NEUROBIOLOGICAL SUBSTRATES INVOLVED IN THE BEHAVIOURAL ALTERATIONS INDUCED BY MDMA AND THC ADMINISTRATION IN MICE

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A totes les persones que m'han ajudat a arribar fins aquí

"Nobody said it was easy
No one ever said it would be this hard"

Coldplay – The scientist

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Abstract

Cannabis and ecstasy are popular recreational drugs that are often consumed together. Hence, the combined use of both drugs makes it difficult to elucidate their selective contribution in the neurobiological alterations observed in this population. We investigated the neural mechanisms underlying the behavioural effects of MDMA (ecstasy) and THC administration separately using animal models. Repeated administration of neurotoxic and non-neurotoxic doses of MDMA induced alterations in the motivation for palatable food, while only neurotoxic doses of MDMA induced durable resistance to extinction and cognitive flexibility, and transient deficits in working memory. A decrease in striatal DAT binding, and lower levels of stimulated dopamine release were observed following repeated neurotoxic doses of MDMA. These findings suggest that MDMA induces alterations in executive functioning that are associated with changes in striatal dopaminergic neurotransmission. In transgenic mice lacking 5-HT_{2A} receptors, we found a decrease in the amnesic, anxiolytic, and pro-social-like effects of THC, as well as the manifestations of THC withdrawal syndrome. In contrast, 5-HT_{2A} receptor deletion did not modulate the acute hypolocomotor, hypothermic, anxiogenic and antinociceptive effects of THC or the reinforcing effects of the cannabinoid agonist, WIN 55,212-2. *In vitro* molecular assays and *ex vivo* studies in mouse brain slices revealed the formation of CB_1-5-HT_{2A} heteromers with specific signaling properties. The disruption of this heteromer with specific transmembrane interference peptides selectively abrogated the memory impairments caused by THC exposure, but not its antinociceptive properties. These findings suggest that the potential therapeutic properties of cannabinoids can be dissociated from their unfavourable side-effects by targeting the CB_1-5-HT_{2A} heteromer.

Resum

El cànnabis i l'èxtasi són dues drogues d'ús recreatiu, les quals usualment es solen consumir de forma conjunta. Degut al seu ús combinat, resulta difícil esclarir les conseqüències que té cada droga a nivell neurobiològic. En aquesta tesi hem investigat els mecanismes neurals involucrats en les respostes conductuals produïdes per l'administració de MDMA (èxtasi) i THC (principi actiu del cànnabis) en models animals. L'administració repetida de MDMA produeix alteracions en la motivació per obtenir menjar apetitós, però és necessària l'administració repetida d'altes dosis que produeixen efectes neurotòxics per produir una manca de flexibilitat cognitiva i dèficits transitoris en la memòria de treball. L'administració de dosis neurotòxiques de MDMA produeix una disminució en els nivells de transportador de dopamina així com un dèficit en l'alliberació d'aquest transmissor. Així doncs, aquests resultats suggereixen que les alteracions en les funcions executives provocades per la MDMA estan associades als canvis observats en la neurotransmissió dopaminèrgica. Per altra banda, mitjançant l'ús de ratolins transgènics als quals els manquen els receptors 2A de serotonina hem revelat que aquests receptors modulen les respostes induïdes pel THC. Aquests animals són menys sensibles als efectes amnèsics, ansiolítics i pro-socials del THC i a més presenten un menor síndrome d'abstinència a aquesta substància. En canvi, l'absència de 5-HT_{2A} no modifica les propietats antinociceptives, hipotèrmiques, hipolocomotores i ansiogèniques del THC, ni tampoc redueix els efectes reforçants de l'agonista cannabinoide WIN 55,212-2. Mitjançant estudis moleculars *in vitro* i assajos *ex vivo* hem descobert i caracteritzat els heteromers CB_1-5-HT_{2A}, els quals presenten unes propietats de senyalització específiques. Pertorbant la formació dels heteromers mitjançant pèptids específics hem aconseguit abolir els efectes amnèsics del THC, mantenint les seves propietats antinociceptives. Aquesta troballa suggereix la possibilitat d'utilitzar els heteromers CB_1-5-HT_{2A} com a diana per dissociar els efectes terapèutics del THC dels efectes indesitjables induïts per la seva administració.

Abbreviations

2-AG: 2-arachidonoylglycerol

5CSRTT: 5 choice serial reaction time task

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

5-HT_{1A}: serotonin 1A receptor

5-HT_{2A}: serotonin 2A receptor

8-OH-DPAT: 8-hydroxy-(din-propylamino) tetralin

Akt: protein kinase B

BiFC: bimolecular fluorescence complementation

BIS: Barrat impulsivity scale

BNST: bed nucleus of the stria terminalis

BRET bioluminescence resonance energy transfer

cAMP: cyclic AMP

CB1: cannabinoid type 1 receptor

CB2: cannabinoid type 2 receptor

CeA: central nucleus of the amygdala

CNS: central nervous system

CPP: conditioned place preference

CPT: continuous performance test

CRF: corticotropin-releasing-factor

CTX: cholera toxin

CYP: cytocrom P450

DAG: diacylglycerol

DAGL: diacylglycerol lipase

DAT: dopamine transporter

DEA: United States drug enforcement administration

DMR: dynamic mass redistribution assay

DOI: 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane

DR: dorsal raphe nucleus

DSM: diagnostic and statistical manual of mental disorders

EMCDDA: European monitoring centre for drugs and drug addiction

EPM: elevated plus maze

ERK 1/2: extracellular signal-regulated kinases 1 and 2

FAAH: fatty acid amine hydrolase

FAN: factor associated with neutral sphingomyelinase

FR5: fixed ratio five

FRET: fluorescence resonance energy transfer

GABA: γ-aminobutiric acid

GBR: GABA B receptor

GPCR: G protein-coupled receptor

GWS: global withdrawal-score

Hcrtr-1: hypocretin receptor 1

Hcrtr-2: hypocretin receptor 2

HHMA: 3,4-dihydroxymethamphetamine

HMMA: 4-hydroxy-3-methoxymethamphetamine

IP3: inositol trisphosphate

IQ: intelligence quotient

IUPHAR: international union of basic and clinical pharmacology

KO: knock-out

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

MDA: methylenedioxyamphetamine

MDMA: 3,4-methylenedioxymethamphetamine

mTOR: mammalian target of rapamycin

NAc: nucleus accumbens

NAPE: N-arachidonoyl-phosphatidylethanolamine

PET: positron emission tomography

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PLA: proximity ligation assay

PLC: phospholipase C

PTSD: posttraumatic stress disorder

PTX: pertussis toxin

RET: resonance energy transfer

SERT: serotonin transporter

SRET: sequencial BRET-FRET

SSRTT: stop-signal reaction time task

THC: Δ^9-tetrahydrocannabinol

TM: transmembrane helix

TPH: tryptophan hydroxylase

VTA: ventral tegmental area

WT: wild-type

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INTRODUCTION

1. Neurobiology of drug addiction

1.1. What is addiction?

Drug addiction is a chronic, relapsing brain disease characterized by compulsive drug seeking and drug taking in spite of harmful consequences, loss of control in limiting drug intake, and the presence of a negative emotional state when the drug access is prevented (Hyman, 2005; Koob and Le Moal, 2008; Cadet et al., 2014). Three categories of drug use are currently considered, which can be seen as a continuum from the occasional, controlled or social use of the drug, to the abuse of drug consumption leading to drug addiction. In this sense, drug use refers to drug consumption to obtain their rewarding and desired effects, sometimes tried out of curiosity. Drug abuse denotes a pattern of drug consumption in which the users takes an amount of the drug consumed or uses a method that is harmful to themselves or others. The consequences of drug abuse can result in developing addiction, which is a disease where drug seeking and consumption is produced despite negative consequences. Recurrent substance use may result in a failure to fulfil responsibilities at work and induce a reduction or even a complete cessation of social or recreational activities due to the seeking and usage of the drug. Drug addiction represents an important public health problem. Almost a quarter of the adult population in the European Union, or over 80 million adults, are estimated to have used an illicit drug at some point in their lives, and at least 1.3 million people received treatment for illicit drug use in Europe during 2012 (EMCDDA European Drug Report 2014). In addition, an American study has revealed that the

direct and indirect economic costs of licit and illicit drug use represent almost 420 billion dollars per year in the United States, which are greater that costs attributable to important diseases such as heart diseases, diabetes or obesity (Bouchery et al., 2011).

An essential goal of drug addiction research is to understand which changes occur in the brain in the transition from controlled drug use to drug addiction. There is some evidence characterizing different behavioural, physiological and even molecular features in the progression of addiction. It is also important to take into account the fact that only certain individuals of those who are exposed to drugs of abuse will develop a substance-related disorder. This has led to an individual-centered perspective in order to explain which factors can represent an increase in individual vulnerability. As a complex disorder, drug addiction cannot be explained by just one or few factors, but it should be addressed as a combination of genetic differences to social factors, which can lead to drug initiation. Individual behavioural differences such as temperament or environmental factors should also be considered, as well as the effects induced by the drug itself, the amount of drug and also the duration of its usage. However, some of these factors can also act in the opposite direction, conveying protection and resilience against substance-related disorders.

1.2. Substance-related and addictive disorders

The recent actualization of the DSM-IV, leading to the publication of DSM-5 in February 2013, has included substantive changes in the chapter of Substance-Related and Addictive Disorders. The most relevant

modification is the combination of the substance abuse and substance dependence categories, which were separated in the DSM-IV, into a single substance use disorder measured on a continuum from mild to severe, depending on the number of criteria endorsed (Table 1). Another relevant change is that the threshold for substance use disorder has been established at two or more criteria in contrast to the previous threshold values found in the DSM-IV, which were one or more criteria for diagnosing substance abuse, and three or more for substance dependence.

A remarkable addition in the substance-related and addictive disorders chapter of the DSM-5 is the gambling disorder as a non-substance-related disorder. This change is consequent with the increasing evidence that some behaviours, such as gambling, can produce the activation of brain reward pathways in a similar way than drugs of abuse do, and the symptoms present in patients suffering from gambling disorders resemble those present in substance use disorders. Although there are other excessive behavioural patterns with also a potential consideration of non-substance disorders such as internet, gaming, sex or exercise addiction, the evidence that identifies these behaviours as mental disorders is still insufficient in peer-reviewed journals.

According to the DSM-5, substance-related disorders are divided into two groups: substance use disorders and substance-induced disorders. The first group refers to the compendium of cognitive, behavioural and psychological symptoms present when an individual continues to use

the substance. The second group includes intoxication, withdrawal and mental disorders induced by substances or medications.

1.2.1. Substance use disorders

A principal characteristic of these disorders is the change produced in brain circuitry that can persist beyond detoxification, especially in the case of those individuals with severe disorders. These changes may explain some of the behavioural effects which lead to intense drug craving and relapse when the individuals are exposed to drug-associated stimuli.

In general, the diagnosis of substance use disorder is based on the presence of behaviours directly related to the substance use, and in the DSM-5 are grouped in different categories, which are listed in Table 1.

Tolerance and withdrawal symptoms can also occur during appropriate medical treatment with prescribed medications. In this specific case, these criteria should not be counted towards diagnosing a substance use disorder. However, inappropriate use of prescription medications can also lead to a substance use disorder, which should be diagnosed according to the presence of other symptoms.

Severity is based on the number of symptoms presented. Therefore, a mild substance use disorder is suggested by the presence of two or three symptoms, a moderate disorder by four to five symptoms and a severe substance use disorder is considered when six or more symptoms are presented.

Table 1. DSM-5 Criteria for the diagnosis of a substance-use disorder.

A. Impaired control over substance use
1. The individual may take the substance in larger amounts or over a longer period of time than was originally intended.
2. The individual may express a persistent desire to cut down or regulate substance use and may report multiple unsuccessful efforts to decrease or discontinue using the drug.
3. The individual may spend a great amount of time obtaining or using the substance or recovering from its effects.
4. Craving is manifested by the individual as an intense urge for the drug that may occur at any time but it is more likely to happen in an environment where the drug has been previously obtained or used.
B. Social impairments, the individual may withdraw from family, activities and hobbies in order to continue using the substance
5. Recurrent substance use may result in a failure to fulfil major role obligations at work, school or home.
6. The individual may continue using the substance despite the appearance of social problems caused by the effects of the substance.
7. Important social, occupational, or recreational activities may be given up or reduced because of substance use.
C. Risky use of the substance, regarding the individual's failure to abstain from using the substance in spite of the difficulty it is causing
8. The individual uses the substance recurrently in physically hazardous situations
9. Substance use is continued despite knowledge of having a psychological problem that is likely to have been caused by the substance
D. Pharmacological criteria
10. A significant increase in the dose of the substance is needed to achieve the desired effect, or the effect produced with the usual dose is markedly reduced. This phenomenon is known as tolerance and it greatly varies between individuals and also between substances. It is important to consider that tolerance to different drug-induced effects could develop at different rates.
11. The individual reports physiological signs when blood or tissue concentrations of substance decline after a prolonged or heavy use of the substance. This is known as withdrawal syndrome. At this point the individual will likely consume the substance with the intention to relieve the symptoms. Withdrawal signs and symptoms vary greatly across the different classes of substance in part based on the effects of substance consumption.

1.2.2. Substance-induced disorders

This category includes intoxication, withdrawal and other substance/medication-induced mental disorders:

- The essential feature in substance intoxication is the development of a reversible syndrome due to the recent ingestion of a substance. The clinically significant behavioural or psychological changes are attributable to the physiological effects of the substance and cannot be a consequence of another medical condition or mental disorder. The most common alterations in intoxication involve disturbances in perception, attention, thinking, judgment and also psychomotor disturbances and alterations on interpersonal behaviour.

- In the course of substance withdrawal, the development of a substance-specific problematic change in behaviours is produced as a consequence of the reduction or the cessation of substance use after a heavy and prolonged consumption. This syndrome causes alterations in cognition which can lead to a significant distress or social and occupational impairments. As in the case of intoxication, symptoms cannot be explained as a result of other medical condition or mental disorder.

- Substance-induced mental disorders refer to those disorders developed in the context of intoxication or withdrawal from substances of abuse, but can also be caused by medications that are taken at the suggested doses. They are potentially severe, but tend to disappear within the first month of substance cessation, although in certain long-duration consumption of particular substances the effects can persist due to the alteration of the central nervous system.

1.3. Transition to drug addiction

The DSM-5 is focused on establishing standard criteria for the diagnosis of mental disorders, including substance-related and addictive disorders. Although these manual is very useful in the clinics, and it will certainly have future consequences in addiction research, the established criteria can be sometimes difficult to apply in research involving animal models. For this reason, in the development of this thesis we have considered more appropriate to take into account the classical theories of addiction.

Thus, addiction is commonly viewed as a transition from acute drug taking to chronic administration, and it is suggested to be a sequential neuroadaptation process. Alterations in several neurobiological processes are involved in this adaptation; including changes in reward and motivation, conditioning and memory, executive function and inhibitory control, emotional interoception, and stress reactivity. In the development of addiction, three different stages have been outlined: binge / intoxication, withdrawal / negative effect, and preoccupation / anticipation (Koob and Le Moal, 1997). However, the initial rewarding stage, is crucial in the initiation process and deserves special attention. Thus, distinguishing between these stages is useful to investigate the alterations and adaptations produced in relevant neuronal circuits at each stage (Koob and Le Moal, 2008).

1.3.1. Reward / Initiation stage

The diagnosis criteria for addiction considered in the DMS-5 are common for all ten classes of drugs of abuse: alcohol; caffeine; cannabis; hallucinogens; inhalants; opioids; sedatives, hypnotics and anxiolytics;

stimulants, and tobacco. All of these drugs produce a direct activation of the brain reward system involved in the reinforcement of behaviours. The pharmacological mechanism by which each class of drugs activates the reward system is different, although all of them produce a feeling of pleasure, referred usually as a "high". Drug consumption is initiated due to the rewarding properties of the drugs, and drug experimentation is often produced as a result of social group pressure.

A reward is an appetitive stimulus, which is considered positive by itself, and can be given to a human or an animal in order to influence their behaviour. In contrast, a reinforcer is something that when presented after a behaviour increases the probability that behaviours paired with it will be repeated. Reinforcing stimulus can be positive or negative, but they always increase the probability that paired behaviours will be repeated. Positive reinforcement strengthens behaviours by being directly linked with positive outcomes, whereas negative reinforcement is based on removing a negative stimulus. These concepts are important in drug addiction, as it is hypothesized that drugs are taken because of their hedonic properties at first, inducing positive reinforcement, whereas after a chronic drug usage, their consumption is based on negative reinforcement in order to avoid the severe dysphoria and negative affect stage of abstinence (Koob, 2004).

Initial brain self-stimulation experiments in rats described a major implication of the midbrain region in reward processes (Olds and Milner, 1954). Specifically, the medial forebrain bundle, which connects the ventral tegmental area (VTA) to the basal forebrain, was considered the

main reward pathway. In the beginning, experimentation was focused on the role of the monoamine systems in this region, first norepinephrine (Stein, 1962) and then dopamine (Crow, 1973; Wise, 1978). The implication of the dopaminergic system in the rewarding action of drugs of abuse was first described by Wise in 1980. In his very first hypothesis, Wise restricted the rewarding pathway merely to the VTA and its projections to the nucleus accumbens (NAc) (Wise, 1980). In accordance, studies performed during last decades have revealed that all drugs of abuse produce an increase in dopamine levels in the NAc (Fuxe et al., 1986; Di Chiara and Imperato 1988; Pettit and Justice, 1989; White et al., 1996; Tanda et al., 1997a; Tanda et al., 1997b ; Ranaldi et al., 1999), although this increase is produced through different mechanisms (Koob, 1992). Recent studies in humans, using positron emission tomography, have revealed that drugs such as nicotine (Brody et al., 2009), alcohol (Boileau et al., 2003), marijuana (Bossong et al., 2009) and stimulants (Volkow et al., 1999; Drevets et al., 2001) also produce an increase in dopamine levels in the striatum, as well as, in the NAc. Nowadays, an expanded and more complex network of neurochemical circuits around the dopamine mesolimbic system is thought to be involved in drug reward, including the opioid, cannabinoid, nicotinic, glutamatergic and γ-aminobutiric acid (GABA)ergic systems (Koob and Volkow, 2010) (Figure 1).

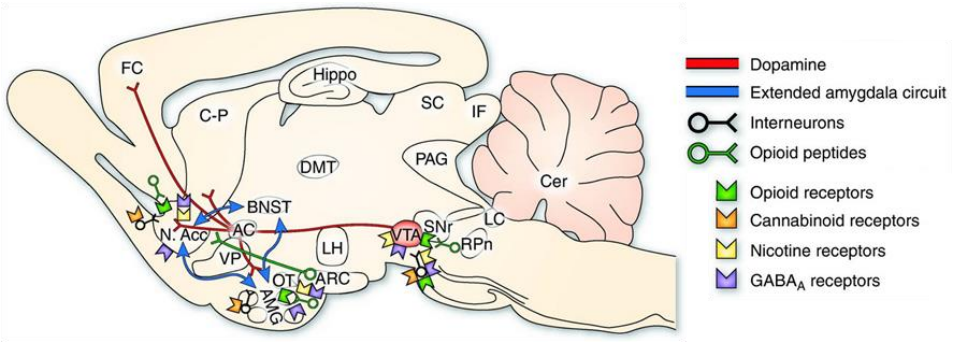

Figure 1. Neurochemical circuits in drug reward. Representation of a sagittal section of a rodent brain illustrating the pathways and systems implicated in the acute reinforcing actions of drugs of abuse. Cocaine and amphetamines activate the release of dopamine in the NAc and amygdala through direct actions on dopamine terminals. Opioids activate opioid receptors in the VTA, NAc and amygdala, facilitating the release of dopamine in the NAc. Alcohol directly activates γ-aminobutyric acid-A receptors in the VTA, NAc and amygdala or indirectly through the release of GABA, also facilitating dopamine release in the NAc. Nicotine activates nicotinic acetylcholine receptors in the VTA, NAc and amygdala either directly or through acting on interneurons. Cannabinoids activate cannabinoid CB_1 receptors in the VTA, NAc and amygdala, facilitating dopamine release in the NAc through an unknown mechanism (Koob and Volkow 2010).

1.3.1.1. The role of dopamine in reward

Although the mesolimbic dopaminergic system has been directly related with the rewarding properties of natural rewards such as food or sex, as well as drugs of abuse, its precise role is still debated. Several different hypotheses have appeared and are subsequently considered.

Activation-sensorimotor hypothesis

The activation-sensorimotor hypothesis sustains that dopamine mediates behavioural activation, effort, movement, action generation and general arousal (Robbins and Everitt, 1982; Stricker and Zigmond, 1986; Salamone et al., 1994) This theory sustains that dopaminergic phasic firing in the NAc is directly related with behavioural activation and might be important for the performance of active responses. A recent

review has put forward the role of NAc dopamine in the activational aspects of motivation (Salamone and Correa, 2012). Thus, in studies where animals are presented with a choice between different types of food or by limiting its accessibility, the administration of dopaminergic antagonists, as well as NAc dopamine depletions induced a shift towards the less effort-requiring behaviour, even at the cost of a preferred reward (Salamone et al., 1991; 1994; Bardgett et al., 2009), indicating that dopamine mediates effort-related decision making.

Hedonia hypothesis

The hedonia hypothesis developed by Wise (Wise, 1980), suggests that brain dopamine systems mediate the pleasure produced by food and other incentives such as sex or drugs of abuse. In 1982, Wise stated that after the administration of dopamine antagonists "all of life's pleasures – the pleasures of primary reinforcement and the pleasures of their associated stimuli– lose their ability to arouse the animal" (Wise, 1982). Although Wise retracted the hypothesis that dopamine blockade reduces pleasure (Wise, 1994), this hypothesis became so widely accepted, that dopamine is very often referred to as the "brain's pleasure neurotransmitter". Subsequently, Berridge focused his research towards understanding how brain systems generate "liking" reactions to pleasant rewards (Berridge et al., 2000). "Liking" is the term used to refer to the psychological and neurobiological events that produce subjective pleasure, but it is just one component of reward (Berridge et al., 2009). In their initial studies, they found that lesions of the dopamine system in the NAc and striatum did not produce any change in "liking" reactions

in animals (Berridge and Robinson, 1998), supporting the idea that dopamine does not mediate subjective pleasure responses. In accordance, studies performed in Parkinsonian patients, who experience deterioration of the dopamine system, revealed normal subjective pleasure ratings when compared to control group (Sienkiewicz-Jarosz et al., 2005). A similar study found that dopamine neurotransmission corresponds better to measures of "wanting" rather than "liking" a reward (Evans et al., 2006). Thus, "wanting" is the quality of a stimulus that makes it a desirable and attractive goal, transforming it from a mere sensory experience into something that commands attention, induces approach, and causes it to be sought out (Berridge et al., 2009). Many neuroscientists now agree that the role of dopamine is more complex than originally stated in the hedonia hypothesis, and that it might mediate other components of reward than just pleasure (Berridge, 2007), and the concept of "wanting" resulted in the next theory, the incentive salience hypothesis.

Incentive salience hypothesis

Formally, the term incentive salience refers to a conditioned motivational response triggered by a reward-related stimulus (Berridge, 2007). According to this hypothesis, dopamine's role is to influence the motivational value of reward in a dynamic way (Berridge and Valenstein, 1991). This theory supports that reward is a construct formed by wanting, learning and liking, and dopamine mediates only the wanting component. This concept was further developed into a theory of addiction called "the incentive sensitization theory" (Berridge and

Robinson, 1998). Studies performed in hyperdopaminergic mutant mice, with enhanced extracellular levels of dopamine, support the incentive salience hypothesis, as these animals appear to "want" rewards more than wild-type (WT) mice in incentive motivation tasks (Peciña et al., 2003; Cagniard et al., 2006; Yin et al., 2006). Therefore, it seems that dopamine mesolimbic activity might be related with incentive motivation and predictive dopamine firing could reflect a conditioned "wanting" response. Accordingly, a new set of electrophysiological experiments performed in dopamine neurons of the ventral pallidum, a brain structure receiving projections from NAc and from the VTA (Zahm, 2000), considered the final common path for mesocorticolimbic reward outputs (McFarland et al., 2004; Zahm, 2006), demonstrated the role of dopamine in "wanting" (Tindell et al., 2004; 2005).

Reward learning hypothesis

The reward learning hypothesis can be seen as a family of several closely related theories suggesting that dopamine mediates learning about a reward. One of these theories states that dopamine is mainly involved in the prediction of errors (Schultz et al., 1993; Schultz, 1998; Schultz and Dickinson, 2000). This theory is based on results obtained in primates, where dopaminergic firing produced as a consequence of reward delivery was only observed during the learning phase of a new task, whereas dopamine firing was lost once the task was already established. Moreover, it has been shown that activity of dopamine neurons changes dynamically over the course of learning rewarding tasks, with burst firing shifting from reward itself to the reward prediction (Schultz, 2002).

Later research suggested that dopamine's role is to indicate the magnitude of reward, since larger rewards resulted in greater dopaminergic neuronal activity (Tobler et al., 2005). Moreover, the role of dopamine in reward learning was also associated with updating the information related to the reward at the moment of receiving it (Bayer and Glimcher, 2005). In this study, dopamine signalling was defined as a predictor value, because firing rates of dopamine neurons were greater when the outcome was larger than expected and lower the other way around (Bayer and Glimcher, 2005). Thus, it has been postulated that the huge dopamine release observed after drug consumption produces an exaggerated expectation of future drug rewards (Redish, 2004), facilitating its continued consumption.

Another theory related to learning is based on the role of dopamine in facilitating stimulus-response or stimulus-stimulus associations. According to this theory, dopamine release causes new stimulus-response habits to be learned and/or modulates the strength of the already learnt habits (Robbins and Everitt, 1999; Everitt et al., 2001). Concerning addiction, since drug consumption induces a strong dopamine release, habits formed as a consequence of drug consumption are stronger than normal learned habits (Miles et al., 2003; Everitt and Robbins, 2005; Nelson and Killcross, 2006). Indeed, drugs of abuse establish strong habits that persist even when the goal or reward is devalued (Schoenbaum and Setlow, 2005). However, experiments performed in hyperdopaminergic mutant mice revealed that these

animals did not persist in stimulus-response habit perseveration more than control animals when reward was devalued (Yin et al., 2006).

An additional theory relies on the role of dopamine in mechanisms involved in memory formation. Persistent changes in striatal function during the progression of addiction might be brought about by mechanisms of long-lasting synaptic plasticity, and in the striatum such mechanisms are strongly regulated by dopamine signalling (Gerdeman et al., 2003). Supporting this view, recent molecular biology studies have demonstrated a role for dopamine in cellular and molecular plasticity mechanisms relevant to memory such as long-term potentiation and long-term depression (Berke and Hyman, 2000; Kelley, 2004). Indeed, further evidence revealed that dopamine manipulations performed soon after a learning trial could alter consolidation or reconsolidation of memories (Fenu and Di Chiara, 2003; Dalley et al., 2005; Hernandez et al., 2005), and recent studies have associated drug exposure with alterations in long-term synaptic strength in dopaminergic neurons of the VTA (Liu et al., 2010; Lee and Dong, 2011; Mao et al., 2011). However, dopamine is not the only mechanism involved in learning, as studies performed in dopamine-deficient mice have demonstrated reward-learning without dopamine (Cannon and Palmiter, 2003, Hnasko et al., 2005; Robinson et al., 2005). In this sense, hyperdopaminergic mutant mice did not present enhanced levels of instrumental learning in comparison with wild-type mice, nor a quicker learning curve (Cagniard et al., 2006).

1.3.2. Withdrawal / Negative affect stage

After the discontinuation of a frequent, repetitive or chronic drug usage, a withdrawal syndrome can appear. All drugs of abuse can produce a motivational withdrawal syndrome characterized by dysphoria, irritability, and emotional distress that can persist for some time (Koob and Le Moal, 1997; 2008). Moreover, interruption of chronic use of opiates, alcohol or sedative hypnotics can trigger an intense physical withdrawal, which in severe cases can be fatal if it is not properly treated.

The mechanisms underlying acute physical withdrawal seem to be drug-specific and are reflected as adaptations in the molecular targets of the different drugs as a consequence of the chronic drug consumption. The extended amygdala has been postulated as a common anatomical substrate implicated in physical withdrawal. The extended amygdala comprises the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), the medial part of the NAc, and a part of the substantia innominata (Alheid and Heimer, 1988). This neuroanatomical entity integrates the brain arousal-stress system and the hedonic processing systems (Koob and Le Moal, 2005). As both systems are altered during the addiction process, the extended amygdala is thought to be involved in the negative emotional states characteristics of the withdrawal stage.

During this stage, neuroadaptation processes play a crucial role in trying to overcome the effects induced by chronic drug administration in order to restore normal functioning. Thus, a decrease in dopaminergic activity

in the mesolimbic system has been found during withdrawal (Diana et al., 1993; 1995; Weiss et al., 1996; Wang et al., 1997). This decrease in dopaminergic activity is directly related with tolerance, which is a process that appears after a continued use of a drug, when the administration of higher doses of drugs is required in order to obtain the same effects. The presence of tolerance is one of the criteria used in the DSM-5 in order to diagnose a substance-related disorder.

Even though the dopaminergic system plays an important role in withdrawal, other relevant systems involved in this stage are the hypothalamic-pituitary-adrenal axis and the brain stress systems. The increase in the levels of stress hormones shows tolerance during chronic drug administration, but is reactivated during drug withdrawal (Aston-Jones and Druhan, 1999). Corticotropin-releasing-factor (CRF) mediates the activation of the brain stress aversive system and it regulates the induction of anxiety-like responses induced by stressors, which are typical of the withdrawal stage. The administration of CRF antagonist reversed the anxiogenic-like effects observed in cocaine withdrawal (Specio et al., 2008), some of the anxiety-like behaviour produced during ethanol withdrawal (Funk et al., 2006), and during precipitated withdrawal in nicotine-dependent rats (George et al., 2007). Norepinephrine is also known to be involved in emotional dysregulation of drug withdrawal, as noradrenergic antagonists blocked place aversion produced during opioid withdrawal (Delfs et al., 2000). Norepinephrine projections to the BNST are known to activate CRF systems, which are key factors in regulating anxiety-like behaviour observed during

withdrawal (Aston-Jones et al., 1999; Delfs et al., 2000). Another neurotransmitter that has been suggested to play a role in the aversive effects of drug withdrawal is dynorphin. Dynorphin is an opioid peptide that increases in the NAc in response to dopaminergic activation. Increased levels of dynorphin have been observed in NAc and amygdala during opioid, cocaine and ethanol withdrawal and can be responsible for decreasing dopaminergic functioning (Carlezon et al., 2006; Koob, 2008).

Therefore, adaptations in the mesolimbic dopamine systems as well as in the brain stress systems are directly associated with drug withdrawal. The extended amygdala which integrates arousal-stress systems with hedonic processes plays a crucial role in this stage. However, the relevance of stress is not restricted only to this stage, as stress is considered to be one of the most important factors for relapse, a key element for defining addiction as a chronic relapsing disorder.

1.3.3. Preoccupation / Anticipation or craving stage

The third stage of the addiction cycle is the craving or preoccupation/anticipation stage, and it has been hypothesized as a key element of relapse in humans. One of the most challenging problems in addiction is the chronic relapse, which can happen after extended periods of drug abstinence, even after the withdrawal symptoms have disappeared. In humans, the most relevant craving-inducing factors are: the presentation of a conditioned stimulus associated with drug consumption, such as the context where drug was taken; the presence of the drug itself; and stressful situations or even negative emotional states.

In order to study the neurobiological mechanisms mediating craving and relapse, animal models of reinstatement to drug-seeking have been developed following a priming injection of the drug, by the presentation of the conditioned stimulus associated with the drug, or by an acute stressor or a residual negative emotional state. These experiments have evidenced the involvement of different brain areas in each reinstatement process. Thus, the medial prefrontal cortex, the NAc and the ventral pallidum are involved in reinstatement induced by drug administration (McFarland and Kalivas, 2001). The basolateral amygdala is crucial for the cue-induced reinstatement (Weiss et al., 2001; Everitt and Wolf; 2002), and CRF and norepinephrine activation on the extended amygdala are the main effectors in the stress-induced reinstatement (Shalev et al., 2002; Shaham et al., 2003). The gradual reorganization in the reward and memory circuits as a consequence of chronic drug consumption is suggested as one of the main mechanisms which subsequently result in drug relapse. During this phase, these reorganizations have been hypothesized to shift behaviour towards focusing on drug-seeking at the expense of natural rewards, consequently inducing an enhanced sensitivity to drug cues and subsequently resulting in relapse. An illustration of the neurocircuitry involved in the different stages of addiction, indicating the brain areas associated with each particular stage is shown in figure 2.

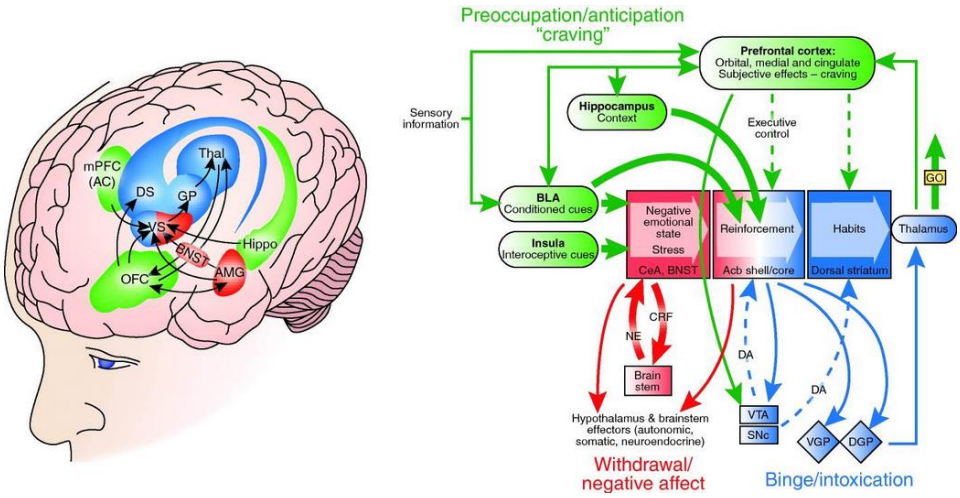

Figure 2. Neural circuitry associated with the three stages of the addiction cycle, which promote drug-seeking behaviour in the addicted state (Koob and Volkow, 2010).

1.4. Addiction from a cognitive point of view

From a psychological and neurological point of view, addiction might be considered as a disorder involving alterations in cognition. In fact, brain regions associated with the addictive processes overlap with those regions involved in cognitive functions such as attention, learning, memory and decision-making. Current evidence shows that drugs are able to induce alterations in structure and functionality in the prefrontal cortex, a brain region involved in executive function, and in the hippocampus, a structure mediating spatial memory (Kalivas and O'Brien, 2008).

1.4.1. Learning and memory in addiction

As previously mentioned, dopamine release in the midbrain has been considered to facilitate learning and memory. When a new event occurs, the generation of a dopaminergic signal is produced, making it possible to learn about this new situation (Schultz, 2010). Therefore, as a

consequence of the sensitization process in the mesolimbic dopamine system, the consumption of drugs of abuse produce artificial learning signals of a greater magnitude and duration than those produced in response to natural events, and this mechanism accounts for the formation of strong drug-stimulus associations (Robinson and Berridge, 2000). Individuals taking drugs perceive their surroundings as highly significant, making strong connections between the pleasant feeling induced by drug and the context where the drug was consumed (Robinson and Berridge, 2000). Indeed, it has been shown that the simply exposure of drug-associated cues to addicts is sufficient for the release of dopamine (Volkow et al., 2006; Yoder et al., 2009). It has been suggested that drugs usurp cognitive processes mediated by dopamine to focus memories on associations between drugs and the actions that can procure them (Hyman et al., 2006).

Drugs such as amphetamine, nicotine, cannabis or cocaine have been shown to produce physiological responses and changes in brain regions related to learning and memory under acute conditions. The acute administration of amphetamine increased performance in attention-demanding tasks in humans (Servan-Schreiber et al., 1998; Silber et al., 2006), and rats (Grilly, 2000). In addition, nicotine has been widely reported to enhance attention; even in laboratory animals, the acute administration of nicotine was able to improve cognitive processes (Lawrence et al., 2002; Kenney and Gould, 2008). Similarly, cocaine self-administration enhanced attention (Devonshire et al., 2007), and improved performance in the morris water maze in rats (Del Olmo et al.,

2007). The administration of opioids induced an increase in performance in the passive avoidance task (Aguilar et al., 1998) although this effect could be attributed to increased motor activity. In contrast, impairments in long-term memory have been revealed after acute cannabis administration in mice (Puighermanal et al., 2009). Finally, the effects of alcohol are biphasic, as high doses of ethanol are able to disrupt cognitive processes (Ryback, 1971), whereas low doses produce a learning enhancement (Gulick and Gould, 2007).

Chronic drug exposure has also been associated with learning and memory alterations. One of the first studies in this respect reported that chronic cocaine facilitated responding for a non-drug paired-cue (Taylor and Horger, 1999). Subsequent studies also demonstrated that chronic nicotine (Olausson et al., 2003; 2004), and chronic cocaine (Olausson et al., 2007) enhanced conditioned reinforcement for natural rewards. Importantly, chronic drug exposure can accelerate habit formation, allowing stereotyped, repetitive motor behaviour to be performed with little or no conscious awareness. Habit formation in food responding was enhanced by chronic amphetamine exposure (Nelson and Killcross, 2006), and habit formation for drugs has revealed to be faster than for natural rewards (Dickinson et al., 2002; Miles et al., 2003). Moreover, habit persistence despite negative outcomes has been revealed after prolonged, but not limited cocaine self-administration (Vanderschuren and Everitt, 2004; Peloux et al., 2007). Remarkably, chronic drug consumption induces adaptive mechanisms in the brain in order to

function in a normal way despite the presence of the drug to overcome its effects.

In abstinent drug abusers, brain imaging studies have shown alterations in frontal regions such as the orbitofrontal cortex, the dorsolateral prefrontal cortex and the cingulate gyrus, all areas involved in inhibitory control and impulsivity (Volkow et al., 2004). Moreover, abstinent cocaine addicts show impaired functions mediated by the medial and orbitofrontal prefrontal cortex including attention, behavioural flexibility and delayed discounting (Bolla et al., 2003; Aharonovich et al., 2006). Besides, impairments were also observed in tasks dependent on the hippocampus such as spatial, verbal and recognition memory tests (Aharonovich et al., 2006). In preclinical studies performed during cocaine withdrawal, rats (Schoenbaum et al., 2004; Calu et al., 2007), and monkeys (Jentsch et al., 2002) showed impaired reversal learning. Additionally, following withdrawal from extended access to cocaine, deficits were revealed in working memory, sustained attention, and novel object recognition tasks in rats (Briand et al., 2008; George et al., 2008).

Taken together, these studies highlight the relevance of the adaptive changes occurring during drug consumption in order to maintain homeostatic conditions, which produce enduring alterations in behaviour when the drug is not present.

1.4.2. Executive functioning in addiction

Executive functions generally refer to "higher-level" cognitive functions involved in the control and regulation of cognitive processes and goal-directed, future-oriented behaviour (Alvarez and Emory, 2006). Executive functions include processes such as attention, working memory (Barcelo and Knight, 2002), reasoning, task flexibility (Sergeant et al., 2002; Welsh, 2002), problem solving (Monsell, 2003), as well as planning and execution (Chan et al., 2008). Prefrontal brain areas of the frontal lobe have been related to executive function processes, although these areas are not solely sufficient for performing all these functions (Alvarez and Emory, 2006). Multiple studies have shown that addicted individuals present alterations in several of the processes involved in executive functioning (George and Koob, 2010), such as attention (Garavan and Hester, 2007), behavioural flexibility (Robbins, 1996), working memory (Baddeley, 2003), behavioural inhibition (Barkley, 1997) and valuing future events (Bechara, 2005).

For instance, cocaine users present deficits in attentional function and also slight visual and working memory deficits in comparison with healthy controls (Jovanovsky et al., 2005). Moreover, methamphetamine users showed a decrease in executive functioning, and information-processing speed, as well as small deficits in attention and working memory (Scott et al., 2007). Moreover, significant neurocognitive impairments have been found in individuals with alcohol use disorder (Bates et al., 2006). However, mixed results have been obtained in studies on chronic cannabis users. It has been reported that heavy marijuana use

is associated with impairments in verbal learning and memory, attention and executive functioning (Bolla et al., 2002; Solowij et al., 2002). In contrast, other studies reported minimal disruptions on attention, working memory, abstract reasoning or in the overall intelligence quotient (IQ) (Fried et al., 2005; Jager et al., 2006).

The discrepancies found across studies could be accounted for by several factors, such as differences in education, IQ, psychiatric co-morbidities, length of abstinence and other features, which are extremely relevant when comparing healthy controls with drug users. Another consideration is whether cognitive impairment is caused by chronic drug use, or the impairment is previous to drug consumption, causing vulnerability to become drug-dependent (Wagner et al., 2013). Moreover, the age of onset of the drug consumption is also critical. For example, starting cannabis consumption in the adolescence produces greater cognitive impairments (Kempel et al., 2003; Pope et al., 2003).

Although it was believed that impairments induced by drugs completely remit after drug abstinence, some evidence suggests that a number of cognitive impairments are not reversible after a period of abstinence. For example, an absence of improvement in cognitive performance was found in methamphetamine dependent individuals following one month of abstinence (Simon et al., 2010). Similarly, in spite of the complete recovery of dopamine transporter deficiency, methamphetamine dependent abstinent individuals displayed persistent neurocognitive deficits (Volkow et al., 2001). Other studies have revealed inhibitory control deficits in cocaine addicts after one (Fox et al., 2007) or four

months of abstinence (Verdejo-Garcia et al., 2007). These executive dysfunctions might be related with treatment failure among patients (Lundqvist, 1995; Verdejo-Garcia et al., 2004).

It has been demonstrated that alterations in executive functioning processes can be related to different types of impulsivity, and some of these impulsive traits are observed among addicts. For example, chronic cocaine and methamphetamine abusers show deficits in response inhibition (Fillmore and Rush, 2002; Monterosso et al., 2005; Li et al., 2006), and alcohol abusers present deficits in attention (Rubio et al., 2009). Similarly, deficits in future planning have been described in amphetamine (Clark et al., 2006), opioid (Ersche et al., 2006a) and cigarette smokers (Yakir et al., 2007). Indeed, there is an overlapping in neurological substrates responsible for executive functioning and those brain areas associated with different types of impulsive behaviour, as represented in figure 3. Thus, behavioural inhibition is associated with the activation of the insula (Cai and Leung, 2011; Hendrick et al., 2012), and prefrontal cortex (Hester and Garavan, 2004; Passarotti et al., 2010), and prefrontal cortex activation has also been related to the valuation of future events and impulsive choice (Cho et al., 2010; Figner et al., 2010). In fact, alterations in these brain areas have been associated with impulsive behaviour in addicted subjects (London et al., 1990; Galynker et al., 2000; Volkow and Fowler, 2000; Volkow et al., 2001; Kaufman et al., 2003; Ersche et al., 2006b; Volkow et al., 2007).

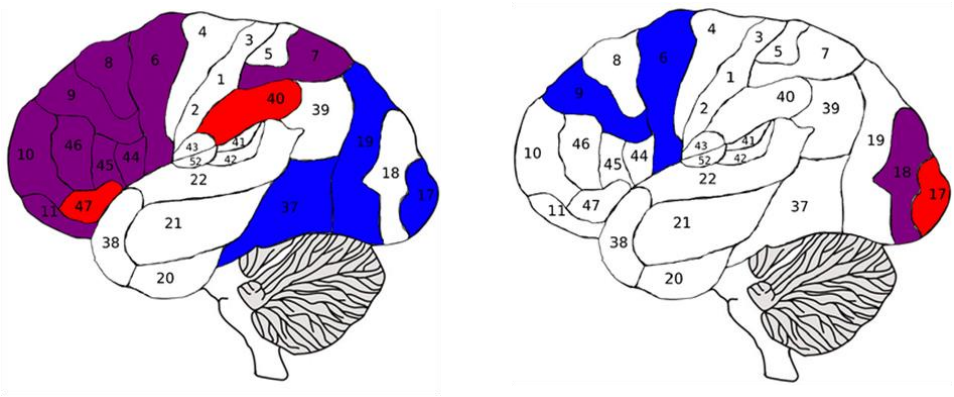

Figure 3. Overlapping of cortical areas involved in executive function and impulsivity. The left panel shows Brodmann's areas color coded wherein lower levels of activation are associated with executive dysfunction (blue), impulsivity (red) or both executive dysfunction and impulsivity (purple). The right panel shows Brodmann's areas color coded wherein higher levels of activation are associated with executive dysfunction (blue), impulsivity (red) or both executive dysfunction and impulsivity (purple) (Bickel et al., 2012).

1.4.2.1. Impulsivity

Impulsivity is defined as the predisposition for premature, poorly planned and risky or inappropriate actions, which often result in undesirable consequences. However, this supposed "maladaptive" behaviour has been conserved across evolution suggesting that manifestations of rapid decision making, quick action, and risk-taking can represent an advantageous behaviour for many species (Dickman, 1990). Nevertheless, in its extreme expression, impulsivity has been associated with a wide range of neuropsychiatric disorders, and substance-related and addictive disorders (Ersche et al., 2010).

One of the most important issues for neuroscientists to address in the field of addiction is why only some individuals exposed to drugs of abuse will develop a substance-related disorder. In this respect, the association of drug addiction with impulsivity or reduced inhibitory control has been well established (Fillmore and Rush, 2002; 2006; Ersche

et al., 2008; 2011; 2012). Thus, impulsivity might be considered as a personality trait which confers vulnerability to develop a substance-related disorder, and could be a predictor for the onset of addiction (Nigg et al., 2006). However, it is still not clear whether impulsivity could be a consequence of the chronic drug exposure, resulting as an outcome of the neuroadaptative processes observed during drug consumption. Supporting this view, it has been suggested that chronic drug consumption causes neuroadaptations on top-down control regions such as the pre-frontal cortex (Jentsch and Taylor, 1999; Everitt and Robbins, 2005; Kalivas and Volkow, 2005), consequently resulting in impulsive behaviour.

At a clinical level, impulsivity is estimated by the use of self-report questionnaires, being the Barratt Impulsivity Scale (BIS-11) (Patton et al., 1995) the most currently used. By analysing the reported answers, three different dimensions of impulsivity could be identified: attentional, motor, and non-planning. The attentional dimension of impulsivity is related to the extent to which an individual can focus on a task. The motor component of impulsivity is exhibited as acting with little or no forethought, and the non-planning dimension of impulsivity reflects the lack in analysing the future consequences of the actions. One advantage in studying impulsivity is the possibility to assess it in an empirical way in the laboratory without using questionnaires. Thus, behavioural tasks that can be used in humans and experimental animals have been developed to measure two components of impulsivity: those measuring impulsive action (motor impulsivity) and those measuring impulsive

choice (attentional and non-planning impulsivity) (Winstanley et al., 2006; Dalley et al., 2008) (Figure 4).

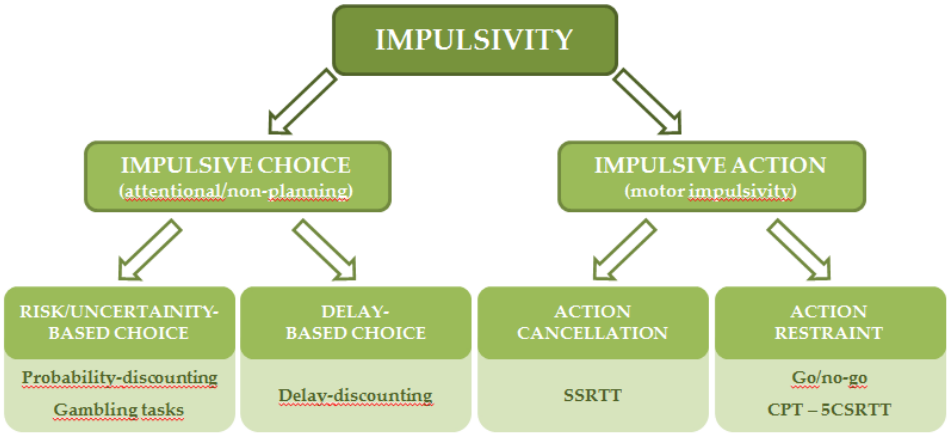

Figure 4. Diagram illustrating distinct aspects of impulsivity and examples of behavioural tests that are used to measure distinct aspects of impulse control. Probability discounting is a test similar to delay-discounting, whereas the adjusted variable is the probability of obtaining a reward. The five choice serial reaction time task (5CSRTT) is a laboratory test performed in operant chambers to measure motor impulsivity (Modified from Winstanley et al., 2010).

The most widely used laboratory tests to study motor impulsivity are the stop-signal reaction time task (SSRTT), the go/no-go paradigm and the continuous performance test (CPT). In the SSRTT, experimental subjects are forced to rapidly respond to the presence of a signal, while on some trials, a stop signal is presented and subjects must inhibit their response. This test has been adapted for laboratory animals such as rodents (Feola et al., 2000) and non-human primates (Liu et al., 2009).

Impulsive choice is measured using the delay-discounting paradigm in both humans and animals. In this test, a choice between an immediate small reward versus a large delayed reward is offered to the experimental subject. Typically, as the delay increases, the preference of the subject for the larger delayed reward decreases, and the subject

changes responding towards the smaller immediate option. Impulsive subjects are more likely to prefer the immediate smaller rewards even with shorter delays, reflecting intolerance to delayed gratification that results in a negative outcome at the end.

An important question raised is whether these tests are able to prove a relationship between addiction and impulsivity. Proving this association in humans is difficult, as clinical studies present a high level of complexity. Apart from just considering the different subtypes of impulsivity, other factors related to subjects such as the phase in the development of addiction or the patterns of drug consumption, as well as their age and gender can also represent a major influence in the behaviours observed. Thus, higher levels of impulsive choice have been observed in crack cocaine consumers in comparison with heroin users (Bornovalova et al., 2005), while impairments in impulsive action were reported in cocaine users (Fillmore and Rush, 2002; Li et al., 2006; 2008). In nicotine addicts, different aspects of craving have been associated with distinct subtypes of impulsivity (Doran et al., 2009). Moreover, long-term amphetamine abusers showed maladaptive decision making on a gambling task, whereas these impairments were not found in opiate abusers (Rogers et al., 1999). A great number of experiments have been performed in animals in order to elucidate the role of impulsivity in addiction. Some of these studies indicate that impulsivity is predictive of addiction-related behaviours. However, this relationship depends on the drug class and impulsivity sub-type. Therefore, rats selected for action impulsivity presented enhanced self-administration of several drugs

such as cocaine (Dalley et al., 2007), nicotine (Diergaarde et al., 2008), alcohol (Radwanska and Kaczmarek, 2012), and methylphenidate (Marusich and Bardo, 2009). Impulsive-like rats also show enhanced conditioned place preference (CPP) to amphetamine (Yates et al., 2012), and increased rates of 3,4-methylenedioxymethamphetamine (MDMA)-primed drug-seeking (Bird and Schenk, 2013), an indicative measure of drug initiation. Moreover, these animals presented higher rates of responding in cue-induce relapse for cocaine seeking (Economidou et al., 2009), as well as, resistance to extinction to both nicotine (Diergaarde et al., 2008) and cocaine (Broos et al., 2012). In non-impulsive animals, the exposure to both heroin (Schippers et al., 2012) and cocaine (Winstanley et al., 2009; Mendez et al., 2010) increased their impulsivity levels. In contrast, cocaine exposure in impulsive rats produces a normalisation of the impulsive behaviour (Dalley et al., 2007). This might potentially sustain, in part, the hypothesis that drug intake in impulsive subjects is a form of self-medication.

Therefore, it seems that the impulsive trait could be somehow related to addictive-like behaviour or even considered as a predictor for addiction in some cases. However, more studies are needed to gather additional evidence for this potential relationship.

Recent studies have addressed the importance of distinguishing between impulsive action and impulsive choice in addiction, as subtle differences can result in major consequences. Thus, studies performed in rats revealed that high reactivity to novelty predicts faster acquisition of d-amphetamine self-administration (Piazza et al., 1989), and faster

acquisition of cocaine self-administration (Belin et al., 2008). However, high-impulsive rats did not acquire cocaine self-administration more rapidly, although these animals displayed compulsive cocaine-seeking behaviour (Belin et al., 2008), and were more prone to relapse to cocaine seeking after abstinence (Economidou et al., 2009). Indeed, these results support the hypothesis that impulsive choice might be important in the initial phases of drug addiction, whereas impulsive action could be crucial in later phases such as compulsive drug use and relapse, as represented in figure 5.

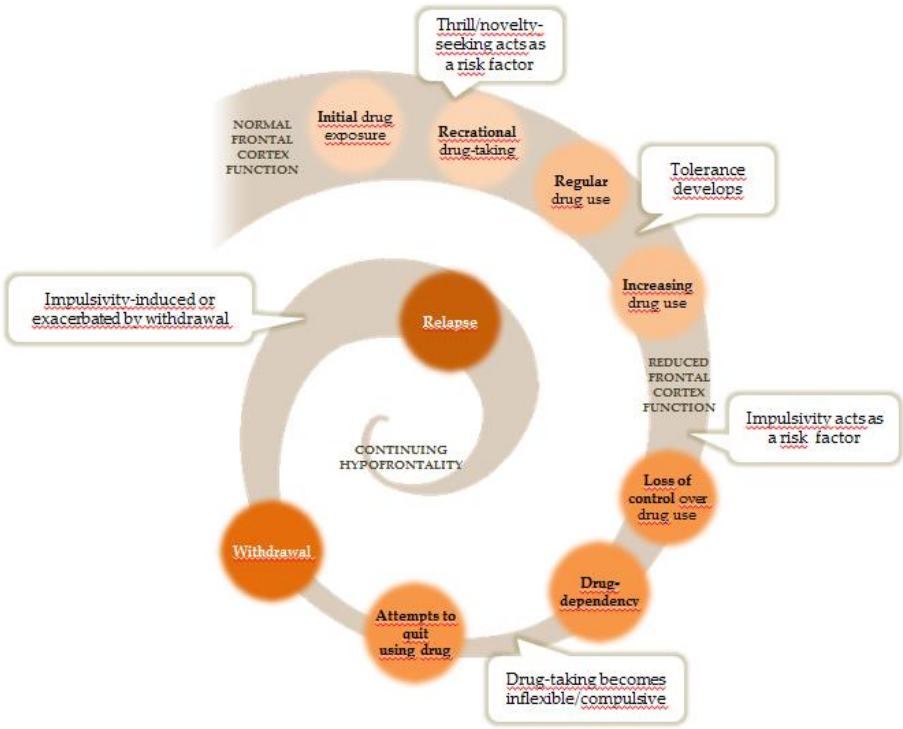

Figure 5. The downward spiral of addiction. Impulsive choice evidenced by novel or thrill sensitivity is though to facilitate initial contact with addictive drugs and the development of regular or recreational drug use. Highly impulsive individuals are more likely to lose control over drug taking, facilitating drug dependency. As drug addiction progresses, drug taking becomes more compulsive and inflexible, and higher levels of impulsivity can precipitate relapse episodes, perpetuating the cycle of addiction. Indeed, fluctuations in prerfrontal cortex activity are observed during this process and can significantly contribute to the behavioural changes produced (Modified from Winstanley et al., 2010)

1.4.2.2. Compulsivity

It has been hypothesized that progress from drug consumption to drug addiction is driven by positive reinforcement followed by a compulsive disorder based on negative reinforcement (Koob et al., 1997). Compulsivity refers to repetitive behaviours that are performed in a stereotypical manner, often with a lack of purpose, and sometimes resulting in undesirable consequences. Nevertheless, there is probably some relationship between impulsivity and compulsivity, as both processes can be viewed as a failure of response inhibition or a malfunction in the top-down cognitive control (Hollander and Cohen, 1996). It has been demonstrated that impulsive animals are more likely to experiment this transition to compulsive drug taking (Belin et al., 2008), and that after continued drug consumption, a "drug habit" is formed becoming increasingly inflexible with longer consumption (Vanderschuren and Everitt, 2004). At a neural level, the change from the initial goal-directed action to the drug consumption maintained by drug-associated stimuli is reflected by a shift in the cerebral regions responsible for controlling drug seeking and taking, namely from the prefrontal cortex to the striatum (Everitt and Robbins, 2005). Moreover, changes within the striatum itself have also been observed. As the process of addiction progresses, the dopamine release produced by drug seeking and drug taking is shifted from the ventral to the dorsal striatum (Belin and Everitt, 2008). This process occurs as a consequence of the so-called "spiralling" connections of the midbrain dopaminergic neurons,

which links the nucleus accumbens with progressively more dorsal regions of the striatum (Haber et al., 2000).

In humans, compulsive-like behaviour has been observed in cocaine dependent subjects (Fernández-Serrano et al., 2012), and alterations in frontostriatal brain systems have been associated with compulsivity in cocaine-dependent individuals (Ersche et al., 2011). In animals, there are few accepted models of compulsive behaviour. One interesting example is provided by reversal learning tasks which offer the possibility to determine perseverative responding (Izquierdo and Jentsch, 2012). It has been shown that this form of compulsion is enhanced after cocaine treatment (Jentsch et al., 2002; Calu et al., 2007). Another attempt to study compulsive drug seeking has been based on measuring the persistence of drug seeking despite negative or aversive outcomes. In this case, compulsive drug seeking only occurs after prolonged periods of cocaine taking. Three different studies have shown that between 15 to 20% of rats with long-term exposure to cocaine will continue to respond for cocaine not only when drug is not available, but also even when responding is punished (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004; Pelloux et al., 2007; Belin et al., 2008). Interestingly, these percentages are similar to the estimated proportion of human subjects vulnerable to develop an addiction among those who take drugs (Anthony et al., 1994; Warner et al., 1995).

1.5. Concluding remarks

One important issue related to the presence of executive dysfunction in drug addicts is that it may be associated with poor response to treatment.

Indeed, cocaine addicts who did not complete treatment performed significantly worse than treatment completers in measures of attention, memory, spatial ability, accuracy, global functioning and cognitive proficiency (Aharonovich et al., 2006). Similarly, cannabis dependent subjects that did not complete treatment performed significantly worse than treatment completers on measures of abstract reasoning, and processing accuracy (Aharonovich et al., 2008). These findings are consistent with other studies suggesting that cognitive impairments, and deficits in inhibitory control tend to be associated with higher drop-out rates (Brewer et al., 2008).

Consequently, pharmacological treatment using cognitive enhancers, and behavioural approaches such as cognitive behavioural therapy, have been suggested as possibilities to overcome the cognitive dysfunctions observed in drug addicts and subsequently increase the utility of addiction treatment (Sofuoglu et al., 2013).

2. 3,4-Methylenedioxymethamphetamine

MDMA, commonly known as ecstasy, is a ring-substituted amphetamine derivative, structurally related to the hallucinogenic compound mescaline (Figure 6). Since the mid-1980s ecstasy has become popular as a recreational drug, frequently associated with "rave" or "techno" parties. MDMA is often presented as a tablet in a diversity of colours and shapes, decorated with a wide variety of designs.

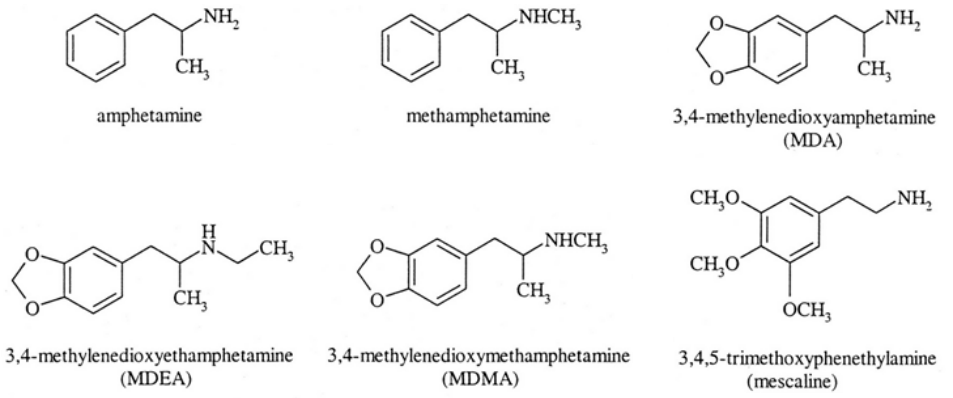

Figure 6. Chemical structures of amphetamine and some of its derivatives, including methamphetamine, MDMA and mescaline.

MDMA was first synthesized by Merck in 1912 and patented in Germany at the end of the same year. Contrary to what is usually maintained in the literature, MDMA was not synthesized as an anorectic drug or appetite suppressor. Indeed, MDMA was synthesized to facilitate the process of synthesis of an existent haemostatic drug called "Hydrastinin" (Freudenmann et al., 2006). Due to its usage as a chemical precursor, MDMA's pharmacological properties were not tested until 1927, when Dr. Max Oberlin focused his research on adrenaline-like substances. In 1952, the first toxicology studies of MDMA were performed in flies, and

Mercks' interest in MDMA was reinstated when the pharmaceutical company focused on producing new stimulants. MDMA was first detected in tablets in the streets of Chicago in 1970 (Gaston and Rasmussen, 1972), and few years later the first studies in humans were reported by Shulgin and co-workers (Anderson et al., 1978; Shulgin and Nichols, 1978). At that moment, a number of psychiatrists and other therapists in the United States and Europe used MDMA as an adjunct to psychotherapy. MDMA was reported to decrease feelings of fear while maintaining a clear-headed, alert state of consciousness, facilitating the psychotherapeutical process (Greer and Tolbert, 1986; Shulgin, 1986). However, due to its lack of clinical application, together with the lack of accepted safety, and its potential toxicity, MDMA was classified as a Schedule 1 drug by the United States Drug Enforcement Administration (DEA) in 1985, and banned in most other countries soon thereafter. MDMA was initially classified as an entactogen, which is a term used to describe a class of psychoactive drugs that produce distinctive social and emotional effects in comparison with other psychoactive drugs (Nichols and Oberlender, 1990). More recently, it has been suggested that MDMA can be useful for being administered in conjunction with psychotherapy in people with chronic, treatment-resistent posttraumatic stress disorder (PTSD). Indeed, a pilot phase II clinical trial has reported significant improvements in PTSD symptoms in the MDMA-assisted psychotherapy group in comparison with the same psychotherapy with placebo (Mithoefer et al., 2011). However, due to the action mechanisms of MDMA, during the neurochemical recovery produced afterwards, the negative moods tend to predominate, and it has been suggested that it

might be counter-productive especially in psychiatrically vulnerable patients (Parrott, 2014).

2.1. MDMA use disorder

In humans, the rewarding properties of MDMA represent the major reason for its consumption. According to the most recent European Drug Report (EMCDDA – Drug Report 2014), it is estimated that 10.6 million Europeans (15 - 64 years) have tried MDMA in their lifetime, and around 1.6 million reported to have used this drug in the last year. Ecstasy consumption is more prevalent in young adults (15 – 34 years) because its consumption has been mainly associated with "rave" or "techno" parties in dance clubs. Last year, 1.3 million young European adults used MDMA (Figure 7).

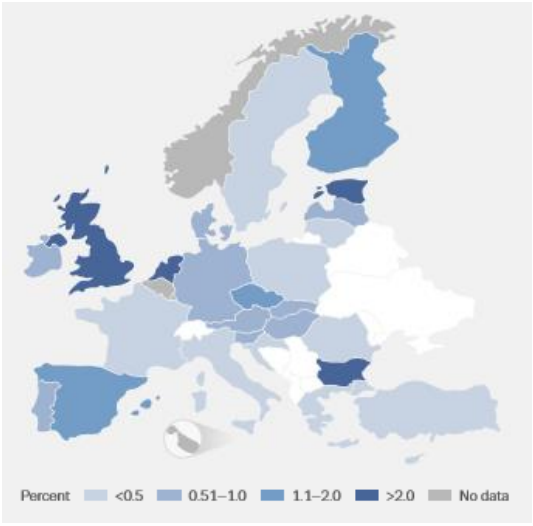

Figure 7. MDMA consumption among young adults in European countries in 2013 (EMCDDA - Drug Report 2014).

According to the DSM-5, MDMA use disorder is currently classified in the "other hallucinogen" chapter despite its strong stimulant properties. Although the structure of MDMA is similar to mescaline-type hallucinogens and amphetamine-type stimulants, MDMA is pharmacologically different from any other substance classes (Nichols, 1986; Fantegrossi, 2008).

MDMA consumption pattern differs from the typical psychostimulant usage, which is characterized by taking another drug dose to re-experience the drug effects, while avoiding withdrawal symptoms. In contrast, MDMA users do not take tablets in succession, as they state that with subsequent use the desired effects declined, and side-effects increased (Merrill, 1996). This could be explained by the acute tolerance to the behavioural effects that characterizes MDMA mainly based on its mechanism of action. MDMA induces a rapid increase in extracellular serotonin followed by a depletion of brain serotonin, and a limitation in serotonin synthesis (Schmidt and Taylor, 1987). Therefore, users stop taking more MDMA tablets because these are no longer effective. Besides this acute tolerance, it has also been described a chronic tolerance phenomena. Reduced subjective effects after repeated usage (Solowij et al., 1992), as well as, an increase in the amount of tablets taken after repeated usage (Steele et al., 1994), confirm this effect.

Potential addictive properties of MDMA are currently controversial mainly because of the criteria for abuse and dependence defined in the DSM-5. Withdrawal symptoms are not included as diagnostic criteria for MDMA use disorder, although more than 68% of MDMA users reported

enough symptoms to be considered as withdrawal, and these symptoms were the second most frequently reported criterion in considering MDMA dependence (Cottler et al., 2009). These data indicate that MDMA should be considered as a separate substance class with its own set of specific criteria for the diagnostic of MDMA use disorder (Cottler et al., 2009).

2.2. Pharmacokinetics

MDMA is metabolised by three major pathways; O-demethylenation, N-demethylation and aromatic hydroxylation, as well as other minor routes such as deamination, glucuronidation and sulfation (Lim and Foltz, 1988; 1991). Studies in rats and humans show that O-demethylenation of MDMA occurs through cytochrome P450 (CYP), and the CYP2D6 has been identified as the main responsible isoenzyme in humans (Tucker et al., 1994; De la Torre et al., 2004). This enzyme isoform catalyzes the conversion of MDMA to 3,4-dihydroxymethamphetamine (HHMA), an unstable and reactive compound in humans (Segura et al., 2001), and also in rats (Lim and Foltz, 1988). Then, HHMA can be O-methylated to form 4-hydroxy-3-methoxymethamphetamine (HMMA), which appears to be a major metabolite in humans (Segura et al., 2001). The second major pathway in MDMA metabolism is N-demethylation, which occurs via CYP1A2 in humans and rats (Maurer et al., 2000), producing methylenedioxyamphetamine (MDA) a potent neurotoxin (Chu et al., 1996; De la Torre et al., 2000) (Figure 8).

Aromatic hydroxylation is the third major metabolic route for MDMA. It consists on the hydroxylation at position 6 of the aromatic ring, producing 6-hydroxy-MDMA which can be demethylenated via CYP2D, generating 2,4,5-trihydroxymethamphetamine (Tucker et al., 1994).

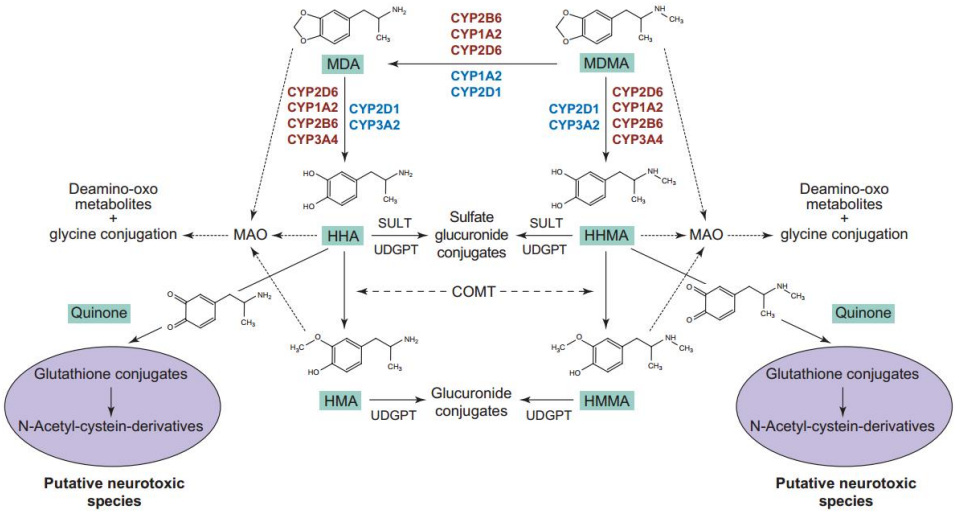

Figure 8. Major pathways of MDMA metabolism in rats and humans. Isoenzymes of CYP involved in the N-demethylation and O-demethylenation metabolic reactions in rats are highlighted in blue, whereas those corresponding to enzymes in humans are shown in red (De la Torre and Farré., 2004).

MDMA metabolism is quite similar in rats and humans. Following administration, MDMA as well as its major metabolites, HHMA and HMMA are present in high concentrations in plasma of both rats and humans. Nevertheless, the main metabolic pathway in humans is O-demethylenation of MDMA to HHMA at any dose tested (De la Torre et al., 2004), whereas in rats the N-demethylation of MDMA to MDA is predominant at low doses (Chu et al., 1996). MDMA can also interact with CYP2D6 to form a complex leading to the inhibition of the enzyme (Wu et al., 1997; Delaforge et al., 1999). In humans, this inhibitory

mechanism underlies the observed non-linear pharmacokinetics of MDMA (De la Torre et al., 2000). Due to this phenomenon, following repeated administration of MDMA, its metabolic disposition is impaired producing an accumulation of MDMA in plasma. Using a model based on physiological observations, it was predicted that the typical recreational MDMA dose could inactivate most hepatic CYP2D6 in 1 hour, and that the return to basal level of CYP2D6 could take at least 10 days (Yang et al., 2006). CYPD26 polymorphisms are found in human population and might confer differences in MDMA metabolism. It has been suggested that poor metabolizers will be more susceptible to acute effects of MDMA as its concentration in plasma will be prolonged, whereas fast metabolizers (subjects with multiple functional copies of the gene encoding for CYP2D6) will be less susceptible to the acute effects of MDMA, but more susceptible to the effects of the different MDMA metabolites (See section 2.5. Neurotoxicity). Although in humans a single dose of MDMA can fully inhibit CYP2D6, in non-human primates several doses of MDMA are required for producing a full inhibition of the enzyme (Bowyer et al., 2003), representing a limitation for MDMA studies in other species.

In contrast, the metabolism of MDMA in mice differs from the one in humans and rats. At comparable doses, concentrations of MDMA and its metabolites are substantially higher in mice, although its clearance is also significantly higher (Mueller et al., 2013). Another difference is that the ratio of MDMA to metabolites is significantly higher in mice (Mueller et

al., 2013), which may account for the different neurotoxic profile observed in mice with respect to other species (See 2.5. Neurotoxicity).

2.3. Pharmacodynamics

The action of MDMA in the central nervous system is complex, as this drug presents several molecular sites of action, and its effect varies across species. MDMA has major effects on serotonin pathways, but it also affects other important neurotransmitters in the brain, namely dopamine, noradrenaline, acetylcholine and histamine.

In rats, MDMA administration induces an acute and rapid increase in extracellular serotonin through different mechanisms, mainly through reversing the membrane serotonin transporter (SERT), and the vesicle monoamine transporter VMAT-2. Serotonin release was initially revealed using microdialysis in the caudate nucleus (Gough et al., 1991), and in the NAc (White et al., 1994). MDMA also inhibits monoamine oxidase, the enzyme responsible for monoamine degradation (Gough et al., 1991; Gudelsky and Nash, 1996), and blocks the activity of tryptophan hydroxylase (Stone et al., 1987), the rate limiting enzyme in serotonin synthesis, restricting its biosynthesis. Therefore, after the initial serotonin increase, a depletion of brain serotonin has been observed in rats and non-human primates, which can be long-lasting depending on the dose administered (Schmidt and Taylor, 1987; Ricaurte et al., 1988a; Green et al., 2003). MDMA also induces a rapid dopamine release in multiple brain regions, as observed in several studies performed in rats (Yamamoto and Spanos, 1988; Marona-Lewicka et al., 1996; Shankaran

and Gudelsky, 1998). The mechanism responsible for dopamine release is controversial as some studies using the dopamine uptake inhibitor, GBR 12909 observed a decrease in dopamine release following MDMA administration (Nash and Brodkin, 1991; Koch and Galloway, 1997), whereas other studies found the opposite effect (Mechan et al., 2002). Therefore, it is possible that other transport mechanisms are involved in the release of dopamine by MDMA. Indeed, promiscuity for dopamine uptake by SERT and norepinephrine transporter has been revealed (Horn, 1973; Raiteri et al., 1977; Schmidt et al., 1987; Morón et al., 2002), and can be altered by MDMA administration (Saldaña and Barker, 2004). In vitro studies have also demonstrated that MDMA induces the release of norepinephrine in rat brain slices (Fitzgerald and Reid, 1990), and this release was also corroborated by microdialysis in the thalamus (Starr et al., 2008; 2012). Finally, studies performed in brain slices of rat striatum revealed that MDMA enhances the release of acetylcholine (Fischer et al., 2000), and this result was recently confirmed using in vivo microdialysis in the pre-frontal cortex, the striatum (Acquas et al., 2001) and the hippocampus (Nair and Gudelsky, 2006).

In mice, MDMA produces an acute release of dopamine as evidenced by measures of dopamine content in striatum (O'Shea et al., 2001), and by in vivo mycrodialysis (Camarero et al., 2002). This increase in dopamine levels has also been confirmed by previous studies performed in our laboratory. Thus, MDMA-induced dopamine release has been revealed using microdyalisis in the NAc (Robledo et al., 2004; Trigo et al., 2007, Orejarena et al., 2009; 2011). Moreover, the administration of MDMA in

mice also induces a release of serotonin (Trigo et al., 2007; Orejarena et al., 2011), and norepinephrine in the prefrontal cortex (Orejarena et al., 2011).

2.4. Acute effects of MDMA

A considerable amount of acute pharmacological effects have been reported following MDMA administration in humans (Farré et al., 2004). These pharmacological responses include subjective effects such as "liking" or "high" feelings, alterations in vision or hearing. In a recent review, a subset of effects has been reported by a large number of subjects across multiple investigations (Baylen and Rosenberg, 2006). Most of these effects were either emotional or somatic although effects on sexual behaviour, cognition, sensory-perception, appetite and sleep were also found. MDMA is consumed for its positive effects, which include euphoria, increased energy, sexual arousal and entactogenic effects among others. However, some MDMA users have also reported undesirable effects such as confusion, mental fatigue, anxiety, depression, and strange thoughts such as feeling immobile, out of control or even dying (Parrott et al., 2007). Dose level, gender, expectancy and the initial emotional state seem to be crucial for the different outcomes of MDMA intake. Furthermore, MDMA consumption has been related to hyperactivity, hypertension, cardiac arrhythmia and hyperthermia (Montoya et al., 2002), or more accurately, a lack of thermoregulation (Dafters, 1994), an important factor as MDMA is usually taken in crowded warm rooms inside the dance clubs. Hyperthermia is an elevated body temperature due to failed thermoregulation. It occurs

when the body produces or absorbs more heat than it can dissipate. When the core body temperature is sufficiently high, hyperthermia is a medical emergency and requires immediate treatment to prevent disability or death. Hyperthermia has been considered the most dangerous clinical symptom associated with acute MDMA intoxication (Kalant, 2001). The first fatal cases reported following MDMA intoxication involved overheating, with core body temperatures above 40 °C (Chadwick et al., 1991; Henry et al., 1992). Since then, hyperthermic adverse reactions to MDMA have continued, although the emergency departments in hospitals now follow optimal procedures involving rapid cooling, so the MDMA fatalities are very unusual (Patel et al., 2005; Greene et al., 2009; Halpern et al., 2011). Apart from mortality, MDMA-induced hyperthermia can cause lasting morbidity due to sequelae such as shock, seizures, acidosis, disseminated intravascular coagulation, acute kidney injury, rhabdomyolysis, and brain injury. Indeed, a recent study reported multiple MDMA overdoses (12) at a rave event, two of them with fatal consequences, and 4 with persistent sequelae as a consequence of hyperthermia (Armenian et al., 2012).

Another major adverse reaction to MDMA is the so called serotonin syndrome, which is a potentially life-threatening drug reaction that may occur as a consequence of excess serotonergic activity at central and peripheral nervous systems. This excess of serotonin activity produces a wide variety of signs and symptoms that may range from barely perceptive to severe. The serotonin syndrome includes cephalea, tachycardia, hypertension, myoclonus, mydriasis, hyperthermia and

hyperreflexia, and in the most severe cases it can produce seizures, metabolic acidosis, and renal failure, which can result in lethal consequences.

2.5. Neurotoxicity

It has been widely demonstrated that MDMA presents a different neurotoxic profile depending on the species analyzed. In rats and non-human primates, repeated administration of MDMA results in a long-term reduction in brain tissue concentrations of serotonin and reductions in serotonin reuptake sites (Ricaurte et al., 2000; Green et al., 2003). Contrastingly, in mice, MDMA-induced neurotoxicity is relatively selective for the dopaminergic system, leaving serotonin concentrations unaffected (Stone et al., 1987; Logan et al., 1988).

In rats, neurotoxicity is evidenced by long-term alterations in serotonergic transmission, such as depletions of serotonin and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the brain (Ricaurte et al., 1988b; Colado et al., 2004), long-term depletions of tryptophan hydroxylase (TPH) activity, reduction in TPH-immunoreactive fibers (Schmidt and Taylor, 1987), and alterations in the density and functionality of SERT (Kovacs et al., 2007; Bonkale and Austin, 2008). However, there is controversy as to whether these changes reflect neuronal damage or not. It has been suggested that initial lesion severity is an important factor for the recovery, since the most severely damaged areas showed the least recovery in both, rats and non-human primates (Fischer et al., 1995). Recent studies performed in rats using low doses of

MDMA, have not revealed neuroanatomical alterations (Wang et al., 2005; Baumann et al., 2007), and no significant alterations in genes associated with neuronal damage (Cuyas et al., 2014).

In addition, the mechanisms by which MDMA induces serotonergic neurotoxicity are still not well established. Several factors have been postulated as underlying causes, such as MDMA metabolites (Esteban et al., 2001), hyperthermia (Fantegrossi et al., 2003; Goñi-Allo et al., 2008), and MDMA-induced dopamine release (Shankaran et al., 1999). The role of MDMA metabolites in MDMA-induced neurotoxicity in rats was demonstrated by the lack of neurotoxic damage observed after direct injection of MDMA into the hippocampus (Esteban et al., 2001). These data suggested that MDMA metabolism could lead to the formation of reactive oxygen and nitrogen species and other toxic oxidation products, which could be responsible for its toxicity (Capela et al., 2009). In addition, MDMA-induced hyperthermia has been associated with serotonergic neurotoxicity, possibly through enhancing free radical formation in the brain (Colado et al., 1998; Malberg and Seiden, 1998; Capela et al., 2006). On the other hand, housing rats under cool conditions has been shown to inhibit MDMA metabolism, which could subsequently reduce MDMA neurotoxicity (Goñi-Allo et al., 2008).

In contrast to what has been revealed in rats, MDMA behaves as a relatively selective dopaminergic neurotoxin in mice, having little effect on serotonergic neurones. Repeated high doses of MDMA produce a sustained loss of dopamine and dopamine metabolite concentrations in the striatum (Stone et al., 1987; Logan et al., 1988; O'Callaghan and

Miller, 1994; O'Shea et al., 2001), a decrease in tyrosine hydroxylase immunoreactive fibers, mainly in the nigrostriatal pathway (Escobedo et al., 2005; Granado et al., 2008), as well as a reduction in the density of dopamine transporter (DAT) binding sites (Mann et al., 1997; Jayanthi et al., 1999; Johnson et al., 2002). These alterations in DAT and tyrosine hydroxylase were reported as early as 1 day after a neurotoxic MDMA administration (20 or 30 mg/kg three times at 3h intervals), but were persistent for 30 days (Granado et al., 2008). A recent study has demonstrated a critical role for dopamine receptor D_1 in neurotoxicity, as knock-out (KO) mice for this receptor are protected against MDMA-induced dopaminergic neurotoxicity (Granado et al., 2014). Interestingly, repeated administration of GBR 12909 before each MDMA injection completely prevented the long term loss of striatal dopamine concentrations. However, this effect was unrelated to GBR 12909 acute actions on dopamine metabolism or MDMA-induced hyperthermia (O'Shea et al., 2001). The evidence for a role of free radicals in producing MDMA-induced neurotoxic in mice was revealed by the attenuation of MDMA-induced oxidative stress in transgenic animals overexpressing human copper/zinc superoxide dismutase (Cadet et al., 1995; Jayanthi et al., 1999). Moreover, two nitric oxide synthase inhibitors were found to protect against MDMA-induced dopaminergic neurotoxicity (Colado et al., 2001), supporting the role of oxidative stress in mice MDMA-induced neurotoxicity.

In non-human primates, serotonergic depletion has also been demonstrated following MDMA administration (Ricaurte et al., 1988b;

Ricaurte and McCann, 1992; Scheffel et al., 1998). These effects were even more pronounced than those observed in rats (Ricaurte and McCann, 1992). Structural damage in non-human primates was revealed by immunocytochemical analysis as a reduction in serotonin-immunoreactive axons throughout the forebrain (Ricaurte et al., 1988b). This reduction in serotonergic axons was still significant two weeks after MDMA administration (Wilson et al., 1989). A recent study performed in squirrel monkeys has revealed that even a single dose of MDMA can induce lasting dose-related serotonergic neurochemical deficits in some brain regions (Mueller et al., 2013).

In humans, recent articles using brain imaging-based techniques have revealed that MDMA use is associated with a serotonergic toxicity revealed by reductions in SERT binding in multiple brain regions, such as the cerebral cortices and the hippocampus (McCann et al., 2008; Kish et al., 2010; Urban et al., 2012), although this decrease was not observed in the midbrain and basal ganglia (Kish et al., 2010), and DAT binding levels were unaffected in MDMA users (McCann et al., 2008). An additional factor, which may be relevant to MDMA neurotoxicity, is the presence of genetic polymorphisms in the CYP2D6 enzyme. As stated before, poor metabolizers could be more susceptible to the acute effects of MDMA due to the accumulation of this compound in plasma. However, their slower metabolism might confer them neuroprotection against neurotoxicity induced by MDMA metabolites. In contrast, rapid metabolizers might exhibit a higher risk of neurotoxicity due to the faster formation of metabolites (De la Torre and Farré, 2004).

2.6. The rewarding and reinforcing properties of MDMA

The rewarding and reinforcing properties of MDMA have been demonstrated in different species. Accordingly, MDMA can induce CPP in rats (Bilsky et al., 1990) and mice (Salzmann et al., 2003; Robledo et al., 2004). Moreover, self-administration of MDMA has been reported in non-human primates (Fantegrossi et al., 2002), in rats (Ratzenboeck et al., 2001; Schenk et al., 2003), and also in mice (Trigo et al., 2006). Since MDMA affects major neurotransmitter pathways, a complex interaction between the different neurotransmitters could account for the effects of MDMA in reward and motivation.

2.6.1. Neurobiological mechanisms involved in MDMA-induced rewarding and reinforcing effects

2.6.1.1. The dopaminergic system

The mesolimbic dopamine system has been implicated in the rewarding effects of MDMA as revealed in electrophysiological and neurochemical studies (Green et al., 2003; Robledo et al., 2004). In rats, the administration of MDMA induced an increase in extracellular levels of dopamine in the NAc and caudate nucleus, in a dose-dependent manner (Yamamoto and Spanos, 1988). Dopamine release in the NAc following MDMA has been associated with its rewarding properties (Marona-Lewicka et al., 1996). In addition, supporting the role of dopamine in MDMA-induced reward, the administration of a dopamine release inhibitor (CGS 10746B) prevented MDMA-induced CPP in rats (Bilsky et al., 1998), and an antagonist of the D_1 dopamine receptor (SCH 23390)

attenuated MDMA reinforcing properties in the self-administration paradigm (Daniela et al., 2004), revealed by a rightward shift in the dose response curve. Likewise, the dopaminergic D_2 receptor antagonist, eticlopride increased responding maintained by several MDMA doses in the self-administration paradigm in rats (Brennan et al., 2009). Therefore, both D_1 and D_2 dopaminergic receptors have been associated with the reinforcing properties of MDMA.

In mice, a recent article published in our laboratory has revealed that repeated low doses of MDMA modify mesolimbic dopaminergic neurotransmission in mice following withdrawal of the drug (Orejarena et al., 2009). In this study, animals that self-administered low doses of MDMA in a contingent or in a non-contingent manner showed lower basal levels of dopamine in the NAc. Moreover, an acute challenge of MDMA did not increase dopamine levels in the NAc in those animals that receive MDMA contingently, although an acute and rapid dopamine release was observed in saline-treated animals. Interestingly, yoked-animals receiving MDMA in a non-contingent manner displayed a reduction in dopamine release in comparison with saline-treated animals after MDMA exposure (Orejarena et al., 2009). Thus, the reduced capacity of MDMA to stimulate dopamine in the NAc could suggest a reduction in the incentive value of the drug. However, mice self-administered MDMA for ten consecutive days without a major fluctuation in this behaviour (Orejarena et al., 2009). Therefore, it seems possible that neuroadaptations in mesolimbic dopaminergic system take place during repeated exposure to MDMA, which could be dependent

on the learning processes observed during drug-seeking behaviour. These adaptations might explain the differences observed between the contingent and non-contingent MDMA administration regarding stimulated dopamine release.

2.6.1.2. The serotonergic system

MDMA increases extracellular levels of serotonin (5-HT) in the NAc of the rat (White et al., 1994). In general, serotonergic antagonists attenuate the rewarding and reinforcing properties of MDMA. Thus, the administration of MDL 72222, a serotonin 3 receptor antagonist, blocked MDMA-induced CPP in rats (Bilsky and Reid, 1991), and the serotonin 2A receptor (5-HT_{2A}) antagonists, ketanserin and MDL 100907 attenuated MDMA self-administration in monkeys (Fantegrossi et al., 2002). Reductions in SERT binding were revealed in several brain regions such as frontal cortex, striatum, hippocampus and brainstem in rats either receiving experimenter-administered or self-administered MDMA (Schenk et al., 2007). This decrease contrasts with previous results obtained in primates that self-administered MDMA (Fantegrossi et al., 2004; Banks et al., 2007), and mice (Orejarena et al., 2009). However, the doses used by Schenk and cols. in both, self- and experimenter-administered conditions were higher in comparison with the other studies. Additionally, studies performed using transgenic mice revealed that MDMA self-administration was abolished in knock-out animals lacking SERT (Trigo et al., 2007), or the 5-HT_{2A} receptor (Orejarena et al., 2011). Recent studies revealed that serotonin 2B receptor KO mice did not exhibit MDMA-induced CPP and the serotonin 2B receptor

antagonist, RS 127445, blocked MDMA-induced reinstatement of this behaviour (Doly et al., 2009).

2.6.1.3. The interaction between the dopaminergic and serotonergic system

There is good evidence to suggest that serotonin is involved in the mechanisms associated with MDMA-induced dopamine release in the striatum. Dopamine release is markedly inhibited in rats by pre-treatment with fluoxetine, a selective serotonin reuptake inhibitor (Koch and Galloway, 1997). It has been suggested that the ratio of dopamine to serotonin released by drugs is a good predictor of self-administration potency (Wee et al., 2005). In agreement, responding for MDMA in the self-administration paradigm is lower during the early days of testing, when MDMA induces a large serotonin release in rats, whereas the response rate is increased in posterior days (Dalley et al., 2007; Schenk et al., 2007). This effect is thought to be produced because continued MDMA exposure results in a decrease of serotonin levels, consequently increasing the dopamine/serotonin ratio. Furthermore, pre-treatment with the 5-HT_2 agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), or 5-methoxy-N,N-dimethyltryptamine potentiates MDMA-induced dopamine release (Gudelsky et al., 1994). Consistent with these data, MDMA-induced dopamine release was inhibited by the 5-$HT_{2A/C}$ antagonist, ritanserin (Yamamoto et al., 1995), and was reduced in 5-HT_{2A} KO mice (Orejarena et al., 2011), suggesting that the activation of these receptors enhanced MDMA-induced dopamine release. Indeed, 5-HT_{2A} KO mice also exhibit lower levels of basal dopamine in the NAc

(Orejarena et al., 2011). These animals were unable to acquire MDMA self-administration behaviour, whereas instrumental responding for natural rewards such as food pellets was unaltered (Orejarena et al., 2011). These results support a specific involvement of 5-HT_{2A} receptors in MDMA reinforcing properties, which could be related to the alterations in dopamine levels.

2.6.2. The effects of MDMA on the motivation for natural rewards

As in the case of other psychostimulants, MDMA also modifies the motivation for natural rewards. Thus, MDMA decreased responding for food on fixed-ratio schedules of reinstatement in pigeons (Nader et al., 1989), in rats (Li et al., 1989; Nagilla et al., 1998) and in mice (Glennon et al., 1987; Miczek and Haney 1994). Acute doses of MDMA also disrupted progressive ratio schedules in monkeys working for food (Frederick and Paule, 1997), and in rats working for water (Laraway et al., 2003). Appetite disturbances have been described as a consequence of MDMA consumption in humans (Vollenweider et al., 1998; Curran et al., 2004), which could also be indicative of a decrease in motivation for this natural reward.

2.7. Cognitive deficits induced by MDMA consumption

Abundant literature has identified cognitive deficits related to acute and long-term MDMA consumption in humans (Rogers et al., 2009) and experimental animals (Frederick and Paule, 1997; Able et al., 2006; Trigo et al., 2008). However, the mechanisms producing these deficits remain

unclear, in part due to the complexity of MDMA-induced effects in different species (Easton and Marsden, 2006).

Studies performed in humans have revealed discrepant finding regarding the cognitive deficits induced by MDMA consumption. The first psychobiological deficits were reported in the early 90s revealing deficits in retrospective memory in abstinent ecstasy users (McCann and Ricaurte, 1991; Krystal et al., 1992). Moreover, impairments in immediate and delayed word recall (Parrott et al., 1998), as well as, poorer prose recall (Morgan, 1999) were also reported. The time since last use of ecstasy varied widely across those studies, from less than a week to more than 6 months. In subsequent years, there have been several conflicting reports regarding the effects of MDMA on memory processes. Thus, differences in life-time MDMA consumption, intensity of drug usage per session, as well as consumption of other psychoactive drugs have been suggested as major source of variance in human studies (Parrott, 2006).

However, in a recent meta-analysis, a significant overall neurocognitive impairment was revealed in ecstasy users compared to drug-naïve controls and to poly-drug abusers using other illegal drugs but not ecstasy (Rogers et al., 2009). In this study, six out of seven dependent variables showed small but significant cognitive deficits for ecstasy-exposed individuals. The only measure where ecstasy users did not differ from drug naïve and poly-drug group controls was in a basic intelligence test (National Adult Reading Test Intelligence Quocient). It has been shown that ecstasy-induced memory deficits are stronger in more cognitively complex tasks, which require interactions between

multiple brain regions (Brown et al., 2010). Therefore, ecstasy users did not present impairments in implicit memory tasks, which only relies on perceptual areas, or in simple tasks such as stem-cued recall and free recall of lists of unrelated words, which principally depend on perceptual areas plus the hippocampus, with low involvement of frontal cortex (Savage et al., 2001).

Animal studies can facilitate or overcome some of the problems encountered in human studies, since factors such as drug purity, drug dosage, and environmental conditions can be monitored. In addition, the use of appropriate controls represents an advantage of animal studies in comparison with human studies when trying to determine the effects of MDMA and its mechanism of action. Thus, MDMA exposure has been associated with persistent cognitive deficits in monkeys (Frederick and Paule, 1997; Taffe et al., 2001; 2003), rats (Able et al., 2006; Skelton et al., 2006; Dalley et al., 2007), and mice (Glennon et al., 1987; Trigo et al., 2008; Nawata et al., 2010).

2.7.1. Neurobiological mechanisms involved in MDMA-induced cognitive deficits

2.7.1.1. The dopaminergic system

The cognitive deficits observed in mice following repeated MDMA administration could be due to dopaminergic neurotoxicity. The dopaminergic system has been associated with reward-related learning, and more specifically, burst firing of dopaminergic neurons might serve as a teaching signal, essential in learning situations (Schultz, 2013;

Steinberg et al., 2013). This effect is suggested by the induction of synaptic plasticity by dopamine bursts, which might facilitate associative learning (Blythe et al., 2009). Moreover, dopamine is an important neuromodulator in fronto-striatal circuits, which connect the most relevant brain regions involved in cognitive flexibility and learning processes (Ragozzino, 2007; Castañé et al., 2010). Damage to these areas has been demonstrated to produce impairments in several cognitive processes such as reversal learning (McAlonan and Brown, 2003; Boulougouris et al., 2007) and attentional set shifting (Dias et al., 1996; Bissonette et al., 2008). Neurotoxic damage to dopaminergic terminals is reflected as a loss in the concentration of dopamine and its metabolites (Colado et al., 2004), as well as a decrease in the density of DAT sites and tyrosine hydroxylase immunoreactive fibers in the striatum and substantia nigra (Granado et al., 2008). Indeed, it was reported that repeated MDMA administration dose-dependently disrupted learning in an active avoidance task, and impaired recall of this task (Trigo et al., 2008). Nevertheless, recall deficits observed using low doses of MDMA (Trigo et al., 2008) were not correlated with DAT reductions (Robledo et al., 2004). Therefore, although dopaminergic changes could, in part, mediate MDMA-induced cognitive deficits in mice, these effects cannot be completely attributed to DAT reductions.

2.7.1.2. The serotonergic system

MDMA-induced neurotoxic damage to serotonergic terminals has been characterized by long-term depletion of serotonin and its metabolite 5-HIAA, reductions in SERT, and reductions in the density of

serotonergic axon terminals (See 2.5. Neurotoxicity). In human abstinent MDMA users, lower 5-HIAA levels were found in the cerebrospinal fluid in comparison with control subjects, and this reduction was correlated with deficits in verbal and visual memory performance (Bolla et al., 1998). In contrast, another study which correlated deficits in working memory with the extent of MDMA use did not reveal reductions in 5-HIAA levels in the cerebrospinal fluid (McCann et al., 1999). Albeit these discrepancies, it was suggested that cognitive deficits observed as a consequence of MDMA use could be attributed to serotonergic nerve terminal dysfunction (Marston et al., 1999).

More recent studies in human ecstasy users have demonstrated that MDMA induces a decrease in SERT expression in several brain areas known to be involved in memory and cognition, such as prefrontal cortex and hippocampus (Kish et al., 2010). This is consistent with previous studies reporting significant correlations between reduced SERT binding and neurocognitive deficits in abstinent ecstasy users (McCann et al., 2008). In addition, MDMA administration produces alterations in 5-HT_{2A} densities in the cerebral cortex in rats and human users (Reneman et al., 2002). Serotonin depletion and destruction of serotonin containing neurons induced an increase in 5-HT_2 receptors in mice (Heal et al., 1985), enhancing head twitching responses, a behaviour which is known to depend on 5-HT_{2A} functionality (Schreiber et al., 1995). Indeed, significant reductions in 5-HT_{2A} in all cortical regions were observed in current MDMA users in comparison with healthy controls (Reneman et al., 2002). This reduction was also observed in MDMA-

treated rats and returned to control values in a time-dependent manner (Reneman et al., 2002). In this study, an increase in 5-HT_{2A} density in the occipital cortex was revealed after a period of MDMA abstinence (Reneman et al., 2000; 2002), and was suggested to occur as a compensatory mechanism due to serotonin depletion, which is more sever in this brain region in both monkeys (Scheffel et al., 1998), and humans (Semple et al., 1999). Moreover, MDMA-induced memory disturbances in humans were correlated with 5-HT_{2A} densities (Reneman et al., 2000), suggesting an important role for these receptors in the cognitive deficits induced by MDMA.

3. Delta-9-Tetrahydrocannabinol

Δ^9-tetrahydrocannabinol (THC) is the main psychoactive component of *Cannabis sativa*, a plant that has been exploited over thousands of years not only for its material properties as a fibre, but also for medicinal and recreational purposes. The earliest archeological evidence of its usage comes from rope imprints in Chinese pottery dated from about 10.000 B.C. Moreover, written documents found in The Shu King, a book dating about 2350 B.C., refer to the use of hemp in clothing. Its psychoactive, mind-altering and medicinal properties were employed by the Assyrians (a major empire of the Ancient Near East) and its wide use in the Middle East has continued ever since. The dual nature of its effects was found in the Chinese classic Ben Ts'ao pharmacopeia (1st century A.D.), where its medical use was recommended for various diseases, but also warned that hallucinations and sickness could appear when taken in excess. Cannabis was introduced in Europe by British physicians returning from India and by Napoleonic soldiers on their way home from Egypt. The psychological effects caused by cannabis preparations were described by the psychiatrist Moreau in his book, "Hashish and Mental Illness" (1845). Numerous psychological phenomena occurring in experimental subjects were detailed: feelings of happiness, excitement, dissociation of ideas, errors in time and space, fluctuations of emotions, delusions, illusions, hallucinations and even delirium or madness. At present, most cannabis users report an increase in relaxation and euphoria, and possibly enhancement of their senses. Apart from these psychoactive effects, cannabis also produces sedation, analgesia, muscle relaxation, and

increases heart rate. It also has antiinflammatory effects, decreases intraocular pressure, stimulates appetite, and inhibits nausea (Hepler and Franck, 1971; Sallan et al., 1975; Merrit et al., 1980; Adams and Martin, 1996; Jones, 2002; Wallace et al., 2007; Nagarkatti et al., 2009; D'Souza et al., 2012; Chiou et al., 2013). However, cannabis consumption has also been associated with impairments in attention and memory, and alterations in motor function and coordination (Ranganathan and D'Souza, 2006; Ramaekers et al., 2006; Solowij and Battisti, 2008; Weinstein et al., 2008; Bosker et al., 2013; Hartman and Huestis, 2013). Modern research relies on quantitative data, and in contrast to cocaine or morphine, which have been isolated and therefore available since the nineteenth century, the psychoactive components of cannabis were not isolated until the 1960s. However, in the last decades, cannabinoid research has experienced a tremendous growth, corresponding to the expansion of its usage.

According to the latest European Drug Report (EMCDDA – Drug Report 2014), cannabis is the most consumed illicit drug in Europe. It is estimated that 73.6 million European adults (15 to 64 years old) have consumed cannabis during their lifetime, which represents a 21.7% of this European population. Data show that 5.3% of Europeans used cannabis during last year and this value duplicates among young people, reaching an 11.2% in young Europeans (15 to 34 years old) (Figure 9), representing a total of 14.6 million young people.

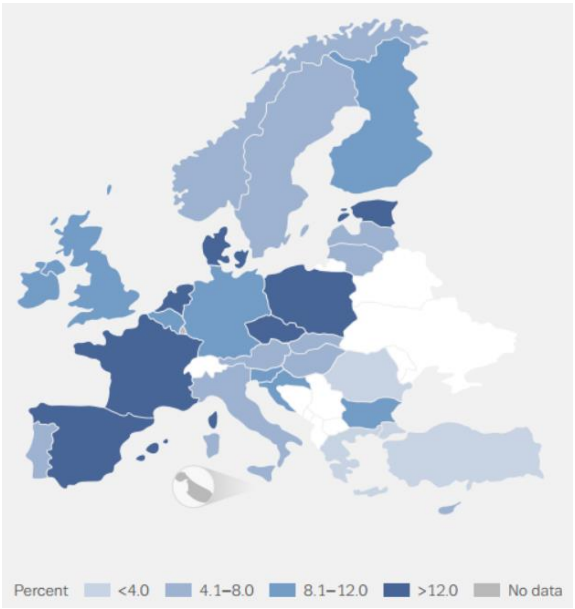

Figure 9. Last year prevalence of cannabis among young adults in European countries (EMCDDA – Drug report 2014).

Currently, cannabis abuse is the principal reason for entering drug treatment for the first time. Its usage has been associated with low academic achievement, unemployment, violence and risk for developing psychiatric disorders (Friedman et al., 2001; Ferdinand et al., 2005; Hall and Degenhardt, 2009). Importantly, the early onset of cannabis consumption is often associated with later problematic use of other drugs and mental health problems (Hall, 2006; Copeland and Swift, 2009).

3.1. Exogenous cannabinoids

The most important exogenous cannabinoid is THC. Albeit the extensive use of cannabis over thousands of years, the chemical structure of THC was not discovered until recently (Gaoni and Mechoulam, 1964). Currently, over 85 different phytocannabinoids have been identified in

Cannabis sativa plant (El-Alfy et al., 2010), and synthetic cannabimimetic drugs have also been produced. This huge amount of phytocannabinoids with closely related structures (Figure 10) and physical properties was probably the main difficulty encountered in the discovery and isolation of THC.

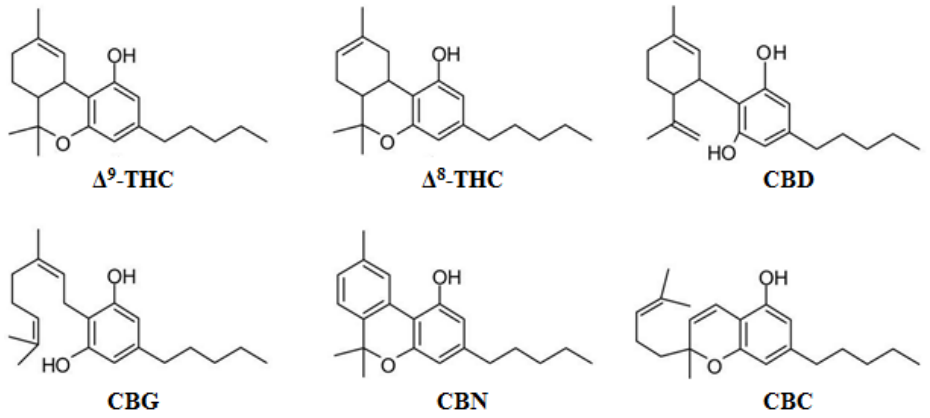

Figure 10. Chemical structure of the most representative phytocannabinoids: THC, Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), and cannabichromene (CBC).

Depending on the effects produced when bound to a receptor it is possible to differentiate between agonists, inverse agonists, and antagonists. By definition, an agonist is a compound that binds to a receptor and activates the receptor producing a biological response, and the elicited response can be maximal (full agonist) or partial (weak or partial agonist). When an inverse agonist binds to the receptor it produces a pharmacological response opposite to the one induced by the agonist. And an antagonist is a compound that does not provoke a response by itself when binds to the receptor, but it is able to block agonist- and inverse agonist-mediated responses.

According to their chemical structure, cannabinoid agonists are classified as classical, non-classical, and aminoalkylindoles (Pertwee et al., 2010), and the chemical structure of the most representative compound of each group is found in figure 11.

- The classical group consists of dibenzopyran derivatives. It includes THC, and HU-210 (Figure 11A). THC possesses affinity for both cannabinoid receptors, although it is considered to be a cannabinoid receptor partial agonist due to its lower affinity, intrinsic activity and potency in comparison with HU-210.
- The nonclassical group contains bicyclic and tricyclic analogues of THC that lack a pyran ring. A well-known member of this group is CP55940 (Figure 11B), a compound which presents lower affinity than HU-210 for both cannabinoid receptors although its intrinsic activity is similar to HU-210.
- Members of the aminoalkylindole grup present a structure that differs markedly from those of the classical and nonclassical group. The most widely used member of this group is WIN 55,212-2 (Figure 11C), which present an intrinsic activity similar to CP55940 and HU-210.

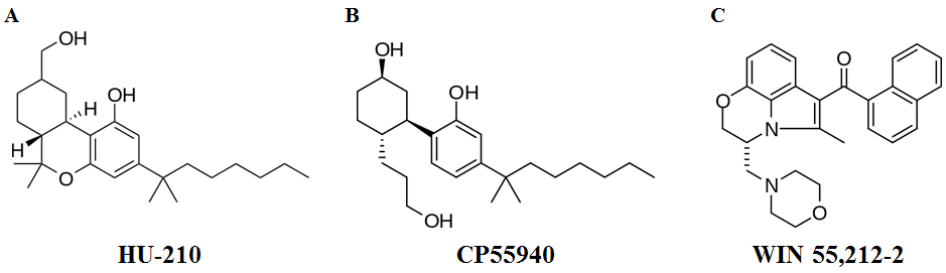

Figure 11. The chemical structure of the cannabinoid agonists the most representative of each group.

Selective antagonists for the different cannabinoid receptors, mainly cannabinoid type 1 (CB_1), and type 2 (CB_2) receptors (See 3.2.1. Cannabinoid receptors) have been synthesized, as the modulation of cannabinoid receptors could be advantageous due to the wide range of effects regulated by the endocannabinoid system. Several compounds such as rimonabant (SR141716A) (Figure 12), AM251, AM281, LY320135 and taranabant can block agonist-induced activation of CB_1 receptor in a competitive manner. These compounds present greater affinity for CB_1 than for CB_2 and are therefore characterized as CB_1-selective competitive antagonists. In some cases, these compounds have been found to act as inverse agonists as they induce opposite effects to those produced by CB_1 agonists (Fong et al., 2007). This may reflect the ability of these compounds to decrease the spontaneous coupling of CB_1 receptors to their effector mechanisms in the absence of any CB_1 agonist. Consequently, the development of neutral antagonists, which only produce the blockade of agonist-induced effects, avoiding the opposite responses has been prioritized. The compound NES 0327 is one example of such a neutral CB_1 antagonist. On the other hand, some compounds can block in a more potent way CB_2 than CB_1 receptor activation. AM630, SR144528 are two examples of CB_2-selective competitive antagonists, although both compounds can produce inverse agonist actions in CB_2 receptors (Rinaldi-Carmona et al., 1998). Neutral

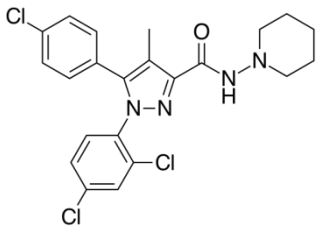

Figure 12. Chemical structure of the CB_1 antagonist rimonabant.

antagonists that selectively target CB_2 receptors have not been developed yet.

In addition, synthetic cannabinoid agonists displaying different activity and selectivity for the cannabinoid receptors have been generated and they represent excellent tools in order to advance towards the understanding of the endocannabinoid system. Three synthetic analogs of anandamide are the most common CB_1 selective compounds used due to their potency and intrinsic activity on CB_1 receptors. These compounds are: R(+)-methanandamide, arachidonyl-2'-chloroethylamide and arachidonylcyclopropylamide (Abadji et al., 1994; Hillard et al., 1999). On the other hand, the most frequently used as CB_2 receptor agonists are JWH-133, JWH-015 and AM1241.

3.2. The endocannabinoid system

For a long time, cannabinoid compounds were supposed to exert their biological effects directly through action into cell membranes, and it was not until the end of 1980s and beginning of 1990s when the cannabinoid receptors were identified. This discovery was followed by the characterization of their endogenous ligands, which are referred to as the endocannabinoids, clearly distinct from the phytocannabinoids or their synthetic analogs. Hence, the endocannabinoid system consists of the endocannabinoids, along with their receptors, as well as the enzymes involved in their synthesis and catabolism. The endocannabinoid system is known to be involved in a large variety of biological functions, including brain development, organogenesis, control of energy

expenditure, as well as, in regulating pain perception and stress responses among others, and it also plays a major role in the modulation of several brain neurotransmitter systems.

3.2.1. Cannabinoid receptors

Initially, it was assumed that cannabinoids produced their effects through a non-specific membrane-associated mechanism. However, it was uncovered that cannabinoids were able to inhibit adenylyl cyclase activity in proportion to their pharmacological effects (Howlett et al., 1986), suggesting that this action was produced through receptors. Shortly after, the existence of cannabinoid binding sites was reported in the brain (Devane et al., 1988), distributed in a consistent manner according to the pharmacological properties of psychotropic cannabinoids (Herkenham et al., 1990). Finally, the orphan G protein-coupled receptor (SKR6) was found to mediate the pharmacological effects of THC, establishing the identity of the first cannabinoid receptor which is known as CB_1 (Matsuda et al., 1990). Three years later, another G protein-coupled receptor (CX5) was identified, the CB_2 as a peripheral receptor initially found in the spleen (Munro et al., 1993).

3.2.1.1. Cannabinoid CB_1 receptors

CB_1 receptors are one of the most abundant seven-transmembrane domain receptors in the central nervous system, and the highest densities of CB_1 receptors have been observed in the basal ganglia, cerebellum, hippocampus, substantia nigra and globus pallidus. They have also been found in other central areas such as cortex, caudate putamen, amygdala,

thalamus and hypothalamus (Herkenham et al., 1991; Pertwee, 1997). Recent studies using positron emission tomography (PET) have been used to characterize CB_1 distribution in the human brain (Burns et al., 2007; Terry et al., 2010) (Figure 13).

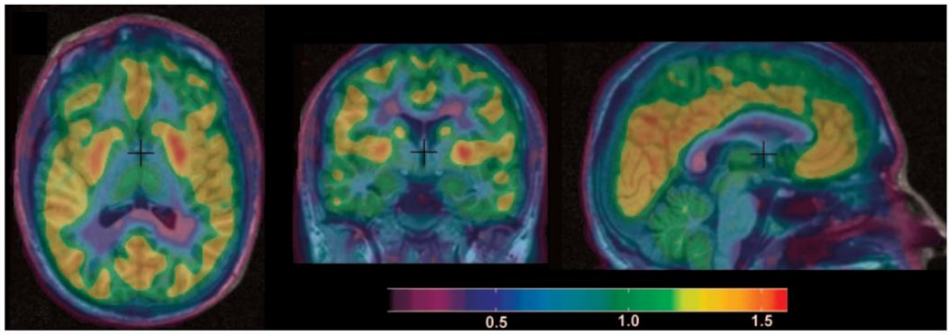

Figure 13. Distribution of the [18F]MK-9470 binding in human brain. [18F]MK-9470 is a selective, high affinity, inverse agonist for the CB1 receptor, specially used for PET studies (Burns et al., 2007).

CB_1 receptor expression is mainly restricted to presynaptic terminals, where they modulate the release of a wide range of neurotransmitters, usually by promoting the inhibition of their release (Vaughan and Christie, 2005; Szabo and Schlicker, 2005), although these receptors have been recently localized in astrocytes (Navarrete and Araque, 2010) and mitochondria (Bénard et al., 2012). CB_1 receptors expressed in glutamatergic terminals are responsible for THC-induced effects on locomotion, body temperature, analgesia and catalepsy (Monory et al., 2007), whereas CB_1 expressed in GABAergic terminals are reported to be crucial for the memory deficits induced by THC (Puighermanal et al., 2009). They are also involved in natural reward processes (De Chiara et al., 2010), as well as, stress mechanisms (Rossi et al., 2008). Although being predominantly expressed in the central nervous system (CNS), CB_1

receptors are also expressed in peripheral tissues, including the heart, lung, liver, adrenal gland, prostate, testis, uterus, ovaries, immune and vascular system, adipocytes and all the tissues involved in the control of metabolism (Pertwee et al., 2010).

3.2.1.2. Cannabinoid CB_2 receptors

Originally, it was reported that CB_2 receptors were expressed exclusively in cells of the immune system, and were thought to be absent in neurons (Munro et al., 1993). However, low levels of CB_2 receptors have been identified throughout the CNS (Van Sickle et al., 2005; Ashton et al., 2006; Onaivi et al., 2008), although the functional role of these central receptors is still not clarified (Atwood and Mackie, 2010). It has been suggested that CB_2 receptors act as a part of a protective system, as their expression is enhanced under some pathological conditions (Pacher and Mechoulam, 2011). Studies in animals using CB_2 antisense oligonucleotides revealed a role for these receptors in anxiety (Onaivi et al., 2006a), locomotion (Onaivi et al., 2006b), and reward (Navarrete et al., 2013). Moreover, it has been shown that the activation of CB_2 receptors inhibits neuronal firing of dorsal root ganglia neurons and dorsal horn neurons in neuropathic rats (Elmes et al., 2004; Sagar et al., 2005). Consistent with these results, studies performed in animals lacking CB_2 receptors show their involvement in neuropathic (Racz et al., 2008a; Racz et al., 2008b), and osteoarthritic pain (La Porta et al., 2013).

3.2.1.3. Other members of the cannabinoid receptor family

Increasing observations show that some cannabinoid responses are mediated by mechanisms different from the known cannabinoid CB_1 and CB_2 receptors, suggesting that additional receptor types should exist to explain ligand activity in a number of physiological processes. The orphan G protein-coupled receptor GPR55 has been suggested as a possible new cannabinoid receptor (Baker et al., 2006; Ryberg et al., 2007). These receptors are mainly expressed in adrenal tissue, ileum, jejunum and also in some brain areas such as the frontal cortex and striatum in a much lower level than CB_1 receptors (Ryberg et al., 2007). There is still controversy regarding their pharmacological properties and signalling pathway.

Moreover, sphingosine-1-phosphate lipid receptors GPR3, GPR6 and GPR12 (Kostenis, 2004), as well as, the transient receptor potential vanilloid type 1 (Di Marzo and De Petrocellis, 2010), have also been suggested to participate in the pharmacological responses induced by cannabinoid compounds.

3.2.2. Endogenous cannabinoids

The discovery of the cannabinoid receptors suggested the presence of endogenous molecules, which may exert their function through activating or inhibiting these receptors. The first endocannabinoid characterized, was named anandamide, from the Sanskrit word *ananda*, which means "pure happiness, interior joy" (Devane et al., 1992). Shortly

after, a second molecule, 2-arachidonoylglycerol (2-AG) was also identified as an endocannabinoid (Mechoulam et al., 1995). Both endocannabinoids present higher affinity for CB_1 than for CB_2, and 2-AG is more potent than anandamide, which has been considered to behave as a partial agonist for both CB_1 and CB_2 receptors. The chemical structure of both compounds is found in figure 14. Since then, other putative endocannabinoids have also been suggested, such as N-arachidonoyl dopamine (Huang et al., 2002), or O-arachidonoylethanolamine (Porter et al., 2002).

Unlike the majority of neurotransmitters, anandamide and 2-AG are not stored in vesicles but rather are synthesized on demand when they are

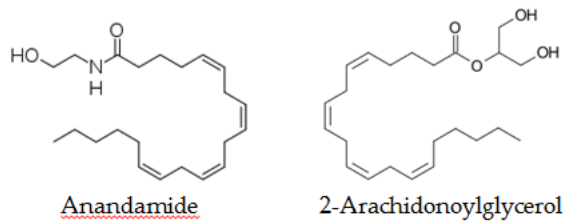

Figure 14. Chemical structures of the most well-known and more studied endogenous cannabinoids.

needed (Di Marzo et al., 2005). In contrast to other neurotransmitters, endocannabinoids are released in postsynaptic terminals, and their action is mostly presynaptic acting like rapid retrograde synaptic messengers in order to regulate the release of other neurotransmitters. According to this rapid neuromodulatory effect, endocannabinoid levels need to be finely regulated through balancing its synthesis and degradation.

3.2.3. Enzymes involved in the synthesis and degradation of endocannabinoids

Different enzymes are involved in the synthesis and degradation of anandamide and 2-AG, as it is represented in figure 15. Anandamide synthesis occurs as a consequence of the hydrolysis of its phospholipid precursor N-arachidonoyl-phosphatidylethanolamine (NAPE) by the action of a specific phospholipase D (Di Marzo et al., 1994). 2-AG is synthesized from diacylglycerol (DAG), which is hydrolysed by a diacylglycerol lipase (DAGL) consequently generating 2-AG. Once released from cells and upon activation of their molecular targets to prevent excessive neuronal activity, therefore maintaining neuronal homeostasis, endocannabinoids need to be rapidly inactivated. However, in order to be hydrolysed, they first need to be cleared away from the receptor and taken up by the cell. This process occurs via rapid diffusion through the cell membrane, although it can also be facilitated by the presence of a membrane transporter by a mechanism not completely characterized (Fu et al., 2012; Marsicano and Chaouloff, 2012). This reuptake system has also been suggested as a possible endocannabinoid release system (Hillard et al., 1997).

After its reuptake in the cell, endocannabinoids are degraded by the effect of specific hydrolases. Anandamide is hydrolyzed to arachidonic acid and ethanolamine by fatty acid amine hydrolase (FAAH) (Cravatt et al., 1996), and 2-AG is mainly hydrolysed by the monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol (Nomura et al., 2008) (Figure 15). The fact that FAAH seems to be most abundant on neurons

postsynaptic to CB_1 receptors, suggest that anandamide might act principally on these neurons (Egertova et al., 2003). Therefore, it is not clear if both endocannabinoids are able to act as retrograde synaptic messengers, or if this effect is only exhibited by 2-AG, which degradation by MAGL is produced at presynaptic level. What seems clear is that the selective inhibition of the endocannabinoid-hydrolysing enzymes can prolong the effects of anandamide (Fegley et al., 2005) and 2-AG (Straiker et al., 2009).

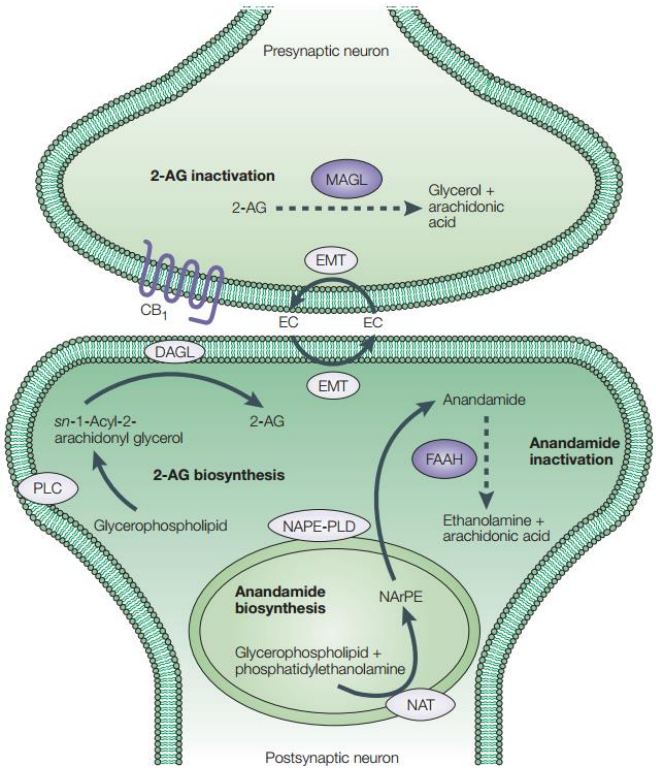

Figure 15. Anabolic and catabolic pathways of endocannabinoids (Di Marzo et al., 2005).

3.3. Behavioural effects of cannabinoids

Due to the extensive distribution of the cannabinoid receptors through the CNS and different peripheral tissues, the endocannabinoid system is involved in numerous physiological processes. The widespread presence of CB_1 receptors at central level is responsible for their variety of effects. Thus, CB_1 expression in the basal ganglia and cerebellum has been related with control of fine and precise movements as well as motor coordination (Rodriguez de Fonseca et al., 1998). Moreover, it has also been associated with the modulation of emotions and motivation (Mechoulam and Parker, 2013), and rewarding properties of both natural (Bellocchio et al., 2010), and non-natural rewards (Maldonado et al., 2011). CB_1 expression in the hippocampus has been widely investigated because of the effects of cannabis on learning and memory (Kano et al., 2009; Puighermanal et al., 2009). The role of endocannabinoid system in anxiety and emotional fear responses has been correlated with the expression of CB_1 receptors in the amygdala (Rubino et al., 2008; McLaughlin et al., 2014; Ratano et al., 2014). Importantly, the endocannabinoid system is also involved in pain modulation, and can be crucial in identifying novel pharmacotherapies for the treatment of pain (Guindon and Hohmann, 2009). Besides, acting at peripheral level, the endocannabinoid system modulates the immune and cardiovascular systems; it affects gastrointestinal motility and metabolism, and also has effects in the liver, the adipose tissue or the reproductive system among others (Grotenhermen and Muller-Vahl, 2003). This section will be focused mainly on the role of endocannabinoid system and the

behavioural effects of cannabinoid compounds in reward, anxiety and cognition.

3.3.1. Cannabinoids in rodents, the "tetrad" test

Little and colleagues (1988) developed a battery of in vivo behavioural tests, which collectively provided a sensitive index of drug affinity and efficacy at centrally located cannabinoid receptors. Although the full battery includes various tests in different species, the primary pool of tests is known as the "rodent tetrad test", because four different effects are produced by prototypical cannabinoid agonists such as THC, WIN 55,212-2 and CP55940: hypoactivity, catalepsy, hypothermia and antinociception. These effects are reversed by SR 141716, providing evidence for the involvement of CB_1 receptors in these behaviours. The exogenous administration of anandamide has been shown to produce similar effects, although with much lower potency and duration (Crawley et al., 1993; Fride and Mechoulam, 1993; Smith et al., 1994). More recent studies with FAAH and MAGL inhibitors have revealed that an increase in endocannabinoid levels can produce similar behavioural responses at this tetrad test. Nevertheless, there are remarkable differences between the behavioural effects of endocannabinoids and CB_1 agonists, probably due to the rapid metabolism of the endogenous compounds.

Among the four effects included in the tetrad test, the antinociceptive properties of cannabinoid compounds are the most relevant for their possible therapeutic application in human patients. Indeed, the

endocannabinoid system has been localized in multiple regions involved in nociceptive responses (Hohmann, 2002). Cannabinoids have demonstrated antinociceptive properties at different levels including supraspinal, spinal and peripheral. The antinociceptive properties of endocannabinoids have also been shown in different types of pain (Hohmann and Suplita, 2006; Guindon and Hohmann, 2009), although these effects differ depending on the dose, the compound and the test used. The blockade of CB_1 receptors has been shown to produce hyperalgesia under specific experimental conditions, which provides support for a physiological role of these receptors on pain modulation (Guindon and Hohmann, 2009). Moreover, CB_2 receptors have also been implicated in neuropathic (Racz et al., 2008a; Racz et al., 2008b), inflammatory (Pini et al., 2012), and osteoarthritic pain (La Porta et al., 2013).

3.3.2. Cannabinoids and anxiety

According to the DSM-5, anxiety is a feeling of fear, worry, and uneasiness, usually generalized and unfocused, or as an overreaction to a situation that is only subjectively seen as menacing. It is often accompanied by muscular tension, restlessness, fatigue, and problems in concentration, and despite being physiological, when continued for a long period of time it can provoke an anxiety disorder. In humans, THC may cause either euphoria and relaxation or dysphoria and anxiety (Wade et al., 2003; D'Souza et al., 2004). These biphasic properties of THC have also been revealed in experimental animals. It has been shown that low doses of THC induce anxiolytic-like effects, whereas higher

doses produce an anxiogenic profile (Viveros et al., 2005; Rubino et al., 2007). The study of anxiety-like behaviour in animals is frequently based on the conflict of two opposing innate motivations: on the one hand, the drive to explore a novel environment (in order to obtain food, water, shelter, escape, or to find mating partners), and on the other hand, the avoidance of potentially dangerous places or situations. The most widely used behavioural paradigms to study anxiety-like responses are the elevated plus maze (EPM), the light-dark box and the open field test. All of these tests measure avoidance of aversive compartments, such as elevated open arms in the EPM, bright lit compartment in the light-dark box or the centre of the arena in the open field. In these paradigms, the administration of anxiolytic drugs shifts the balance between approach and avoidance towards approach responses, and the administration of cannabinoid compounds have also been found to modify anxiety-like behaviour in these animal models. Several studies in animals using specific inhibitors of endocannabinoid-hydrolyzing enzymes have been performed to study the role of endocannabinoids on anxiety. Potent and selective FAAH inhibitors (Kathuria et al., 2003) had anxiolytic properties in different anxiety tests (Moreira et al., 2008). Consistent with this result, the direct injection of methanandamide into the prefrontal cortex of rats leaded to anxiolytic-like responses (Rubino et al., 2008). Moreover, the release of anandamide has been observed in the amygdala in response to anxiogenic situations (Gaetani et al., 2003), confirming the regulatory role of endocannabinoids on anxiety. Regarding the role of 2-AG, it has also been shown that increasing the levels of 2-AG with a

MAGL inhibitor produced anxiolytic-like effects in rats (Sciolino et al., 2011). Furthermore, the effects of the FAAH inhibitor URB597 were mediated through a CB_1-dependent mechanism, whereas the anxiolytic-like effects induced by MAGL inhibitor JZL184 were dependent on CB_2 receptors (Busquets-Garcia et al., 2011). Moreover, mice overexpressing CB_2 receptors showed lower anxiety-like behaviour in different paradigms (García-Gutiérrez and Manzanares, 2011) confirming the crucial role of CB_2 receptors on the modulation of anxiety behaviour.

Thus, the putative anxiogenic-like effects induced by blockade of CB_1 receptors had remarkable consequences for rimonabant, a CB_1 antagonist developed by Sanofi-Aventis to combat obesity. Albeit its promising results in body weight loss and reduction of several cardiometabolic risk factors in obese patients, rimonabant was suspended in Europe, and not even approved by the food and drug administration in the USA because of its important emotional side effects. Increased incidence of depression-related mood disorders, anxiety problems and suicidal tendencies were found in rimonabant-treated patients (Christensen et al., 2007). It was suggested that rimonabant's drawbacks were a consequence of its inverse agonist properties on CB_1 receptors at central level. Hence, novel approaches using peripheral CB_1 receptor antagonists and pure neutral CB_1 receptor antagonists are being currently considered.

3.3.3. Cannabinoids and cognition

Cannabis affects cognitive performance, attention, working memory and cognitive flexibility in humans (Lundqvist, 2005). Additionally, a transient impairment in short-term episodic and working memory, as

well as, deficits in consolidating these short-term into long-term memories have been reported under the effects of THC (Hall et al., 1999; Ilan et al., 2004; Lundqvist, 2005). However, retrieval of previously encoded memories is not affected (Ranganathan and D'Souza, 2006). Importantly, the deficits observed differ in severity depending on the quantity and duration, as well as, the age of the onset of cannabis use (Crean et al., 2011). These factors along with the use of widely differing methodologies to determine potential cognitive-deficits, participant selection strategies, and the lack of appropriate controls, make it difficult to draw conclusions from the different studies performed in humans. For instance, some studies conclude that chronic marijuana use is associated with dose-related cognitive impairments (Solowij and Battisti, 2008), whereas other reports indicate that few impairments on cognition are produced after years of heavy cannabis use (Dregan and Gulliford, 2012). Indeed, there is one unique study that has been performed in "pure" cannabis users (Fried et al., 2005). These authors, conducted a longitudinal examination in young adults before and after marijuana use, and found that cannabis-induced cognitive impairment was present in current heavy users, but not in individuals that had already ceased cannabis consumption. Concerning the effects of chronic cannabis consumption, a recent meta-analysis including several longitudinal studies, revealed a clear and consistent association between the frequency of cannabis use during adolescence and adverse young adult outcomes, such as a lower educational achievement, lower income attainment, and unemployment. The authors suggested that adolescent

cannabis use is linked to difficulties in successfully completing the tasks that mark the transition to adulthood (Silins et al., 2014).

Therefore, experimental investigation on the effects of cannabinoids on various cognitive processes such as learning and memory rely heavily on animal models. Additionally, these models can be extremely useful to determine the role of the endocannabinoid system in such cognitive processes. Acute administration of CB_1 receptor agonists impairs short-term and working memory in animal models without affecting retrieval of previously acquired memories (Mechoulam and Parker, 2013). One of the cognitive tests used in rodents is the novel object recognition task, which does not rely on prior operant training, but on the natural preference of rodents to explore novel objects. In this test, amnesic-like effects were induced by acute (Puighermanal et al., 2009), and chronic (Puighermanal et al., 2013) THC administration, and these effects were blocked by rimonabant. Other studies have focused on evaluated using the Morris water maze and the 8-arm radial maze. The Morris water maze is the most common test to study spatial memory. In this test, animals navigate in a pool of water to locate a hidden platform by learning its location based on the presence of visual cues in previous trials. The administration of THC disrupted both, working and reference memory in this test (Varvel et al., 2001). This effect was also observed in rodents treated with WIN 55,212-2 and methanandamide, and was subsequently blocked by the administration of rimonabant (Varvel and Lichtman, 2002). The involvement of CB_1 receptors in the memory impairment produced by cannabinoids was confirmed in genetic studies

using CB_1 knock-out mice (Varvel and Lichtman, 2002). Interestingly, animals treated chronically with THC did not develop tolerance to its effects on memory (Boucher et al., 2009; Puighermanal et al., 2013). The 8-arm radial maze requires previous training sessions for the rats to learn which arm contains food reward. Using this test, it has been revealed that low doses of THC produced an increase in the number of errors counted as arm-reentries, and these effects are also blocked by antagonizing CB_1 receptors (Lichtman and Martin, 1996). Recent studies with chronic THC administration in adolescent rats revealed an impairment in spatial working memory even when the 8-arm radial test was performed 30 days after the treatment (Rubino et al., 2009).

The decrement in memory produced by cannabinoids appears to be directly related with their action in the hippocampus. As previously mentioned, this structure is one of the brain regions with a higher density of CB_1 receptors, and most of the experimental paradigms where cannabinoids produce memory impairments are known to be hippocampal-dependent (Davies et al., 2002; Zanettini et al., 2011). Accordingly, intracranial administration of CB_1 agonists directly into the hippocampus produces impairments in working memory performance in several tests such as the Morris water maze (Abush and Akirav, 2010), the 8-arm radial test (Lichtman et al., 1995; Wegener et al., 2008), T-maze alternation (Suenaga et al., 2008), and object recognition memory (Clarke et al., 2008). At a cellular level, CB_1 receptor expression in different cell types (Marsicano and Lutz, 1999; Kawamura et al., 2006), might represent a crucial element for the effects of cannabinoids in cognition.

Moreover, CB_1 receptors are more abundant on GABAergic inhibitory terminals than on glutamatergic excitatory synapses (Kawamura et al., 2006; Bellocchio et al., 2010). In addition, THC acts as a full agonist at CB_1 receptors present on GABAergic terminals, whereas it acts as a partial agonist on glutamatergic terminals (Laaris et al., 2010), and glutamatergic CB1 receptors are more sensitive to agonist-induced activation (Ohno-Shosaku et al., 2002; Lee et al., 2010), and more effective in terms of G protein coupling (Steindel et al., 2013), than CB1 receptors in GABAergic neurons. In this regard, studies using mutant mice specifically lacking CB_1 receptors in glutamatergic or GABAergic neurons (Monory et al., 2006) have reported that THC-induced amnesic-like effects are dependent on CB_1 receptors present in GABAergic neurons, as GABA-CB_1 KO animals did not present the memory impairments induced by THC (Puighermanal et al., 2009). However, another study has also revealed that CB_1 receptors expressed in astroglial cells were crucial for the THC-induced impairment of working memory (Han et al., 2012). According to the differences in CB_1 receptor expression levels and sensitivity, it has been suggested that the balance between GABAergic and glutamatergic activity is crucial at least in the disrupting effects of THC in recognition memory (Puighermanal et al., 2009).

The findings that CB_1 receptor agonists impair working memory suggest that blocking these receptors might lead to an enhancement of short-term memory. Accordingly, CB_1 receptor antagonism was reported to produce memory enhancement in mice in an olfactory recognition task (Terranova et al., 1996), and in the 8-arm radial maze (Lichtman, 2000). In

addition, CB_1 knock-out mice are able to retain memory in the novel object recognition task for at least 48h whereas wild-type animals fail at object retention after 24h (Reibaud et al., 1999). In contrast, other studies have revealed that rimonabant did not produce any improvements in memory (Mallet and Beninger, 1998a). One possible explanation for these mixed findings is the different temporal requirements of the tasks used. It has been suggested that rimonabant may facilitate memory retention by prolonging the duration of memory without facilitating learning (Varvel et al., 2009). Therefore, tasks requiring rapid learning of new information will not be affected by blockade of CB_1 receptors, whereas memories tested long time after its retention might be enhanced.

However, when emotional memory processes are involved, the effects of cannabinoids do not seem to follow this pattern of action (Chhatwal and Ressler, 2007; Lutz, 2007). Indeed, the infusion of CB_1 agonists directly into the basolateral amygdala enhanced consolidation of inhibitory avoidance learning (Campolongo et al., 2009), and a similar effect happens in extinction learning, where the administration of cannabinoid agonists facilitated extinction of contextual fear memory (Pamplona et al., 2006). In contrast, CB_1 knock-out mice and wild-type mice treated with rimonabant showed impaired extinction in fear-conditioning tests (Marsicano et al., 2002). These data indicate that the endocannabinoid system plays a distinct role in brain structures mediating different types of memory processes.

3.4. Involvement of the serotonergic system on cannabinoid-induced behavioural effects

Several findings have linked the serotonergic and the endocannabinoid systems during the last decade. In this line, studies performed in cells stably expressing 5-HT_{2A} receptors, and brain cells from the inferior olive, which present a high expression of 5-HT_{2A} receptors, revealed that the activation of 5-HT_{2A} receptors induces endocannabinoid release (Parrish and Nichols, 2006; Best and Regehr, 2008). The serotonergic system has been involved in several cannabinoid-induced effects. Indeed, hypothermia induced by THC in rats was potentiated by the subcutaneous administration of the serotonin 1A receptor (5-HT_{1A}) antagonist, WAY 100635 (Malone and Taylor, 2001). However, neither WAY 100635, nor the 5-$HT_{1A/7}$ agonist 8-hydroxy-(din-propylamino) tetralin (8-OH-DPAT) have any significant effect on THC-induced hypothermia when microinjected in the dorsal raphe nuclei. In contrast, when microinjected in the median raphe nuclei, WAY 100635 potentiated, and 8-OH-DPAT significantly inhibited THC-induced hypothermia (Malone and Taylor, 2001), revealing the involvement of 5-HT_{1A} in the modulation of this THC effect.

Catalepsy, which is another THC-induced behaviour included in the "tetrad", is also modified by the serotonin system. Thus, the administration of 8-OH-DPAT inhibited THC-induced catalepsy in mice, and this effect was reversed by WAY 100635, but not by the selective 5-HT_7 antagonist, SB 269970 (Egashira et al., 2006), suggesting the involvement of 5-HT_{1A} receptors in THC-induced catalepsy. Moreover,

another study from the same laboratory also demonstrated the involvement of 5-HT_{2A} receptors in THC-induced catalepsy, as the administration of the 5-$HT_{2A/2C}$ receptor agonist, DOI significantly inhibited THC-induced catalepsy in mice. The THC-induced catalepsy inhibition by DOI was reversed by the 5-HT_{2A} antagonist, ketanserine, but not by the selective serotonin 2C receptor antagonist, SB 242084 (Egashira et al., 2007). In this study, ketanserine enhanced the catalepsy-like effect induced by THC, suggesting that 5-HT_{2A} receptors might also be involved in THC-induced catalepsy.

THC-induced impairment of spatial memory could also be mediated by 5-HT_{2A} receptors. The administration of DOI in rats significantly attenuated the spatial memory impairment induced by THC in the 8-arm radial maze (Egashira et al., 2002), and a posterior study performed in the same laboratory revealed that the administration of 8-OH-DPAT also prevented this impairment (Inui et al., 2004). Importantly, it has been revealed that the interaction between the serotonin and the endocannabinoid system could be reciprocal. Thus, the administration of cannabinoid compounds reduced DOI-induced behaviours such as head twitches and ear-scratching responses in mice in a dose-dependent manner (Darmani, 2001). Moreover, animals lacking CB_1 receptors presented a reduced number of DOI-induced head twitches (Mato et al., 2007). Taken together, evidence support a bilateral interaction between the endocannabinoid and serotonergic systems, with a prominent role for serotonin 5-HT_{1A} and 5-HT_{2A} receptors, which will be further developed in the discussion.

3.5. Cannabinoids and reward system

The rewarding properties of cannabinoids are well documented. Human subjects report feelings of "high", well-being and euphoria following the administration of THC or cannabis extracts (Ward et al., 1997; Haney et al., 1997; Hart et al., 2005). As previously stated, a key feature of all known drugs of abuse is the ability to stimulate mesolimbic dopamine neurotransmission, which is thought to mediate their rewarding properties. It has been shown that cannabinoids produce this effect in rats (Tanda et al., 1997; Tanda and Goldberg, 2003), and recently, it has also been confirmed that THC induces dopamine release in the human striatum (Bossong et al., 2009). Regardless of this evidence, rewarding effects of THC or other cannabinoid agonists have been difficult to demonstrate in animal models.

Conditioned Place Preference

Studies using the CPP paradigm reported that THC was either rewarding or aversive depending on the dose, and on the regimen of administration in rats (Lepore et al., 1995; Braida et al., 2001). Several studies reported aversive properties of cannabinoid agonists in rats (Parker and Gillies, 1995; McGregor et al., 1996; Mallet and Beninger, 1998b; Cheer et al., 2000). However, a more recent study has reported that rats housed under environmental enrichment shifted preference towards a WIN 55,212-2 associated compartment in comparison with animals housed in standard conditions (Bortolato et al., 2006). An advance in the understanding of the rewarding and aversive properties of cannabinoids in mice was reported by Valjent and Maldonado (2000).

In this study, pre-exposing the animals with a low THC dose was necessary to establish CPP in mice, suggesting that the first administration of even a low dose of THC to mice could induce aversion preventing subsequent CPP.

Self-administration of cannabinoids

Early research in monkeys suggested that THC was not reinforcing (Harris et al., 1974), failing to demonstrate that intravenous drug self-administration could be maintained by THC or other cannabinoid agonists in this species (Carney et al., 1977; Takahashi and Singer, 1979; 1980; Mansbach et al., 1994). In 1998, the first evidence of self-administration behaviour of the cannabinoid agonist WIN 55,212-2 was reported in mice (Martellotta et al., 1998). However, this experiment was performed under restraining conditions and it was not until recently, that self-administration was demonstrated in catheterized mice during repeated daily testing (Mendizabal et al., 2006). In rats, reliable self-administration of WIN 55,212-2 was reported in Long Evans (Fattore et al., 2001), and in Sprague-Dawley strains (Lecca et al., 2006). THC self-administration directly into the posterior part of the VTA or into the shell of the NAc was also maintained by operant responding in rats (Zangen et al., 2006). In non-human primates, the first report of THC self-administration was obtained in squirrel monkeys with a history of cocaine self-administration (Tanda et al., 2000), and later in naïve animals without drug experimental history (Justinova et al., 2003).

Drug discrimination

Another experimental model used to characterize the abuse-related effects of cannabinoids is the two-lever choice drug discrimination procedure, which measures subjective effects (Jarbe and Henriksson, 1974; Wiley et al., 1995; Burkey and Nation, 1997; Alici and Appel, 2004). Although discriminative stimulus effects of drugs are not a direct measure of reward, these procedures appear to have good predictive validity for self-administration of drugs of abuse (Solinas et al., 2006). Given the difficulties of establishing replicative models of cannabinoid self-administration and CPP in rodents, drug discrimination could be considered as a useful alternative.

3.5.1. Rewarding effects of endocannabinoids

Several preclinical studies have investigated whether endocannabinoid compounds produce rewarding effects. Like THC, anandamide and its synthetic analogue methanandamide are intravenously self-administered in squirrel monkeys (Justinova et al., 2005), and this effect was sensitive to pharmacological blockade of CB_1 receptors. Recently, the self-administration of 2-AG has also been reported by the same group (Justinova et al., 2011). Using CPP, neither anandamide (Mallet and Beninger, 1998b) nor the FAAH antagonist URB 597 (Gobbi et al., 2005) produced place conditioning effects. The effects of rimonabant in CPP are not clear as some studies have found no effects after administering the CB_1 antagonist in rats (Chaperon et al., 1998) or mice (Mas-Nieto et al., 2001), whereas in other studies, the administration of rimonabant

induced CPP in rats (Sañudo-Peña et al., 1997; Mallet and Beninger, 1998b; Cheer et al., 2000). However, it is not possible to discern whether the rewarding effect of rimonabant was produced by blocking CB_1 receptors or by acting as an inverse agonist (Landsman et al., 1997). The studies performed using the drug discrimination paradigm revealed that synthetic analogues of anandamide, but not anandamide were able to substitute for the effects of THC in rats (Burkey and Nation, 1997), indicating that anandamide's fast metabolic inactivation could account for its lack of effect. Indeed, anandamide produced THC-like discriminative effects in rats, when its metabolism was blocked by URB 597 (Solinas et al., 2007). Remarkably, the administration of URB 597 by itself did not produce any THC-like effect in drug discrimination (Gobbi et al., 2005).

3.5.2. Neurobiological mechanisms involved in the rewarding properties of cannabinoids

Dopaminergic system

THC and other cannabinoid agonists are able to stimulate dopaminergic mesolimbic neurotransmission (Tanda et al., 1997). This stimulation has been shown to correspond with an increase in the firing rate of VTA dopamine neurons (French et al., 1997; Gessa et al., 1998; Wu and French, 2000). Cannabinoids also increase phasic dopamine neurotransmission (Cheer et al., 2004). Thus, transient increases in dopamine release are a consequence of high-frequency bursts of dopamine neural activity (Gonon, 1988; Sombers et al., 2009). THC and WIN 55,212-2 both

increased the frequency of bursts, as well as, the number of spikes during each activity burst (Gessa et al., 1998). One of the most recent models to explain the modulation of the dopamine system by cannabinoids suggests that cannabinoids increase dopamine release by indirectly disinhibiting dopamine neurons (Lupica and Riegel, 2005). Supporting this model, the administration of WIN 55,212-2 in rat VTA slices decreased GABAergic inhibitory postsynaptic currents (Szabo et al., 2002). CB_1 receptors are located presynaptically on both glutamatergic and GABAergic neurons in the VTA, and thus modulate excitatory and inhibitory inputs on the mesolimbic dopaminergic neurons.

Endogenous opiod system

The endogenous opioid system is comprised of three receptors, mu-, delta- and kappa-opioid receptors, and of several endogenous ligands derived from three different precursors, proopiomelanocortin, proenkephalin, and prodynorphin. An important role for the opioid receptors and their endogenous ligands has been demonstrated in brain reward processes, as well as, in the modulating behavioural and neurochemical effects of several drugs of abuse including cannabinoids (Van Ree et al., 1999; 2000; Trigo et al., 2010). Thus, the discriminative effects of THC were enhanced after systemic administration of heroin or morphine, and attenuated with the opioid receptor antagonist naltrexone (Solinas and Goldberg, 2005). However, a recent study performed in monkeys did not find any modification in THC discriminative effects after heroin or morphine administration (Li et al., 2008). The rewarding effects of THC revealed by CPP were not modified either in delta- or

kappa-opioid receptor knock-out mice (Ghozland et al., 2002). Nevertheless, it was abolished in mice lacking mu-opioid receptors (Ghozland et al., 2002), and in the double knock-out for mu- and delta-opiod receptor (Castañé et al., 2003). In contrast to the involvement of opioid receptors in cannabinoid rewarding properties, kappa-opioid receptors might mediate the aversive effects of THC and other cannabinoids (Ghozland et al., 2002; Mendizabal et al., 2006). Thus, dynorphin deficient mice did not develop conditioned place aversion with high doses of THC in comparison to wild-type mice (Zimmer et al., 2001), and show a shift to the left in the dose-response curve in WIN 55,212-2 self-administration paradigm (Mendizabal et al., 2006), indicating increased reinforcement.

Cannabinoid and opioid receptors, especially mu-opioid receptors, show similar brain distributions, co-localize in brain areas involved in motivation (Braida et al., 2001), and share similar second-messenger cascades (Reisine et al., 1996; Howlett, 2002). Moreover, recent demonstrations of allosteric modulation of mu- and delta-opioid receptors (Kathmann et al., 2006), and reductions of CB_1 signalling in mu-opioid receptors knock-out mice (Berrendero et al., 2003), might indicate an interaction of these receptors at cell membrane level. Indeed, the heterodimeric formation of mu-opioid receptors with CB_1 receptors has been recently discovered (Hojo et al., 2008).

Hypocretin / Orexin system

The hypocretin/orexin system consists of two hypocretin receptors (Hcrtr-1 and Hcrtr-2), and their endogenours ligands, hypocretin 1 and hypocretin 2 (Sakurai et al., 1998). Hypocretins are lateral hypothalamic neuropeptides that project through the brain (Peyron et al., 1998). This system has been reported to play a pivotal role in the reward circuits and the reinforcing properties of several drugs of abuse, including cannabinoids (Aston-Jones et al., 2010; Plaza-Zabala et al., 2012; Flores et al., 2014). The pharmacological blockade of Hcrtr-1, but not Hcrtr-2 reduced WIN 55,212-2 self-administration in mice (Flores et al., 2014), and this effect was also observed in knock-out mice for Hcrtr-1. Hypocretin signalling in the VTA seems to be important in the regulation of drug reward-seeking behaviour (Mahler et al., 2012), and high density of Hcrtr-1 is observed in this brain area (Narita et al., 2006; Borgland et al., 2009). In addition, the release of dopamine in the NAc induced by THC was blocked in animals lacking Hcrtr-1 (Flores et al., 2014). A recent article has revealed the formation of heteromers between the CB_1 and the Hcrtr-1 receptors (Ward et al., 2011), suggesting the existence of an important cross-talk between both systems, although further investigations are needed to unveil the potential implications.

3.6. Cannabinoid receptor signalling

Stimulation of cannabinoid receptors produces a wide variety of effects through the activation of several signal transduction pathways (Bosier et al., 2010). When stimulated, both CB_1 and CB_2 receptors mainly mediate their biological effects by activating heterotrimeric $G_{i/o}$ type G proteins.

Their activation reduces cyclic AMP (cAMP) levels by inhibiting the adenylyl cyclase activity, and decrease protein kinase A (PKA) activity (Howlett, 2005). In order to maintain the focus of this thesis, this section will be solely based on the signalling following CB_1 activation.

In spite of CB_1 activation mediates their effects by activating Gi/o type G proteins, CB_1 receptors can also be coupled to other Gα proteins (Figure 16). Indeed, under certain circumstances CB_1 receptor may be able to couple to G_s and G_q (Glass and Felder, 1997; Lauckner et al., 2005), although CB_1 receptors can also exert their actions in a G-protein independent manner, such as coupling to the factor associated with neutral sphingomyelinase (FAN) (Sánchez et al., 2001).

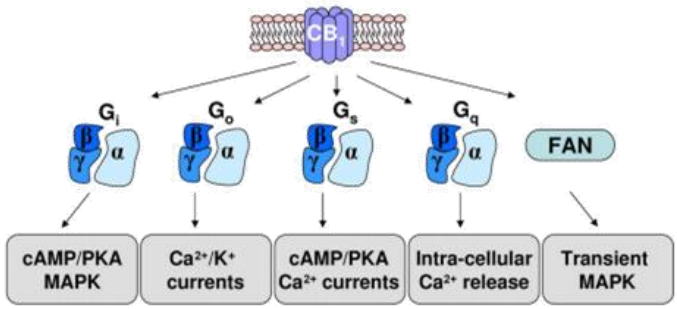

Figure 16. Main signalling pathways downstream the activation of the different Gα proteins by CB_1 (Bosier et al., 2010).

As mentioned, one of the most characterized CB_1 mediated effects through $G_{i/o}$ type G proteins is the inhibition of the adenylyl cyclase, producing a decrease in cyclic AMP production, accompanied by a decrease in PKA activity. Moreover, the activation of CB_1 receptors can stimulate the phosphorilation and activation of various members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 in addition to c-Jun N-

terminal kinase (Bouaboula et al., 1995; Howlett, 2005). The activation of CB_1 receptors can also stimulate the phosphatidylinositol 3-kinase (PI3K) (Bouaboula et al., 1995; Gómez del Pulgar et al., 2000). The stimulation of PI3K can induce the activation of protein kinase C (PKC) through an effect mediated by the action of phospholipase C (PLC) (Hillard and Auchampach, 1994), and the activation of protein kinase B (Akt) and glycogen synthase kinase-3 signalling pathway (Ozaita et al., 2007). Another signalling pathway downstream of Akt activation is the modulation of the mammalian target of rapamycin (mTOR) / ribosomal p70 S6 kinase pathway, which has been related to the regulation of protein synthesis (Puighermanal et al., 2009). By acting on G proteins, CB_1 receptor modulates the activity of several ion channels in the cell surface. It activates the inward-rectifying K^+ and A-type K^+ channels, triggering the repolarization of the plasmatic membrane (Deadwyler et al., 1995; Vásquez et al., 2003). Moreover, CB_1 inhibits N, P/Q and L-type voltage-gated Ca^{2+} channels, leading to a decrease in Ca^{2+} influx (Howlett et al., 2002). A simplification of the signalling pathways activated by the cannabinoid receptor is represented in figure 17.

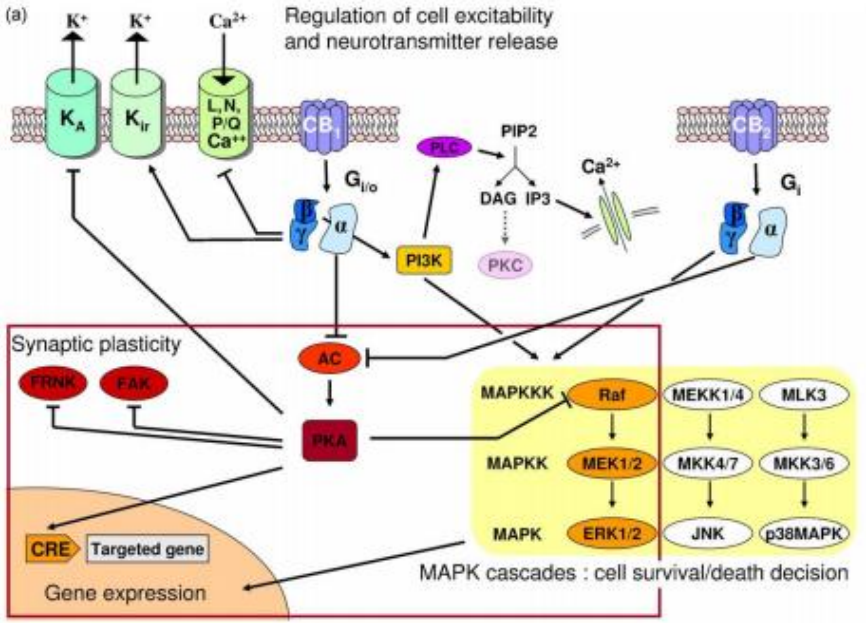

Figure 17. Representation of the cannabinoid receptor signalling pathways.

Finally, the activation of the receptor is able to modulate the lipid composition of the cellular membrane in the surroundings of the receptor (Maccarrone, 2010), and in a higher order of complexity, this receptor can form heteromeric complexes with other G protein-coupled receptors (GPCR) (Pertwee et al., 2010).

3.6.1. CB_1 heteromerization

Traditionally, a single GPCR is thought to be activated by the presence and binding of an agonist and this stimulated receptor will trigger its signalling through G protein complexes. However, some studies have determined that GPCRs can also exist in the form of homodimers, heterodimers and even more complex oligomeric structures, which might entail variations in the GPCR properties.

Concerning homomerization, at least two molecules of GPCR seem to be needed to interact and activate G protein signalling (Liang et al., 2003; Herrick-Davis et al., 2005). Robust support for homodimerization has been obtained from morphological studies of the rhodopsin receptor, using atomic force microscopy (Fotiadis et al., 2003), and protein crystallography for metabotropic glutamate receptors (Kunishima et al., 2000). Moreover, CB_1 homomerization has also been demonstrated in rat brain membrane preparations, using immunoprecipitations with an antibody that preferentially recognized the dimerized form of the receptor (Wager-Miller et al., 2002; Mackie, 2005).

Besides the formation of GPCR homomers, there is clear evidence for the formation of heteromeric complexes between different GPCRs. However, we are just beginning to understand their functional significance (Ferré et al., 2007a; Franco et al., 2008). Two different types of heterodimeric formation are found. First, there are examples of GPCRs that require interaction with other GPCRs in order to function in a proper manner. This is the case of the $GABA_B$ receptors (GBR). In this case, the ligand-binding site is present in the GBR_1, although this monomer needs the presence of another monomer, the GBR_2 in order to be expressed in the cell surface. On the other hand, GBR_2 can be expressed in the cell surface alone, but it is not capable of GABA binding itself. Therefore, the interaction of GBR_1 with GBR_2 is essential for triggering the intracellular response associated with GABA signalling (Kuner et al., 1999).

Second, there are cases where the heteromerization of GPCR is not essential for their function. In these situations, heteromerization provides

biochemical properties that are different than those of the individual GPCRs. For instance, in the dopamine D_1-D_2 receptor heteromer, selective ligands for D_1 receptor can also activate D_2 receptors in the heteromer (Rashid et al., 2007). Sometimes, activation of one receptor of the heteromer induces changes in the binding properties of the other, inducing a cross-talk phenomenon (Agnati et al., 2003; Ferré et al., 2007a). These changes in binding properties generate signalling alterations, which are frequently observed in heteromeric formations, and can be used as a biochemical fingerprint, allowing the identification of heteromers in brain tissue (Franco et al., 2007). Another common property of GPCR heteromers is their switch to a new type of G protein coupling. The heteromer formed by CB_1 and D_2 receptor is a nice example of such a switch. Both CB_1 and D_2 receptors are coupled to $G_{i/o}$ protein, and inhibit adenylyl-cyclase activation. However, co-stimulation of both receptors in the CB_1-D_2 heteromer results in an activation of adenylyl-cyclase dependent on G_s signalling (Jarrahian et al., 2004).

Currently, a wide variety of techniques are used to determine the formation of heterodimers, both in cells and tissues. Several different methods using immunoprecipitation, protein complementation and direct observation using special microscopes are being used. In addition, techniques such as bioluminescence or fluorescence resonance energy transfer (BRET and FRET, respectively) are based on the principle of energy transmission, and allowed the development of useful techniques that are applied to the study of heteromer formation.

Thus, resonance energy transfer (RET) was first described by Förster (1948), and is characterized by the transfer of energy from an excited donor to an acceptor molecule, and the efficiency of this process is highly dependent on the distance between the donor and the acceptor, and on their relative orientation with respect to each other (Förster, 1948; Stryer and Hangland, 1967; Lackowitz, 1983). In most RET-based assays, the typical effective distance between the donor and the acceptor is 10 to 100 angstroms, which importantly correlates with most biological interactions, making RET-based techniques excellent tools for monitoring biological interactions. The most widely used techniques based on this technology are FRET and BRET. In FRET, both the donor and the acceptor are fluorescent molecules, whereas in BRET, the donor is a bioluminescent molecule and the acceptor is a fluorescent one (Figure 18).

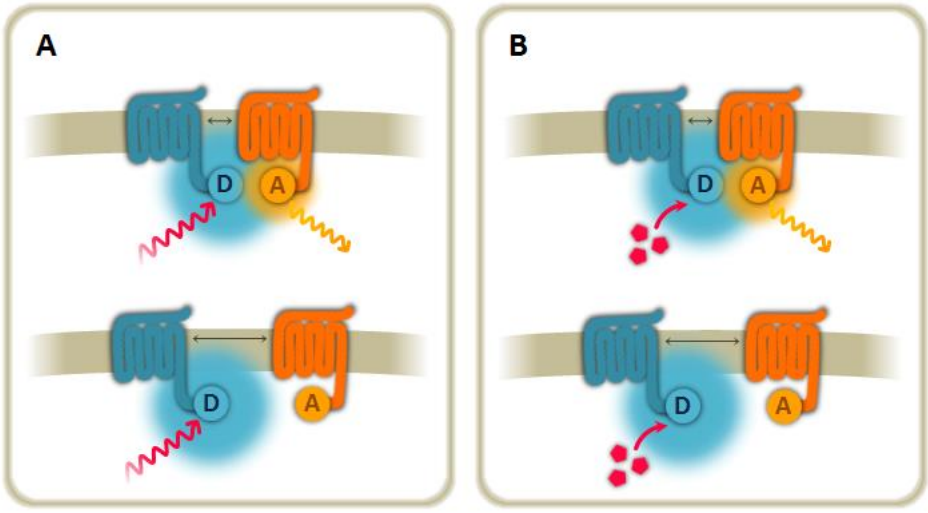

Figure 18. Schematic representation of FRET and BRET. (A) In FRET, two fluorophores are used (Donor and Acceptor), excitating the donor with light induces its activation, allows the energy transfer to the acceptor when it is in the close proximity. Then, the activation of the acceptor can be

detected by the emission of fluorescence by the acceptor. (B) In BRET, the donor is a bioluminiscent protein, which is activated by the presence of a specific ligand. Following the activation of the donor, the transfer of energy to the acceptor might happen when both molecules are sufficiently close. In this case, the acceptor activation is also evidenced by the emission of fluorescence.

Several heteromeric entities containing the CB_1 receptors have been described in the last years, although their biological functionality and relevance remains largely unknown. CB_1-D_2 receptor heteromerization has been demonstrated in co-transfected cells by co-immunoprecipitation and FRET techniques (Kearn et al., 2005; Marcellino et al., 2008), and their existence in the striatum has been confirmed by electron microscopy, which corroborated their overlapping subcellular distribution (Pickel et al., 2006). In a recent double immunohistochemical confocal analysis in rat striatal sections, a strong colocalization of CB_1 and A_{2A} receptors has been observed (Carriba et al., 2007), and the presence of heteromeric CB_1-A_{2A} complexes was then confirmed by BRET in co-transfected cells (Carriba et al., 2007). Considering that CB_1 can form heteromers with both A_{2A} and D_2 receptors, and A_{2A} can also interact with D_2 forming A_{2A}-D_2 heteromers (Ferré et al., 2007a;b), the existence of a macromolecular complex including all three receptors has been suggested using Sequential BRET-FRET (SRET) analysis in co-transfected mammalian cells (Carriba et al., 2008). Albeit the direct interaction of CB_1 and mu-opioid receptor has been known for some time (Vaysse et al., 1987), the heteromerization of these receptors was not demonstrated until recently in co-transfected cells using BRET (Rios et al., 2006) and FRET (Hojo et al., 2008). In addition, a recent study has demonstrated the formation of CB_1-delta-opioid receptor heterodimers (Rozenfeld et al., 2012). A high degree of co-localization between CB_1 and

delta-opioid receptor was found in mouse primary cortical cells (Rozenfeld et al., 2012), and using co-immunoprecipitation, interacting complexes between both receptors were found in cell membranes, supporting previous BRET results showing that CB_1 and delta-opioid receptors existed in close proximity to directly interact in cells (Rios et al., 2006). Moreover, the existence of CB_1-Hcrtr-1 heteromer has been recently described (Ward et al., 2011). Previous studies have inferred this interaction based on alterations in selective agonist functionality (Hilairet et al., 2003), and FRET studies in intracellular structures (Ellis et al., 2006). Recently, the direct interaction of CB_1 and Hcrtr-1 in cells was demonstrated using co-immunoprecipitation (Ward et al., 2011).

Therefore, the recent discovery of the GPCR heteromeric formation might produce a shift in understanding of GPCR properties. Albeit, their functional relevance and behavioural significance is still unknown, further studies of these new entities might represent a major advance in pharmacology and therapeutics. Thus, specifically targeting a heterodimer while avoiding actions on the separate receptors might be considered a whole new approach to treating specific disorders or it could be useful to avoid some undesired side effects.

OBJECTIVES

General objective

To investigate the neurobiological substrates involved in the behavioural alterations induced by the administration of MDMA and THC in mice, focusing on the dopaminergic and the serotonergic systems.

Specific objectives

1. To evaluate the acute and long-lasting effects of repeated treatment with neurotoxic and non-neurotoxic doses of MDMA on the motivation for a highly palatable food reward using fixed and progressive ratio schedules of reinforcement (Article 1).

2. To analyse the effects of MDMA administration on extinction training and reinstatement of palatable food-seeking (Article 1).

3. To examine the long-lasting effects of repeated administration of neurotoxic and non-neurotoxic doses of MDMA on executive functioning related to working memory, response inhibition and behavioural flexibility (Article 2).

4. To set up two cognitive tasks in mice based on instrumental responding for a positive reinforcement. For this purpose, we adapted an operant model to study working memory and central inhibitory processes, the delayed alternation task, and an operant-based set-shifting paradigm to evaluate behavioural flexibility (Article 2).

5. To investigate the relevance of the dopaminergic system in the behavioural alterations observed following MDMA administration (Article 1 and Article 2).

6. To determine the specific role of 5-HT_{2A} receptors in the modulation of THC-induced behavioural and electrophysiological responses related to CB_1 receptor activation using genetically modified mice (Article 3).

7. To uncover the underlying molecular mechanism responsible for the putative interaction between 5-HT_{2A} and CB1 receptors (Article 3).

8. To characterize the cross-talk phenomenon observed between CB_1 and 5-HT_{2A} receptors at a cellular level and in tissue from WT and 5-HT_{2A} KO animals (Article 3).

9. To describe the signalling properties, the functionality and the localization of the newly discovered receptor entity, the CB_1-5-HT_{2A} heteromer, using *in vitro* and *ex vivo* assays (Article 3).

10. To determine whether it is possible to disrupt the heteromer by the administration of specifically directed interference peptides *in vitro* and *in vivo*, and examine the behavioural effects occurred upon CB_1-5-HT_{2A} heteromeric disruption (Article 3).

RESULTS

ARTICLE 1

Effects of repeated MDMA administration on the motivation for palatable food and extinction of operant responding in mice

Plaza-Zabala A, Viñals X, Maldonado R, Robledo P.

Psychopharmacology (2010) 208: 563-573

OBJECTIVE:

To evaluate the acute and long-lasting effects of repeated treatment of MDMA on the motivation for highly palatable food rewards, extinction training, and reinstatement of palatable food-seeking.

RESULTS:

Under acute conditions, the administration of high doses of MDMA impaired instrumental responding on a fixed ratio schedule of reinforcement. Although responding levels returned to control values 24 h after MDMA withdrawal, residual effects on the motivation for palatable food were revealed by significant decreases in the breaking point in the progressive ratio test. Repeated administration of MDMA produced resistance to extinction, whereas the reinstatement of palatable food-seeking behaviour was similar between all treatment groups. Long-lasting alterations in the dopaminergic system were found in animals treated with high doses of MDMA, revealed by the reduction in DAT binding levels one month after drug treatment.

CONCLUSION:

Repeated treatment with MDMA decreases incentive motivation for a palatable reward and induces long-lasting dopaminergic toxicity in the striatum, which increases resistance to extinction.

Plaza-Zabala A, Viñals X, Maldonado R, Robledo P. Effects of repeated MDMA administration on the motivation for palatable food and extinction of operant responding in mice. Psychopharmacology (Berl). 2010 Mar;208(4):563-73. doi:10.1007/s00213-009-1750-x.

ARTICLE 2

Effects of repeated treatment with MDMA on working memory and behavioural flexibility in mice

Viñals X, Maldonado R, Robledo P.

Addiction Biology (2013) 18: 263-273

OBJECTIVE:

To examine the long-lasting effects of MDMA on executive functioning related to working memory, response inhibition and behavioural flexibility.

RESULTS:

MDMA disrupted performance of a previously acquired operant alternation task, and this impairment was apparent for several days before it completely recovered. Repeated administration of MDMA impaired behavioural flexibility as revealed by an increase in perseverant responding in the attentional set-shifting task. Basal levels of striatal dopamine were not altered following repeated MDMA treatment, although a challenge with MDMA failed to increase dopamine release in MDMA-treated animals.

CONCLUSION:

Repeated treatment with neurotoxic doses of MDMA decreases the levels of stimulated dopamine release in the striatum, which may contribute to the lasting impairments in recall and reduce cognitive flexibility in mice.

Viñals X, Maldonado R, Robledo P. Effects of repeated treatment with MDMA on working memory and behavioural flexibility in mice. Addict Biol. 2013 Mar;18(2):263-73. doi: 10.1111/j.1369-1600.2011.00421.x.

ARTICLE 3

Cognitive impairment induced by THC occurs through heteromers between cannabinoid CB_1 and serotonin 5-HT_{2A} receptors

Viñals X, Moreno E, Lanfumey L, Cordomi A, Pastor A, de la Torre R, Gasperini P, Howell L, Pardo L, Lluís C, Canela EI, McCormick PJ, Maldonado R, Robledo P.

PLOS Biology (under review)

OBJECTIVE:

To determine the specific role of 5 HT_{2A} receptors in the modulation of pharmacological responses induced by THC in mice.

RESULTS:

5-HT_{2A} receptors modulate several THC pharmacological effects such as amnesia, anxiety and social interaction, whereas they do not influence antinociceptive, hypothermic or hypolocomotor responses caused by THC. In vitro and in vivo assays revealed that CB_1 and 5-HT_{2A} receptors form heteromers with specific signalling and functional properties. CB_1-5-HT_{2A} receptor heteromers are expressed in the brain, and their formation can be prevented using transmembrane interfering peptides.

CONCLUSION:

CB_1-5-HT_{2A} heteromers mediate the detrimental properties of THC, implying that it may be possible to target this complex for dissociating the potential therapeutic properties of cannabinoids from their unfavourable side-effects.

Viñals X, Moreno E, Lanfumey L, Cordomí A, Pastor A, de La Torre R, Gasperini P, Navarro G, Howell LA, Pardo L, Lluís C, Canela EI, McCormick PJ, Maldonado R, Robledo P. Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors. PloS Biol. 2015 Jul 9;13(7):e1002194. doi: 10.1371/journal.pbio.1002194

DISCUSSION

Cannabis and ecstasy are popular recreational drugs of abuse that are often consumed together (Sala and Braida, 2005; Wish et al., 2006; Black et al., 2009). One of the main reasons given by recreational drug users to consume both drugs is that cannabis helps to relieve the dysphoric symptoms following MDMA use (Winstock et al., 2001; Strote et al., 2002). This pattern of poly-drug abuse may represent an important confounding factor when studying the neurobiological alterations induced by each drug individually (Daumann et al., 2004; Parrott et al., 2007). Indeed, both of these substances may induce cognitive deficits and mood alterations in human subjects (Croft et al., 2001; Dafters et al., 2004; Parrott et al., 2003; 2004), and complex interactive effects have been described in experimental animals (Morley et al., 2004; Robledo et al., 2007; Touriño et al., 2007; Touriño et al., 2010). Therefore, experimental investigation relies heavily on the use of animal models, where most conditions can be precisely controlled in order to understand the neural mechanisms underlying the specific effects of each drug individually.

In this thesis we investigated the neurobiological substrates involved in the behavioural alterations induced by MDMA and THC separately. In the first part, we examined the effects of MDMA on cognition and motivation, focusing on the dopaminergic system. In the second part, we investigated the therapeutic and detrimental effects of THC focusing on the interaction between the endocannabinoid and the serotonergic systems, and more specifically, examining the role of 5-HT_{2A} receptors in these responses.

1. The role of dopamine in the cognitive and motivational alterations induced by repeated MDMA treatment in mice

MDMA is a popular recreational drug of abuse among young adults worldwide. Although there is abundant literature revealing deleterious effects of MDMA on several neurophysiological processes such as reward, cognition, temperature regulation and food consumption, the mechanisms that produce these effects remain unclear. This could in part be related to the complexity of MDMA-induced effects on brain neurotransmitter systems, which are different across species (Easton and Marsden, 2006).

Repeated administration of high doses of MDMA induces long-lasting alterations in the serotonergic system in rats and non-human primates (Battaglia et al., 1987; 1988; Insel et al., 1989; Li et al., 1989; Mayerhofer et al., 2001; Mechan et al., 2006). In humans, repeated exposure to MDMA also produces alterations in this system, such as SERT decreases in several brain areas (McCann et al., 2008; Kish et al., 2010; Urban et al., 2012). In mice however, repeated administration of MDMA predominantly produces changes in the dopaminergic system consisting of depletion in brain dopamine and its metabolites (Colado et al., 2004), decreases in the density of DAT binding sites (Trigo et al., 2008), and reduction of dopaminergic fibers in the striatum and substantia nigra (Granado et al., 2008). In the first phase of this thesis, we aimed to elucidate the long-lasting impact of repeated treatment with neurotoxic and non-neurotoxic doses of MDMA in mice on behaviours related to dopaminergic function. We first examined the effects of these treatments

on the motivation of mice to obtain a highly palatable food, and on the extinction and reinstatement of food-seeking behaviour. Subsequently, we evaluated whether this exposure induced deficits in cognitive processing related to specific subsets of executive functioning, and determined extracellular levels of dopamine in the striatum using *in vivo* microdialysis.

1.1. MDMA-induced dopaminergic neurotoxicity and its effects on the motivation for palatable food and extinction of operant responding

In our first manuscript, we investigated the effects of repeated treatment (two daily administrations during 4 days) of neurotoxic (30 mg/kg) and non-neurotoxic (3 mg/kg) doses of MDMA on the motivation of mice to obtain a highly palatable food, and on the extinction and reinstatement of food-seeking behaviour. In this work, we found that in animals trained under a fixed ratio 5 (FR5) schedule of reinforcement, MDMA administration at dose of 30 mg/kg produced a significant decrease in responding on the first and third day of repeated treatment (Article 1), while the lower dose (3 mg/kg) did not modify this behaviour. These results were in agreement with previous studies, which reported that the acute administration of high doses of MDMA decreased operant responding in pigeons (Nader et al., 1989; LeSage et al., 1993), mice (Glennon et al., 1987), rats (Nagilla et al., 1998), and primates (Goodwin et al., 2013). However, other studies reported that acute MDMA increased fixed interval, but not fixed ratio responding in mice (Miczek

and Haney, 1994), and it has been recently reported that chronic administration of MDMA at low doses increased instrumental responding in mice (Olausson et al., 2006). Together, these data suggest that the effects of MDMA on food-reinforced operant responding may depend on the dose administered and on the species tested. Moreover, the acute administration of MDMA induces hyperactivity in a wide range of species (Gold et al., 1988; Slikker et al., 1989; Spanos and Yamamoto, 1989; Scearce-Levie et al., 1999). In our study, we also found significant increases in locomotor activity, which might represent a confounding factor when analysing the effects of MDMA on responding for food under the acute effects of the drug. For this reason, we focused our study in the persistent alterations following a repeated MDMA administration. We found that mice recovered operant responding 24 h after the last MDMA administration. Thus, responding rate returned to pre-treatment values and no residual effects of MDMA were observed in comparison with vehicle treatment.

Despite the recovery of responding observed in FR5 schedule of reinforcement, MDMA induced a significant reduction in the incentive motivation to work for a highly palatable food. This effect was revealed by a decrease in the breaking point achieved on a progressive ratio schedule of reinforcement in MDMA-treated animals at both doses tested (3 and 30 mg/kg). In this case, MDMA-induced motor deficits were excluded as contributors because neither residual effects on locomotor activity nor in motor coordination were observed under the same experimental conditions used to evaluate operant responding. This result

was consistent with previous findings in monkeys, where MDMA induced a decrease in motivation for palatable food (Frederick et al., 1995). Moreover, a reduction in breaking point in rats responding for water was also reported as a consequence of MDMA administration (Laraway et al., 2003). Contrastingly, a recent study performed in rats reported an increase in breaking point values after a chronic low-dose of MDMA (Olausson et al., 2006). The differences observed between these studies could be explained by discrepancies in the drug regimens or the species used. Thus, the effects observed by Frederick and cols. (1995) in monkeys and Laraway and cols. (2003) in rats were obtained after acute MDMA administrations, whereas in the study of Olausson and cols. (2006) MDMA was chronically administered during 15 days before behavioural testing.

In our first study, we also examined the effects of MDMA on the extinction of operant responding. We showed that mice treated with the neurotoxic regimen of MDMA exhibited a higher resistance to extinction. This result could be attributed to an MDMA-induced increase in motivation to seek palatable food. However, the results obtained in the progressive ratio paradigm indicated just the opposite. A more plausible explanation could be that MDMA administration induced perseverant responding in mice. This reasoning is consistent with previous studies associating MDMA with perseverative behaviour. In fact, studies in humans revealed that MDMA users made more perseverative responses in different tasks evaluating executive functioning in comparison with non-users (Fox et al., 2001; Montgomery et al., 2005), or in comparison

with abstinent MDMA users (von Geusau et al., 2004). Moreover, acute administration of MDMA also increased perseverative behaviour in monkeys (Verrico et al., 2008). Perseverative responding is thought to be a consequence of altered response inhibition, and impairments in inhibitory control are apparent among drug users as a result of heavy drug consumption (Smith et al., 2014). Current associative learning theories propose that extinction is an active learning process, distinct from acquisition (Myers and Davis, 2002). In accordance, during extinction, existing memories need to be rearranged, a process involving synaptic changes (Almeida-Corrêa and Amaral, 2014). Thus, it is possible that the observed resistance to extinction following MDMA administration is due to a learning impairment. However, when reinstatement of palatable food-seeking behaviour was tested after extinction training, all animals increased responding when the conditioned cues were presented, regardless of the treatment received. This result indicates that repeated MDMA exposure does not disrupt the reinstatement of food-seeking behaviour by the presentation of the conditioned cues.

In our study, a significant reduction in DAT binding was observed in the striatum 25 days after MDMA treatment with the high, but not with the low dose of the drug. In accordance with our results, it has been reported that the administration of a neurotoxic regimen of MDMA decreased DAT binding sites in the striatum and substantia nigra of mice, as early as 1 day after drug administration, and was still observed 1 month after the treatment (Granado et al., 2008). Previous studies from our group,

also found reductions in DAT binding sites in mice striatum 4 days after the last administration of high (30 mg/kg twice a day during 4 days) (Trigo et al., 2008), but not lower MDMA doses (10 mg/kg twice a day during 5 days) (Robledo et al., 2004).

Our data showing reduced dopaminergic function in the striatum of mice exhibiting deficits in response inhibition suggest that this brain structure participates in some aspects of executive functioning. Since many decades, the role of frontal lobes in behavioural regulation has been proposed (Luria, 1966). Indeed, behavioural inhibition has been related to frontal lobe integrity mainly based on studies in patients and animals with lesions in this brain region (Brutkowski and Mempel, 1961; Mishkin, 1964; Drewe, 1975). Current models of inhibitory control also support the critical contribution of prefrontal areas to response inhibition, and there are plenty of studies indicating that depending on the specific task, different cortical and subcortical regions are involved in response inhibition (Bari and Robbins, 2013). However, the striatum, which receives most inputs from basal ganglia, is considered an important brain region for stop signal responses (Vink et al., 2005; Padmala and Pessoa, 2010). In fact, the inhibitory difficulties observed in addictive processes resulting from excessive drug consumption are thought to be majorly mediated by fronto-striatal circuitry (Morein-Zamir and Robbins, 2014). Particularly, the role of striatal dopamine in cognition seems to be related with behavioural flexibility (Crofts et al., 2001; Darvas and Palmitier, 2011). Moreover, studies in Parkinson's disease patients have also confirmed the participation of striatal

dopamine in behavioural flexibility (Cools et al., 2010), and lower striatal DAT binding has also been related with deficits in executive function in these patients (Siepel et al., 2014). Thus, the higher resistance to extinction observed in our study, following the administration of high doses of MDMA, could be associated with the alterations in dopaminergic functioning revealed by decreases in DAT binding.

However, MDMA treatment also blunted the motivation for a palatable reward at doses that do not reduce DAT levels in the striatum. The fact that non-neurotoxic doses of MDMA at striatal level also induced deficits in motivated behaviour suggests that MDMA may be altering the functionality of other brain areas participating in the reward circuit. Accordingly, we have previously shown that repeated self-administration of MDMA during 10 days (from 1.25 mg/kg to 2.5 mg/kg intravenously) reduced basal and MDMA-evoked dopamine levels in the NAc of mice (Orejarena et al., 2009), indicating that repeated low doses of MDMA can produce changes in NAc dopaminergic function. Therefore, it is possible that the deficits in motivation for food rewards observed in our study were due to a decrease in mesolimbic dopamine activity. This notion is in accordance with the role of this neurotransmitter system in reward-related behaviours and incentive motivation (Palmiter et al., 2007; Kenny, 2011; Salamone and Correa, 2012).

1.2. MDMA-induced dopaminergic neurotoxicity and its effects on working memory and behavioural flexibility

Based on our previous findings, we have further analysed the effects of repeated MDMA administration on cognitive performance in our second manuscript. For this purpose, we examined the long-lasting effects of repeated administration of neurotoxic and non-neurotoxic doses (30 and 3o mg/kg twice a day during 4 days, respectively) of MDMA on executive functioning related to working memory, response inhibition and behavioural flexibility. For this purpose, we started by setting up in the laboratory several cognitive tasks based on instrumental responding for a positive reinforcement namely, a highly palatable food-reward. The first procedure was the delayed alternation task, designed to evaluate working memory, as well as central inhibitory processes related to striato-cortical functionality (Granon et al., 1994; Goldman-Rakic, 1995). This procedure was adapted to mice from the one originally designed for rats (Dunnett et al. 1999). Briefly, in this operant paradigm animals need to alternate responses between two nose pokes, and withhold responding for a period of time (2-10 sec) signalled by a light in order to obtain palatable food rewards. The ratio of correct responses and the learning curve obtained during training sessions are used to study working memory. Additionally, the analysis of premature responses is used as an indication of impulsive-like behaviour, directly related with central inhibitory processes.

In our study, repeated MDMA administration did not produce alterations in acquisition of delayed alternation at any of the doses

tested. The highest dose administered, known to induce dopaminergic neurotoxicity, disrupted performance of the task once it was already acquired, and this impairment persisted for several days after the last drug administration (Article 2). In concordance with our results, previous studies in our laboratory revealed that dopaminergic neurotoxicity induced by MDMA impaired recall of an active avoidance task, a test which relies on fear-motivated behaviour, and negative reinforcement (Trigo et al., 2008). In contrast with our findings, MDMA also impaired acquisition of the active avoidance task, an effect which we did not observe in a positive-reinforcement based task. These acquisition impairments were particularly relevant in the latest phase of training, when a greater effort was needed. In agreement, it has been shown that the cognitive deficits observed in ecstasy consumers are larger in complex tasks that require interactions between multiple brain regions (Brown et al., 2010). Additionally, MDMA administration in rats induced persistent acquisition deficits in a test similar to delayed alternation, but with longer delays (0-30 sec) (Marston et al., 1999).

The second operant paradigm used was the set-shifting procedure, recently described in rats (Floresco and Jentsch, 2011), that we successfully adapted to mice. In this procedure, mice are previously trained to perform a specific response (See Figure 19A) and then animals are required to change their response strategy and use previously irrelevant information in order to correctly perform the task (See Figure 19B). This test allows the examination of behavioural flexibility, which is crucial for adapting to changing conditions. Accordingly, perseveration

on the initial response strategy is indicative of compulsive-like behaviour, and MDMA repeated administration at high doses induced an increase in perseverative responding. Thus, MDMA-treated mice failed to shift their behaviour to the new response strategy necessary to obtain a food pellet reward.

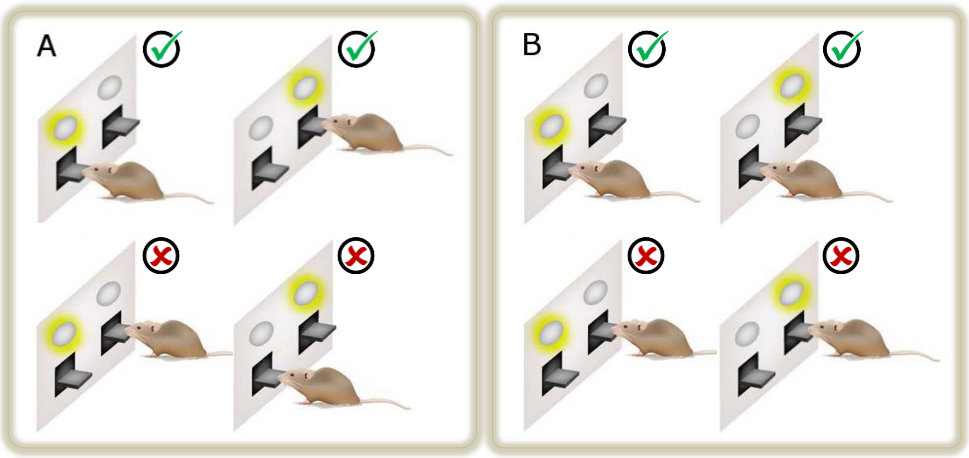

Figure 19. Diagrams of the operant chamber disposition for the procedures used to study behavioral flexibility. (A) Visual-cue discrimination task, where animals are trained to press levers according to the presentation of a light stimulus above the lever lever. (B) Set-shifting task. In this step, a shifting in behaviour is necessary as mice need to press a lever according to its position, with light being irrelevant.

In agreement with our results, repeated amphetamine administration in rats induced impairments in a set-shifting task (Fletcher et al., 2005), and rats with a history of methamphetamine self-administration presented this type of deficits as well (Parsegian et al., 2011). Remarkably, MDMA has been associated with perseverative behaviour in humans. As previously stated, MDMA users exhibit enhanced perseverant behaviour in several tasks (Fox et al., 2001; von Geusau et al., 2004; Montgomery et al., 2005), and deficits in reversal learning are observed in MDMA-treated primates (Verrico et al., 2008). Moreover, a study performed in

alcoholic patients revealed cognitive flexibility deficits, also using the attentional set-shifting task (Trick et al., 2014). This cognitive inflexibility observed in human addicts as perseverative or habit responding could also reflect a difficulty in inhibiting reward-related responses associated with drug consumption (Groman et al., 2009). Although the role of impulsivity and compulsivity in addiction has been widely studied, it is still not clear if it is a cause or a consequence of drug consumption. Impulsive decision making may facilitate initial contact with drugs, and in the case of alcoholism, impulsive choice seems to be a vulnerability factor (Poulos et al., 1995; 1998; Wilhelm and Mitchell, 2008; Oberlin and Grahame, 2009). However, we also showed that MDMA administration evokes an increase in perseverative responding, indicating that impulsivity or compulsive behaviour can also be a consequence of drug consumption. Therefore, although impulsivity might be considered a vulnerability factor for drug addiction, it can also be a consequence of drug consumption, suggesting that both points of view can be right. Recent experiments based on the classification of animals as highly impulsive versus low impulsive (Perry et al., 2005; 2007; Dalley et al., 2007; Belin et al., 2008) can be very useful in elucidating such an important issue.

A complex behavioural task such as attentional set-shifting involves the activity of multiple brain structures, including the medial frontal cortex, dorsal striatum and amygdala among others (Roberts et al., 1994; Crofts et al., 2001; Clarke et al., 2008; Bissonette and Powell; 2012). Moreover, the ability to shift behaviour has been related to mesocortical dopamine

function (Roberts et al., 1994; Floresco et al., 2006; Floresco and Jentsch, 2011). The interaction of medial prefrontal cortex with the NAc and the striatum is crucial in facilitating set-shifting behaviour (Floresco 2006; Block et al., 2007). Thus, alterations in dopaminergic functionality in the striatum observed as a consequence of repeated MDMA administration (Trigo et al., 2008; Article 1) could also account for this deleterious effect on behavioural flexibility. Although numerous studies performed in a wide range of species associated MDMA exposure with cognitive deficits (humans (Rogers et al., 2009), non-human primates (Frederick and Paule, 1997; Taffe et al., 2001), rats (Young et al., 2005; Dalley et al., 2007; Schenk et al., 2011), and mice (Glennon et al., 1987; Trigo et al., 2008; Nawata et al., 2010), the underlying mechanisms producing these deficits remain unclear, in part due to the complexity of MDMA-induced effects in the different species (Easton and Marsden, 2006).

In order to investigate the neurochemical correlates of the alterations found in behavioural flexibility, we measured extracellular levels of dopamine in the striatum when behavioural testing was performed. Unexpectedly, basal levels of dopamine were similar between saline- and MDMA-treated animals at both doses tested (3 and 30 mg/kg twice a day during four consecutive days). The similar levels of basal extracellular dopamine in MDMA-treated animals in comparison with the control group could be explained by the action of compensatory mechanisms following MDMA repeated administrations. However, differences between groups were significant when dopamine release was induced by an acute challenge with MDMA. Thus, extracellular dopamine levels in

animals treated with the highest dose of MDMA did not increase after being challenged with MDMA, possibly indicating a reduction in DAT functionality, or a lack of dopamine availability due to the absence of dopamine storage. These results are in concordance with previous results obtained in our laboratory, showing reductions in MDMA-induced dopamine release in mice that self-administered MDMA in a contingent manner during 10 days (Orejarena et al., 2009), supporting an alteration in dopaminergic system as a consequence of MDMA administration. Remarkably, it was revealed that the blockade of MDMA-induced dopamine release was less pronounced in those animals that receive the same dose of the drug in a non-contingent way (Orejarena et al., 2009), suggesting other alterations in the dopaminergic system than those induced by the drug itself. We also showed that the basal levels of dopamine did not differ between experimental groups seven days after the last MDMA administration, and MDMA challenge induced an increase in dopamine levels in animals treated with MDMA as well as in those animals treated with saline, revealing a recovery of the dopaminergic availability in the striatum at this time point. Interestingly, our behavioural results correlate with these neurochemical findings, as MDMA administration induced impairments in task performance of an already acquired task, and in shifting behaviour, which lasted for several days before returning to control levels. Thus, the recovery of dopaminergic functionality in the striatum observed one week after MDMA repeated administration sustains the hypothesis that dopaminergic impairment could be a cause for impaired cognition.

Indeed, changes in mesocorticolimbic dopamine neurotransmission, including alterations in dopmaine levels, DAT density, and DAT trafficking have been related to the cognitive alterations observed following repeated administration of psychostimulants (Kalivas and Volkow, 2005). In addition, a role of serotonin in the attentional set-shifting has also been demonstrated in humans (Homberg, 2012), as tryptophan depletion induced impairments in this task (Rogers et al., 1999; Borg et al., 2009), and increased levels of extracellular serotonin as a result of an allelic variant in the serotonin transporter gene (Kalueff et al., 2010) improved attentional set-shifting in humans (Bosia et al., 2010). Thus, the alterations observed in behavioural flexibility in primates and humans could be related to the MDMA-induced effects on serotonergic system.

In this study we have also determined whether MDMA induced anhedonic effects which could interfere with food consumption and motivation. For this purpose, we used a set of experimental boxes equipped with a food and drink monitoring systems which have been developed by our laboratory in collaboration with Panlab and Harvard Apparatus (Bura et al., 2010). First, we found that MDMA does not alter saccharin preference either during the MDMA treatment or after it, indicating a lack of MDMA-induced anhedonic-like effect. In contrast, MDMA administration induced a significant decrease in caloric intake during the treatment days. MDMA-induced lack of appetite or weight loss has been described in earlier studies performed in human volunteers (Vollenweider et al., 1998) and rats (De Souza et al., 1997; Kobeissy et al.,

2008). In our first study, a transient suppression of appetite may have caused the reduction in operant responding observed following MDMA administration under acute conditions. However, in our second study we found a deleterious effect in performance, which is not attainable by just a decrease in the rate of responding, indicating that cognitive alterations observed are not explained by just the MDMA-induced appetite suppression.

1.3. General conclusions obtained from both studies

Taking together the data obtained in both studies, we can corroborate that the repeated administration of MDMA at high doses induces alterations on different cognitive levels. First, under acute conditions, MDMA not only perturbs behaviour by decreasing operant responding for food but it also induces cognitive disturbances in a previously learned task. These effects gradually recover once the MDMA administration finishes, as operant responding for food returned to control levels 24 h after treatment and cognitive impairment was present only for a few days after finishing drug treatment. On the other hand, repeated administration of high doses of MDMA induced long-lasting effects in terms of behavioural flexibility. This effect was observed as perseverant responding through extinction training in our first study, and with perseverative responding on the initial strategy in the attentional set-shifting task in our second study. Moreover, we showed that MDMA induced a decrease in motivation for food when a great

amount of effort was required, using a progressive ratio schedule of reinforcement.

Thus, considering the neurochemical evidence reported in our and previous manuscripts we can suggest that the acute effects of MDMA observed in our studies could be directly related with the extensive effects of the drug over brain neurotransmitter systems. More precisely, these effects could be explained by the alterations in monoamine levels, being the dopamine levels particularly important at least in mice. However, based on the different duration of monoamine levels alteration (MDMA-induced dopamine release is restored one week after MDMA treatment, Article 2), and neurotransmitter systems disturbances (significant reductions in DAT can be observed one month after the drug treatment, Granado et al., 2008), we propose that long-lasting disturbances are consequence of neuronal damage or alterations in monoamine transporters and receptors. In fact, the involvement of other mechanisms than alterations in DAT binding levels cannot be discarded as MDMA-induced recall deficits have been reported even in the absence of DAT binding reductions (Trigo et al., 2008). Therefore, although we have reported neurochemical correlates which can explain at least part of the MDMA-induced effects on behaviour, more research is needed to further elucidate the complex consequences of MDMA administration on neurotransmitter systems, and better characterize the mechanisms that account for the long-lasting effects induced by this drug.

2. The role of serotonin 2A receptors in the behavioural effects induced by THC administration in mice

2.1. Bilateral interactions between the endocannabinoid and the serotonergic system

Cannabis is the most consumed illicit drug all over the world, and its usage among young people is alarming due to its potential harmful effects on brain development and educational outcome. Moreover, early onset cannabis use has been associated with increased odds of later cannabis dependence, worse prognosis the possibility of developing substance use related disorders, and worse prognosis of future potential substance use disorders, and increased risk of psychosis. Cannabis is mostly taken for its relaxing, euphorigenic and hedonic properties, but it also has therapeutic effects such as antinociception, anxiolysis and neuroprotection. Indeed, cannabinoid-based compounds have been approved to treat spasticity in multiple sclerosis patients, chemotherapy-induced nausea and vomiting, anorexia and cachexia in patients with HIV-AIDS, and its compassionate use for pain has been recently approved in Catalonia. However, it has also been associated with undesirably consequences such as memory impairments, anxiogenic effects, alterations in motor coordination and dependence. Thus, identifying a mechanism to dissociate the therapeutic from the detrimental actions of cannabis is one of the major challenges in cannabinoid research.

Recent evidence has linked the behavioural responses of the endocannabinoid system with the activation of 5-HT_{2A} receptors. Thus, THC-induced catalepsy was significantly inhibited by the administration of the 5-HT_{2A} agonist DOI in mice, and this inhibition was blocked by the 5-HT_{2A} angatonist, ketanserine (Egashira et al., 2007). In addition, THC-induced impairment of working memory in the 8-arm maze in rats was attenuated by DOI (Egashira et al., 2002). Conversely, DOI-induced behaviours were reduced by several cannabinoid compounds in a dose-dependent manner (Darmani, 2001), indicating that the interaction between these systems could be reciprocal. Besides these results, experiments performed in CB_1 knock-out animals confirmed the interactions between these systems, as these mutant animals presented a reduction in DOI-induced effects (Mato et al., 2007).

Interestingly, both systems have been involved in the pathophysiology of schizophrenia. Indeed, atypical antipsychotics, which are a group of drugs used to treat psychiatric conditions, present a higher binding affinity for cortical 5-HT_{2A} receptors than for striatal D_1 or D_2 receptors (Meltzer et al., 1989; Seeman, 2002). Thus, their administration produces less extrapyramidal side effects than those induced by the "classical" antipsychotic drugs. In addition, reductions in 5-HT_{2A} receptor levels, as well as 5-HT_{2A} receptor polymorphisms have been associated with cognitive deficits or psychosis in Alzheimer's disease patients (Nacmias et al., 2001; Rocchi et al., 2003; Hasselbalch et al., 2008; Marner et al., 2012). Moreover, atypical antipsychotics have also been used to treat psychotic manifestations in Parkinson's disease patients (Fernandez et

al., 2004; Pollak et al., 2004), and alterations in 5-HT_{2A} receptor binding were found in patients with first-episode schizophrenia (Rasmussen et al., 2010). Conversely, cannabinoids have not only been associated with the exacerbation of disease symptoms in individuals with psychotic disorders (Mathers and Ghodse, 1992; Wilson and Nicoll, 2002; D'Souza et al., 2005; Henquet et al., 2010; Rentzsch et al., 2011), but also cannabis consumption can induce acute episodes of psychosis in healthy individuals (Chopra and Smith, 1974; D'Souza et al., 2004; Morrison et al., 2009). Additionally, various studies have associated early and heavy exposure to cannabis with a higher risk for psychotic outcomes, including schizophrenia (Ferdinand et al., 2005; Henquet et al., 2005; McGrath et al., 2010; Radhakrishnan et al., 2014).

Importantly, CB_1 and 5-HT_{2A} receptors are commonly expressed in several brain areas involved in the regulation of emotions, learning and memory including the cerebral cortex, amygdala and hippocampus (de Almeida and Mengod, 2007; Bombardi and Di Giovanni, 2013; Mechoulam and Parker, 2013). Figure 20 represents the distribution of both receptors throughout the rodent brain, revealing the existence of a remarkable overlapping, and further supporting a possible interaction between both systems.

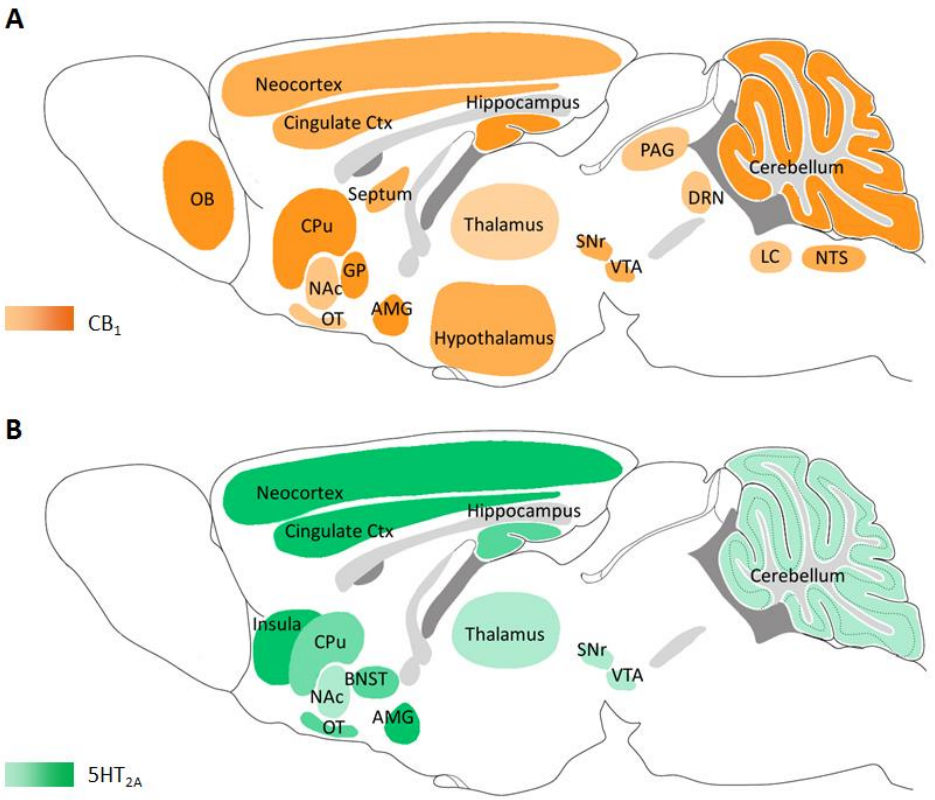

Figure 20. Schematic representation of the main areas expressing CB_1 and 5-HT_{2A} receptors in the mouse brain. (A) CB_1 receptor distribution. (B) 5-HT_{2A} receptor distributions. AMG, amygdala; CPu, caudate putamen; Ctx, cortex; DRN, dorsal raphe nucleus; GP, globus pallidus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal gray; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area.

2.2. 5-HT_{2A} receptors mediate the detrimental, but not the beneficial effects of THC

Using mutant mice lacking 5-HT_{2A} receptors, we have revealed a dissociation between the beneficial and the detrimental effects of THC (Article 3). The most relevant finding is that animals lacking 5-HT_{2A} receptors are less sensitive to the amnesic-like effects induced by the administration of THC, whereas the antinociceptive properties of this

drug remain intact. Thus, the memory impairment induced by THC (3 mg/kg) was significantly reduced in 5-HT_{2A} KO animals in comparison with the wild-type group in the novel object recognition task. This result suggests that 5-HT_{2A} receptors are necessary for the full expression of THC-induced amnesic-like effects. Nevertheless, when a higher dose of THC was administered (10 mg/kg), the amnesic-like properties of this drug were shown in both WT and KO animals. These findings can be contrasted with a previous study showing that DOI administration significantly attenuated the impairment induced by THC on working memory using the 8-arm radial maze in rats (Egashira et al., 2002). The mechanism responsible for the effect of DOI was not clarified, although the authors suggested a possible interaction between CB_1 and 5-HT_{2A} receptors in this effect.

Besides the implication of 5-HT_{2A} receptors in THC-induced amnesic-like effects, we also analysed the effects of these receptors in THC-induced anxiolytic and anxiogenic effects. 5-HT_{2A} receptors modulate behavioural responses to novelty and threat behaviours, which usually reflect anxiety (Millan, 2003; Weisstaub et al., 2006). However, in basal conditions, we did not observe any differences between WT and 5-HT_{2A} KO animals in the anxiety-like responses measured using the EPM paradigm. KO animals spent a similar amount of time in the open arms when compared to WT animals, and the number of entrances to these arms was also comparable. Pharmacological studies using 5-HT_{2A} ligands have reported contradictory effects in different anxiety paradigms, and their role in modulating anxiety has not been completely elucidated. In one hand, the

administration of DOI induced anxiolytic-like responses (Onaivi et al., 1995; Nic Dhonnchadha et al., 2003a; Ripoll et al., 2006; de Paula Soares and Zangrossi, 2009), an effect mediated by 5-HT_{2A} receptors (Nic Dhonnchadha et al., 2003b). On the other hand, the activation of 5-HT_{2A} receptors has been related to a facilitatory activity on the hypothalamo-corticotropic axis, activating the stress pathways (Hemrick-Luecke and Evans, 2002). Thus, although the involvement of 5-HT_{2A} receptors in anxiety seems reasonable, inconsistencies between studies are obtained. These differences could appear because of using different experimental paradigms, animal models or experimental conditions, as anxiety-like behaviour is sometimes difficult to discern from fear, threat, or novelty behaviours.

Using the EPM paradigm, we revealed that the administration of 0.3 mg/kg of THC induced a decrease in anxiety-like behaviour, whereas the dose of 3.0 mg/kg produced an anxiogenic effect in WT animals. This effect was revealed by an increase or a decrease in the time spent in the open arms, respectively. The biphasic effect of cannabinoids in anxiety-like responses has been previously reported in animals. Indeed, low doses of cannabinoid agonists induced anxiolytic-like effects in the elevated plus maze or in the light/dark box (Berrendero and Maldonado, 2002; Valjent et al., 2002; Marco et al., 2004), whereas the administration of high doses of these compounds resulted in anxiogenic-like effects (Onaivi et al., 1990; Arevalo et al., 2001; Genn et al., 2004; Marco et al., 2004), and this biphasic effect has also been recently confirmed in our laboratory (Puighermanal et al., 2013). In our study, we revealed that

5-HT$_{2A}$ receptors were necessary for the THC-induced anxiolytic-like response, as KO animals did not present this effect, while the anxiogenic-like effects of THC remained unaltered in these animals. In accordance with this finding, we obtained similar results using the social interaction test, where an increase in social interaction is indicative of an anxiolytic-like effect (File and Seth, 2003). The administration of a low dose of THC (0.3 mg/kg) did not modify social behaviour in 5-HT$_{2A}$ KO animals in contrast with the significant increase in interaction time produced in WT. The dorsal raphe nucleus (DR) is the main source of 5-HT innervation to limbic structures involved in anxiety, including the central and basolateral nuclei of the amygdala, the paraventricular hypothalamus, the bed nucleus of the stria terminals, and the infralimbic and insula cortices (Kiyasova et al., 2011). Early studies suggested that different serotonergic efferent pathways from the DR controlled defensive behaviours associated with anxiety and fear (Deakin and Graeff, 1991), and a recent study has corroborated that DR is indeed an aggregate of different subpopulations of neurons that are morphologically and functionally different (Calizo et al., 2011). Concerning the involvement DR 5-HT neurons in anxiety-like behaviour and the modulatory role of 5-HT$_{2A}$ receptors on this behaviour, we used and electrophysiological approach in collaboration with the group of Laurence Lanfumey to analyse the effects of THC on these neurons. Our results revealed that 5-HT$_{2A}$ KO animals presented a similar pattern of DR 5-HT neuron discharge under basal conditions. This result was unexpected as we hypothesized that the absence of 5-HT$_{2A}$ receptors

would induce a decrease in basal serotonergic firing due to the predominant effect of 5-HT_{1A} on these neurons, leading to a decrease in firing. In order to obtain a regular firing of DR 5-HT neurons, we stimulated these cells by adding the α1 adrenoceptor agonist, phenylephrine to the bath solution before starting firing recordings. This evoked stimulation could mask any effects due to the absence of 5-HT_{2A} receptors in DR 5-HT neuronal firing. We have also revealed that 5-HT_{2A} KO animals were less sensitive to the decrease in neuronal firing induced by a low dose of THC (1 nM), whereas DR 5-HT neurons from KO animals responded similarly than WT with a higher dose (10 nM). Thus, we hypothesized that the differences observed between 5-HT_{2A} KO and WT animals in both anxiolytic-like effects and DR 5-HT neuronal activity as a consequence of the administration of a low dose of THC might be related. Therefore, the decrease in DR 5-HT neuronal activity induced by THC could be associated with its anxiolytic-like properties, and it appears to be under the control of 5-HT_{2A} receptors, as KO animals did not present any of these effects.

We also tested whether these receptors were involved in the "rodent tetrad test", a battery of behavioural responses characteristics of CB_1 cannabinoid agonists. The administration of THC induced the expected dose-response curve in the different tests, confirming the antinociceptive properties of THC, as well as its hypothermic and hypolocomotor potential. Interestingly, we revealed that 5-HT_{2A} receptors were not implicated in THC-induced antinociception, hypoactivity, or hypothermia, as results obtained in THC-treated KO animals did not

differ from WT mice at any of the dose tested (0, 0.3, 1, 3 and 10 mg/kg). Moreover, the absence of differences between KO and WT animals in basal conditions was also indicative that 5-HT$_{2A}$ receptors were not mediating such effects. The involvement of 5-HT$_{2A}$ receptors in THC-induced catalepsy, which is the other behaviour included in the "tetrad" test of cannabinoids, was previously examined by Egashira and coleagues (2007). In that study, they revealed that DOI administration attenuated catalepsy-like immobilization induced by THC (Egashira et al., 2007). Thus, it may be possible that 5-HT$_{2A}$ receptors are involved in mediating THC-induced catalepsy.

We also addressed the role of 5-HT$_{2A}$ receptors in mediating the reinforcing effects of cannabinoids by using the WIN 55,212-2 self-administration paradigm (Mendizabal et al., 2006). Both WT and KO mice learnt to discriminate between the active and inactive nose pokes, and similarly self-administered the drug. Thus, 5-HT$_{2A}$ receptors do not seem to be involved in the reinforcing properties of the cannabinoid agonist WIN 55,212-2. This result is in accordance with previous findings where 5-HT$_{2A}$ receptors were not involved in mediating cocaine reinforcing properties in rats (Fletcher et al., 2002), whereas is in contrast with previous findings reporting the involvement of 5-HT$_{2A}$ receptors on the reinforcing properties of MDMA in mice (Orejarena et al., 2011), probably due to the direct involvement of these receptors in MDMA-induced effects.

Finally, we determined the involvement of 5-HT_{2A} receptors in THC withdrawal syndrome after a chronic exposure to high doses of THC (20 mg/kg twice daily during five days). We evaluated several somatic signs of abstinence following rimonabant treatment, and calculated a global withdrawal-score (GWS) by giving each sign a proportional weight. Interestingly, 5-HT_{2A} KO animals presented a significant attenuation in withdrawal signs such as paw-tremor and sniffing. Moreover, a significant reduction in the GWS was also revealed in KO when compared to WT group. These results indicate that 5-HT_{2A} receptors are necessary for the full expression of THC withdrawal. We also evaluated CB_1 receptor levels in the hippocampus and cerebellum at the end of this chronic treatment in WT and KO animals, and we found a significant decrease of CB_1 levels in both areas, which is consistent with previous findings reporting CB_1 receptor desensitization and downregulation after chronic exposure to the drug (Breivogel et al., 1999; Rubino et al., 2000). Interestingly, the receptor downregulation observed in the hippocampus was greater in KO than WT animals.

Together, all these findings encouraged us to further analyse the involvement of 5-HT_{2A} receptors in CB_1-mediated mechanisms. Thus, we focused on studying the possible molecular mechanisms involved in this interaction.

2.3. CB_1 and 5-HT_{2A} receptor signalling

CB_1 and 5-HT_{2A} receptors are members of the GPCR family. CB_1 signalling pathway has already been vastly covered in introduction (See

3.6. Cannabinoid receptor signalling). One of the most characterized effects upon stimulation of these receptors is the activation of heterotrimeric $G_{i/o}$ type G proteins, which induce the inhibition of the adenylyl cyclase, producing a decrease in cAMP production, accompanied by a decrease in PKA activity. Moreover, CB_1 receptor activation triggers the phosphorylation and stimulation of various members of the mitogen-activated protein kinase family, including ERK1/2 (Howlett, 2005). The activation of CB_1 receptors can also stimulate the phosphorylation of Akt and mTOR through a PI3K-dependent mechanism (Ozaita et al., 2007; Puighermanal et al., 2009). Using cells stably expressing CB_1 receptors, we performed the dynamic mass redistribution assay (DMR), which allows measuring GPCR signalling in a non-invasive way (Schroder et al., 2011). The stimulation of cells with WIN 55,212-2 induced a change in optical density which was reverted by the administration of pertussis (PTX), but not cholera (CTX) toxin, confirming that under these conditions CB_1 receptors signal through G_i (Sim-Selley, 2003). Moreover, WIN 55,212-2 reduced the forskolin-induced cAMP, and this effect was also blocked by PTX and not CTX. The stimulation of cells with WIN 55,212-2 also produced an increase in the phosphorylation of ERK1/2 and Akt. Moreover, WIN 55,212-2-induced phosphorylation of ERK1/2 was also observed in brain slices from several brain regions in both 5-HT_{2A} KO and WT mice, suggesting that CB_1 signalling in these animals was not altered.

On the other hand, 5-HT_{2A} receptor activates PLC through G_q type G proteins. This induces the cleavage of membrane-bound

phosphatidylinositol biphosphate producing the accumulation of the second messengers inositol trisphosphate (IP3) and DAG and activates the PKC (Hoyer et al., 1994). Cytoplasmic IP3 increase causes a release of intracellular calcium from the endoplasmic reticulum, which is the main characteristic of the activation of GPCRs signalling trough G_q subunits. However, the activation of 5-HT_{2A} receptors can also activate other signalling cascades depending on the activating ligand, a specific phenomenon called functional selectivity (Urban et al., 2007). 5-HT_{2A} receptors was one of the first receptors characterized by this phenomenon (Berg et al., 1998), and revealed that stimulation of 5-HT_{2A} receptor leaded to the production of at least three distinct biochemical signals, namely IP3/DAG, arachidonic acid, and the endocannabinoid 2-AG (Kurrasch-Orbaugh et al., 2003). In addition, different studies in several cell lines have linked 5-HT_{2A} receptor stimulation with changes in levels and activity of various molecules such as calmodulin, nitric oxide, ERK1/2 or Akt among others (Quinn et al., 2002; Johnson-Farley et al., 2005; Turner and Raymond, 2005; Göoz et al., 2006; Schmid and Bohn, 2010). Thus, apart from the major PLC, IP3/DAG/PKC pathway, activation of 5-HT_{2A} receptors can trigger other signalling cascades depending on the cell line and the ligand used to stimulate the receptors (See Figure 21).

We have confirmed that 5-HT_{2A} receptor signals through G_q protein using the dynamic mass redistribution assay in cells stably expressing 5-HT_{2A} receptors. Thus, changes in optical density induced by the administration of DOI were not reverted by PTX or CTX, but they were

indeed reverted by the G_q protein inhibitor YM-254890. We have also reported that DOI stimulation induced an increase in ERK1/2 and Akt phosphorylation in cells and in several brain areas in WT mice. As expected, the increase in ERK1/2 and Akt phosphorylation induced by DOI was not observed in any of the brain areas in 5-HT_{2A} KO animals.

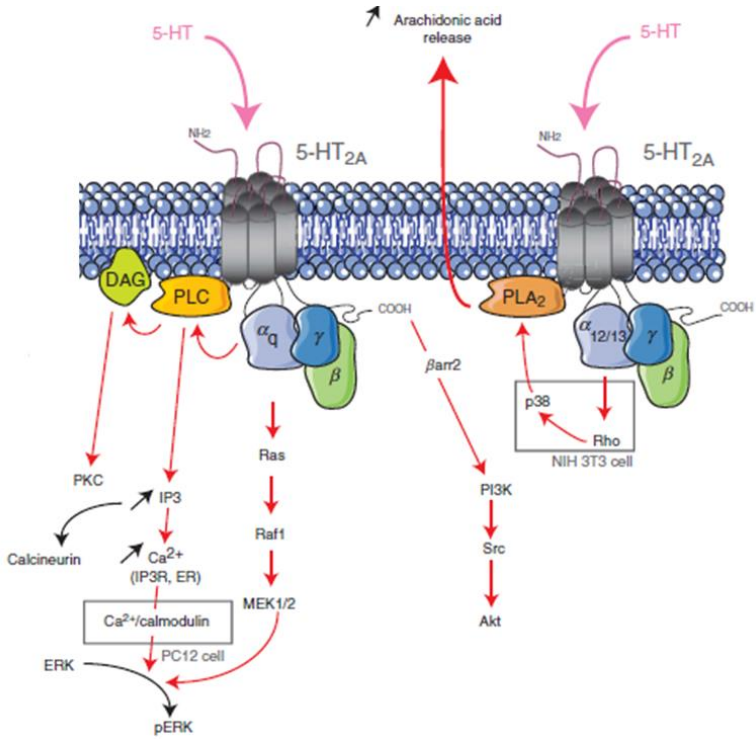

Figure 21. 5-HT_{2A} receptor signalling pathways in neurons. 5-HT2A receptor signalling through Gq induces the activation of PLC/DAG/PKC pathway, the production of 2-AG, and the release of intracellular calcium from the endoplasmic reticulum. Moreover, the activation of MAPK pathways is also produced, as well as the activation of Akt through β arrestin II and PI3 kinase. In addition, 5-HT2A receptor signalling through G12/13 protein leads to the release of arachidonic acid as a consequence of PLA2 activation.

Both of these receptors have been traditionally considered as monomeric structural units that are coupled to intracellular heterotrimeric G proteins. However, recent evidence suggests that they can also assemble into homomers (Guo et al., 2008) or heteromers (Milligan, 2009). In fact,

previous studies have identified the formation of heteromeric complexes involving 5-HT_{2A} receptors, such as 5-HT_{2A}R-mGlu2R and 5-HT_{2A}R-D_2R (González-Maeso et al., 2008; Borroto-Escuela et al., 2010). Similarly, CB_1 receptors can form heteromers with dopamine D_2, adenosine A_{2A}, μ and δ opioid, and hipocretin Hcrtr-1 receptors (See 3.6.1. CB_1 heteromerization). Thus, because of the co-localization of CB_1 and 5-HT_{2A} receptors in key brain structures underlying cognition and mood processing, and the involvement of 5-HT_{2A} receptors in mediating several THC-induced effects, we analysed whether the hypothetical interaction between these receptors was possible.

2.4. Discovery of a new receptor entity: the CB_1-5-HT_{2A} heteromer

We used three different techniques in Human Embryonic Kidney 293 (HEK) cells and in mouse brain tissue to explore the possible formation of 5-HT_{2A}-CB_1 heteromers. First we used BRET, a technique based on the transfer of energy between two different molecules depending on their spatial proximity. BRET has already been used to analyse GPCR oligomerization (Bouvier et al., 2007), and to monitor protein-protein interactions (Issad and Jockers, 2006). With this technique, we demonstrated the existence of 5-HT_{2A}-CB_1 complexes in HEK cells. Further evidence supporting this interaction was provided by using the Bimolecular Fluorescence Complementation (BiFC), a technique which relies on the formation of a protein by complementation of two truncated segments (Hu et al., 2002), that has been successfully applied in the study

of GPCR heteromerization (Guitart et al., 2014). Both BRET and BiFC assays indicated that 5-HT_{2A} and CB_1 receptors were in very close proximity, which is required for the receptors to interact. Moreover, we also corroborated the existence of 5-HT_{2A}-CB_1 heteromers by using Proximity Ligation Assays (PLA) in mouse brain slices. This technique allows the direct detection of molecular interactions between two proteins. In this case, both receptors need to be in close proximity in order to allow two different complementary antibody-DNA probes to anneal, forming double stranded segments. Then, these segments are amplified by *in situ* Polymerase Chain Reaction in the presence of fluorescent nucleotides to allow detection using a microscope. Thus, 5-HT_{2A}-CB_1 complexes have not only been observed *in vitro* in a controlled environment, but also *in vivo* in brain slices under physiological conditions.

2.4.1. Specific functionality of CB_1-5-HT_{2A} heteromers

At a functional level, there are several putative consequences as a result of heteromer formations. These consequences might include changes in G protein binding, alterations in downstream signalling upon co-stimulation of both receptors, and the possibility of cross-antagonism. We found that the co-expression of CB_1 and 5-HT_{2A} receptors in cells causes a switch to another type of G protein coupling. Using the DMR assay, we showed that the DMR signal induced by WIN 55,212-2 was inhibited by PTX, indicating that in case of co-expression of both receptors WIN 55,212-2 signalling was still produced through the

activation of Gi proteins, suggesting that CB_1 signalling was unaffected. However, when those cells were stimulated with DOI, the effects obtained in the DMR were also inhibited by PTX, indicating that DOI signalling was also produced through Gi proteins when both receptors are co-expressed. Under these circumstances where the formation of CB_1-5-HT_{2A} heteromers has been confirmed, the stimulation of 5-HT_{2A} receptors with DOI no longer induces the activation of Gq, but it activates Gi. This change in G protein signalling was also corroborated analysing alterations in cAMP production, one of the main effectors of Gi signalling. Stimulation with forskolin lead to an increase in cAMP, which was reduced with the administration of WIN 55,212-2 and this reduction was sensitive to PTX, revealing the involvement of an activation of Gi. Supporting the results obtained in the DMR assay, the administration of DOI also induced a reduction in cAMP production as WIN 55,212-2 did. This reduction was also prevented with the administration of PTX, confirming that when CB_1 and 5-HT_{2A} receptors are co-expressed, stimulating 5-HT_{2A} receptors induces Gi signalling instead of the usual Gq signalling. These results indicate that the heteromer presents a signalling profile different from the single receptors, and suggests that in this case blocking Gi might be sufficient to block the signalling produced by the CB_1-5HT_{2A} heteromer. This switch in G protein coupling has already been observed with other GPCR heteromers. For example, a similar result was obtained with the $mGluR_2$-5-HT_{2A} heteromer, where 5-HT_{2A} receptor signalling was also mediated through Gi under the heteromeric form (Fribourg et al., 2011).

We also demonstrated alterations in downstream signalling upon dual stimulation of the receptors in the heteromer, which has been reported as a common consequence of heteromerization (González et al., 2012; Kern et al., 2012; Baba et al., 2013). In cells co-expressing both CB_1 and $5\text{-}HT_{2A}$ receptors, we compared the effects obtained after stimulation with WIN 55,212-2 or DOI with those produced by co-stimulation. Stimulation with WIN 55,212-2 and DOI induced a decrease in forskolin-induced cAMP, an increase in ERK1/2 and Akt phosphorylation and a recruitment of β-arrestin II. Co-stimulation with both agonists did not modify the levels of ERK1/2 or Akt phosphorylation in comparison with single stimulation. In addition, co-stimulation led to a greater reduction of cAMP production, and to a decrease in recruitment of β-arrestin II when compared with the effects caused by the administration of the single agonists. Taken together, these results suggest that co-stimulation of the CB_1-$5\text{-}HT_{2A}$ heteromer leads to a reduction in cell signalling. Indeed, this reduction was confirmed when p-ERK1/2 levels were analysed in isolated brain slices from cortex, hippocampus and dorsal striatum. Co-stimulation with WIN 55,212-2 and DOI did not induce an increase in p-ERK1/2 levels in comparison with single stimulation and were even lower. Remarkably, this reduced signalling was not observed in all of the brain regions studied. Hence, co-stimulation induced a significant increase in p-ERK1/2 in the NAc in comparison with single agonist stimulation, suggesting that the CB_1-$5\text{-}HT_{2A}$ heteromer may be expressed in selective brain regions where it could modulate specific THC-induced responses.

Another effect observed in some GPCR heteromers is the so-called cross-antagonism, which is the ability of an antagonist of one of the receptors to antagonize the signalling of the other receptor in the heteromer (Moreno et al., 2011; 2014). This phenomenon requires direct protein-protein interactions, as antagonists do not signal on their own. In our study we revealed that this effect was also occurring, as the administration of CB_1 receptor antagonist, rimonabant not only blocked WIN 55,212-2-induced effects, but also blocked the effects of DOI. Rimonabant pre-treatment prevented the decrease in cAMP, the recruitment of β-arrestin II, as well as the phosphorylation of ERK1/2 and Akt induced by both, WIN 55,212-2 and DOI. Correspondingly, the administration of the $5\text{-}HT_{2A}$ antagonist, MDL 100907 also prevented both DOI- and WIN 55,212-2-induced effects, indicating that the cross-antagonism observed in $CB_1\text{-}5\text{-}HT_{2A}$ heteromer was bidirectional. Further evidence supporting the cross-antagonism was reported using two different approaches in mice. First, we analysed the cross-antagonism in brain slices from WT and KO animals, and then we tested whether it could also occur at a behavioural level. Levels of p-ERK1/2 were increased by both WIN 55,212-2 and DOI in all the brain areas tested (cortex, hippocampus, dorsal striatum and NAc). Bidirectional cross-antagonism was observed in the brain of WT animal in the cortex, hippocampus and dorsal striatum, but not in the NAc. The absence of cross-antagonism in the NAc together with the differential p-ERK1/2 induction upon co-stimulation suggested a possible lack of heteromeric formation in this brain area. As expected, no cross-antagonism was

observed in 5-HT2A KO mice. Finally, cross-antagonism was also revealed in our behavioural experiments, since pre-treating WT animals with MDL 100907 prevented the amnesic- and anxiolytic-like effects induced by THC, further supporting the role of CB_1-5-HT_{2A} heteromers in the amnesic-like effects induced by THC.

In order to provide some guidance in defining the physiological relevance of GPCR heteromers, the International Union of Basic and Clinical Pharmacology (IUPHAR) released some recommendations (Pin et al., 2007). The IUPHAR established that at least two out of the three following criteria should be met before the acceptance of new heteromers by the scientific community: (1) Evidence of physical association in native tissue or primary cells. This may be achieved by co-immunoprecipitation experiments with selective antibodies or using energy transfer techniques. (2) Evidence for specific properties of the heteromer such as heteromer-specific signalling properties, the presence of heteromer-selective ligands, or the presence of allosteric ligand binding properties. (3) Supporting evidences from KO animals or RNA interference technologies to validate the functionality of the heteromers *in vivo*. Therefore, according to these recommendations, the results presented in our study are sufficient to accept the formation of CB_1-5-HT_{2A} heteromers since we have evidenced the physical interaction of CB_1 and 5-HT_{2A} using FRET and PLA. Moreover, we have also demonstrated that CB_1-5-HT_{2A} heteromers present signalling properties different from the single receptors, and we have confirmed our results using mutant animals lacking 5-HT_{2A} receptors (Article 3).

2.5. Molecular basis of cross-antagonism in CB_1-5-HT_{2A} heteromers and its validation

The huge increase in the number of solved GPCR structures bond to agonists, antagonists, or in complex with G protein complexes have facilitated the understanding of the molecular basis of GPCR activation (Venkatakrishnan et al., 2013). It has been reported that agonist binding induces small structural changes in the extracellular side (Rasmussen et al., 2011a), which are translated into larger re-arrangements in the intracellular structure (Rasmussen et al., 2011b). Specifically, agonists increase receptor signalling through the movement of transmembrane helices (TMs) 5 and 6, facilitating the interaction with G proteins. The molecular basis of bidirectional cross-antagonism in terms of structural changes has not been described yet. However, a novel model of dimerization has been revealed using the crystal structure of the μ-opioid receptor (Manglik et al., 2012). In this model, TMs 5 and 6 of one receptor form a very stable four-helix bundle with TMs 5 and 6 of the other receptor, because of a high surface complementarity. Based on these findings and considering that GPCRs exist in a dynamic equilibrium between a continuum of conformations with close energetic states, which are stabilized by the presence of different ligands (Kobilka and Deupi, 2007), we hypothesized that antagonist binding to one of the receptors could prevent the required re-arrangements in TMs 5 and 6 by stabilizing the four-helix bundle conformation. In order to test this hypothesis, we investigated whether we could disrupt the CB_1-5-HT_{2A} heteromerization using synthetic peptides with the sequence of TMs 5

and 6 of CB_1 receptor. This approach has already been used successfully in recent studies (Guitart et al., 2014; Kabli et al., 2014; Lee et al., 2014). Using these interfering peptides we were able to disrupt the CB_1-5-HT_{2A} heteromer not only in cells, but also in tissue from WT animals, an effect revealed by BiFC and PLA respectively. The administration of a control peptide with the sequence of TM 7 of CB_1 receptor did not disrupt the formation of the heteromer neither in cells nor in tissue. In addition, with the administration of TMs 5 and 6 peptides we also confirmed that the heteromer was necessary for the cross-antagonism. Thus, pre-treatment with the peptides abolished the cross-antagonism in cells when cAMP production was analysed as well as when p-ERK1/2 and p-Akt levels were measured. As a consequence of TMs 5 and 6 pre-treatment, rimonabant was no longer able to block DOI effects and conversely, MDL 100907 did not antagonise WIN 55,212-2 effects. The administration of TM 7 had no consequences in terms of cross-antagonism, leading to the conclusion that TMs 5 and 6 were crucial for the formation of CB_1-5-HT_{2A} heteromers, and for their functionality. Our behavioural experiments in animals also revealed that the intracerebroventricular administration of TM 5 and 6 peptides prevented the amnesic- and anxiolytic-like effects induced by THC, whereas animals pre-treated with TM 7 presented both THC-induced effects. Importantly, the antinociceptive properties of THC were not altered as a consequence of the peptide pre-treatment, corroborating that the disruption of heteromers could be differently affecting some of the effects induced by THC, highlighting their relevance.

2.6. Relevance of the CB_1-$5HT_{2A}$ heteromers

The discovery and the functional characterization of CB_1-5-HT_{2A} heteromers represents a major finding in the field of cannabinoid research, as it points to a possible way for dissociating some of the beneficial effects induced by THC from its undesirable effects. Moreover, due to their unique signalling properties, CB_1-5-HT_{2A} heteromers might represent a novel pharmaceutical target of interest in those disorders associated with alterations of CB_1 and 5-HT_{2A} receptors since compounds acting on these receptors can also potentially signal through the heteromer. Indeed, it is known that not all receptors present in the cell membrane show the same state of heteromerization (Ward et al., 2011), which highlights the importance of the simultaneous signalling through single and heteromer receptors when a drug is administered. Therefore, potential modulation could be achieved by either specifically targeting the heteromer with novel compounds, while avoiding acting on the single receptors, or by disrupting the heteromer so that only single receptors can be activated. In our study, we have seen that the disruption of the heteromer, or its absence, in the case of KO animals, leads to a significantly different profile of THC actions. Interestingly, the modulation in the opposite direction has also been revealed (Darmani, 2001; Aso et al., 2009). This suggests that potential therapies targeting the heteromer could also be designed to modulate the undesirable effects of current antipsychotic drugs.

An issue that has not been addressed in our study is the specific sub-cellular locations of the CB_1-5-HT_{2A} heteromer. The differential

distribution of CB_1 in glutamatergic and GABAergic neurons (Marsicano and Lutz, 1999; Kawamura et al., 2006) suggests that CB_1-5-HT_{2A} heteromers could also be differentially expressed in those neuronal types. It is known that the specific location of CB_1 receptors in either GABAergic or glutamatergic neurons is critical in some of the effects induced by THC. In this regard, studies using mutant mice specifically lacking CB_1 receptors in glutamatergic or GABAergic neurons (Monory et al., 2006) have been very valuable. Indeed, THC-induced amnesic-like effects are dependent on CB_1 receptors present in GABAergic neurons, as GABA-CB_1 KO animals did not present the memory impairments induced by THC (Puighermanal et al., 2009). In addition to its presence in neurons, CB_1 receptors have also been found in brain astroglial cells (Navarrete and Araque, 2010), and the impairment in short-term working memory induced by THC has been associated with the presence of CB_1 in this specific cell type (Han et al., 2012). Besides, modulation of anxiety-like behaviour by THC has also been associated with the location of CB_1 receptors in specific neuronal populations. Thus, CB_1 receptors located at glutamatergic terminals mediate the cannabinoid-induced anxiolytic-like effects, whereas CB_1 receptors present in the GABAergic terminals are required for the anxiogenic-like properties of cannabinoids (Rey et al., 2012). Based on our studies, we can speculate that CB_1-5-HT_{2A} heteromers could be expressed in both GABAergic and glutamatergic terminals. In fact, we showed that the presence of heteromers was crucial for the amnesic-like effects of THC, which are related with the presence of receptors in GABAergic terminals (Puighermanal et al., 2009),

although we have also observed alterations in the anxiolytic-like properties of THC, an effect that has been related with CB_1 receptors expressed on glutamatergic terminals (Rey et al., 2012).

Moreover, CB_1 receptors expressed on glutamatergic neurons are more sensitive to agonist-induced activation (Ohno-Shosaku et al., 2002; Lee et al., 2010), and more effective in terms of G protein coupling (Steindel et al., 2013), than CB_1 receptors in GABAergic neurons. Thus, another important issue that has yet to be determined is the role of CB_1-5-HT_{2A} heteromers in the activity of GABAergic and glutamatergic neurotransmission under physiological conditions. We could speculate that depending on the expression pattern of CB_1-5-HT_{2A} heteromers, the normal balance between these neurotransmitter systems could be altered, leading to major functional consequences.

CONCLUSIONS

The main conclusions of the work presented in this thesis are:

1. Under acute conditions, neurotoxic doses of MDMA transiently decrease responding for a palatable food reward under a fixed ratio schedule of reinforcement. On the other hand, residual alterations in the motivation for palatable food were observed following treatment with both neurotoxic and non-neurotoxic regimens of MDMA evidenced by lower breaking points in a progressive ratio schedule of reinforcement.

2. Repeated administration of neurotoxic doses of MDMA disrupts performance on the previously acquired operant alternation and visual-cue discrimination tasks under a drug-free state. However, MDMA does not impair posterior learning processes.

3. Long-lasting impairments in cognitive flexibility are observed as a consequence of the repeated administration of neurotoxic doses of MDMA. This effect is not only evidenced as a marked resistance to extinction, but also as an increase in perseverant responding in the attentional set-shifting task.

4. Repeated administration of neurotoxic doses of MDMA induces a long-lasting decrease in DAT binding in mice, which may be related to the lower levels of stimulated dopamine release observed. These alterations are associated with the temporary impairments in memory and recall, and may also contribute to the lack of behavioural flexibility.

5. The amnesic, anxiolytic, and pro-social-like effects induced by THC are reduced in 5-HT_{2A} KO mice, as well as the manifestations of THC withdrawal syndrome. In contrast, 5-HT_{2A} receptor deletion does not modulate the acute hypolocomotor, hypothermic, anxiogenic and antinociceptive effects of THC or the reinforcing effects of the cannabinoid agonist, WIN 55,212-2.

6. In vitro molecular assays and ex vivo studies using PLA in mouse brain slices revealed a direct interaction between CB_1 and 5-HT_{2A} receptors, and the formation of CB_1-5-HT_{2A} heteromers.

7. The formation of the CB_1-5-HT_{2A} heteromer complex induces a shift in G-protein coupling by 5-HT_{2A} receptors from Gq to Gi, generating a unique signalling profile different from the one observed in each single receptors.

8. CB_1-5-HT_{2A} heteromers are present in specific brain structures involved in THC responses regulated by 5-HT_{2A} receptors such as cortex, hippocampus and striatum, but not in the nucleus accumbens, a key structure of the reward circuit.

9. The administration of specific transmembrane interference peptides disrupts CB_1-5HT_{2A} heteromerization in vitro and in vivo, leading to a selective abrogation of memory impairments caused by exposure to THC.

10. Targeting the CB1-5-HT2A heteromer may serve to dissociate the potential therapeutic properties of cannabinoids from their unfavourable side-effects.

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ANNEX

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