is well-known that structural plasticity modifications could spring from neuronal activities. Therefore, PP1α expression changes, a protein involved in synaptic plasticity, were also analyzed and an opposite regulation in the expression of this protein was found between HCP and ST in addict-like mice. This protein seems to be important for triggering LTD through the DA-induced phosphorylation of DARPP-32 (Yan et al. 1999). The DA DARPP-32 signalling pathway integrates glutamate and DA signals in midbrain DA neurons regulating fronto-striatal functions and plasticity, and it was related to several behavioural alterations including drug addiction (Albert et al. 2002; Svenningsson et al. 2005; Fernandez et al. 2006).

An overexpression of DGKζ protein was selectively observed in HCP and ST of chocolate trained mice obtaining 0 criteria. DGKζ seems to modulate the cannabinoid signalling (Gantayet et al. 2011). This could lead to an opposite expression regulation between

DGKζ and CB1R in specific brain areas under our experimental conditions, although more studies are needed to confirm this hypothesis.

Although the relative importance for these protein adaptations largely remains to be determined, they likely represent the sustained molecular changes underlying the behavioural outcome of repeated training with palatable food. These adaptations could play important roles in the development of the compulsive food-seeking and –taking behaviours characteristic of addiction.

In conclusion, our research validated for the first time an operant model of eating addiction in a heterogenic mouse population allowing to identify extreme subpopulations, vulnerable or resistant to addiction. We detected in these subpopulations specific epigenetic and proteomic alterations in HCP, ST, NAc and PFC. Changes in DNA methylation at CNR1 gene promoter and its encoding transcript were observed in PFC. The involvement of the CB1R in the development of this addictive-like behaviour was also demonstrated by using genetic and pharmacologic approaches. Moreover, we identified proteins expressed in different subpopulations of mice that have allowed formulating novel hypotheses on the molecular mechanisms orchestrating eating addiction. These changes could participate in the biological substrate underlying the behavioural alterations that could eventually lead to eating-related disorders and provide an important advance in understanding the mechanisms engaged in hedonic aspects of food consumption furthering eating-addiction.

Our previous study revealed a crucial involvement of the CB1R in the addictive-like behaviour promoted by palatable food. In our following study (article 4), we investigated the possible implication of CB2R in the compulsive palatable food consumption using the same behavioural model. Previous studies reported that CB2R might be associated with addiction vulnerability due to its possible involvement in the modulation of the reward system (Zhang et al. 2014). It was hypothesized that CB2R could be present in local DA terminals of the NAc and here it may inhibit DA release (Morales & Bonci 2012). Alternatively, CB2R may influence the activity of NAc resident GABA medium spiny neurons or cholinergic neurons, as well as glutamatergic inputs (from the PFC, HCP and others) that could regulate either release of DA or the activity of the GABA medium spiny neurons (Morales & Bonci 2012). Another possibility is that modulation of the secretion of cytokines by activation of CB2R in microglia may influence DA levels or neuronal signalling (Morales & Bonci 2012). In this regard, emerging evidence suggests that under normal conditions microglia plays an important role in remodelling synaptic circuits, influencing synaptic structure and establishing dynamic interactions with presynaptic and postsynaptic neuronal elements (Tremblay et al. 2011). However, whether and which neurons express CB2R under normal conditions is still a matter of debate. Further studies are required to determine how CB2R modulates DA neurotransmission and finding the precise distribution of CB2R within the NAc is crucial to answer this question. Accordingly, contradictory results have been reported with regards to the rewarding effects mediated

by CB2R with different drugs of abuse. Indeed, attenuation of nicotine seeking-behaviour was revealed in mice lacking CB2R (Navarrete et al. 2013), although no modification of this behaviour after selective CB2R agonist or antagonist injections was reported in rats (Gamaleddin et al. 2012b), maybe due to the use of different experimental conditions. Moreover, the down-regulation of CB2R expression in midbrain appeared to facilitate alcohol reinforcing effects (Onaivi et al. 2008; Ishiguro et al. 2007) and polymorphisms in the CB2R gene could influence alcoholism vulnerability (Ishiguro et al. 2007). Furthermore, reduction of cocaine-induced place preference and self-administration were reported by transgenic mice overexpressing CB2R (Aracil-Fernández et al. 2012) and intra-NAc local administration of JWH133, a selective CB2R agonist, dose-dependently inhibited intravenous cocaine self-administration, cocaine-enhanced locomotion, and cocaine-enhanced NAc extracellular DA in wild-type, but not in CB2R knockout mice (Xi et al. 2011b). In addition, activation of CB2R by JWH133 inhibited VTA DA neuronal firing during cocaine self-administration in wild-type mice (Zhang et al. 2014). The possible explanation of these last findings could be that the inhibitory effect of JWH133 on cocaine consumption may be mediated by activation of CB2R at the presynaptic level on the ventral DA projection in the NAc. Our results using a natural reward showed that the genetic disruption or overexpression of CB2R had not major consequences in the operant behaviour maintained by chocolate-flavoured pellets during the FR1 and FR5 schedule of reinforcement. In this line, no significant changes in food intake within 1 hour after administration

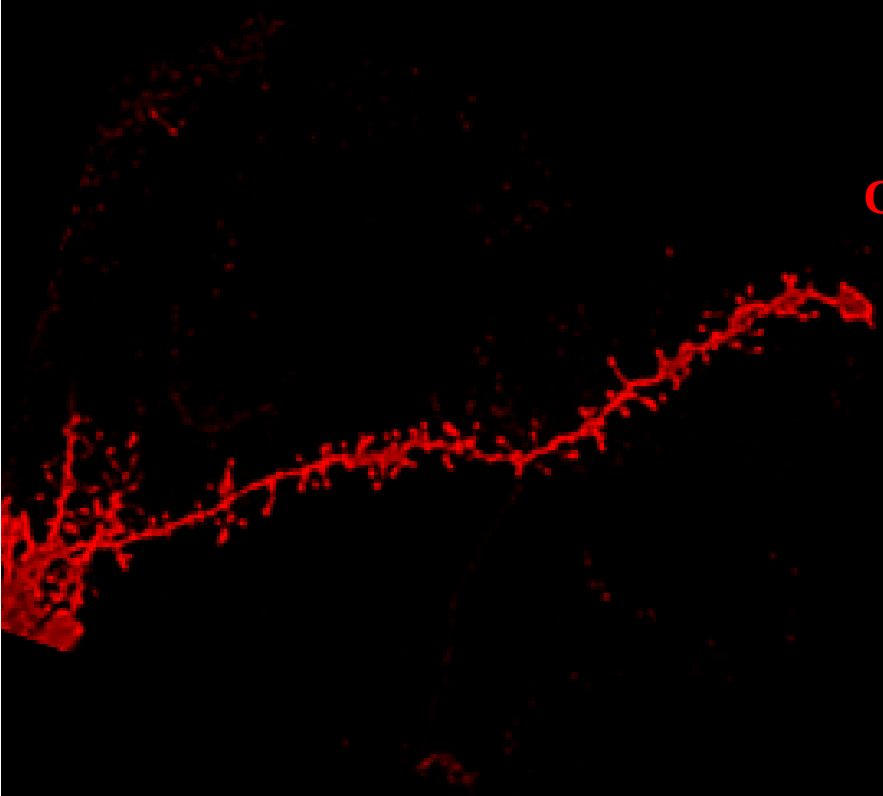
of the CB2R antagonist SR144528 in mice deprived of food for 24 hours were found in a previous study (Wiley et al. 2005). However, a significant increase in food intake was reported in rats that received intra-cerebroventricular injections of the CB2R antagonist AM 630 following a 12-hr fast was described (Werner & Koch 2003). In our experimental conditions, we show that CB2R does not seem particularly involved in the reinforcing properties of palatable food, although a trend to increase active responses was reported only by CB2R knockout mice. Although no significant differences in the acquisition were revealed between genotypes, this does not mean that this receptor is not involved in addictive-like behaviour. In fact, addiction is not just the taking of the drug, but it is also measured by the appearance of drug-related behavioural problems and high motivation for the drug (Piazza & Deroche-Gamonet 2013). When all groups of mice were tested for the three criteria of addiction (Mancino et al, 2015), significant differences were obtained during the early period for mice overexpressing or deficient of the CB2R compared to the control group. The main differences were obtained during the early training period when a higher percentage of CB2R knock-out and CB2R Tg reaching the 2-3 criteria (64% and 67% respectively) was revealed in comparison to wild-type mice (34%). In addition, significant differences in the percentage of mice subpopulations reaching the 0 criteria between the CB2R knockout (0%) and control group (60%) were reported during this early period. This result suggests that the deletion of CB2R may predispose to develop addictive-like behaviour during the early training period, decreasing the percentage of mice resistant

to this behaviour. Specifically, this finding means that CB2R could participate in the vulnerability to addiction. Nevertheless, addiction is a behaviour appearing only after an extended access to drugs (Deroche-Gamonet et al. 2004) and this high vulnerability revealed in CB2R knockout mice to develop addictive behaviours just during the early training period could be an ambiguous phenomenon. Indeed, after long operant training maintained with palatable food a similar percentage of addict-like mice was revealed between CB2R knockout and the control group (36% and 33% respectively), although a trend to decrease the percentage of CB2R-deficient mice reaching 0 criteria (18%) was revealed when compared to the 0 criteria control mice (40%). This result could suggest that the deletion of the CB2R is involved in producing the adaptive process occurring during the transition to addiction, making animals more vulnerable to loss of control over food intake. However, the lack of significant results between the two groups in the late phase of the operant training could be influenced by compensatory mechanisms activated in mice with genetic manipulations of CB2R that could minimize the differences between genotypes. Moreover, it should also be considered that constitutive CB2R knockout mice presented increased vulnerability to stressful stimuli, whereas transgenic mice overexpressing CB2R resistant to these stressful stimuli, as revealed in the light-dark box and elevated plus-maze test (Ortega-Alvaro et al. 2011). This vulnerability to stressful stimuli could have affected the performance of CB2R knockout mice in our operant paradigm. In summary, the present findings support the notion that the activity of CB2R could decrease the vulnerability to develop aadictive-like

behaviours promoted by palatable food, and this effect was more pronounced during the early period of the operant training. More studies are needed to clarify the exact involvement of CB2R in eating addictive-like behaviour after long-term palatable food operant training.

Finally, our results demonstrated that CB1R could participate in the biological substrate underlying the behavioural alterations during eating-addictive disorders, although the precise CB1R circuits involved must be still clarified. Moreover, our study also revealed the participation of CB2R in palatable food reward properties. However, numerous questions remain open with regards to possible mechanisms involved in this response. The generation of conditional mutants with selective CB2R deletion in particular neurons will be essential to further advance in this topic and to investigate novel possible therapeutic approaches for this brain disorder.

CONCLUSIONS



The results obtained in the present thesis allow to draw the following conclusions:

1. The endogenous opioid system is critically involved in cocaine reinforcing effects and reinstatement of cocaine-seeking behaviour.

2. Opioid peptides derived from PENK acting on DOR have an important role in cocaine reinforcing properties.

3. MOR and DOR, and endogenous opioid peptides different from enkephalins are crucial for cue-induced reinstatement of cocaine-seeking behaviour by modulating neuronal activation of brain areas involved in the control of motor, motivation, and memory processes, while opioid peptides derived from DYN have an opposite role to MOR and DOR in the control of cocaine reinstatement.

4. The absence of basal tone of MOR, DOR, PENK and PDYN in mice did not modify high-fat food-seeking behaviour.

5. DOR seems implicated in the reinforcing effects, motivation and impulsive behaviour induced by operant training with palatable chocolate pellets.

6. DOR mediates structural plasticity changes in PFC, HCP and NAc shell triggered by prolonged operant training with chocolate palatable food.

7. Training with palatable food induced structural plasticity changes in the mesocorticolimbic circuit similar to those produced by addictive drug exposure. However, these alterations require more time to take place than those produced by drugs of abuse.

8. We validated an animal model of eating addictive-like behaviour based on the DSM-5 substance use disorder criteria using operant conditioning maintained by chocolate pellets in an outbred mouse population allowing to identify subpopulations of addict-like and non-addict-like mice.

9. Specific proteins in the HCP, ST and PFC regions related to impulsive-like behaviour (α-Syn), synaptic plasticity (PP1α, DCalmK 2) and cannabinoid signalling (DGKζ) could be involved in the neurobiological changes leading to eating addictive-like behaviour.

10. Reduction in DNA methylation at CNR1 gene promoter in PFC of addict-like mice represents an important adaptive mechanism to long-term training with palatable food.

11. CB1R is involved in the development of addictive-like behaviour promoted by palatable food. Indeed, CB1R deletion or pharmacological antagonism leads to reduced percentage of mice that accomplish addiction criteria after long-term training to palatable food.

12. CB2R could be involved in the loss of control over consumption promoted by the operant training with palatable food.

13. The deletion of CB2R could predispose to generate addictive-like behaviour decreasing the percentage of mice resistant to develop this behaviour.

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