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Study of the interaction between 3,4-methylenedioximethamphetamine and the endocannabinoid system

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CERTIFY:

That the current PhD project entitled “Study of the interaction between MDMA and the endocannabinoid system” presented by Clara Touriño Raposo, B.S. degree in Biology, has been performed under their direction, and fulfills the necessary requestes to be evaluated.

In witness whereof, and for such purposes as may arise, the following certification is signed on December 1st 2008

Olga Valverde Granados Rafael Maldonado López

A la memoria de mi yayo, el sastrecillo valiente

Agradecimientos

Este trabajo de tesis no solo me ha aportado gran cantidad conocimientos científicos sino también una serie de valores personales que me han transmitido todas aquellas personas que han estado a mi lado durante estos años. Todos vosotros habéis contribuido de una manera o otra a mi crecimiento personal.

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A todos gracias.

2-AG	2-Arachidonoylglycerol
5-HT	Serotonin
AEA	Anandamide
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine-5'-monophosphate
CBD	Cannabidiol
CNS	Central nervous system
CEA	Central nucleus of the amygdala
CPP	Conditioned place preference
CREB	cAMP response element binding protein
CRF	Corticotrophin-releasing factor
DA	Dopamine
DAT	Dopamine transporter
DAG	Diacylglicerol
DSI	Depolarization-induced suppression of inhibition
FAAH	Fatty acid amide hydrolase
FR	Fixed ratio
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
HPA	Hypothalamic-pituitary-adrenal
HHMA	3, 4-dihydroxymethamphetamine
HMMA	4-hydroxy-3-methoxymethamphetamine
IEG	Immediate early genes
IFN-γ	Interferon- γ
IL-1β	Interleukin-1β
LTD	Long-term depression
LTP	Long term potentiation
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinases
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
NA	Noradrenalin
NAc	Nucleus accumbens

NAPE	*N*-arachidonoyl phosphatidylethanolamide
NO	Nitric oxide
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
PFC	Prefrontal cortex
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
SERT	Serotonin transporter
THC	Δ^9-tetrahydrocannabinol
TNF-α	Tumor necrosis factor-α
VP	Ventral pallidum
VTA	Ventral tegmental area

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Introduction

1. Neurobiology of addiction

Drug addiction is a chronic, relapsing disorder in which compulsive drug-seeking and drug-taking behavior persists despite serious negative consequences. In addict subjects, drug taking become an essential issue in their lives. The drug-centered existence of addicts can cost them their jobs, personal relationships, financial standing, happiness, and in some cases their lives. Drug addicts often appear to have lost the ability to make choices that promote their own happiness and survival. Many drug addicts who seek treatment report that they realize the destructive nature of their addiction but are unable to alter their compulsive behavior (Koob and Le Moal, 2006). Drug addiction is the endpoint of a series of transitions; (1) initial drug use, when a drug is voluntarily taken because it hedonic effects, (2) drug abuse or harmful use, (3) loss of control over this behavior, such that it becomes compulsive. However, not all drug users become addicts. Vulnerability to develop a drug addiction is influenced by a combination of genetic and environmental factors, which determine drug-induced effects and influence the progression of the different phases of addiction. Each step in the progression of addiction is characterized by different behavioral, physiological and molecular features (Kalivas, 2005).

1.1. Acute drug taking.

Drugs of abuse are highly diverse chemical substances with particular mechanisms of action (Camí and Farré, 2003). Accordingly, each drug binds to different protein targets located at the synapse. Drugs such as opioids and cannabinoids bind to G-coupled

protein receptors, nicotine or phencyclidine act on ligand-gated ion channels, and drugs such as cocaine or amphetamines inhibit monoamine transporter (Hyman and Malenka, 2001). Each of these drugs elicits a different combination of behavioral and physiological effects upon acute administration. However, the acute administration of all drugs with abuse potential shares the induction of both rewarding and reinforcing effects. A reward is a stimulus that the brain interprets as intrinsically positive, whereas a reinforcing stimulus is one that increases the probability that behaviors paired with it will be repeated. Pleasant stimuli such as food, sex or social acknowledgement are rewarding, and usually reinforcing. However, non-rewarding stimuli may also increase the probability that behaviors paired with it will be repeated, such as pain healing or punishment removal. Thus, the withdrawal of aversive stimuli is also reinforcing.

The neural substrates that underlie the perception of reward and the phenomenon of positive reinforcement are a set of interconnected forebrain structures called brain reward pathway or mesocorticolimbic dopamine (DA) system (Fig. 1). Mesocorticolimbic DA projections originate in the ventral tegmental area (VTA) of the ventral midbrain and project through the medial forebrain bundle to limbic and forebrain structures. The DA projections that extend from the VTA to the nucleus accumbens (NAc; the major component of the ventral striatum) are the best-established substrate for reward. All addictive drugs cause selective elevation of extracellular DA levels in the medial subdivision of the NAc (NAc shell). The rewarding properties of drugs of abuse are related to their ability to enhance DA release in the NAc. Drug-induced pleasurable states are important motivators of initial drug use, and DA neurons projecting from the VTA to the NAc are essential in producing this pleasure. However, the motivational component that promotes drug seeking is mediated by DA projections from VTA to prefrontal cortex (PFC). All these connections are part of a complex neural circuit that regulates limbic emotional-motivational information and extrapyramidal regulation of motor behavior (Nestler et al., 2001) (Fig. 1). Exposure to drugs of abuse releases not only DA but also glutamate in the mesolimbic pathway. The release of glutamate promotes changes in synaptic plasticity that after repetitive drug taking may lead to addiction in vulnerable individuals (Kauer and Malenka, 2007).

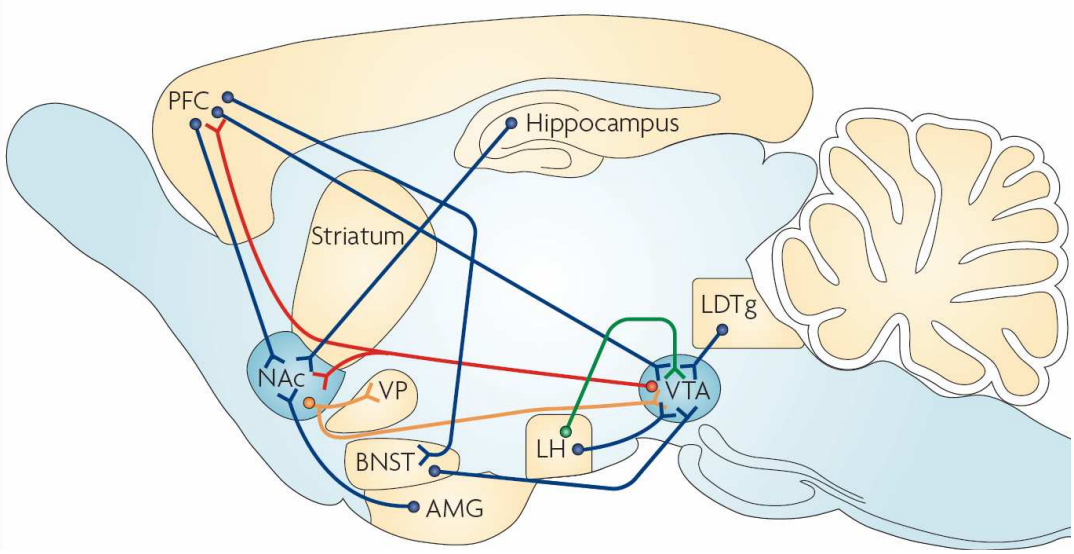

Figure 1. Mesolimbic DA system circuitry. Simplified schematic of the circuitry of the mesolimbic DA system in the rat brain highlighting the major inputs to the nucleus accumbens (NAc) and ventral tegmental area (VTA) (glutamatergic projections, blue; DA projections, red; GABAergic projections, orange; orexinergic projections, green). AMG, amygdala; BNST, bed nucleus of the stria terminalis; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VP, ventral pallidum (from Kauer and Malenka, 2007).

The molecular responses to acute drug administration induced by DA and glutamate release are generally short-lived. DA and glutamate receptors activation causes the alteration of post-receptor intracellular messenger pathways. An outstanding intracellular consequence after the acute exposure to several addictive drugs is the inhibition of the functional activity of the cyclic adenosine-5'-monophosphate (cAMP) pathway by inhibiting adenylyl cyclase, the enzyme that catalyzes the synthesis of cAMP (Chao and Nestler, 2004). Together with other second messengers, cAMP modulates protein kinases and phosphatases, leading to the induction of several transcription factors, including immediate early genes (IEG). IEG are a class of genes whose expression is induced within minutes of exposure to a stimulus. Acute exposure to drugs of abuse rapidly induces proteins such as Fos family transcription factors, Homer1a, NAC-1 and Narp, which are involved in neural activation. These proteins respond to drugs of abuse with a characteristic sharp upregulation followed by a quick decline to basal levels within hours (Hope et al., 1992; Kalivas, 2005). Unfortunately, it is still unclear which of these changes are antecedent to habitual drug use.

1.2. Habitual drug use and drug dependence.

After repeated administration the effects of a drug diminish, and is necessary to increase the dose to produce the same effect. This process is known as *tolerance*. Tolerance may develop to some of the effects of a drug but not to others. The reverse situation occurs when repeated administration of the same drug dose elicits escalating effects. This process is known as *sensitization*. Repeated drug administration induces the development of an adaptive state known as *dependence*. When unmasked by drug cessation, this adapted state can result in the production of cognitive, emotional or 'physical' withdrawal symptoms. Dependence resulting from long-term drug use may have both a somatic component manifested by physical symptoms and an emotional-motivational component, manifested by dysphoria and anhedonic symptoms that occur when the drug is discontinued. While, dysphoria is observed after discontinuation of all drug of abuse, physical dependence occurs only with certain drugs. More is known about adaptations that produce physical dependence than about adaptations related to emotional and motivational aspects of dependence (Nestler et al., 2001).

1.2.1. Physical dependence and withdrawal.

So-called 'physical dependence' on drugs of abuse results from adaptations that occur in brain circuits that control directly observable physiological functions, such as heart rate or blood pressure. Withdrawal symptoms associated with physical dependence vary depending on the type of drug that has triggered dependence. Physical dependence is believed to result from homeostatic regulatory processes in the body, which, in the continued presence of the drug, counteract the effect of drug presence and return the system to a normal level function. The emergence of withdrawal signs in response to cessation of drug use in a physically dependent individual indicates that the body has adapted to the presence of the drug and requires it for normal function. During withdrawal, the overcompensated system is suddenly unopposed by the drug. Consequently, withdrawal symptoms are generally opposite to the acute effects produced by drug exposure. The molecular adaptations that underlie tolerance and physical dependence are well known for repeated opioid administration (Gellert and Holtzman, 1978), and take place primarily in the locus coeruleus (Maldonado, 1997). It is also known that alterations in cAMP signaling pathways in the cerebellum importantly contribute to cannabidnoid physical dependence (Hutcheson et al., 1998; Castañé et al., 2004; Gonzalez et al., 2005). Some of these neuronal and molecular adaptations have also been described for nicotine (Balfour, 1994) and alcohol (Littleton, 1998). However, physical dependence is not a necessary component of drug addiction since

highly addictive drugs such as cocaine or amphetamines do not produce a withdrawal syndrome. Furthermore, the presence of physical dependence alone is not sufficient evidence of addiction because many drugs that are nonaddictive (e.g. β-adrenergic antagonists, tricyclic antidepressants, and antipsychotics) produce physical dependence and withdrawal symptoms but do not lead to compulsive use. This emphasizes the emotional motivational aspects of dependence. Nevertheless, physical dependence is an important component in the abuse of drugs as opioids, cannabis, ethanol and nicotine (Nestler et al., 2001).

1.2.2. Emotional and motivational aspects of dependence.

The emotional and motivational component of drug dependence is manifested by withdrawal symptoms such as anhedonia, depression, anxiety and negative motivational states. The fact that these symptoms can be characterized as opposite to the initial effects of addictive drugs suggests that they may result from counteradaptations to prolonged drug exposure. While the reinforcing effects of addictive drugs are believed to be related to actions on brain reward circuitry, emotional and motivational aspects of dependence may be related to drug-induced adaptations in the same circuitry. These adaptations within reward pathway may be potential mediators of drug withdrawal dysphoric states. Actually, reduced mesolimbic DA activity is associated with drug withdrawal, inducing emotional-motivational symptoms. Up-regulation of the cAMP pathway in the NAc is a common adaptation to long-term exposure to several types of addictive drugs, including opiates, cocaine, and ethanol, but is not a feature associated with the use of nonaddictive drugs. Adaptations in the cAMP pathway consist of increased levels of adenylyl cyclase and protein kinase A, and decreased levels of Gi/o proteins. Up-regulation of the cAMP pathway in the NAc in response to long-term drug administration may contribute to negative motivational states that characterize withdrawal. Up-regulation of the cAMP pathway occurs in the γ-aminobutyric acid (GABA) interneurons that innervate DA projections in the NAc. Activation of cAMP pathway in these cells during withdrawal may lead to increased GABA release and consequently reduced firing of the DA cells. Such activity may account for the reduction in DA neurotransmission from the VTA to the NAc occurring during withdrawal, and that is believed to contribute to aversive withdrawal states. Long-term drug use has also been found to alter the function of cAMP response element binding protein (CREB), a member of the bZIP superfamily of transcription factors. CREB may mediate the effects of drug exposure on several neuropeptide genes known to contain CRE sites such as prodynorphyn. Prodynorphin contributes to

the generation of aversive states during withdrawal by decreasing DA release in the NAc through an action of kappa opioid receptors located on presynaptic DA nerve terminals in these regions.

Moreover, several studies have implicated CRF system in the mediation of many of the anxiogenic and aversive aspects of drug withdrawal (Sarnyai et al., 2001). Corticotropin-releasing factor (CRF) is a neuropeptide that is expressed in neuroendocrine cells of the hypothalamus, where stimulates the release of drenocorticotropic hormone in the pituitary gland, or in neurons of the central nucleus of the amygdala, where it acts as a neurotransmitter. It plays an important role in stress response and anxiety states. Increased release of CRF, particularly in the central nucleus of the amygdala, occurs during withdrawal from ethanol, opiates, cocaine and cannabinoids. Accordingly, CRF antagonists have successfully reversed the aversive effects of cocaine, ethanol, and opiate withdrawal in laboratory animals.

1.3. Transition to addiction .

1.3.1. Compulsive drug seeking and craving.

The transition from recreational drug use to an addicted state is marked by a dramatic escalation in the amount of drug consumed and by profound increases in craving and compulsive drug-seeking behavior (Self, 2004). The pleasure (or relief of dysphonic moods) produced by drugs that motivates initial drug use often habituates. The symptoms of both physical and emotional-motivational withdrawal subside relatively rapidly after drug use is terminated and cannot account for the high incidence of relapse among users of addictive drugs, particularly after signs of withdrawal have long finished. Drug-taking behavior, in certain individuals, evolves into compulsive patterns of drug-seeking and drug-taking behavior that take place at the expense of most other activities. This pattern of behavior is the consequence of a desire to reexperience the effect of the psychoactive substance and is known as *craving*. Robinson and Berridge (1993) proposed that, in addiction, compulsive drug-seeking and drug-taking behavior is often not motivated by either the desire to obtain pleasure or by the desire to relieve withdrawal. As opposed to drug social use, the dominant emotional response to drug is no longer ‘liking’, but an intense drug urge or ‘wanting’. Thus, although rewarding effects disappear during repeated drug taking the anticipatory expectancy of drug is progressively enhanced increasing the reinforcing effects of drugs. The underlying sensitization of neuronal structures persists for long periods of time, making addicts vulnerable to relapse.

Under normal circumstances, drug consumption produce rewarding effects by DA circuit activation. After repeated drug taking DA circuits undergo a number of neuroadaptations, and as a consequence addict subjects are no longer capable of experiencing drug-induced reward. Instead of that, an anticipatory reward occurs, and transient firing of DA neurons take place just before drug administration. The rewarding response to the drug itself habituates and, instead, DA neurons firing occurs before drug administration triggered by the presence of drug associated-cues. However, compulsive drug intake, intense drive to take the drug, and loss of control that characterize drug addiction are not explained by the anticipatory reward. Changes in other pathways different from the reward circuits are necessary to mediate addiction. Therefore, circuits involved with drive and preservative behavior may play a major role in drug addiction. Representation of goals, assignment of value to them, and selection of actions based on the resulting valuation are functions under control of PFC. Normal subjects value many goals, making it necessary to select among them, but addict subjects show a pathological narrowing of goal selection to those that are drug related (Self, 2004; Hyman, 2005). Within the PFC, the OFC is networked with the amygdala, dorsal striatum, NAc, hypothalamus, insula, and medial prefrontal cortex and it is then in a position to integrate emotional and motivational information with object representations held in working memory (Schoenbaum et al., 2006) (Fig. 2).

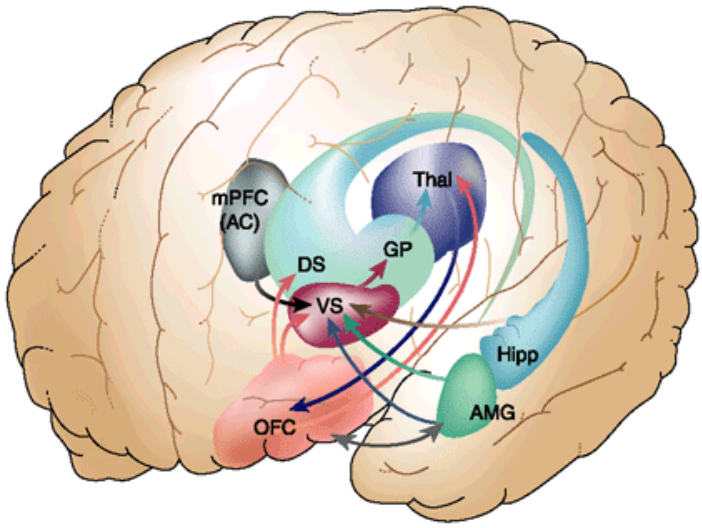

Figure 2. Circuits involved with the transition to craving in the human brain. (Acb, nucleus accumbens; AMG, amygdala; BLA, basolateral amygdala; CeN, central nucleus of the amygdala; VTA, ventral tegmental area; SNc, substantia nigra pars compacta. GP, globus pallidus (D, dorsal; V, ventral); Hipp, hippocampus; mPFC, medial prefrontal cortex; AC, anterior cingulate cortex; OFC, orbitofrontal cortex; VS, ventral striatum; DS, dorsal striatum; Thal, thalamus) (Everitt and Robbins, 2005).

Similar to the NAc, the PFC receives DA innervation from the VTA. In line with its posited role in reinforcement learning, phasic DA release has been hypothesized to gate the updating of information in the PFC such that appropriate new goals can be encoded and selected (Cohen et al., 2002b; Montague et al., 2004). As in the NAc, addictive drugs would be expected to produce a distorted and excessive DA signal in the OFC and other regions of the PFC because of their ability to elevate DA by their direct pharmacologic action. This distorted DA signal has been hypothesized to produce overlearning of drug-related cues, thus leading to the valuation of drugs above other goals (Montague et al., 2004).

In addition to distorting valuations of goals and narrowing the focus of behavior, drug taking has been hypothesized to impair the self-control and willpower by producing pathological adaptations in the PFC (Kalivas, 2005). In general, impairments in executive function and thus increased impulsivity have been correlated with the diminished ability to recruit the PFC in regular drug users. Together, PFC dysfunction results in the compulsive behavior and the exaggerated motivation to administer the drug regardless of its adverse consequences typical of addicted subjects (Volkow and Fowler, 2000) (Fig. 3).

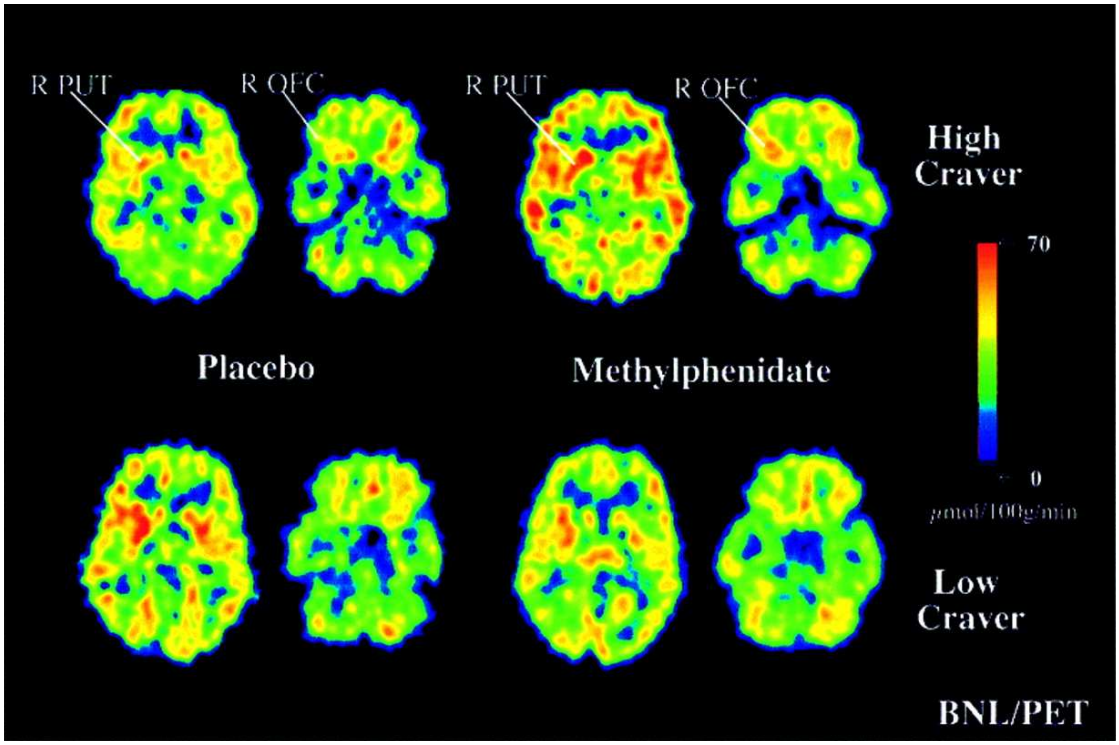

Figure 3. Regional brain metabolic images of a cocaine abuser in whom methylphenidate induced intense craving and one in whom it did not. Notice the activation of the right orbitofrontal cortex (R OFC) and of the right putamen (R PUT) in the subject reporting intense craving (Volkow and Fowler, 2000).

Whereas NAc DA plays a critical role in the establishment of drug-seeking behaviors, the dorsal striatum progressively takes on a central role as drug seeking becomes well established. NAc shell is required for the initial acquisition of drug consumption. However, as drug seeking and drug taking becomes well established, there is a progressive shift from motivated seeking of goals dependent on the NAc, to stimulus-response habits, which are dependent on the dorsal striatum. Such a shift would help explain cue activated–automatized or habit-like drug seeking in addicted subjects and the resistance of drug-seeking habits to treatment interventions, in line with the overall resistance of well-ingrained habits to disruption (Everitt and Robbins, 2005; Vanderschuren et al., 2005).

1.3.2. Relapse.

A basic definition of relapse is the return to drug-seeking and drug-taking behavior after a prolonged period of abstinence. High rates of relapse to drug-taking are widely reported following drug detoxification. For that reason, relapse remains the primary problem in treating drug abuse. After prolonged abstinence, 3 different types of stimuli may precipitate drug relapse and craving in animals and humans; reexposure to the drug itself, cues (people or environments) associated with previous drug use, and stress. There is evidence that the neuronal mechanisms underlying drug priming-, cue- or stress-induced relapse involve different brain areas and neurotransmitters (Shalev et al., 2002).

Neural activity in the VTA, PFC, NAc and ventral pallidum (VP) is necessary for drug-induced relapse and DA and D2 receptors appear to play a critical role in priming-induced relapse. Drug-triggered relapse is induced by the stimulation of D2 receptors in the NAc.

In cue-induced relapse, midbrain DA neurons show a complex changing response to reward. The response to reward itself habituates and, instead, the DA neurons fire in response to the predictors or cues. DA neurons receive highly processed information from the PFC and amygdala. Basolateral amygdala (BLA) is implicated in assigning environmental stimuli with affective value and consolidate memory for emotionally arousing events (Everitt et al., 2003). In drug addicts, the exposure to drug-associated cues evokes drug-related emotional memories stored in the amygdala, bringing back the desire to consume and inducing relapse.

Stress is also a common precipitant of relapse. Two neurotransmitter systems and two brain structures are critically involved in stress-induced, but not in drug- or cue-

induced reinstatement. Corticosterone releasing factor (CRF) and noradrenaline (NA) systems are released at the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) (Shaham et al., 2000; Erb et al., 2001). The BNST and CeA are interconnected with the shell of the NAc and the ventral pallidum, modulating emotional responses, including those associated with addiction (Koob et al., 1993; McFarland et al., 2004). NA neurons project from the locus ceruleus to the CeA and BNST. Stress induces release of NA in the BNST and CeA leading to the activation of CRF and mediating relapse (Aston-Jones and Harris, 2004). Important contribution of glutamatergic projection from the PFC to the NAc, and the amygdala on all types of reinstatement has been also shown (Shalev et al., 2002; McFarland et al., 2004).

Relapse can arise months and even years after detoxification, indicating that persistent neuroadaptations occurred after the repeated administration of drugs. These enduring plastic changes induced by the chronic exposure to drugs are due to modifications in multiple proteins in the reward–related brain regions. Among this high amount of proteins, the transcription factor ΔFosB has been shown to contribute to certain features of addiction. ΔFosB belongs to the family of immediate early genes and is a truncated splice variant of full-length FosB. Unlike other Fos family products with sharp upregulation and quick decline, ΔFosB isoforms are very stable and persist for weeks. As a result, ΔFosB levels gradually accumulate with repeated drug exposure most prominently in the NAc and PFC, but to a lesser extent in other brain regions important in addiction, including the amygdala. This expression is significantly induced in response to chronic exposure to most of abused drugs (Perrotti et al., 2008). Thus, changes induced by ΔfosB at the level of gene expression could explain the longevity of neural and behavioral adaptations responsible for drug addiction (Nestler and Aghajanian, 1997). Indeed, ΔFosB modulates the synthesis of certain cell-signaling enzymes and AMPA glutamate receptor subunits, indicating the involvement of synaptic plasticity in the addictive process.

1.4. Synaptic and structural plasticity and drug addiction.

The fact that vulnerability to relapse in addicts can persist after years of abstinence implies that addiction is caused by long-lasting changes in brain function as a result of repeated drug use. A ubiquitous property of all synapse is their ability to undergo activity-dependent long-term changes in synaptic strength, that is, synaptic plasticity. Long-term synaptic plasticity consists in two phenomena; long-term potentiation (LTP) and long-term depression (LTD). Drugs of abuse can produce synaptic plasticity in

brain circuits involved in reinforcement and reward processing. Indeed, an influential hypothesis is that addiction represents a pathological form of learning and memory (Hyman and Malenka, 2001). Although the brain circuitry underlying addiction is complex, VTA, NAc, PFC and associated limbic structures are critical substrates for the neural adaptations that underlie addiction. It is clear that interactions between addictive drugs and synaptic plasticity in different brain regions contribute to specific aspects of addiction such as craving, withdrawal and, most importantly, relapse. Addiction is not triggered instantaneously upon exposure to drugs of abuse. It involves multiple, complex neural adaptations that develop with different time courses ranging from hours to days to months. Synaptic plasticity takes place in the VTA in the early behavioral response following initial drug exposure and persists for long time periods (Kauer, 2004). It has been found that a single exposure to different drugs of abuse can cause LTP in the VTA DA cells. There is evidence that drugs of abuse, but not other drugs, elicit LTP at excitatory synapses on VTA DA neurons. These findings suggest that this synaptic adaptation can be directly related to the addictive properties of drugsd of abuse. By contrast, chronic but, not acute administration of addictive drugs followed by withdrawal causes LTD in medium spiny neurons of the NAc. The major cell type in the NAc is the GABAergic medium spiny neuron, which make inhibitory connections with cells in the ventral pallidum and VTA, and receive excitatory inputs from PFC, amygdala and hippocampus. Thus, LTP in VTA and LTD in the NAc GABAergic neurons lead to a potentiation of the mesolimbic pathway. Drugs of abuse also provoke synaptic plasticity in BNST and amygdala. As mentioned above, the BNST is considered a component of the extended amygdala and has a role in stress-and reward-related limbic circuitry. It contributes to stress-induced relapse to drug seeking and its neurons project to VTA and NAc. LTP can be triggered in the BNST by administration of drugs of abuse, but only occurs when an operant task is performed to obtain a reward. The amygdala is known to be involved in forms of memory that involve strong emotional component. Drug-associated cues that induce craving in addicts robustly alter neuronal activity in the amygdala. It has been found that LTP at excitatory synapses increase in the central amygdala weeks after withdrawal. However, this effect was not observed just after last drug injection, indicating that LTP in amygdala increases with time of withdrawal. Additionally, CRF, which increases during withdrawal, is essential for LTP in the amygdala. Enhanced synaptic plasticity in the amygdala may be a cellular adaptation contributing to the CRF-dependent signaling that appears to cause anxiety and stress responses during withdrawal from drugs of

abuse, correlating with the aversiveness of the withdrawal syndrome (Kauer and Malenka, 2007).

Long-lasting behavioral consequences of repeated exposure to drugs of abuse in adulthood are accompanied not only by changes in synaptic plasticity but also by structural plasticity. Repeated exposure to different drugs of abuse such as cocaine, amphetamine, methamphetamine, morphine or nicotine have long-lasting effects in the structure of dendrites and dendritic spines in brain regions thought to mediate drug-induced changes in incentive motivation and reward (such as the NAc) and in cognitive function (such as PFC). The most extensive data available are from studies with the psychostimulant drugs, amphetamine and cocaine. (Robinson and Kolb, 2004) used Golgi-stained material to quantify the structure of dendrites and the density of dendritic spines after treatment with several drugs of abuse. They described that stimulant drugs increased spine density and branching, whereas depressant drugs decreased spine density and branching in the NAc and mPFC. The molecular mechanisms responsible for structural plasticity associated to drug addiction are not well known, but they are though to involve changes initiated by calcium entry via glutamate NMDA receptors, activation of numerous intracellular signaling cascades that alter gene expression and eventually to changes in growth factors, citoskeletal and adhesion molecules, and many other proteins needed to form new synapses. Structural plasticity is present still after the discontinuation of drug administration, suggesting that drugs of abuse produce persistent reorganization in synaptic connectivity in regions associated with drug addiction. It is not known how these structural changes alter the function of cells and circuits, but presumably the reorganization of these brain regions contributes to some of the persistent consequences associated with repeated drug use.

1.5. Animal models to evaluate responses related to addiction.

1.5.1. Conditioned place preference; an animal model of reward.

Conditioned place preference (CPP), or place conditioning, is a procedure for assessing the rewarding efficacy of drugs using a classical or Pavlovian conditioning procedure. In a simple version of the place preference paradigm, animals experience two distinct neutral environments that are paired spatially and temporally with distinct drug and nondrug states. Animals then are given an opportunity to choose to enter and explore either environment, and the time spent in the drug-paired environment is considered an index of the rewarding value of the drug. Animals exhibit a conditioned preference for an environment associated with drugs that produce pleasurable effects, which means that animals spend more time in the drug-paired compartment compared to the placebo-paired compartment. This procedure permits to asses the rewarding

properties of drugs. The apparatus used in conditioned place preference experiments consists of two environments differentiated from each other on the basis of color and texture and pattern (Fig. 4). The distinctiveness of the environments is essential for the development of conditioning (Maldonado et al., 1997; Valverde et al., 2005).

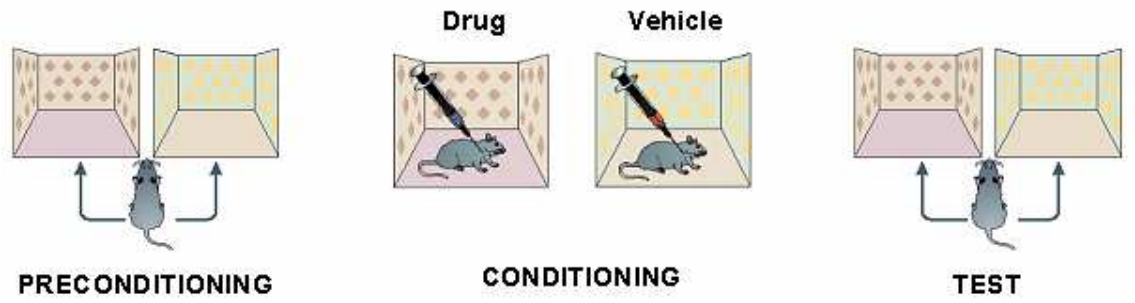

Figure 4. Place conditioning paradigm. Animals experience two distinct neutral environments paired spatially and temporally with distinct unconditioned stimuli (drug or drug antagonist vs. saline). At the end of the conditioning phase animals are given the opportunity to enter either environment, and the time spent in each environment is used as an index of the rewarding or aversive value of each unconditioned stimulus. These time values are often compared to a baseline preference for each environment measured at the first day.

1.5.2 Intravenous drug self-administration.

1.5.2.1.Acquisition and maintenance of intravenous drug self-administration. Animal model of reinforcement.

Drugs that are self-administered by animals correspond well with those that have high abuse potential in human, and intravenous drug self-administration is considered a predictive animal model of abuse potential (Collins et al., 1984). Intravenous drug self-administration has also proven to be a powerful tool for exploring the neurobiology of drug positive reinforcement. For intravenous self-administration, animals are intravenously implanted with a catheter and trained to self-administer the drug in a Skinner box. The chamber is provided with an active and an inactive lever or nosepoke. Responding on the active manipulanda will activate a pump delivering an intravenous infusion of drug. Active lever pressing can be paired with unconditioned stimuli such as a light or a tone, which improves learning of the operant behavior. Activation of the inactive lever will have no consequences, but will provide important control procedures for nonspecific motor and motivational actions such as increases in exploratory activity and locomotion (Fig. 5). Rodents on a simple schedule of continuous reinforcement, such as fixed ratio 1 schedule, where one lever-press or one

nosepoke delivers one drug infusion, will develop a highly stable pattern of drug self-administration in a limited access situation (Caine and Koob, 1993). The use of a progressive ratio schedule, where the schedule of reinforcement to deliver a single infusion is progressively increased, can provide information of the reinforcing strength of the drug. The higest schedule of reinforcement reached by a subject in a progressive ratio is known as breaking point. Therefore, the higher the breaking point, the more reinforcing is the drug.

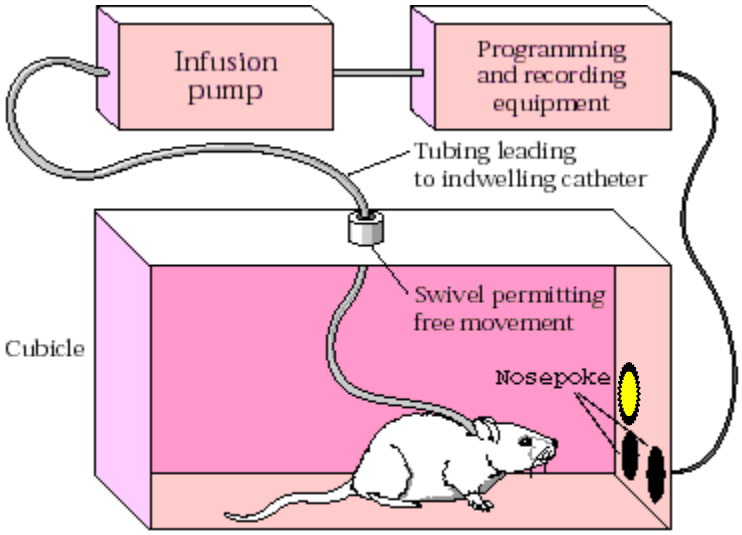

Figure 5. Intravenous self-administration procedure. Animal implanted with an intravenous catheter in the jugular vein and trained to self administer drug. The chamber is provided with two nosepokes. The number of responses in each nosepoke will be recorded by equipment. Responses on the active nosepoke will deliver a drug infusion and activate a light, while responses of the inactive lever will have no consequences.

1.5.2.2. Resistance to extinction associated with drug self-administration. Animal models of craving.

Extinction procedures can provide measures of the motivational properties of drugs by assessing the persistence of drug-seeking behavior in the absence of response-contingent drug availability. In an extinction paradigm, subjects are trained to self-administer a drug until stable self-administration patterns are achieved, and then the drug is removed (Schuster and Woods, 1968). Extinction testing sessions are identical to training sessions except that no drug is delivered after completion of the response requirement. Measures provided by an extinction paradigm reflect the degree of resistance to extinction, including the duration of extinction responding, and the total

number of responses emitted during the entire extinction session. Resistance to extinction and high responding rate are related with highly reinforcing stimuli, such as addictive drugs with high seeking behavior-inducing potential.

1.5.2.3. Reinstatement of frug-seeking behavior. Animal models of relapse.

The reinstatement model has been widely used to study relapse in animals. Reinstatement refers to the reinitiation of drug seeking in animal models after the extinction of previous drug administration (Shalev et al., 2002). In the reinstatement model, laboratory animals are initially trained to self-administer in operant conditioning chambers. Subsequently, the drug-reinforced behavior is extinguished by substituting the drug solution with saline or by disconnecting the infusion pumps. After extinction of the drug reinforced behavior, the ability of drug priming, drug-associated cues and stress to trigger reinstatement is determined.

Noncontingent drug injections administered after extinction can induce reinstatement of a drug seeking behavior. Drug priming effect on reinstatement has been reported in cocaine-, heroin-, ethanol- and nicotine-trained animals (Chiamulera et al., 1996; Self and Nestler, 1998; Le et al., 1998; Stewart, 2000; Soria et al., 2008).

Drug-associated cues or conditioned reinforcers can be defined as motivational neutral stimuli, which acquire motivational properties through associations with a primary reinforcer. In this paradigm, responses on the active lever result in the presentation of a brief stimulus (light or tone) followed by drug infusion. This previously neutral stimuli can acquire conditioned reinforcing properties. Thus, drug associated stimuli can elicit drug-seeking behavior in experimental animals. Subsequent re-exposure after extinction to a drug-associated stimulus produces strong recovery of responding at the active lever in the absence of any further drug availability.

Animal models of stress reinstatement show that stressors elicit strong recovery of extinguished drug-seeking behavior in the absence of further drug availability (Ahmed and Koob, 1997). The most frequently used stressor is intermittent footshock, but other stressors such as food deprivation are also able to reinstate drug seeking behavior.

1.5.3. Animal models to evaluate drug withdrawal.

1.5.3.1. Animal models of physical withdrawal syndrome.

The chronic administration of some drugs (opioids, cannabinoids, alcohol or nicotine) produces a counteradaptation by homestatic changes in the system. When abstinence is precipitated in these animals, (spontaneously or by a drug antagonist) they show several somatic signs that are usually opposite to the acute initial actions of the drug.

Many of these overt physical signs associated with withdrawal can be easily evaluated and quantified. Withdrawal symptoms associated with physical dependence vary depending on the type of drug that has triggered dependence and the use or not of antagonists. Each drug withdrawal syndrome shows different quantitative and qualitative somatic signs (Table 1).

Table1. Quantitative and qualitative signs of antagonist-precipitatedwithdrawal syndrome to different drugs of abuse.

Morphine		Cannabinoids		Nicotine	
Quantitative signs	**Qualitative signs**	**Quantitative signs**	**Qualitative signs**	**Quantitative signs**	**Qualitative signs**
jumping	teeth chattering	wet dog shakes	tremor	wet dog shakes	ptosis
wet dog shakes	body tremor	paw tremor	ptosis	paw tremors	genital licks
paw tremor	ptosis	sniffing	teeth chattering	sniffing	tremor
sniffing	piloerection		piloerection	scratches	piloerection
	diarrhea		ataxia		teeth chattering
			hunched posture		
			genital licks		

1.5.3.2. Motivational measures of withdrawal.

While the physical signs of withdrawal are measured easily and may provide a marker for the study of neurobiological mechanisms of dependence, motivational measures of withdrawal have validity for understanding the dysphoric effects of withdrawal. Place conditioning paradigm has been used to measure the aversive effects of withdrawal (Hand et al., 1988; Stinus et al., 1990). In contrast to conditioned place preference discussed above, in the conditioned place aversion paradigmanimals are exposed to a particular environment while undergoing precipitated withdrawal. These animals suffer a dysphoric state and spend less time in the withdrawal paired environment when they have to choose between such an environment and an unpaired environment. To observe this abstinence-induced conditioned place aversion, animals are chronically treated with a drug and administered with an antagonist in one compartment, and with placebo in the other compartment during the conditioning phase (Fig. 4).

2.Cannabinoids

2.1. The endocannabinoid system.

2.1.1. Cannabinoid receptors.

In the early 1990s, two distinct subtypes of cannabinoid receptor were identified by molecular cloning; CB_1 and CB_2 cannabinoid receptors (Fig. 6). Nevertheless, compelling evidence supports the existence of novel cannabinoid receptors, such as endothelial cannabinoid receptor or GPR-55 (Begg et al., 2005; Pertwee, 2007). The CB_1 (Matsuda et al., 1990) and CB_2 cannabinoid receptor (Munro et al., 1993) subtypes belong to the seven transmembrane domain receptor family with seven α-helices spanning the cell membrane. The intracellular loops of the receptor protein are involved with G-proteins that cause the inhibition of adenylate cyclase activity, responsible for the production of cAMP in the cell.

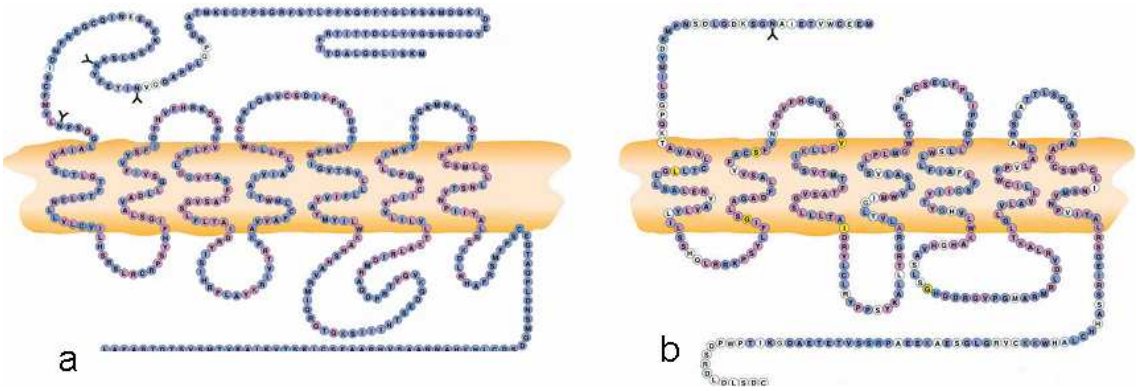

Figure 6. CB_1 (a) and CB_2 (b) receptor structure with seven α-helices transmembrane domains.

2.1.1.1. Cannabinoid receptor signaling.

Stimulation of CB_1 and CB_2 cannabinoid receptors activates a number of signal transduction pathways mainly via the Gi/o family of G proteins. When cannabinoid receptor is activated, G protein β and γ subunits are released from the α subunit, and Giα subunit liberate GDP and binds GTP, becoming active and inhibiting the enzyme adenylate cyclase. Inactive adenylate cyclase is unable to stimulate cAMP production from ATP, and thus subsequent protein kinase A (PKA) activity is inhibited. The consequent reduction of phosphorilation by PKA modulates several signaling pathways, such as that of ion channels and focal adhesion kinase, which is important for integrating cytoskeletal changes with signal transduction events, perhaps playing a role in synaptic plasticity.

On the other hand, free Gβγ dimmer activates G protein-gated inwardly rectifying K^+ channels, and inhibits voltage-gated Ca^{2+} channels, regulating synaptic transmission. Cannabinoid receptor-mediated $G_{i/o}$ release of βγ subunits also leads to activation of phosphatidylinositol-3-kinase (PI3K), resulting in tyrosine phosphorylation and activation of Raf-1, and subsequent mitogen-activated protein kinases (MAPK) phosphorilation. Stimulated MAPK activity was associated with phosphorilation of cytoplasmatic phospholipase A_2, and subsequent synthesis of prostaglandin E_2 that participates in a wide range of body functions, such as contraction and relaxation of smooth muscle, dilation and constriction of blood vessels, control of blood pressure, and inflammation inhibition. The activation PI3K by cannabinoid agonists led to activation of protein kinase B/Akt. Protein kinase B phosphorilation and inhibition of glycogen synthase kinase-3 could account for increased glycogen synthase and activity, and increased glycolysis.

Cannabinoid receptors also activate G protein-independent signaling pathways. Data supported a pathway that utilizes the adaptor protein Fan (factor associated with neutral sphingomyelinase) to couple CB_1 receptor stimulation to sphingomyelinase activation, release of ceramide and subsequent activation of Raf-1/MAPK cascade. In a second mechanism, ceramide activated carnitine palmitoyltransferase to stimulate ketogenesis and fatty acid oxidation. MAPK activation can be linked to expression of IEG, as Krox-24, c-fos or c-jun. There is also evidence of a signal transduction pathway leading to the regulation of nitric oxide synthase (NOS) (Howlett et al., 2002) (Fig. 7).

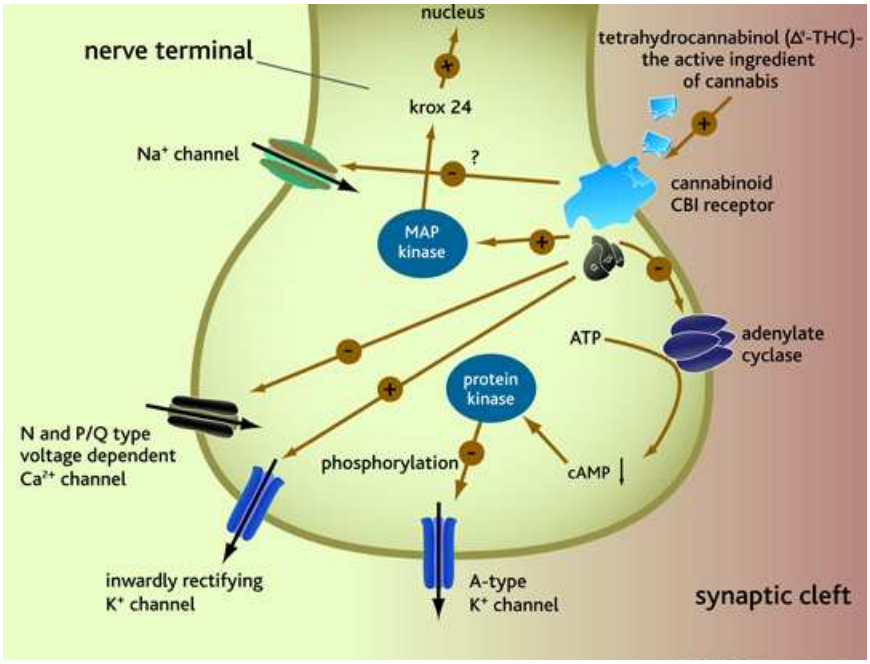

Figure 7. Cannabinoid receptor signaling pathways in neurons.

2.1.1.2 CB_1 cannabinoid receptor: Distribution and properties of the CB_1 cannabinoid receptor.

The CB_1 receptor is one of the most abundantly expressed neuronal receptors. Its distribution has been well characterized in rats (Herkenham et al., 1991; Tsou et al., 1998) and humans (Westlake et al., 1994). CB_1 exhibits a widespread distribution in the brain, which correlates well with the functions attributed to cannabinoids. The highest density of CB_1 receptors has been found in the basal ganglia and the molecular layer of the cerebellum. The high density of cannabinoid receptors in the basal ganglia is consistent with the marked effects that cannabinoids exert on locomotor activity (Compton et al., 1990). High densities were also found in the hippocampus, and layer I and IV of the cortex, indicating that cannabinoids are involved in the control of cognition and memory. The expression of CB_1 receptor was also observed in the limbic system, including the amygdala, hippocampus, hypothalamus, prefrontal and anterior cingular cortex, and nucleus accumbens (Tsou et al., 1998), what explains the role attributed to cannabinoid system in emotional behavior. In addition, CB_1 receptors in the nucleus accumbens are associated with brain reward, and its expression in the hypothalamus correlates with cannabinoids thermoregulation and food intake control (Cota et al., 2003). CB_1 immunoreactive fibers have been detected in periaqueductal gray as well as in the thalamus, and dorsal horn and lamina X of the spinal cord (Tsou et al., 1998). Both, in turn, are important sites in ascending and descending pain transmission (Behbehani, 1995), and the CB_1 receptor in these regions is expected to be involved in the

antinociception induced by cannabinoids. CB_1 receptors are also expressed in peripheral tissues. At the peripheral level, the most relevant effects of CB_1 receptor are on adipose tissue (Cota et al., 2003), liver (Osei-Hyiaman et al., 2005b), gastrointestinal tract (Calignano et al., 1997), pancreatic beta cells (Bermudez-Silva et al., 2008) and vascular endothelium (Liu et al., 2000).

2.1.1.3 CB_2 cannabinoid receptor: Distribution and properties of the CB_2 cannabinoid receptor.

CB_2 cannabinoid receptor, as well as CB_1 receptor is a G-protein coupled receptor with seven transmembrane domains, but shows only a 44% amino acid sequence identity to the CB_1 receptor (Munro et al., 1993). There are considerable differences in the size of the CB_1 and the CB_2 receptor. While the human CB_1 receptor consists of 472 amino acids acids and has molecular weight of 60 kDa, the human CB_2 receptor consists of only 360 amino acids and has molecular weight of 40 kDa (Matsuda et al., 1990).

The localization of the CB_2 receptor differs markedly from that of the CB_1 receptor. The occurrence of the CB_2 receptor is mainly restricted to the periphery, although it has been found in brainstem neurons related to vomit control (Van Sickle et al., 2005). CB_2 receptor expression was identified for the first time in immune system cells (monocytes, B-cells, T-cells) (Matsuda et al., 1990; Munro et al., 1993; Galiegue et al., 1995). The localization of CB_2 receptors in immune tissues is responsible for the antiinflammatory effects of cannabinoids (Lunn et al., 2006; Ashton and Glass, 2007). Recently, CB_2 receptor expression has been observed in other peripheral tissues such as osteoblasts, osteocytes, and osteoclasts (Ofek et al., 2006), liver (Julien et al., 2005) and somatostatin secreting cells in the pancreas (Bermudez-Silva et al., 2007).

2.1.1.4 Non-CB_1/non-CB_2 cannabinoid receptors.

Endothelial cannabinoid receptor.

The first indication that cannabinoid receptors other than CB_1 or CB_2 may exist came from studies of the mesenteric vasodilator effect of cannabinoids. The endocannabinoid anandamide (AEA) elicited long-lasting vasodilation, whereas synthetic cannabinoids or THC potent at both CB_1 and CB_2 receptors did not have a dilator effect (Wagner et al., 1999). Although the vasodilator response to AEA could be inhibited by the selective CB_1 receptor antagonist rimonabant, somewhat higher concentrations were needed than concentrations sufficient to inhibit CB_1 receptors (Wagner et al., 2001). In addition, "abnormal cannabidiol" (Abn-cbd), a neurobehaviorally inactive cannabinoid that does not bind to CB_1 receptors, yet causes hypotension and mesenteric vasodilation in wild-type

mice and in mice lacking CB_1 receptors or both CB_1 and CB_2 receptors. Hypotension by Abn-cbd is also inhibited by cannabidiol, which does not influence anandamide- or HU-210-induced hypotension. These findings suggest that Abn-cbd and cannabidiol are a selective agonist and antagonist, respectively, of an endothelial receptor for AEA (Jarai et al., 1999). As a result, it was proposed that an endothelial site distinct from CB_1 or CB_2 receptors was involved in the vasodilator effect of AEA.

GPR-55.

The orphan receptor GPR-55 (Sawzdargo et al., 1997) has been proposed as a new cannabinoid receptor. GPR-55 binds to and is activated by the cannabinoid ligand CP-55,940. In addition, endocannabinoids including AEA and virodhamine activate GTPγS binding via GPR-55. Ligands such as cannabidiol which exhibit no CB_1 or CB_2 activity are believed to function at GPR-55 (Ryberg et al., 2007). However, GPR-55 is apparently not expressed in the vascular endothelium and is unsensitive to HU-210, a potent synthetic cannabinoid with vasorelaxant properties (Wagner et al., 1999). This suggests that GPR-55 is not the abnormal cannabidiol-sensitive endothelial receptor. Mice deficient in GPR-55 will help in defining the biological functions of this novel cannabinoid-sensitive receptor (Pacher et al., 2006).

2.1.2. Cannabinoid endogenous ligands.

(Devane et al., 1992) isolated from porcine brain the lipid arachidonoyl ethanolamide, named AEA, which bound to the brain cannabinoid receptor with reasonably high affinity and mimicked the behavioral actions of THC when injected into rodents. Three years later a second endocannabinoid, 2-arachidonoylglycerol (2-AG), was discovered (Mechoulam et al., 1995; Sugiura et al., 1995). Since then, a number of related endogenous lipids with endocannabinoid-like activity have been reported (Fig. 8). Despite growing interest in endocannabinoids and their roles as retrograde neurotransmitters (Wilson and Nicoll, 2002; Chevaleyre et al., 2006), the mechanism of their release is not well understood. Like prostanoids, endocannabinoids are not stored but generated on demand in response to a depolarization-induced rise in intracellular calcium or activation of various metabotropic receptors (Pacher et al., 2006). Thus, efficient machinery for the synthesis transportation and degradation of endocannabinoids is essential.

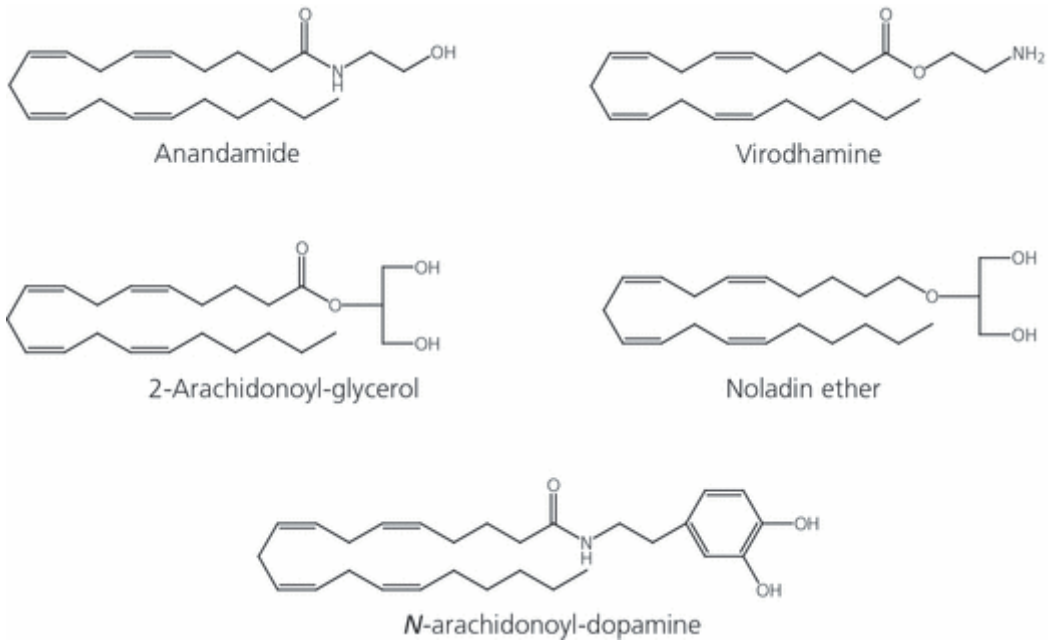

Figure 8. Chemical structure of endogenous cannabinoids (Bisogno, 2008).

2.1.2.1. Endocannabinoid ligands: Syntesis, reuptake and degradation.

AEA is a partial or full agonist of CB_1 receptors, depending on the tissue and biological response measured. Although AEA also binds to CB_2 receptors, it has very low efficacy and may act as an antagonist (Gonsiorek et al., 2000). The *in vivo* biosynthesis of AEA (Fig. 9) is believed to occur through the enzymatic hydrolysis catalyzed by a phospholipase D of a membrane lipid precursor, *N*-arachidonoyl phosphatidylethanolamide (NAPE) (Schmid et al., 1983), which itself is generated from arachidonic acid (Di Marzo et al., 1994). Although a specific transacylase for the latter reaction has not yet been identified, a NAPE-specific phospholipase D has been cloned (Okamoto et al., 2004). Indeed, there may be parallel pathways for the generation of AEA from NAPE. AEA present in the extracellular space is accumulated by neurons and other cells by facilitated diffusion. This process is driven by its transmembrane concentration gradient, is saturable and temperature-dependent, and does not require ATP or sodium ions. AEA uptake is selectively inhibited by a variety of structural analogs, which suggests the existence of a saturable cellular component involved in AEA transport (Beltramo et al., 1997). However, a specific AEA transporter protein has yet to be cloned. Fatty acid amide hydrolase (FAAH) has been proposed as AEA putative transporter protein (Glaser et al., 2003), but its mechanism is not well known. In contrast, the role of FAAH in the degradation of AEA has been extensively documented (McKinney and Cravatt, 2005). The phenotype of FAAH knockout mice displayed 10 to 15 times elevated levels of AEA across the brain, supersensitivity to the actions of exogenous AEA, and the appearance

of tonic signaling by endogenous AEA, resulting in CB_1 receptor-mediated hypoalgesia (Cravatt et al., 2001; Lichtman et al., 2004), reduced anxiety (Kathuria et al., 2003), antidepressant activity (Gobbi et al., 2005), and lowering of blood pressure in different models of experimental hypertension (Batkai et al., 2004). Interestingly, other amidohydrolase catalyzing the same reaction as FAAH has been identified and cloned (Tsuboi et al., 2005). 2-AG is generated from diacylglycerol (DAG) by a selective DAG lipase (Fig. 9). DAG, an intracellular second messenger that activates protein kinase C, can be generated from phosphoinositides by a phosphoinositide-specific PLC or from phosphatidic acid by phosphatidic acid phosphohydrolase (Bisogno et al., 2005). Two DAG lipase isozymes, α and β, have been cloned (Bisogno et al., 2003). In the adult brain they are localized in the postsynaptic membrane, in line with their putative role in generating 2-AG involved in retrograde transmission. Basal levels of 2-AG in the brain are approximately 2 orders of magnitude higher than the levels of AEA (Giuffrida et al., 1999). Although 2-AG is also hydrolyzed by FAAH under *in vitro* conditions (Goparaju et al., 1998; Lang et al., 1999), it is not a substrate of FAAH *in vivo*, as indicated by the unchanged brain levels of 2-AG in WT and FAAH KO mice (Osei-Hyiaman et al., 2005a). 2-AG is hydrolyzed *in vivo* by a monoacylglycerol lipase (MAGL) (Dinh et al., 2002; Saario et al., 2004). MAGL is localized in presynaptic axon terminals, whereas, FAAH is located postsynaptically (Gulyas et al., 2004). The postsynaptic localization of MAGL as well as DAG makes 2-AG an important retrograde transmitter involved in synaptic plasticity (Katona et al., 2006).

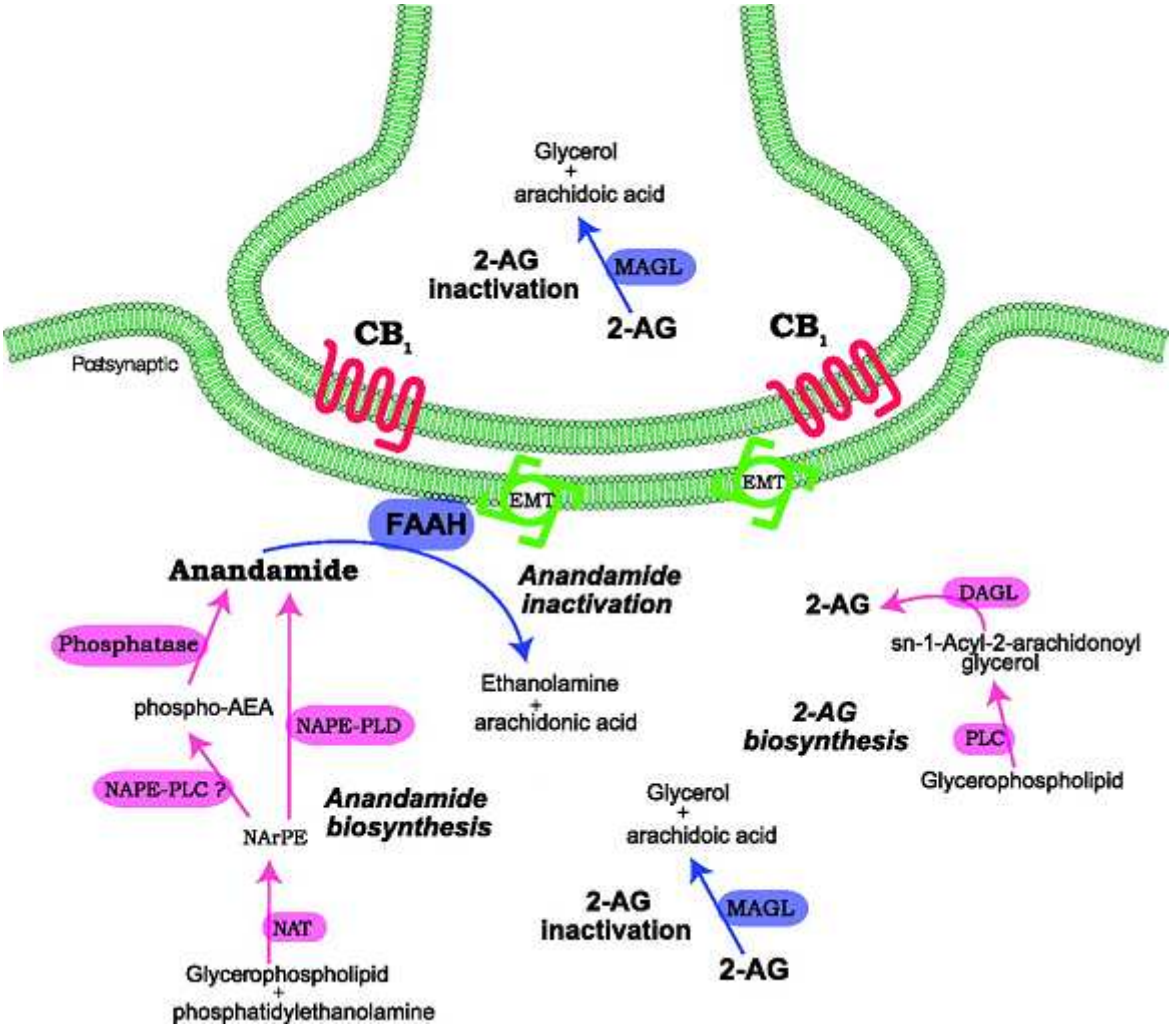

Figure 9. Schematic representation of the endocannabinoid system in pre- and postsynaptic neurons. The presynaptic terminal is located in the top, whereas the postsynaptic neuron is located in the bottom. EMT, endocannabinoid membrane transporter; MAGL, monoacylglyceride lipase; DAGL, DAG lipase; AEA, anandamide; NArPE, *N*-arachidonyl phosphatidylethanolamine; NAT, *N*-acyltransferase (Pacher et al., 2006).

2.2. Cannabinoid compounds.

2.2.1. *Cannabis sativa*.

Cannabis is a plant that grows throughout the temperates and tropical zones of the world. Cannabis is a form of hemp originated in Central Asia, and was a familiar agricultural crop from the beginning of civilization. Hemp is a tall herb with tough fibers used for cords and ropes. Hemp plant has been used as far back as the late Neolithic era by Chinese as a medicinal herb. Marijuana is the dry shredded mixture of flowers, stems, seeds, and leaves of the hemp plant *Cannabis sativa*. The dried mixture can be smoked like a cigarette (termed a *joint*) or in a pipe. Other preparations include hash or hashish, the dried sticky resin of the flowers of the female plant, or hash oil, a sticky black liquid.

2.2.2 Phytocannabinoids.

Up until the last two decades, marijuana research was a rather esoteric field, of interest to a small number of scientists. The first important breakthrough that ultimately led to a rejection of this concept was the identification by (Gaoni and Mechoulam, 1964) of the chemical structure of the main psychoactive ingredient of marijuana, Δ^9-THC, which is one of approximately 60 cannabinoids present in the plant (Dewey, 1986). In the plant, THC content is highest in the oil from the flowering tops and lowest in the seeds. After inhalation, THC is detectable in plasma within seconds, with peak concentrations 3-10 min after smoking. THC has a peculiar distribution because of its high lipophilicity compared to other drugs of abuse and as a result, rapidly enters highly vascularized tissues. Significant accumulation of cannabinoids occurs later in less vascularized tissues and body fat. THC is broken down in the liver by enzymes of the cytochrome P450 system. The elimination half-life for THC is estimated to be in the range of 20-60 h. THC is excreted as acid metabolites, and these metabolites can be detected in urine for 27 days on average in chronic users. Tolerance develops readily to most of the effects of cannabinoids in humans and is largely attributed to pharmacodynamic (brain neuroadaptation), not pharmacokinetic (metabolic distribution) changes. Cannabis dependence in humans has long been described anecdotically, but is not generally accepted by the medical community. However, an accumulation of data from both inpatient and outpatient studies has led to a proposal for cannabis dependence criteria in humans. The most common symptoms associated with cannabis withdrawal syndrome were decreased appetite/weight loss, irritability, nervousness, anxiety, anger, aggression, restlessness, and sleep disturbances. A substantial percentage of heavy marijuana users showed these symptoms. Onset of withdrawal typically occurs within 1-3 days, and most symptoms last 4-14 days. The long onset of THC withdrawal appears to be directly related to the long half-life and slow decline of blood THC levels (Koob and Le Moal, 2006).

THC, which acts as an agonist at both CB_1 and CB_2 receptors, and partially mimics the actions of the endogenous cannabinoids (Howlett et al., 2002). Besides Δ^9-THC, Δ^8-tetrahydrocannabinol and cannabinol are also plant cannabinoids with psychotropic properties. However, plant derivatives such as cannabidiol, which is not a ligand for CB_1 or CB_2 receptors, also shows important cannabimimetic actions attributed to antioxidant properties, inhibition of AEA degradation, and interactions with other cannabinoid receptors still unidentified (Mechoulam et al., 2007) (Fig. 10).

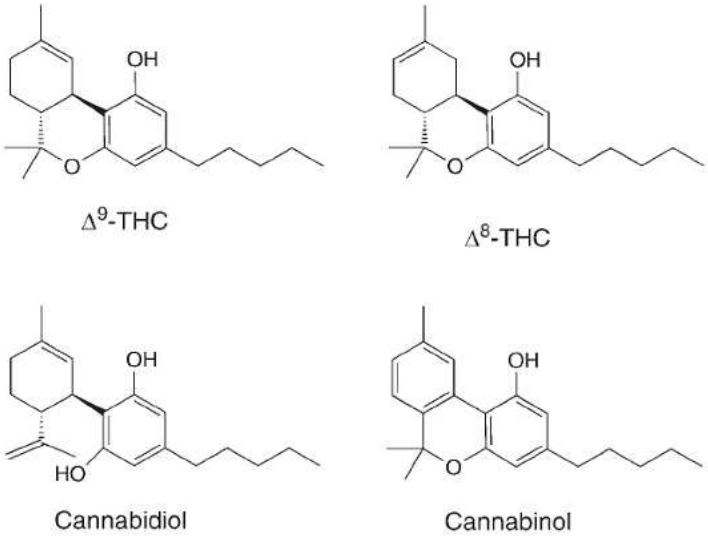

Figure 10. Chemical structure of phytocannabinoids (Howlett et al., 2002).

2.2.3. Synthetic ligands.

The discovery of phytocannabinoids' structure in 1964 stimulated the generation of a whole range of synthetic analogs in the 1970s that included not only compounds structurally similar to phytocannabinoids (Fig. 10) but also analogs with different chemical structures, including classic and non-classic cannabinoids, and aminoalkylindoles (Fig. 11), as well as the subsequently discovered endogenous arachidonic acid derivatives or endocannabinoids (Fig. 8) (Howlett et al., 2002), which will be discussed in more detail.

The synthetic cannabinoid receptor ligands are characterized by a wide chemical diversity (Fig. 11). They are usually classified into the following categories: classical cannabinoids, non-classical cannabinoids, aminoalkylindoles, and eicosanoids. It should be noted that several agonists (JTE 907, BAY 38-7271) do not fall into these standard classes. The classical cannabinoid agonists retain the tricyclic diterpene structure of THC. This category includes compounds like HU-210, HU-243, nabilone and ajulemic acid, which act at both cannabinoid receptors. Selective agonists for CB_2 receptors have been developed too. These agonists include compounds like JWH-133, JWH-1051, JWH-308, L-759633, and L-759656, which could be useful in the treatment of pain and inflammation (Palmer et al., 2002). In non-classic cannabinoid agonists, one of the rings of the tricyclic Δ^9-THC structure is open. This group of compounds includes CP-55,940, CP-47,497, CP-55,244 and the selective CB_2 agonist HU-308 (Howlett et al., 2002; Gomez-Ruiz et al., 2007). Finally, the aminoalkylindoles are less related to phytocannabinoids. This category includes compounds like WIN-55,212-2 and selective CB_2 agonists JWH-015, L-768242 and AM-1241. There are a number of synthetic eicosanoids (mainly derived from AEA structure) that improve several pharmacokinetic (they are metabolically more stable than

AEA) or pharmacodynamic (they provide more selectivity for the different cannabinoid receptors) properties. Examples of these analogs are R-methananadamide (AM356), arachidonoyl-2-chloroethylamide (ACEA), arachidonoyl-clyclopropylamide (ACPA), O-1812, retroanandamide, arvanail, O-1861, O-585 and O-689 (Howlett et al., 2002; Gomez-Ruiz et al., 2007).

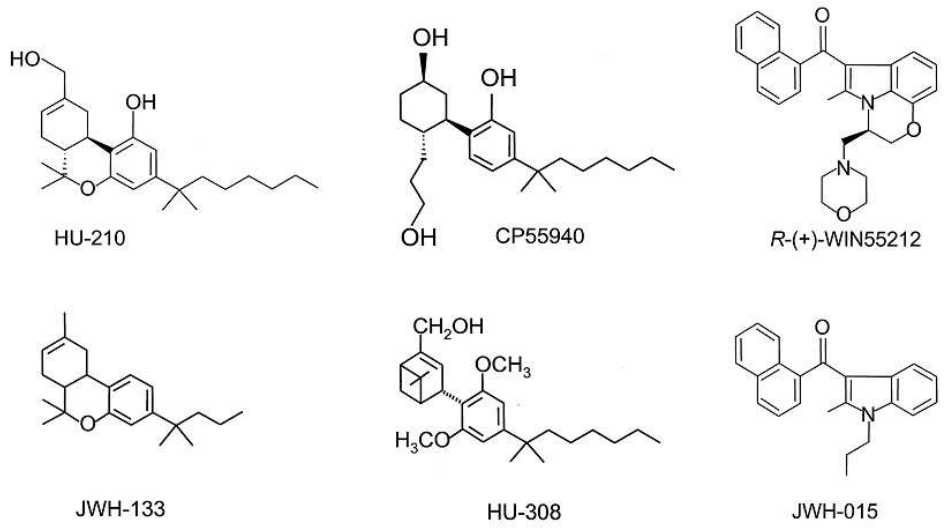

Figure 11. Chemical structure of synthetic cannabinoid agonists (Howlett et al., 2002).

2.2.4. Cannabinoid antagonists.

Cannabinoid receptor antagonists were initially synthesized as a pharmacological tool for the study of the endocannabinoid system. Rimonabant was the first selective CB_1 antagonist to be developed. Rimonabant behaves as CB_1 inverse agonist as well as other CB_1 antagonists, including SLV-326, LY-320135, and AM-251. Examples of neutral antagonists are O-2654 and AM-5171. Several specific CB_2 receptor antagonists such as AM-630, SR-144528, and O-1184 have also been synthesized (Gomez-Ruiz et al., 2007) (Fig. 12).

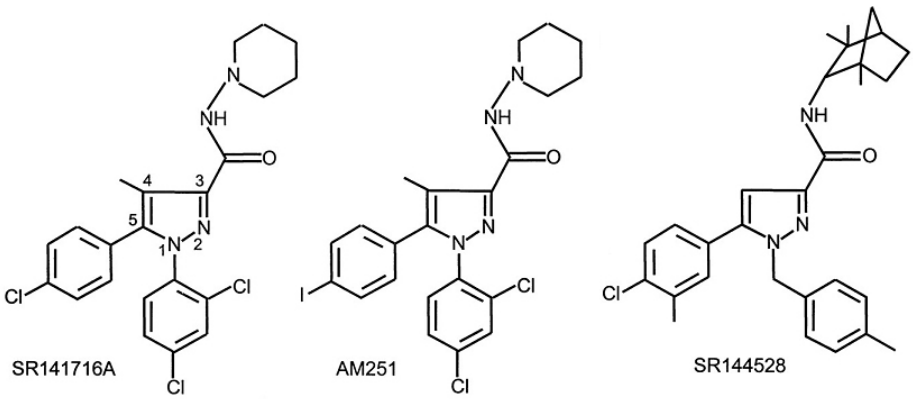

Figure 12. Chemical structure of cannabinoid antagonists/inverse agonists (from Howlett, 2002).

2.2.5. Endocannabinoid enzyme inhibitors.

The therapeutic potential of endocannabinoids, particularly AEA and 2-AG, is clearly established. Nevertheless, their use as therapeutic agents would imply that sufficient concentrations could be reached at the level of their biological targets. Since both AEA and 2-AG are quickly metabolized *in vivo*, a promising way to promote their therapeutic potential is increasing their half-life. Several potent selective inhibitors of endocannabinoids metabolizing enzymes FAAH and MAGL have been synthesized (Fig. 13). The carbamate compound URB597 (Fig. 13) is a very potent and irreversible FAAH inhibitor, which elevates endocannabinoid tone. Unlike direct cannabinoid receptor agonists, URB597 does not produce some of the side effects, such as catalepsy or hypothermia, and has been widely studied in several pain models (Piomelli et al., 2006; Jhaveri et al., 2006). Another FAAH inhibitor, OL-135, which inhibits the enzyme in a reversible manner, has also been tested for its possible analgesic effect (Saario and Laitinen, 2007). The therapeutic potential of MAGL inhibitors is still unknown, but URB602 has been utilized *in vivo* to provide evidence for the involvement of 2-AG in pain suppression (Comelli et al., 2007). One advantage of enzyme inhibition over the direct administration of cannabinoid agonists could be the higher selectivity, as the intervention would increase the activity of the endocannabinoid system locally, at sites where endocannabinoids are produced (Vandevoorde, 2008). Another alternative to enhance endocannabinoid tone is inhibiting AEA uptake. The AEA uptake inhibitor AM404 has been used for reducing different pain models (Mitchell et al., 2007; La Rana et al., 2008), anxiety (Bortolato et al., 2006) and ethanol consumption (Cippitelli et al., 2007). UCM707, another AEA uptake inhibitor, has also been reported to improve symptoms in animal models of Huntington's disease and multiple sclerosis (de Lago et al., 2006).

Figure 13. Chemical structure of FAAH and MAGL inhibitors (Vandevoorde, 2008).

2.3. The endocannabinoid system as therapeutic target in central nervous system disorders.

The particularly high density of CB_1 receptors in the cortex, cerebellum, hippocampus, and basal ganglia had drawn early attention to the use of cannabinoid drugs in the

treatment of diseases affecting movement, mood and anxiety disorders, and conditions related to altered brain reward mechanisms, as well as processes of memory and learning. For the better understanding of the work developed, we will focus on the participation of endocannabinoid system as well as the therapeutic potential of cannabinoids on addictive and anxiety disorders. Additionally, the neuroprotective role of cannabinoids in several central nervous system (CNS) disorders will be discussed. Although endocannabinoid system has been proposed as a pharmacological target for many other central and peripheral diseases, they will not be discussed in the current revision.

2.3.1. Mental Disorders.

The well-known psychotropic effects of cannabinoids and the distribution of cannabinoid receptors across important emotional circuits in the brain suggest that the endocannabinoid system may be involved in various psychiatric disorders such as schizophrenia, anxiety, depression or addictive disorders (Vinod and Hungund, 2005).

2.3.1.1. Anxiety and depression.

The high level of CB_1 receptors in the hippocampus, amygdala, prefrontal and anterior cingular cortex, key regions in the regulation of anxiety, suggests that the endocannabinoid system plays a role in the control of anxiety (Valverde, 2005). Further support of this theory came from studies using CB_1 receptor antagonists or CB_1 receptor knockout mice. Most of the studies describe anxiogenic-like properties of rimonabant (Navarro et al., 1997; Arévalo et al., 2001), although anxiolytic-like effects of rimonabant were also reported (Haller et al., 2002). In agreement, CB_1 knockout mice displayed increased anxiety and aggressiveness, and marked alterations in the hypothalamic-pituitary-adrenal (HPA) axis (Martin et al., 2002; Haller et al., 2002). A majority of evidence supports a role for CB_1 receptors in the control of emotional behavior and suggests the existence of an anxiolytic endocannabinoid tone. Inhibiting the degradation of endocannabinoids may be therapeutically exploited, as indicated by the reduced anxiety-like behavior and antidepressant-like effects in mice and rats treated with a FAAH or AEA transport inhibitor (Kathuria et al., 2003; Gobbi et al., 2005; Bortolato et al., 2006). The mechanisms responsible for the effects of cannabinoids on anxiety-related responses involve modulation of HPA axis through the release of CRF (Rodriguez de Fonseca et al., 1996; Valverde, 2005). Cannabinoids also modulate GABAergic transmission and the release of the peptide cholecystokinin, which may contribute to both anxiolytic and anxiogenic effects (Marsicano and Lutz, 1999). Thus, pharmacological

modulation of the endocannabinoid system holds considerable promise in the treatment of both anxiety and mood disorders.

According to the fact that CB_1 receptors are widespread in limbic-related areas, THC exerts anxiolytic, antidepressant, and hypnotic effects under certain doses and conditions. However, under different conditions and at higher doses, cannabis or THC can produce dysphoric reactions, anxiety, panic, paranoia, and psychosis (Hall and Solowij, 1998). CBD possesses anxiolytic, antipsychotic and anticonvulsant properties, which are not mediated by classic cannabinoid receptors (Pacher et al., 2006). The mode of action of CBD is not completely understood. It may involve blockade of AEA reuptake (Bisogno et al., 2001), inhibition of the enzymatic hydrolysis of AEA (Mechoulam et al., 2002), or an interaction with as yet unidentified receptors (Haller et al., 2002; Pertwee et al., 2002). Animal studies yielded further support to the biphasic and bidirectional effects of cannabinoids on anxiety, with low doses being anxiolytic and high doses being anxiogenic. Indeed, low doses of CP-55,940, nabilone, and THC exerted anxiolytic-like effects in the light-dark crossing test and in the elevated plus-maze in adult rodents. In contrast, at medium to high doses, CP-55,940 and HU-210 displayed anxiogenic-like effects in the same or other experimental paradigms. Several hypotheses have been proposed to explain the biphasic effects of cannabinoids on anxiety, including distinct receptors or neuroanatomically separated CB_1 receptors with a differential sensitivity to the anxiolytic versus anxiogenic effects of cannabinoids (Viveros et al., 2005).

2.3.1.2. Drug addiction disorders.

The positive reinforcing effect of natural rewards, such as those derived from eating, drinking, work, sexual activity, as well as the hedonic effects drugs of abuse are mediated by the brain's reward circuitry (see chapter 1). A common denominator among different addictive drugs interacting with distinct receptors is their ability to activate the mesolimbic DA reward pathway to and increase DA levels in the NAc, which is believed to be responsible for their addictive properties (Koob, 1992; Wise, 2004). Similar to other drugs of abuse, THC increases extracellular DA levels in the NAc via activation of CB_1 receptors (Chen et al., 1990; Tanda et al., 1999) and decreases the reward threshold for electrical brain stimulation (Gardner et al., 1988), a phenomenon known to involve activation of the mesolimbic DA system. THC also increases the firing rate of the VTA-NAc DA neurons via CB_1, but not opioid receptors (French, 1997), and withdrawal from THC increases CRF levels in the central nucleus of the amygdala (Rodriguez de Fonseca et al., 1997), another hallmark of drugs of abuse. THC and related synthetic cannabinoid agonists also fulfil the reward-related behavioral criteria for drugs of abuse: they induce CPP under

appropriate conditions (Lepore et al., 1995; Valjent and Maldonado, 2000), they are self-administered intravenously or intracerebrally (Martellotta et al., 1998), and they reinstate cocaine- or heroine-seeking behavior in rats previously extinguished from self-administration (De Vries et al., 2001). An issue of intense interest is the location of the CB_1 receptors mediating these effects. In the VTA, CB_1cannabinoid receptors are located on presynaptic glutamatergic and GABAergic neurons. By contrast, VTA dopaminergic neurons do not synthesize CB_1 cannabinoid receptors. Activation of CB_1 receptors in the VTA by endocannabinoids produces inhibition of GABA release, thus removing the inhibitory effect of these GABAergic cells on dopaminergic neurons. In addition, the increase of dopaminergic neuron activity induces release from the dopaminergic cells of endocannabinoids that, acting in a retrograde manner on presynaptic CB_1 receptors, inhibit both inhibitory GABAergic and excitatory glutamatergic inputs to VTA dopaminergic neurons. Glutamatergic projections from the BLA and hippocampus, which are involved in motivation and memory processes related to drug rewarding effects, are also under the control of CB_1 receptors, through an inhibitory effect on presynaptic inhibitory neurons that release both GABA and cholecystokinin. In the NAc, endocannabinoids behave as retrograde modulators acting mainly on CB_1 receptors on the axon terminals of glutamatergic neurons. The subsequent inhibition of glutamate release inhibits the GABAergic neurons that originate in the NAc and project to the VTA, thus indirectly activating VTA dopaminergic neurons. Endocannabinoids have also been demonstrated to participate in synaptic plasticity in the NAc. Thus, repetitive activation of prelimbic glutamatergic afferents to the NAc results in long-term depression (LTD) of this excitatory transmission that depends on endocannabinoids and CB_1 receptors (Robbe et al., 2002). Chronic (Hoffman et al., 2003) or even a single (Mato et al., 2004) THC exposure modifies this form of synaptic plasticity, which is important for the development of the addictive process. Endocannabinoid release in the VTA participates in the modulation of drug rewarding effects (Lupica and Riegel, 2005), which would explain the involvement of CB_1 receptors in the rewarding properties of opiates, ethanol, THC and nicotine. Hence, CB_1 receptors would not participate in the primary rewarding effects of psychostimulants because they essentially act on dopaminergic axon terminals in the NAc. Nevertheless, somatodendritic dopamine release induced by psychostimulants in the VTA could promote endocannabinoid release in this brain area (Melis et al., 2004). Finally, CB_1 receptors on the glutamatergic projections from the prefrontal cortex (PFC) would be important to modulate motivation to seek the drug. These findings suggests that endocannabinoid activation of CB_1 receptors in the mesolimbic reward pathway may be part of a common pathway of drug reward and as a consequence play an important role

in the neurobiology of addiction (De Vries and Schoffelmeer, 2005; Maldonado et al., 2006). Examples of the involvement of endocannabinoid system on the rewarding and addictive properties of prototypical drugs of abuse are discussed below (Fig. 14).

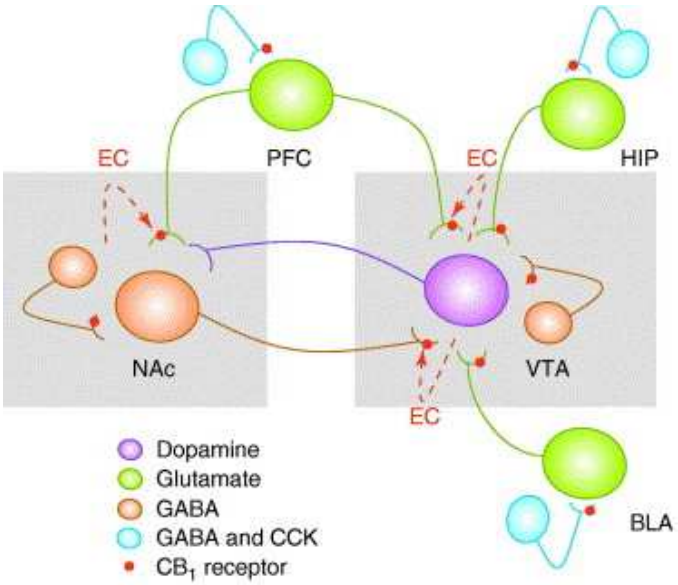

Figure 14. Possible sites of endocannabinoid action in modulation of drug rewarding effects (VTA, ventral tegmental area; NAc, nucleus accumbens; PFC, prefrontal cortex; Hip, hippocampus; BLA, basolateral amygdala; EC, endocannabinoids (Maldonado et al., 2006).

Opioids.

There is a large body of evidence indicating reciprocal relationships between the endocannabinoid and the endogenous opioid system in drug dependence (Maldonado and Valverde, 2003). Moreover, both systems participate in the common circuits involved in the addictive properties of different drugs of abuse (Maldonado and Rodriguez, 2002). This fact is not surprising, since opioids and cannabinoids have a similar pharmacological profile at both the behavioral (e.g., analgesia, hypothermia, catalepsy, and motor impairment) and cellular/molecular level (both CB_1 and opiate μ receptors are predominantly presynaptic, they are coupled to and share the same pool of G_i/G_o proteins, and have an overlapping brain distribution). CB_1 cannabinoid receptors have and important role in the rewarding properties of opioid drugs. Thus, morphine-induced CPP (Martin et al., 2000) and intravenous self-administration (Ledent et al., 1999; Cossu et al., 2001) were abolished in mice lacking CB_1 receptor. In agreement, reduced opioid self-administration and CPP was observed in rodents treated with rimonabant (Navarro et al., 2001; De Vries et al., 2003). Rimonabant also prevented heroin-seeking behavior after a long period of extinction, and the cannabinoid agonist HU-210 reinstated such a seeking behavior (De Vries et al., 2003; Solinas et al., 2003; Fattore et al., 2003). A neurochemical correlate of the previous observations is the lack of morphine-induced DA

release in the NAc of CB_1 receptor knockout mice (Mascia et al., 1999) and animals treated with a CB_1 antagonist (Rubino et al., 2000). Rimonabant also precipitated abstinence in morphine-dependent rats, indicating that CB_1 cannabinoid receptors are involved in opioid physical dependence (Navarro et al., 1998). Similarly, a robust attenuation in the severity of naloxone-precipitated morphine withdrawal was reported in CB_1 knockout mice (Ledent et al., 1999).

Nicotine.

Nicotine is the main psychoactive component in tobacco and is responsible for its addictive properties. Nicotine addiction is a complex neurochemical process that involves many neurotransmitters, including endocannabinoid system. The role of the endocannabinoid system in the rewarding effects of nicotine was first revealed by the absence of nicotine-induced CPP in CB_1 knockout mice (Castañé et al., 2002), although the acquisition of nicotine self-administration was not affected by the lack of CB_1 receptors in another study using an acute reinforcement paradigm (Cossu et al., 2001; Castañé et al., 2002). On the other hand, rimonabant was reported to decrease nicotine operant self-administration (Cohen et al., 2002a) and nicotine-induced CPP in rats (Le Foll and Goldberg, 2004; Forget et al., 2006). Accordingly, sub-effective doses of nicotine and THC showed positive synergism, as revealed by the rewarding effects in the CPP paradigm (Valjent et al., 2002). Supporting behavioral data, rimonabant also inhibited nicotine-induced DA release in the NAc shell (Cohen et al., 2002a). Nicotine relapse induced by associated environmental stimuli is also mediated by activation of the endocannabinoid system. Hence, rimonabant attenuated the influence of environmental cues in nicotine seeking behavior in rats (Cohen et al., 2005; De Vries et al., 2005). However, CB_1 receptors do not seem to participate in the development of nicotine physical dependence since rimonabant did not precipitate a withdrawal syndrome in nicotine-dependent animals (Balerio et al., 2004) and the severity of nicotine abstinence was not modified in CB1 knockout mice (Castañé et al., 2002). Several clinical trials, including STRATUS programme, were carried out in order to evaluate the efficacy of rimonabant on tobacco smoking cessation and relapse. Although high doses of rimonabant rimonabant may slightly increase the probability of quitting, the evidence for rimonabant in maintaining abstinence is inconclusive (Cahill and Ussher, 2007).

Alcohol.

Several lines of evidence indicate the involvement of the endocannabinoid system in alcohol drinking behavior (Colombo et al., 2005). Many studies showed the effectiveness

of rimonabant in reducing voluntary ethanol intake in rodent models of ethanol drinking (Arnone et al., 1997; Wang et al., 2003), whereas cannabinoid agonists promoted drinking (Gallate et al., 1999). The possible role of the endocannabinoid system in ethanol preference was further indicated by observations of reduced voluntary ethanol drinking in CB_1 knockout compared to wild-type mice (Hungund et al., 2003). The reduced voluntary ethanol intake in CB_1 knockout mice was associated with reduced alcohol-induced CPP (Houchi et al., 2005), a further indication of the role of CB_1 receptors in the rewarding effects of alcohol. Similar to other drugs of abuse, alcohol intake also results in increased DA release in the NAc (Weiss et al., 1993). The reported absence of such release in CB_1 knockout mice and the ability of rimonabant to block ethanol-induced DA release in wild-type mice further suggest the involvement of endocannabinoids in the reinforcing effects of ethanol (Hungund et al., 2003). A role of CB_1 receptor in stress-induced alcohol drinking and ethanol withdrawal has also been reported using knockout mice (Racz et al., 2003). Finally, CB_1 receptors are also involved in the mechanisms mediating alcohol relapse. Therefore, the exposure to the cannabinoid agonist WIN 55,212-2 or THC promotes the relapse of alcohol use in abstinent rats (Lopez-Moreno et al., 2004; McGregor et al., 2005), and rimonabant reduces conditioned reinstatement of ethanol-seeking behavior in rats (Cippitelli et al., 2005). According to these results, chronic alcohol intake increases endocannabinoid levels in the limbic forebrain (Gonzalez et al., 2002) and decreases CB_1 receptor binding and downstream signalling (Basavarajappa and Hungund, 2002).

Cocaine.

Unlike THC, opiates and nicotine, cocaine does not directly increase the activity of DA neurons in the VTA, but elevates synaptic levels of DA in the NAc by blocking DA reuptake at the DA transporter (Giros et al., 1996). CB_1 receptor does not appear to participate in the acute rewarding properties of cocaine, as indicated by the preserved cocaine-induced CPP and acute cocaine self-administration in CB_1 knockout mice (Martin et al., 2000; Cossu et al., 2001) or in mice treated with rimonabant (Tanda et al., 2000). Therefore, it is not surprising that cocaine-induced increases in DA in the NAc were found to be unaffected by genetic ablation of CB_1 receptors (Soria et al., 2005). In addition, rimonabant treatment did not affect the threshold-lowering effect of cocaine in the intracranial self-stimulation paradigm either (Vlachou et al., 2003). However, other studies indicate that CB_1 receptors may participate in the reinforcing effects of cocaine. Indeed, the acquisition of and operant behavior response to self-administer cocaine was impaired in CB_1 null mice, mainly, when the effort required to obtain a cocaine infusion was

enhanced. Thus, the breaking point achieved on a progressive ratio schedule of reinforcement was significantly reduced in CB_1 knockout mice, whereas self-administration was only slightly attenuated on an FR1 schedule. A similar result was obtained on the progressive ratio schedule after the blockade of these receptors using rimonabant (Soria et al., 2005). Rimonabant treatment also decreased the sensitivity of rats to the reinforcing effects of cocaine in an intracranial self-stimulation paradigm (Deroche-Gamonet et al., 2001). Furthermore, prior use of cannabis was found to enhance the "high" elicited by subsequent use of cocaine in humans (Foltin et al., 1993) and to hasten relapse in abstinent former cocaine users (Rawson et al., 1986). In agreement, treatment with HU-210 promoted reinstatement of cocaine-seeking behavior in rats, whereas treatment with rimonabant prevented cue-and priming-induced reinstatement (De Vries et al., 2001). Thus, the endocannabinoid system may be involved in the acquisition and consolidation of cocaine addiction as well as in relapse, through mechanisms other than a direct effect on the cocaine-induced increase in DA transmission in the NAc. These latter studies also predict the possible effectiveness of rimonabant in the treatment of cocaine addiction.

Several studies confirm the role of endocannabinoid system on the rewarding and addictive properties of other psychostimulants. Thus, the CB_1 antagonist AM251 decreased methamphetamine self-administration (Vinklerova et al., 2002). Amphetamine-induced long-term synaptic depression in the amygdala was blocked by the same CB_1 antagonist, mimicked by the agonist WIN 55,212-2, and occluded by the transport inhibitor AM404, suggesting that amphetamine-induced LTD and related behavioral effects may be mediated via endocannabinoid release (Huang et al., 2003).

Ecstasy.

3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy) is a drug abused for its entactogen and psychostimulant properties. MDMA shows a predominant effect on serotonin (5-HT) release, which provides particular effects to this psychostimulant drug. Although no classical addictive properties have been reported for ecstasy, rewarding effects of this drug have been widely described (see section 3.5 for detail). According to the results obtained with cocaine, CB_1 receptor knockout mice exhibited similar MDMA-induced CPP than wild-type littermates, but MDMA self-administration was completely abolished in these mutants (Tourino et al., 2007). However, treatment rimonabant abolished CPP and enhanced MDMA self-administration (Braida and Sala, 2002; Braida et al., 2005). In addition, cross-interaction between cannabinoid agonists and MDMA were reported. THC potentiated the rewarding and reinforcing effects of MDMA (Robledo

et al., 2007), although intracerebral self-administration of MDMA was reduced in the presence of the cannabinoid agonist CP-55,940 (Braida and Sala, 2002), and THC abstinence was reduced by MDMA in a dose-dependent manner (Touriño et al., 2007).

2.3.2. Neurotoxicity, pain and inflammation.

The endocannabinoid system plays an important neuroprotective role in acute neuronal injury (e.g., traumatic brain injury, stroke, and epilepsy), chronic neurodegenerative disorders (e.g., multiple sclerosis, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Alzheimer's disease), and pain (Ramos et al., 2005). Although the underlying mechanisms are not fully understood, multiple cannabinoid receptor-dependent as well as receptor-independent processes have been implicated. Cannabinoid receptors are present on immune cells, and stimulation of immune cells by bacterial toxins such as lipopolysaccharide (LPS) increases the cellular levels of endocannabinoids and their degrading enzymes. Moreover, cannabinoid agonists modulate immune function both *in vitro* and *in vivo* via CB_1 and CB_2 cannabinoid receptor-dependent and -independent mechanisms in neurons, astrocytes, microglia, macrophages, neutrophils and lymphocytes. The anti-inflammatory effects of cannabinoids are complex and may involve modulation of cytokine (e.g., TNF-*α*, IL-12, IL-1, IL-6, and IL-10) and chemokine production (e.g., CCL2, CCL5, CXCL8, and CXCL10), modulation of adenosine signalling (Carrier et al., 2006), expression of adhesion molecules (e.g., ICAM-1, P-intercellular adhesion molecule-1 and P-selectin), and migration, proliferation and apoptosis of inflammatory cells (Walter and Stella, 2004). In addition to the involvement of endocannabinoid system on the immune response, cannabinoids exert a neuroprotective effect through the activation of cytoprotective signalling pathways (Grigorenko et al., 2002) such as protein kinase B/Akt (Kim et al., 2005) or neurotrophic factors (Khaspekov et al., 2004), modulation of excitability (Robbe et al., 2001) and calcium homeostasis (Zhuang et al., 2005), antioxidant properties (Marsicano et al., 2002), and direct hypothermic effects (Leker et al., 2003).

One of the earliest uses of cannabis was to treat pain (Mechoulam and Hanus, 2000). Cannabinoids are effective in several kinds of acute and chronic pain. In acute pain, AEA, THC, cannabidiol, and synthetic cannabinoids such as CP-55,940 and WIN-55,212-2 are effective against chemical, mechanical, and thermal pain stimuli (Buxbaum, 1972; Sofia et al., 1973). Recent animal studies indicate that AEA and cannabinoid ligands are also effective against chronic pain of both neuropathic (Herzberg et al., 1997) and inflammatory origin (Tsou et al., 1996). Moreover, endocannabinoids and synthetic cannabinoids exert synergistic antinociceptive effects when combined with commonly

used nonsteroid anti-inflammatory drugs, which may have utility in the pharmacotherapy of pain (Guindon and Beaulieu, 2006). Cannabinoids exert their antinociceptive effects by complex mechanisms on the CNS, spinal cord, and peripheral sensory nerves. This is consistent with the anatomical location of CB_1 receptors in areas relevant to pain in the brain, spinal dorsal horn, dorsal root ganglia, and peripheral afferent neurons (Herkenham et al., 1990). In addition to the role of CB_1 receptors, there is recent evidence implicating CB_2 receptors in the antihyperalgesic activity of cannabinoids in models of acute and chronic pain, mainly of neuropathic and inflammatory origin (Clayton et al., 2002; Ibrahim et al., 2003; Melis et al., 2004; Racz et al., 2008). In humans, the analgesic activity of THC and other cannabinoids is less clear-cut. There are numerous case reports on the beneficial effects of cannabis or synthetic derivatives of THC in pain associated with multiple sclerosis, cancer, neuropathies, and HIV infection (Burns and Ineck, 2006).

On the other hand, endocannabinoid system has been found to be altered in several neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer disease (Pacher et al., 2006), although the cause of these adaptations remains still uncertain. Nevertheless, cannabinoid agonists were found to be useful for the treatment of neurodegenerative diseases and the relief of their symptoms. One of the first findings about the neuroprotective effect of cannabinoids came from the field of stroke research (Pacher et al., 2006). Cannabinoid agonists have also been proposed as a treatment for spasticity, tremor, or hyperkynesia in motor related disorders such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, or Huntington's disease (Sagredo et al., 2007). Furthermore, cannabinoid agonists may neuroprotect against the progression of these neurodegenerative diseases, rather than simply relieving their negative symptoms. Hence, cannabinoids can delay the progression of multiple sclerosis and amyotrophic lateral sclerosis, by decreasing oxidative stress, neuroinflammation, autoimmunity and glutamate toxicity (Arévalo-Martin et al., 2003; Raman et al., 2004). Similarly, in other progressive neurodegenerative disorders such as Alzheimer's disease or HIV encephalitis, cannabinoids seem to prevent the progression of the disease through the blockade of microglial activation (Benito et al., 2005; Ramirez et al., 2005).

Given the widely reported neuroprotective effects of cannabinoids, some authors also propose them as potential neuroprotective agents against the neurotoxic effects of amphetamine derivatives. Thus, anti-oxidant and anti-inflammatory properties of cannabinoids may attenuate MDMA neurotoxicity, which is mediated by the production of highly reactive oxygen species. In addition, the ability of THC to produce hypothermia has been shown to be neuroprotective (Hayakawa et al., 2004). This hypothesis is in

agreement with the results found in our laboratory revealing that the pre-treatment with THC completely reversed the activation of microglia and astrocytes induced by a neurotoxic regimen of MDMA. Animals treated with MDMA showed a mild increase in body temperature, whereas animals treated with THC had a marked hypothermia, and animals receiving both drugs suffered a more severe hypothermia than THC-treated animals. Mice lacking CB_1 receptors did not show hypothermia or neuroprotection induced by THC, suggesting that THC protects against MDMA neurotoxicity by decreasing body temperature through a CB_1 receptor-mediated mechanism. However, an anti-inflammatory and antioxidant mechanism cannot be discarded.

3. Psychostimulants

3.1. Types of psychostimulants.

Psychostimulants are drugs that produce behavioral activation accompanied by increased arousal, alertness, and motor activity. Stimulant drugs have medical uses but also considerable abuse potential. Regarding to their pharmacodynamic properties, there are two major classes of psychomotor stimulants; direct or indirect sympathomimetics, such as cocaine and amphetamine, and non-sympathomimetics, such as caffeine or nicotine (Table 2).

Table 2. Psychomotor stimulant drugs.

Direct sympathomimetics	Indirect sympathomimetics	Non-sympathomimetics
Isoproterenol	Amphetamine	Caffeine
Epinephrine	Methamphetamine	Teobromine
Norepinephrine	MDMA	Teophiline
Phenylephrine	Cocaine	Tein
Pheniylpropanolamine	Methylphenidate	Nicotine
Apomorphine	Phenmetrazine	Pentylenetetrazol
	Pipradrol	
	Tyramine	
	Pemoline	
	Modafinil	

This section will just describe the indirect sympathomimetics, since these compounds produce the highest addictive and abuse potential among psychostimulants. Indirect sympathomimetic compounds such as cocaine and amphetamines share a common molecular structure, a benzene ring with an ethylamine side chain (Fig. 15). Sympathomimetics reproduce the peripheral actions of NA activating the sympathetic nervous system, and either directly or indirectly activate monoamine receptors. Indirect sympathomimetics mimic this action by acting on neuronal mechanisms that do not involve direct activation of postsynaptic receptors, but an increase in the release of monoamines in the synaptic cleft.

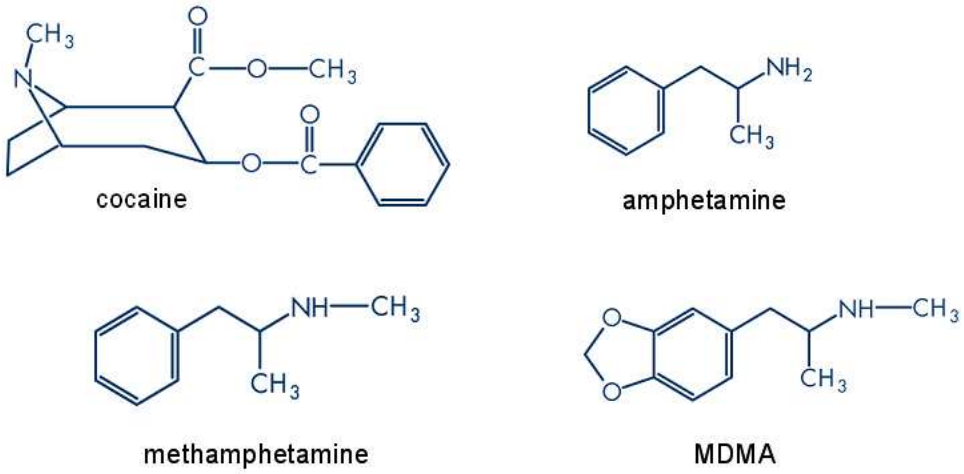

Figure 15. Chemical structure of some psychostimulant drugs.

3.2. History of psychostimulant use.

Cocaine is derived from the coca plant (*Erythroxylon coca*) and has a long history as a stimulant compound. Coca plant is native from northwestern South America. The plant plays a significant role in traditional Andean culture. Cocaine has been used for centuries to relieve fatigue, sustain performance, and treat a large variety of illnesses. Presumably because cocaine was used in numerous tonics, there was extensive cultivation of cocaine in South America and exportation to the United States and Europe at the beginning of 20th century. Widespread use followed, and the first restriction of coca products started in 1914. In the 1960's, a rise in the smuggling and use of cocaine started on the basis of the high monetary profit involved in its illegal trafficking, which culminated in an epidemic of cocaine use in the 1980's. In the 1970's cocaine was usually administered intranasally in a powder form (cocaine hydrochloride), but freebase cocaine use developed. Cocaine freebase is generally prepared from its hydrochloride salt by one of two techniques. In one procedure, the hydrochloride salt first is mixed with buffered ammonia, then the alkaloidal cocaine is extracted from the solution using ether, and finally the ether is

evaporated to yield cocaine crystals. When heated, the crystals release vaporized cocaine that can be inhaled. The hydrochloride salt by itself cannot be smoked because it is quickly destroyed at high temperatures. Cocaine freebase melts at 98 ºC and vaporizes at 260 ºC, so it is usually smoked. The crystals also make a popping sound when heated; it is this characteristic sound that has been hypothesized to be the origin of the term 'crack' cocaine. Later, crack cocaine was sold in small, ready-to-smoke 'rocks'. Cocaine hydrochloride was also combined with baking soda (sodium bicarbonate), and the solution was heated until a solid was formed. This is the preferred method to produce smokable cocaine because it is simpler and safer that the ether extraction method.

Amphetamines are synthetic compounds with widespread medical use in the treatment of narcolepsy and a variety of other disorders until 1940's. Methamphetamine was first synthesized in Japan in 1893 and came into widespread use during World War II for increasing endurance and performance of military personnel. The most common manufacturing process uses ephedrine or pseudoephedrine in a reduction process with a mixture of iodine and red phosphorous to yield both the dextro (D) and the levo (L) isomers of methamphetamine. The D-isomer is 5-10 times more potent than the L-isomer in producing CNS effects. The L-isomer, called desoxyephedrine, is the active ingredient in some nasal decongestant inhalers. Crystal methamphetamine, which is the D-isomer, is called 'ice', allegedly because of its resemblance to ice crystals and can be smoked or snorted (Koob and Le Moal, 2006).

MDMA is another synthetic psychostimulant drug that was originally patented for use as an appetite suppressant. In the 1980's, MDMA started to be used in psychotherapy and was said to increase patient self-esteem and facilitate therapeutic communication. In 1985 MDMA was classified as a *Schedule I* drug (list of hazardous drugs to the public safety made by U.S Drug Enforcement Administration) due to its high abuse potential, lack of clinical application and evidence that 3, 4-methylenedioxyamphetamine (MDA), a related compound and major metabolite of MDMA, induced serotonergic nerve terminal degeneration in rat brain. Nevertheless, since the mid 1980's it has become popular as a recreational drug, often being taken at 'rave' or 'techno' parties particularly in large dance clubs. MDMA comes in a variety of colors, shapes and sizes of tablet, which are decorated with a wide variety of designs or logos and may also be available in capsule form. As with any illicit drug, both doses and purity vary greatly, but tablets have regularly been found to contain between 80 and 150 mg of MDMA.

3.3. Behavioral effects of psychostimulants in humans.

Cocaine administered intranasally produces stimulant effects similar to those of amphetamines, but with much shorter delay and duration (20-45 min) that include feelings of having much energy, fatigue reduction, a sense of well being, increased confidence, and increase talkativeness. When taken intravenously or smoked, cocaine produces an intense euphoria sometimes followed by a crash. Cocaine has many of the same stimulant effects and produces the same subjective and physiological effects of amphetamines, including sustained performance in situations of fatigue. Amphetamines in recreational dose ranges produce stimulant effects, but the most dramatic effects are observed in situations of fatigue and boredom. Beneficial effects include increase stimulation, improved coordination, increased strength and endurance, and increased mental and physical activation, with mood changes and boldness, elation and friendliness. Amphetamines enhance performance in simple motor and cognitive tasks, including measures of reaction time, speed, attention and performance. Amphetamines also can significantly improve athletic performance with low doses. Nevertheless, stimulants such as the amphetamines and cocaine fail to improve intellectual functioning in complex tasks.

Other acute actions of amphetamines and cocaine include a decrease in appetite for which these drugs have been used therapeutically and to which tolerance develops. Amphetamines also produce decreases in sleepiness, increased latency to fall asleep, increased latency to the onset of REM sleep, and reduction in the proportion or REM sleep. Finally, amphetamines and cocaine have long been reported to heighten sexual interest and prolong orgasm. However, systematic studies of the effects of amphetamines and cocaine on sexual behavior show that they can lead to significant decrease in sexual performance with prolonged use of the drugs.

The stimulant effects of cocaine and amphetamines depend on the route of administration. As noted above, intravenous or inhaled freebase preparations produce marked, intense, pleasurable sensation characterized as a ‘rush’, that is thought to be a powerful motivation for the abuse of these drugs. Smoked cocaine in the freebase form has absorption characteristics similar to intravenous administration. Intranasal administration of cocaine also produces euphoric and stimulant effects that last approximately 30 min. Cocaine has less powerful effects administered orally, presumably due to a markedly slower absorption rate. Intranasal or oral administration of D-amphetamine produces stimulant effects similar to cocaine. Intranasal absorption is faster with more intense effects than oral administration, and the stimulant effects of amphetamines last considerably longer (up to 4-6 h) (Koob and Le Moal, 2006).

3.4. Abuse and addiction potential of psychostimulants.

Amphetamine and cocaine have high abuse potential and can produce addiction. While 80-85% of users do not become addicted to the drug, clinical observations indicate that controlled use often shifts to more compulsive use, either when there is increased access to the drug or when a more rapid route of administration is employed. Initial experiences with cocaine produce intense euphoria, followed by dysphoria. With chronic use, dose required to produce euphoria increases, and the subjective high decreases. As cocaine use and duration increases, the positive reinforcing effects are diminished while the resulting dysphoria increases. Compulsive use results in an exaggeration of the binge stage where a user characteristically re-administers the drug every 10 min for up to 7 days but usually averaging 12 h. Euphoria is replaced by dysphoria, including agitation, anxiety, and even panic attacks. Stereotyped movements such as teeth grinding and pacing may appear, as well as hyperactivity and labile emotions. High doses may cause paranoia and hallucinations in some users, and in some subjects, heavy cocaine use may cause psychotic symptoms.

Withdrawal from chronic or high dose cocaine use in humans is not associated with physical signs, but a number of motivationally relevant symptoms such as dysphoria and depression, anxiety, anergia, insomnia, and craving for the drug usually occur. Several phases have been identified in outpatient studies of compulsive users:

- Phase 1 consists of a ‘crash’ phase, which last up to 4 days where there is a rapid lowering of mood and energy and acute onset of both an agitated and retarded depression. Craving for the drug, anxiety and paranoia peak and then are replaced by hyperphagia and insomnia.
- Phase 2 has been described as a period of prolonged dysphoria, anhedonia and lack of motivation, and increased craving that can last up to 10 weeks; relapse is highly possible during this phase.
- Phase 3 is characterized by episodic craving and last indefinitely. The withdrawal syndrome contributes to a vicious circle where cessation of cocaine use leads to withdrawal symptoms, then the associated dysphoria combined with craving leads to relapse (Koob and Le Moal, 2006).

3.4.1. Neurobiological mechanism of psychostimulant addiction.

Indirect sympathomimetics such as amphetamine and cocaine are known to act enhancing the amount of monoamines available within the synaptic cleft of synapses in the CNS. Cocaine and amphetamines block the reuptake of DA, 5-HT and NA in the brain, but the action at DA transporter (DAT) is the main responsible for motor activation and reinforcing properties of these psychostimulants. However, MDMA effects are mainly related to 5-HT system, despite sharing DA related behaviors with cocaine and amphetamine. Although the DAT is a site of action shared by all psychostimulants, there are differences in the molecular mechanisms by which each specific drug interact with DAT. Amphetamine acts as a false substrate and is transported into the cytoplasm whereas cocaine binds the DAT at a site distinct from DA and is not internally transported. Hence, while cocaine just inhibits DA uptake, amphetamine reverses transport of DA from the cytoplasm to the extracellular space (Koob and Le Moal, 2006).

Brain DA neurons are organized into three major pathways that originate in the midbrain and project to numerous forebrain and cortical regions. The mesocorticolimbic pathway originates in the VTA and projects to the ventral forebrain, including the NAc, olfactory tubercle, septum and PFC. This pathway has been related to rewarding and reinforcing properties of psychostimulants (Fig 1). The nigrostriatal DA system arises primarily in the substantia nigra and projects to the corpus striatum, and has been associated with both motor stimulant action of cocaine and amphetamine. The tuberoinfundibular pathway refers to a population of DA neurons in the arcuate nucleus of the mediobasal hypothalamus (the 'tuberal region') that project to the median eminence (the 'infundibular region'). DA released at this site regulates the secretion of prolactin from the anterior pituitary gland. Therefore, reproductive system disfunctions and hyperprolactinemia are often associated with chronic cocaine abuse. These effects are consequence of the activation of DA receptors, D1-like (D1, D5) and D2-like (D2, D3, D4). D1-like receptor family signaling is mediated chiefly by the heterotrimeric G proteins $G_{\alpha s}$, which cause sequential activation of adenylate cyclase, cylic AMP-dependent protein kinase, and the protein phosphatase-1 inhibitor DARPP-32. The increased phosphorylation that results from the combined effects of activating cyclic AMP-dependent protein kinase and inhibiting protein phosphatase 1 regulates the activity of many receptors, enzymes, ion channels, and transcription factors. D1 receptor also signals via phospholipase C-dependent and cyclic AMP-independent mobilization of intracellular calcium. D2-like receptor signaling is mediated by the heterotrimeric G proteins $G_{\alpha i}$ and $G_{\alpha o}$. These pertussis toxin-sensitive G proteins regulate some effectors, such as adenylate cyclase,

via their G_{α} subunits, but regulate many more effectors such as ion channels, phospholipases, protein kinases, and receptor tyrosine kinases as a result of the receptor-induced liberation of $G_{\beta\gamma}$ subunits. In addition to interactions between dopamine receptors and G proteins, other protein:protein interactions such as receptor oligomerization or receptor interactions with scaffolding and signal-switching proteins are critical for regulation of dopamine receptor signaling (Neve et al., 2004) (Fig. 16).

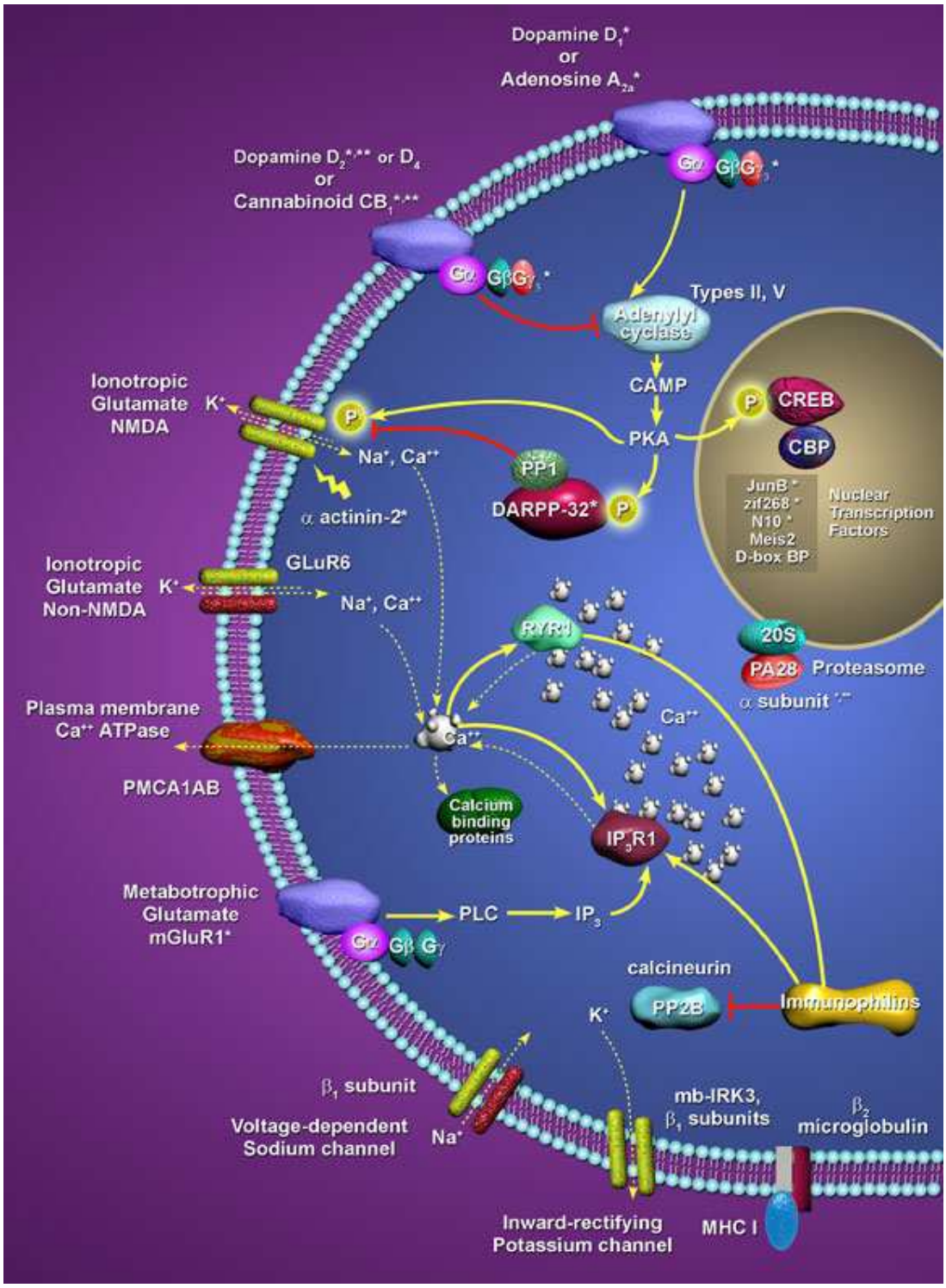

Figure 16. Dopamine receptor signaling.

Both D1-like and D2-like receptors in dorsal and ventral striatum have been involved in psychostimulant-induced hyperactivity, although D1 receptors seem to play a principal role in these responses (Xu et al., 1994; Reimer and Martin-Iverson, 1994; Baker et al., 1996). The NAc is also the primary site involved in the maintenance of psychostimulants reinforcement, which again relies primarily on DA D1-like receptors (Self et al., 1996). Neurophysiological studies indicate that in the NAc, both D1- and D2-like receptors decrease the release of glutamate from afferents derived from PFC, hippocampus and amygdala (Kalivas and Duffy, 1997). The modulation of glutamate release by psychostimulants explains synaptic plasticity changes that take place after repeated psychostimulant use and leads to the sensitizing and addictive properties of these drugs (see section 1.3).

The repeated use of psychostimulants can result in alterations in the behavioral effects of acute drug administration, which emerge over time and endure after long periods of abstinence. These alterations include the emergence of drug craving and sensitization to psychostimulant-induced hyperactivity (Martin-Iverson and Burger, 1995; Pierce and Kalivas, 1997). The behavioral alterations associated with psychostimulant abuse are related with several neuroadaptations in VTA DA neurons. Most of them are relatively transient, but may be necessary to trigger other alterations responsible for the maintenance of addiction related behaviors (White, 1996). These transient neuroadaptations include DA autoreceptors subsensitivity, reduction of inhibitory G-protein levels, enhanced basal levels of extracellular DA, enhanced sensitivity of AMPA receptors on VTA DA neurons, and increased expression of glutamate receptors (AMPA and NMDA) (Kalivas and Duffy, 1998). Each of these changes suggests an enhancement in the basal activity of DA neurons. Repeated treatment with psychostimulants also lead neuroadaptations in NAc neurons, such as enhanced pre- and post-synaptic D1-like receptors by signaling (Henry and White, 1995).

The enhancement of DA activity in the NAc after a chronic administration of psychostimulants increases the motivational value of the drug. This DA enhancement also takes place in other brain regions related to addiction. Thus, increase of DA activity in the dorsal striatum turns motivation for drugs into a habit, and loss of DA inhibition in PFC enhances NAc and amygdala activation, leading to impulsivity in drug consumption. Hypersensitized D1 receptors in NAc also facilitate glutamatergic transmission from PFC and BLA to NAc producing LTP in these circuits. The potentiation of these circuits is essential for the setting of a strong motivation for drug seeking (where NAc plays a critical role) as well as abnormal motor habits of seeking behavior (Vanderschuren and Kalivas, 2000).

Nevertheless, not all the psychostimulant drugs show the same addictive profile. While cocaine and methamphetamine produce addiction among their consumers, no such effects have been clearly observed in MDMA consumers (Murray, 2001). The prolonged activation of D1 and D2 receptors is necessary for the development of addiction, as explained above. Therefore, the ability of a drug to enhance DA release (as well as its pharmacokinetics) is directly related to its addictive properties. MDMA enhances DA release reversing the activity of DAT, but unlike addictive psychostimulants such as cocaine, amphetamine or methamphetamine this effect is much more evident on 5-HT transporter (SERT), producing a stronger enhancement of 5-HT release and 5-HT receptor activation (Cole and Sumnall, 2003).

3.5. MDMA.

MDMA is a ring-substituted amphetamine derivative that is also structurally related to the hallucinogenic compound mescaline, since methylenedioxy ring substitution enhances potency for 5-HT release. It was first patented in Germany in 1914. In the 1980's MDMA started to be used in psychotherapy and was said to increase patient self-esteem and facilitate therapeutic communication. In such settings it was administered orally (75-175 mg) and noted to produce acute sympathomimetic effects. In the mid 1980's MDMA was illegalized due to its high abuse potential, lack of clinical application, lack of accepted safety, for use under medical supervision, and evidence that 3, 4-methylenedioxyamphetamine (MDA), a related compound and major MDMA metabolite, induced 5-HT nerve terminal degeneration in rat brain (Ricaurte et al., 1985). Nevertheless, it has become a popular recreational drug, often being taken at 'rave' or 'techno' parties, particularly in large dance clubs. Ecstasy comes in a variety of colors, shapes, and sizes of tablet, which are decorated with a wide variety of designs or logos and may also be available in capsule form. As with any illicitly prepared and obtained recreational drug, both, doses and purity vary greatly, but tablets have regularly been found to contain between 80 and 150 mg of MDMA. The onset effect can take 20 to 60 min to occur, the peak occurring 60 to 90 min after ingestion, and the primary effects last for 3 to 5 h (Green et al., 2003; Koob and Le Moal, 2006).

3.5.1. Pharmacokinetics of MDMA.

MDMA is usually present in two optical isomers with the dextrorotatory form, S- (+)-MDMA being more potent in the CNS. MDMA is metabolized to MDA (which is sometimes used recreationally), 4-hydroxy-3-methoxymethamphetamine (HMMA), and 3, 4-dihydroxymethamphetamine (HHMA). The more active S- (+)-MDMA isomer is

metabolized faster and more extensively than the levorotatory form (Mas et al., 1999; Segura et al., 2001). Assessment of plasma levels of MDMA and MDA indicated a nonproportional dose-dependent kinetics. While there was no significant difference between doses with regard to urinary clearance of MDMA, non-renal clearance was dose-dependent, and was reduced at high doses, indicating an impairment of hepatic clearance of the drug (Mas et al., 1999; Segura et al., 2001).

MDMA metabolism is rather complex and includes 2 main metabolic pathways: (1) O-demethylenation followed by catechol-O-methyltransferase-catalyzed methylation and/or glucuronide/sulfate conjugation; and (2) N-dealkylation, deamination, and oxidation to the corresponding benzoic acid derivatives conjugated with glycine (de la Torre et al., 2000; de la Torre et al., 2004). Different CYP450 isozymes are involved in the different metabolic pathways of MDMA. O-demethylenation of MDMA is catalyzed by CYP1A2, CYP2D6 and CYP2B6 to form HHMA. N-dealkylation of MDMA is catalyzed by CYP1A2, CYP2D6, CYP2B6 and CYP3A4 in humans (de la Torre and Farré, 2004). The elimination half-life of MDMA is about 8 to 9 h (de la Torre et al., 2000) (Fig. 17).

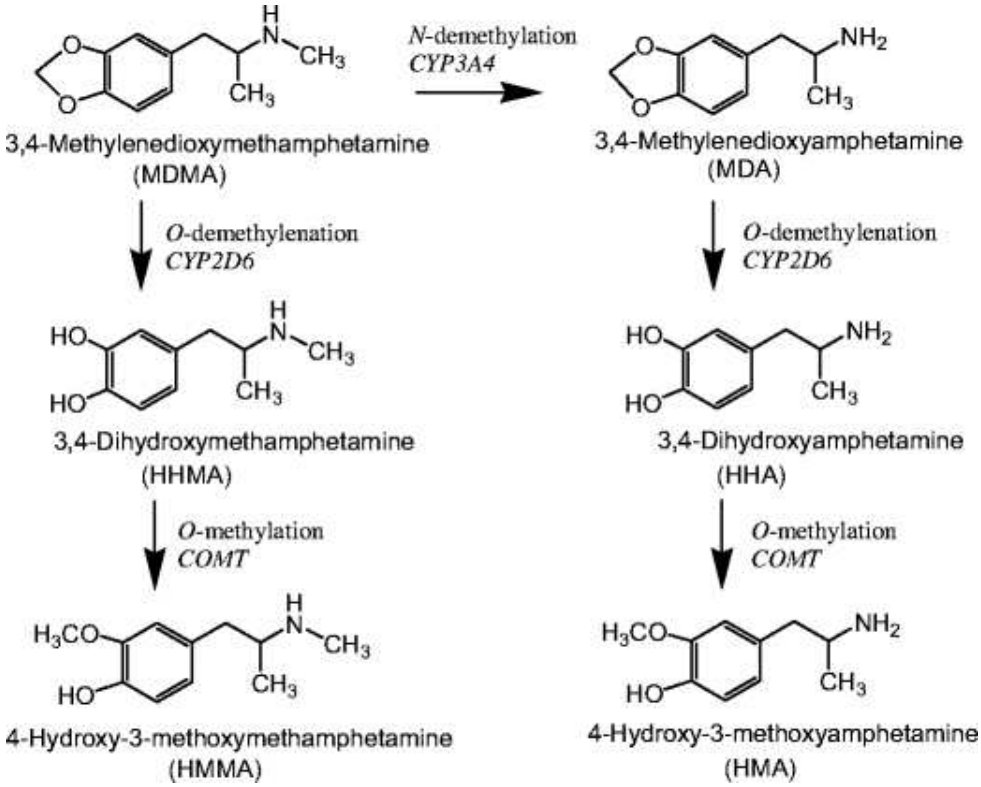

Figure 17. Metabolism of MDMA in humans (de la Torre et al., 2004).

3.5.2. MDMA pharmacology in humans.

MDMA binds to plasma membrane monoamine transporters and is translocated into the cytoplasm. MDMA is a substrate for DAT, SERT and NA transporter (NET). When

compared to amphetamine and methamphetamine, the major effect of methylenedioxy ring substitution is enhanced potency for 5-HT release and reduced potency for DA release. MDMA releases 5-HT about ten times more potently than methamphetamine, whereas MDMA releases DA about six times less potently than methamphetamine. However, MDMA has a very low affinity for post-synaptic serotonin receptors. Consistent with *in vitro* results, in vivo microdialysis experiments demonstrate that MDMA increases extracellular 5-HT and DA, with effects on 5-HT being greater in magnitude (Cole and Sumnall, 2003). There is also an increase of extracellular acetylcholine and NE in the brain, but most of the psychological effects of MDMA are mediated by 5-HT (El-Mallakh and Abraham, 2007; Baumann et al., 2007).

The popularity of ecstasy may be explained by the desirable effects that recreational users report, including friendliness, euphoria, increased energy and sexual arousal. Rewarding and reinforcing properties MDMA have been widely reported, as with many drugs of abuse. However, unlike other psychostimulant drugs such as cocaine amphetamine or methamphetamine, addiction to MDMA has never been reported in humans. MDMA has also been associated with undesirable effects (e.g. confusion, defensiveness, mental fatigue, anxiety, depression) and unhealthy medical consequences (e.g. hyperthermia, cardiac arrhythmia, hypertension). The acute somatic, emotional and cognitive effects that occur during the peak period after MDMA ingestion are listed in table 3.

Table 3. Acute effects of ecstasy (Green et al., 2003; Baylen and Rosenberg, 2006).

Somatic	Emotional	Cognitive
Body temperature changes	Euphoria	Loss of memory
Elevated blood pressure and heart rate	Elation	Increased alertness
Nausea	Increased energy	Concentration difficulty
Chills	Happiness	
Sweating	Exhilaration	
Tremor	Warmth	
Jaw clenching	Friendliness	
Bruxism	Affection	
Hyperreflexia	Calmness	
Urinary urgency	Improved mood	
Muscle aches or tension	Decreased defensiveness	
Nystagmus	Moodiness	
Insomnia	Anxiety	
Dry mouth	Depression	
Dilated pupils	Irritability	
	Panic attacks	
	Paranoid delusions	

Effects on sexual behavior (e.g. sexual arousal, increased sensual awareness, improved sex and enhanced lubrication in women), sensory perception (e.g. visual effects, sound hallucinations, enhanced sense of touch), sleeplessness, and decreased appetite were also reported by users.

Long-term psychological effects that can result from chronic use of MDMA have been reported to persist time long after cessation of drug use. Visual hallucinations and paranoid delusions can appear in the peak effect of the drug, but may sometimes persist for days or weeks together with anxiety, depression, panic disorders, cognitive impairment and other behavioral alterations. Regular use of MDMA has been reported to result in chronic psychosis in a small number of subjects. Heavy ecstasy poly-drug users scored more highly on phobic anxiety, obsessive-compulsive behavior, anxiety, psychoticism, and somatization than control subjects. However, problems did not appear to be specific to MDMA use as they were also seen in other recreational poly-drug users. There is substantial evidence that some recreational MDMA users display selective cognition deficits, and those deficits continue in the drug-free state and may be more pronounced in heavy drug users. The cognitive deficits appear to be more apparent in tasks known to be sensitive to temporal functioning. MDMA users have shown to display impaired immediate and delayed verbal memory and working memory, sometimes correlating to 5-HT deficits (Green et al., 2003; Baylen and Rosenberg, 2006).

3.5.3. MDMA pharmacology in animals.

Large number of animal studies have been undertaken to clarify the effects of MDMA in humans. Although not all the animal data can be translated to humans, most of the acute physiological and behavioral effects are similar. Several somatic effects produced by MDMA are observed in both rodents and humans. Elevated blood pressure is observed in humans after MDMA use (Baylen and Rosenberg, 2006). Similarly, MDMA is well known to produce a range of effects on cardiovascular function including tachycardia, arrhythmia, and enhanced vasoconstriction in rats (Gordon et al., 1991; Baylen and Rosenberg, 2006). Cardiovascular effects are also observed with cocaine and amphetamine, indicating that they are produced by the sympathomimetic properties of psychostimulants. Acute MDMA administration to humans rats and mice alters body temperature with most studies showing marked hyperthermia, although hypothermic responses have been observed in certain strains of rats and mice (Green et al., 2003; Baylen and Rosenberg, 2006). Moreover, ambient temperatures influence the effect of MDMA and other amphetamines on body temperature. Rats kept at high ambient temperature show an increased hyperthermic and neurotoxic response to MDMA

(Malberg and Seiden, 1998; Mechan et al., 2001). This hyperthermia is mediated by the MDMA-induced increase of 5-HT (Colado et al., 1993) and DA (Mechan et al., 2002). The effect of ambient temperature is an important issue in subjects consuming MDMA. Dance club conditions, where MDMA is usually used, have high ambient temperature, and may enhance neurotoxic and hyperthermic effects of this substance.

Increased physical and emotional energy reported by MDMA users is directly related to hyperactivity observed in animals. The hyperactivity induced by MDMA is complex in neurochemical terms and both 5-HT and components DA are involved (Gold and Koob, 1988; Daniela et al., 2004). Anxiety-related behaviors have been also observed in animal models, although heterogeneous results have been reported depending on the dose, frequency of administration and behavioral test used. In the elevated-plus maze (an anxiety test evaluating response to open spaces) anxiogenic-like effects of MDMA are usually observed. However, low doses of MDMA have been observed to be anxiolytic in tests involving social interaction (Morley and McGregor, 2000) in rats. This late result is in agreement with the feeling of friendliness and affection described by MDMA users. Social interaction test is not an appropriate test to use in mice due to the aggressive nature of these animals. Therefore, only anxiogenic effects of MDMA have been reported in this specie (Lin et al., 1999).

Cognitive deficits have been widely reported in animals chronically treated with MDMA, which show impaired performance in tasks involving cognitive ability (Marston et al., 1999), learning (Moyano et al., 2004; Trigo et al., 2008) and memory (Piper and Meyer, 2004). In primates, MDMA disrupted processes associated with learning/acquisition of new information, although short-term memory was less sensitive to the drug effects. Most authors suggested that the behavioral effects observed could be primarily attributed to 5-HT nerve terminal dysfunction.

Rewarding and reinforcing effects of MDMA have been widely reported in animal models. MDMA established CPP in both rats (Bilsky et al., 1990) and mice (Robledo et al., 2004), and decreased reward threshold lon intracranial self-stimulation (Hubner et al., 1988). Furthermore, MDMA self-administration has also been observed in rats (Schenk et al., 2003), mice (Trigo et al., 2006) and monkeys (Beardsley et al., 1986; Lamb and Griffiths, 1987). As mentioned above, MDMA administration induces a strong 5-HT release, but in a lesser extent, MDMA also induces DA release in rodents. Thus, MDMA rewarding effects observed in animals are mediated by the enhanced release of DA in the NAc (Colado et al., 2004; Touriño et al., 2007).

3.5.4. Neurotoxicity.

MDMA is toxic to 5-HT neurons in rats, guinea pigs monkeys and humans, whereas produces DA toxicity in mice due to the different isoenzymes involved in their MDMA metabolism between species (de la Torre and Farré, 2004). There is wide evidence of the neurotoxic effects produced in MDMA users. Poorer verbal or visual memory, prospective everyday memory, and executive cognitive functioning were reported in ecstasy users compared to controls. Moreover, brain imaging studies have reported deficiencies in cerebral metabolism, serotonin transporter densities, and N-acetyl aspartate (NAA, a marker of cellular health) in the frontal cortex, but not in the parietal or occipital cortex of ecstasy users (Reneman et al., 2001). The prominent 5-HT activity in frontal cortex makes this region the main target for MDMA neurotoxicity. These deficiencies have been observed in animal as well, where repeated high doses of MDMA caused signs of damage to 5-HT axons in primates and rats, and to DA axons in mice (Lyvers, 2006).

3.5.4.1. Mechanisms of neurotoxicity.

The long-term adverse effects of MDMA on 5-HT system have attracted substantial interest. Studies in rats and nonhuman primates show that high doses of MDMA produce persistent reductions in markers of 5-HT nerve terminal integrity (tryptophan hydroxylase activity, depletions of brain tissue 5-HT, and reductions in SERT binding). These 5-HT deficits are observed in various regions of rat forebrain, including frontal cortex, striatum, hippocampus, and hypothalamus. Immunohistochemical analysis of 5-HT in cortical and subcortical areas reveals an apparent loss of 5-HT axons and terminals in MDMA-treated rats arising from dorsal raphe nucleus. Moreover, 5-HT axons and terminals remaining after MDMA treatment appear swollen and fragmented, suggesting structural damage (Baumann et al., 2007). MDMA has a very different neurotoxic profile in mice in comparison to that observed in rats. MDMA is a relatively selective DA neurotoxin in mice leaving 5-HT concentrations unaffected, which is exactly opposite to the effect of MDMA in rats. Long-lasting effects of MDMA in the striatal DA neurons are reflected by a loss of DA concentration, a reduction in the density of DA uptake sites, a decrease in tyrosine hydroxilase activity, and an elevation in the level of glial fibrillary acidic protein (GFAP), a marker of astrogliosis. Nevertheless, although MDMA damages 5-HT neurons in rats and primates, and DA neurons in mice, the mechanisms of neurotoxicity are similar between species. MDMA metabolites (Monks et al., 2004) and excessive extracellular DA concentrations (Quinton and Yamamoto, 2006) may be oxidized enzymatically and nonenzymatically to form highly reactive DA quinones and reactive oxygen species. They may be uptaken into the cell via the monoamine transporter, generating reactive oxygen

and reactive nitrogen species, and leading to increase in oxidative stress inside the cell. MDMA increases hydroxyl radical formation, and produces lipid peroxidation, events that usually accompany free radical formation. In addition to reactive oxygen species, reactive nitrogen species also appear to play a major role in mediating MDMA-induced neurotoxicity. Several studies show that nitric oxide synthase inhibitors provide neuroprotection against MDMA-induced 5-HT depletion in rats and DA depletion in mice brain (Itzhak and Ali, 2006). In addition to the increased oxidative stress produced by MDMA, more recent evidence suggests an important role in the mitochondrial electron transport chain (Quinton and Yamamoto, 2006). Despite the accumulating evidence that reactive oxygen and nitrogen species are responsible for amphetamine-related damage, the manner by which MDMA causes oxidative stress, or the cellular source of the reactant species is still unknown.

3.5.4.2. Glial cells and MDMA neurotoxicity.

Microglial cells are the only resident immune cells of the CNS. They are tissue macrophages, which originate from specific white blood cells called monocytes. Monocytes and macrophages are phagocytic cells, acting in both non-specific defense (or innate immunity) and specific defense (or cell-mediated immunity) of vertebrate animals. Their role is to phagocytate cellular debris and pathogens either as stationary or mobile cells, and to stimulate lymphocytes and other immune cells to respond to pathogens. Furthermore, macrophages are antigen-presenting cells, and play a crucial role in initiating an immune response. After digesting a pathogen, a macrophage will present the antigen to a corresponding helper T cell. The presentation is done by integrating the antigen into the cell membrane and displaying it attached to a major histocompatibility complex class II (MHC II) molecule, indicating to other white blood cells that the macrophage is not a pathogen, despite having antigens on its surface. Eventually, the antigen presentation results in the production of antibodies by B cells, which attach to the antigens of pathogens, making macrophages easier to adhere and phagocytate pathogens. Macrophages are also secretory cells, vital to the regulation of immune responses and the development of inflammation. They release an amazing array of powerful chemical substances including cytokines, enzymes, complement proteins, and regulatory factors. At the same time, they express receptors for other signals that allow them to be activated (Table 4).

Table 4. Cell-associated Molecules and Soluble Factors of Activated Microglia.

Cell surface receptors	Cytokines	Neurotransmitters & intracellular molecules
B7.1 (CD80/CD28R)	Growth regulated oncogene (GROα)	Glutamate
B7.2 (CD86/CD28L)	IL-1α, 1β, 3, 6, 8, 10, 12, 15, 18	Nitric oxide
C3bi receptor (CR3/CD11b/Mac1)	Interferon-γ inducible protein-10	Reactive oxigen species
CD14/TLR (LPS receptor/signaling complex)	Monocyte chemoattractant protein-1	Granule cell death-10 gene
CD68	M-CSF	Iba-1 calcium binding protein
Immunoglobulin receptors	Macrophage derived chemokine	Microglia response factor
Intracellular cell adhesion molecule-1 (ICAM-1/CD54)	Macrophage inflammatory protein-1α, 1β, 2, 3β	
Leucocyte common antigen (LCA/CD45)	Transforming growth factor (TGFβ)	
Major histocompatibility complex II (MHC II)	Tumor necrosis factor (TNFα)	
Multiple receptors for cyto- and chemokines	Regulated on activation, normal T-cell expressed and secreted (RANTES)	

Microglial cells show variable phenotypes depending on the conditions of the surrounding nervous tissue (Fig. 18). Microglial cells with ramified morphology and sparse expression of molecules associated with macrophage function are found in healthy nervous tissue, and have been associated with a resting phenotype. However, resting microglia is not dormant. Stationary microglia scan their environment without disturbing fine-wired neuronal structures. The random scanning rapidly changes to a targeted movement towards the site of an injury when microlesions are induced. This response and its directional guidance apparently depend on purinoreceptor stimulation and may involve assistance from astrocytes. Microglial activation involves the transformation of resting microglia to a reactive profile to cope with altered homeostasis. Chemotactic reorientations can occur in minutes to seconds, and even massive induction of complex gene sets is achieved within a few hours. Many molecules and conditions can trigger a transformation of resting cells to activated microglia. Two important signaling principles organize microglial responsiveness. The sudden appearance of factors that are not usually seen (i.e. microbial structures, intracellular constituents) are sensed by an array of receptors (Table 4). But also the disruption of constitutive signaling causes alert and activation (i.e. impairment of neuronal integrity) (Hanisch and Kettenmann, 2007). When microglia is fully activated, they release reactive oxygen species, NO, or TNFα at quantities and in combinations that potentially can damage neurons. However, microglia does not acquire such pathological state in all condition. Thus, microglial activation can exacerbate damage or protect under different pathological conditions, and its activation can exacerbate or protect under different pathological conditions.

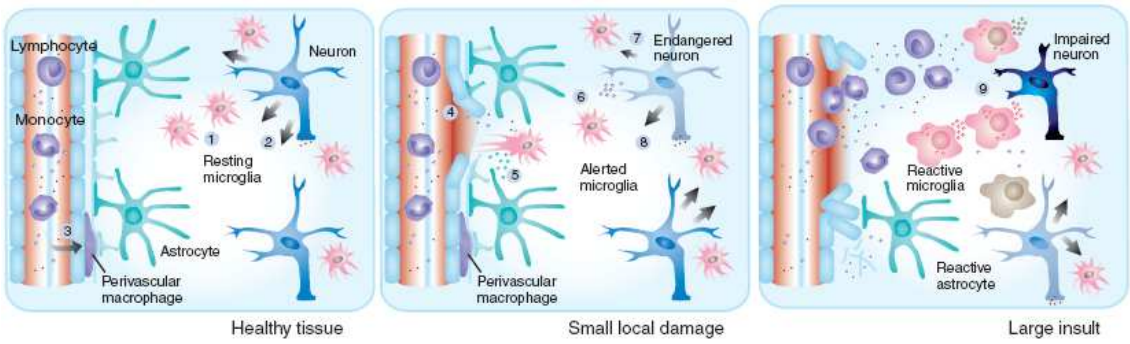

Figure 18. Activity states of microglia. Left, microglial cells in normal tissue constantly screen the environment. Middle, upon detection small homeostatic disturbances microglia can rapidly respond with a directed reorganization and a change in the activity profile. Right, stronger insults to the CNS may trigger more drastic changes in the functional phenotype of microglia. Excessive responses of microglia may lead to substantial impairment of neurons and glia (Hanisch and Kettenmann, 2007).

Few studies describe microglial activation after treatment with MDMA (Thomas et al., 2004b). However, the beneficial or deleterious role of microglia in this condition is not clear. Microglial activation has been shown to be pathological in a model of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced DA neuronal death (Mount et al., 2007). MPTP interferes with complex I of the electron transport chain (a component of mitochondrial metabolism), causing the buildup of free radicals and toxic molecules and leading to cell destruction. DA cell damage by MPTP and by toxic amphetamines is very similar, since both involve increased oxidative stress. Moreover, levels of proinflammatory cytokines such as TNF-α and IL-1β have been observed in both Parkinson disease (Mandel et al., 2000) and toxic amphetamine treatment (Orio et al., 2004), suggesting that microglial activation after MDMA administration may be deleterious rather than beneficial.
Astrocytes activation has been proved to be a sensitive indicator of chemical-induced injury of the CNS even when there is no damage in neuronal soma. Astrocytes are important for maintenance of the extracellular potassium concentration and provide metabolic support to neurons. After an injury, astrocytes are mobilized together with microglia. Astrocytic activation is characterized by an increased expression of GFAP, enlarged cell body, and projections in the injured area. Astrocytes are stimulated by various cytokines such as interferon- γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), released by microglia (Schubert and Rudolphi, 1998). For that reason, both types of glial cells show a similar activation time-course. Microglial activation following MDMA administration is usually accompanied by astrogliosis (O'Callaghan and Miller, 1994), and together represent an important marker of MDMA-induced neurotoxicity.

3.6. Interaction between cannabinoids and MDMA.

3.6.1. Clinical studies.

Most recreational ecstasy users are polydrug users, and they report to take a range of other psychoactive compounds. These include other illicit stimulants such as cocaine or amphetamine, hallucinogens such as LSD, and non-illicit drugs such as alcohol and nicotine. However, many surveys have also revealed that cannabis is one of the most widely taken drugs among recreational ecstasy users (90-98 % of MDMA users also take cannabis). Males are more likely to have taken ecstasy, but amongst the ecstasy users, cannabis was taken almost universally by both genders.

There are several possible reasons for this particular form of polydrug usage. In terms of psychosocial aspects, gateway theory suggests that once individuals had decided to use one illicit drug, they are most likely to take another proscribed substance. However, genetic studies suggest that a common risk factor model would better explain the co-use of cannabis and other drugs rather than the gateway theory (Agrawal et al., 2004). Accordingly, the same risk factors that lead people to become ecstasy users will also increase the risk for taking other psychoactive drugs including cannabis. Several studies show a link between the trait of sensation seeking, and polydrug use. Hence, ecstasy users showed higher novelty seeking and lower harm avoidance scores than non-user controls. In terms of functional aspects, one of the main reasons given by recreational users to consume cannabis is that it provides symptomatic relief against the feelings of anhedonia and depression following MDMA use (Winstock et al., 2001). This symptomatic relief model would explain why cannabis is often used in the immediate post-MDMA period. However, it should be emphasized that there is no empirical data on the effectiveness of this apparent strategy. Many ecstasy users also report taking cannabis during the initial acute stimulatory phase in order to improve its effects.

The objective psychobiological effects also need to be considered, together with the subjective aspects, to understand the consequences of MDMA and cannabis combination. Cerebral activation patterns during working memory performance were compared in pure MDMA users, poly-drug ecstasy users who took other drugs including cannabis and control subjects. Pure ecstasy users presented somewhat different activation patterns compared to the control and polydrug users, whereas polydrug users did not differ from controls (Daumann et al., 2003). These findings provided support for the notion that cannabis may serve to protect ecstasy users from neurotoxicity.

The influence of several types of co-drug usage has also been investigated on the psychobiological profiles of ecstasy users. As expected, the co-use of other

psychostimulants such as amphetamine, cocaine and nicotine, heightened some of the adverse psychobiological profiles. In marked contrast, cannabis was associated with significantly higher positive moods, lower depression, better interpersonal sensitivity, and less total negative symptoms. Nonetheless, in ecstasy poly-drug users heavy cannabis consumption has been associated with higher paranoid symptoms compared with cannabis mild users. In addition, former heavy cannabis users were the most likely to complain of a variety of ecstasy-related long-term problems. These finding indicate that heavy cannabis and ecstasy use is associated with long-term psychological problems, while moderate cannabis use can ameliorate ecstasy related symptoms (Milani et al., 2005). In contrast, other recent studies have suggested that psychopathology of ecstasy users was predominantly attributable to regular cannabis use, and abstinence from cannabis and not ecstasy seemed to be a predictor for remission of psychological complaints in ecstasy users (anxiety, depression, interpersonal sensitivity and obsessive-compulsive behavior) (Daumann et al., 2004). On the other hand, several studies have shown impairment of learning and memory tasks, deficits in short-term memory, working-memory, and executive functions in the abstinent ecstasy users as mentioned above. Cannabis is also linked to a range of motivational, memory and other neurocognitive problems (Hall and Solowij, 1998). The majority of studies employing objective measures of neurocognitive function reported additional impairment on cognitive and memory performance in subjects consuming both cannabis and ecstasy together (Gouzoulis-Mayfrank et al., 2003).

The recreational use of ecstasy and cannabis is associated with neurocognitive and psychobiological deficits, but their adverse effects are not always additive. Instead, the two drugs can display a more interactive profile. The acute effects of cannabis (hypothermic, relaxant, antioxidant and anti-inflammatory) contrast markedly with those of MDMA (hyperthermic, stimulatory and neurotoxic). The profile of both drugs suggests that the use of cannabis should reduce the oxidative stress and hyperthermia caused by MDMA. However, the notion that cannabis may provide some degree of neuroprotection against the damaging effects of ecstasy is currently a working hypothesis. Several studies in animal models have been carried out to elucidate the neurobiological bases of the interaction between these two drugs.

3.6.2. Animal studies.

Interactions between cannabinoids and ecstasy have been reported in animal models. Several parameters such as acute pharmacological responses, alterations on mood and reward, and cognitive abilities were studied after the combination of MDMA and

cannabinoids. Thus, the co-administration of THC or CP 55,490 prevented MDMA-induced hyperthermia in rats. Interestingly, the concomitant administration of THC with MDMA induced greater hypothermia than THC given alone. Similarly, THC and CP 55,490 attenuated MDMA-induced locomotor stimulation. Additionally, brief exposure to MDMA also increased anxiety-like behavior in the emergence and social interaction tests. THC reversed the anxiogenic-like effects of MDMA in the social interaction test, but not in emergence test (Morley et al., 2004). By using mice lacking CB_1 receptor, we have also revealed the important role played by CB_1 cannabinoid receptor in MDMA acute effects. Both, MDMA-induced hyperthermia and hyperlocomotion were attenuated in CB_1 knockout mice. Likewise anxiogenic-like effects of MDMA were completely abolished in these mutant animals (Touriño et al., 2008).

Further animal studies described that the concomitant administration of both MDMA and cannabinoids modify the rewarding, reinforcing and dependence induced by these drugs when given alone. The combination of non-rewarding doses of MDMA with THC induced CPP and intravenous self-administration in mice (Robledo et al., 2007). Moreover, the administration of ecstasy dose-dependently reduced the withdrawal syndrome precipitated after the chronic administration of THC (Touriño et al., 2007). The participation of endocannabinoid system on rewarding and reinforcing effects of MDMA has also been described. The effects of CB_1 cannabinoid receptor blockade on MDMA CPP were reported in two different studies with opposite results. While MDMA-induced CPP was abolished in animals treated with the CB_1 receptor antagonist rimonabant (Braida et al., 2005), both CB_1 knockout mice and wild-type displayed a similar place conditioning after MDMA (Touriño et al., 2008). On the other hand, the participation of CB_1 receptor on the reinforcing effects of MDMA has been reported in animals treated with rimonabant (Braida and Sala, 2002) and mutant mice lacking CB_1 receptor (Touriño et al., 2008) in a self-administration paradigm with contradictory outcome. While genetic ablation of CB_1 receptor completely abolished MDMA self-administration (Touriño et al., 2008), pretreatment with rimonabant was reported to enhance it (Braida and Sala, 2002). However, the different species, dose, route of administration and methodological design used may explain this apparent contradiction.

The investigation of how MDMA and THC coadministration affects cognitive abilities (Young et al., 2005) revealed that both drugs together synergistically disrupted memory. These results are in agreement with those reported in humans, where additional impairment on cognitive and memory performance was observed in subjects consuming both cannabis and ecstasy together (Gouzoulis-Mayfrank et al., 2003).

Finally, the putative neuroprotective role that cannabinoids could exert on MDMA neurotoxicity remains a relatively unexplored issue. Only a single report describes a partial protective role of cannabinoids on 5-HT depletion. This effects was suggested by a recovery of 5-HT and its metabolite 5-HIAA in MDMA/cannabinoid-treated relative to the MDMA-treated rats (Morley et al., 2004). In order to clarify this important issue, an exhaustive study in our laboratory described how THC prevents MDMA neurotoxicity, and how this neuroprotective effect was mainly mediated by CB_1 cannabinoid receptors.

Objectives

Objective 1. To establish the role played by CB_1 cannabinoid receptor on the pharmacological and rewarding effects of MDMA. For that purpose, locomotor activity, body temperature, anxiety-like responses, DA release in the NAc, CPP and intravenous self-administration were evaluated in mice lacking CB_1 cannabinoid receptor after exposure to different doses of MDMA.

Article 1

Touriño C, Ledent C, Maldonado R, Valverde O (2008). "CB_1 Cannabinoid Receptor Modulates 3,4-Methylenedioxymethamphetamine Acute Responses and Reinforcement". *Biol Psychiatry* 63: 1030-8.

Objective 2. To evaluate the effects of acute and chronic MDMA administration on THC physical dependence and withdrawal. The mechanisms involved in this process were also studied. For that reason, participation of 5-HT and DA in this mechanism were studied. The interaction between MDMA and rimonabant were also evaluated to determine if reduced the effects of physical dependence or the triggering of withdrawal syndrome.

Article 2

Touriño C, Maldonado R, Valverde O (2007). "MDMA attenuates THC withdrawal syndrome in mice". *Psychopharmacology* 193:75-84.

Objective 3. To determine the neuroprotective properties of THC on the neurotoxic effects of MDMA in mice. Body temperature, oxidative stress markers, inflammation, DA terminal loss and motor coordination were investigated.

Article 3

Touriño C and Valverde O. "THC protects against the neurotoxic effects of MDMA in mice. *In preparation.*

Article 1

<u>Touriño C</u>, Maldonado R, Valverde O (2007). "MDMA attenuates THC withdrawal syndrome in mice". *Psychopharmacology* 193:75-84.

Objectives

- To study the effects of acute and chronic administration of MDMA on rimonabant-precipitated THC withdrawal syndrome.
- To study the mechanisms by which MDMA may attenuate THC withdrawal syndrome and physical dependence.

Results

- Acute and chronic administration of MDMA dose-dependently attenuated rimonabant-precipitated THC withdrawal syndrome in mice.
- 5-HT but not DA release were enhanced in THC abstinent mice after the administration of MDMA.
- Rimonabant did not modify MDMA-induced hyperlocomotion or 5-HT release.

Conclusions

- MDMA attenuated THC withdrawal syndrome by increasing 5-HT release.
- MDMA attenuated THC withdrawal syndrome compensating somehow de effects of the chronic administration of THC but not reducing the effects of rimonabant.

Touriño C, Maldonado R, Valverde O.
MDMA attenuates THC withdrawal syndrome in mice.
Psychopharmacology (Berl). 2007 Jul;193(1):75-84. Epub 2007 Mar 27.

Article 2

<u>Touriño C</u>, Ledent C, Maldonado R, Valverde O (2007). "CB_1 Cannabinoid Receptor Modulates 3,4-Methylenedioxymethamphetamine Acute Responses and Reinforcement". *Biol Psychiatry* 63: 1030-8.

Objectives

- To study the involvement of CB_1 cannabinoid receptor in the hyperlocomotion induced by MDMA.
- To study the involvement of CB_1 cannabinoid receptor in the hyperthermia induced by MDMA.
- To study the involvement of CB_1 cannabinoid receptor in the anxiogenic-like responses induced by MDMA.
- To study the involvement of CB_1 cannabinoid receptor in the rewarding properties of MDMA.
- To study the involvement of CB_1 cannabinoid receptor in the reinforcing properties of MDMA.
- To study the involvement of CB_1 cannabinoid receptor in the release of DA in the NAc induced by MDMA.

Results

- MDMA-induced hyperactivity and hyperthermia are attenuated in CB_1 receptor deficient mice.
- MDMA-induced anxiogenic-like effects are abolished in CB_1 knockout mice.
- Rewarding effects and DA release in the NAc induced by MDMA are similar between CB_1 knockout mice and their wild-type littermates.
- Reinforcing effects of MDMA are absent in CB_1 knockout mice.

Conclusions

- CB_1 cannabinoid receptor modulates the hyperlocomotive, hyperthermic and anxiogenic-like effects of MDMA.
- CB_1 cannabinoid receptor does not participate in the rewarding effects and the DA release in the NAc induced by MDMA.
- CB_1 cannabinoid plays an essential role in the reinforcing properties of MDMA.

Touriño C, Ledent C, Maldonado R, Valverde O.
CB1 cannabinoid receptor modulates 3,4-methylenedioxymethamphetamine acute responses and reinforcement.
Biol Psychiatry. 2008 Jun 1;63(11):1030-8. Epub 2007 Oct 24.

Article 3

<u>Touriño C</u>, Zimmer A, and Valverde O (2008). "THC prevents MDMA-induced neurotoxicity in mice". *In preparation*.

Objectives

- To determine whether THC can exert neuroprotective effects on MDMA-induced neurotoxicity in mice.
- To study the mechanism by which THC may protect against the neurotoxic effects of MDMA.

Results

- THC attenuates MDMA-induced hyperthermia at both room and high-ambient temperature.
- THC attenuates MDMA-induced microglia and astrocytes activation.
- THC is unable to prevent MDMA-induced microglia and astrocytes activation in CB_1 and CB_1-CB_2 knockout animals, and just partially prevented it in CB_2 knockout mice.
- The CB_2 cannabinoid agonist JWH-133 is unable to attenuate MDMA-induced microglia and astrocytes activation.
- THC prevents nNOS overexpression induced by MDMA.
- THC prevents the DA terminal terminal loss induced by MDMA at high ambient temperature.
- THC prevents motor coordination impairment induced by the administration of MDMA at high ambient temperature.

Discussion

- The activation of CB_1 receptor by THC attenuates MDMA-induced hyperthermia and prevents MDMA neurotoxicity.
- Mocroglial inhibition through the activation of CB_2 receptor by THC may also contribute to attenuate MDMA neurotoxicity.

Article 3

THC prevents MDMA-induced neurotoxicity in mice

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Abbreviations: 3, 4 - methylenedioxymethamphetamine (MDMA), Dopamine (DA), Serotonin (5-HT), D9-tetrahydrocannabinol (THC), Area under the curve (AUC), nitric oxide (NO), nitric oxide synthase (NOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), Dopamine transporter (DAT), tyrosine hydroxilase (TH), glial fibrillary acidic protein (GFAP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Abstract

The majority of recreational MDMA users also consume cannabis. The prolonged use of MDMA and cannabis is functionally damaging, but in certain neuropsychobiological aspects their combination may be interactive rather than additive. MDMA usually causes hyperthermia, oxidative stress and serotonin (5-HT) or dopamine (DA) terminal loss, especially at high ambient temperature. On the contrary, Δ_9-tetrahydrocannabinol (THC), induced hypothermia in animals, and has neuroprotective, anti-inflammatory and antioxidant properties. Hence, THC may exert a protective effect against the neurotoxicity induced by MDMA. To clarify this issue, mice receiving a neurotoxic regimen of MDMA (20 mg/kg x 4) were pretreated with THC (3 mg/kg) at room (21ºC) and at high ambient (27ºC) temperature, and several markers of neurotoxicity were evaluated. THC prevented hyperthermia, and motor coordination impairment observed in mice after treatment with MDMA. Moreover, THC also prevented nitric oxide synthase (NOS) expression, microglia and astrocytes activation, DA terminal loss, induced by MDMA in mice striatum. To determine the mechanism through which THC protects against MDMA neurotoxicity, mice lacking CB_1, CB_2 or both cannabinoid receptors were treated with the same regimen of MDMA and THC, and microglia and astrocytes activation was evaluated. THC could not protect CB_1 and double knockout mice against MDMA glial activation, and partially reversed microglial activation in CB_2 knockout mice. These results indicate that THC protected against hyperthermia, neuronal damage and inflammation, dopaminergic loss and motor impairment induced by MDMA neurotoxicity. These neuroprotective actions are primarily mediated by the activation of CB_1 receptor, although CB_2 receptors also contributed to this process.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA), commonly known as ecstasy, is a widely used recreational drug with low addictive potential, but with severe neurotoxic effects after prolonged use (Schmidt, 1987). MDMA produces a long-term loss of 5-HT nerve terminals when administered to primates or rats (Colado et al., 1997; Hewitt and Green, 1994), and a strong degeneration of DA nerve terminals in mice (Logan et al., 1988). Nevertheless, the mechanisms of neurotoxicity are similar in both species. There is evidence to suggest that oxidative stress plays an important role in this process (Colado et al., 1997; Quinton and Yamamoto, 2006). MDMA increases the formation of hydroxyl radicals (Colado et al., 1997; Shankaran et al., 1999) and increases lipid peroxidation (Sprague and Nichols, 1995). Reactive oxygen and nitrogen species are involved in the neurotoxicity produced by amphetamine analogs (Cadet et al., 2007; Itzhak and Ali, 2006). Nitric oxide has been implicated as a mediator of neurotoxicity (Cerruti et al., 1995), and several studies have demonstrated that nitric oxide synthase (NOS) inhibitors provide protection against MDMA-induced dopaminergic (DA) and serotonergic (5-HT) neurotoxicity in rodents (Darvesh et al., 2005; Itzhak and Ali, 2006). MDMA-induced neuronal damage activates microglia (Thomas et al., 2004) and astrocytes (O'Callaghan and Miller, 1994). MDMA-activated microglia secretes an array of pro-inflammatory mediators such as cytokines (IL-1b or TNF-α) (Orio et al., 2004), prostaglandins or nitric oxide (NO) that exacerbate the damage to neuronal tissue (Hanisch, 2002).

It is well established that MDMA causes hyperthermia and disrupts thermoregulatory ability in animals (Nash, Jr. et al., 1988; O'Callaghan and Miller, 1994) and humans (McCann et al., 1996). Hence, high ambient temperature strongly increases MDMA-induced hyperthermia (Malberg and Seiden, 1998). This hyperthermia enhances MDMA-induced neurotoxicity by increasing MDMA metabolism (Goñi-Allo et al., 2008; Malberg and Seiden, 1998), and as a consequence reactive oxygen species (Halliwell, 1992). This is of clinical interest because MDMA is frequently consumed in dance clubs with warm environment (Green et al., 1995), where the neurotoxic effects of the drugs may be exacerbated.

The majority of recreational MDMA users also consume cannabis (Strote et al., 2002; Winstock et al., 2001). Several reasons have been hypothesized for this particular form of drug usage, including genetic susceptibility, reduction of post-MDMA aversive effects, or enhancement of MDMA effects (Parrott et al., 2007). Despite the adverse effects of these drugs, the effects of their combination do not always fit a simple additive factor model. Instead, the two drugs can often display a more interactive profile (Daumann et al., 2004; Parrott et al., 2007). Indeed, several animal studies have already described interactions

after the administration of both MDMA and Δ9-tetrahydrocannabinol (THC), the main psychoactive compound of cannabis (Morley et al., 2004; Robledo et al., 2007; Touriño et al., 2007). The effects of MDMA markedly contrast with those of THC. THC is a substance with a wide reported range of neuroprotective properties, both receptor dependent and independent (Sarne and Mechoulam, 2005), while MDMA produces neurotoxicity and nerve terminal degeneration. THC showed antioxidant properties through a mechanism not mediated by cannabinoid receptor, but by its phenolic structure (Chen and Buck, 2000). Moreover, THC also attenuates inducible nitric oxide synthase (iNOS) reducing oxidative stress (Jeon et al., 1996) by activating CB_2 cannabinoid receptor (Munro et al., 1993), whereas MDMA increases the formation of oxygen and nitrogen reactive species, enhancing oxidative stress. CB_2 receptor activation by THC also prevents the activation of microglial cells and the release of pro-inflammatory mediators such as cytokines, chemokines and NO by a mechanism, exerting neuroprotective effects (Walter and Stella, 2004). However, MDMA-induced cell damage activates astrocytes and microglia, and promotes the release of proinflammatory mediators. Finally, THC induces hypothermia by CB_1 receptor activation (Ledent et al., 1999), and previous reports describe that THC reduces MDMA-induced hyperthermia in rats (Morley et al., 2004). The opposite properties of both drugs suggest that THC may attenuate the oxidative stress, the microglial and astrocytes activation and the hyperthermia caused by MDMA and protecting against terminal loss and neuronal degeneration.

Materials and methods

Animals

9- to 12-week-old male C57BL/6 mice, obtained from Charles River, mice with a deletion of the CNR1 and CNR2 genes (CB_1–/– and CB_2–/– mice) (Zimmer et al., 1999; Buckley et al., 2000) and CB_1−/−/CB_2−/− double knockout mice (Járai et al., 1999) were used in these experiments. Mice were housed in a temperature (21° ± 1℃), humidity (55% ± 10%), and light-cycle controlled room, except for a group of animals housed at 27º ± 1℃ from the beginning of the treatment. Food and water were available ad libitum. Light was on between 8:00 am and 8:00 pm, and the experiments took place during the light phase. All animal care and experimental procedures were conducted according to the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Drugs

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland) dissolved in 0.9% physiological saline, and administered at 20 mg/kg, i.p. for four times every 2h. THC (THC Pharm, Frankfurt, Germany) was dissolved in a solution of 5% ethanol, 5% cremophor EL (Sigma Chemical, Madrid, Spain) and 90% distilled water, and administered at 3 mg/kg, i.p. 1h before each MDMA injection. JWH-133 (Tocris Bioscience, Ellisville, MS) was dissolved in TocrisolveTM 100 (Tocris Bioscience, Ellisville, MS), and administered at 3 mg/kg, i.p. 1h before each MDMA injection. All these drugs were administered in a volume of 0.1 ml/10 g. Ketamine hydrochloride (100 mg/kg; Imalgène 1000®, Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg/kg; Sigma Chemical Co., Madrid, Spain) were mixed and dissolved in ethanol and water (1:9). This anesthetic mixture was injected in a volume of 0.2 ml/10g body weight i.p.

Body temperature

Rectal temperature was measured in animals housed at both 21 ± 1℃ or 27 ± 1℃ and treated with MDMA (20 mg/kg, i.p. x 4). An electronic thermocouple flexible rectal probe (Panlab, Madrid, Spain) was placed in the mice rectum for 10 s. Basal temperature was measured 1 hour before treatment, and 30 min after each THC or MDMA injection. Area under the curve (AUC) was calculated by using a standard trapezoid method (Gibaldi and Perrier, 1975). The following equation was used:

$$AUC= [(0.5 * (T_0+T_1) * t] + [(0.5 * (T_1+T_2) * t] +\ldots + [(0.5 * (T_8+T_9) * t]$$

where T_n is the temperature increase values, and t is the time (min) between the consecutive measurements.

Immunostaining

Microglia and astrocytes from mouse striatum were identified by immunohistochemisty in animals housed at 21 ± 1℃ and treated with MDMA (2 0 mg/kg, i.p. x 4) and THC (3 mg/kg, i.p. x 4) or JWH-133 (3 mg/kg, i.p. x 4). Mice were anesthetized 48 h after last injection with a ketamine/xylazine mixture and transcardially perfused with 0.1 M PB buffer containing 4% paraformaldehyde. Brains were removed and postfixed in the same solution for four hours and cryoprotected in 30% sucrose overnight. After freezing in dry ice brains were sliced into 30-µm thick coronal sections containing striatum.

Sections were preincubated for 30 min in 2% H2O2, and then incubated for 2 h in a solution of 3% normal goat serum and 0.3% triton x-100. Activated microglia was detected with rat anti-mouse CD11b (Serotec, Oxford, UK), and astrocytes were detected with polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark). To visualize primary antibodies, a biotinylated secondary antibody to rat or rabbit Igs were applied for 1 h, followed by avidin/biotin reagent (Vector Laboratories, Inc., Burlingame, CA) for 2 h before incubation and staining with diaminobenzidine-HCl (DAB) and H2O2. Quantification of immunostaining was carried out with NIH Image J software. Briefly, background was subtracted by adjusting detection threshold density, considering just the signal density above the threshold. The software then automatically measures the number of pixels per area, and determines the percentage of stained area.

Western blot analysis

Analysis of dopamine transporter (DAT), tyrosine hydroxilase (TH), neuronal nitric oxide synthase (nNOS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels in the striata of animals housed at 21 ± 1℃ or 27 ± 1℃ were analyzed by western blot. Animals were sacrificed 48 h after last injection, and striatum was dissected. Samples from all animal were processed in parallel to minimize inter-assay variations. Frozen brain areas were dounce-homogenized in 30 volumes of lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 µg/mL aprotinin, 1 µg/mL leupeptine, 1 µg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-glycerolphosphate) plus 1% Triton X-100. After 10 min incubation at 4℃, samples were centrifuged at 16 000 g for 30 mi n to remove insoluble debris. Protein

contents in the supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain), following manufacturer's instructions.

Equal amounts of brain lysates were mixed with denaturing 5x Laemmli loading buffer and boiled for 5 min at 95 ºC. Samples with equal amounts of total protein (20 µg per lane) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Afterwards, membranes were incubated for 2 h with monoclonal anti-DAT (1:1000; Chemicon, Temecula, CA), monoclonal anti-TH (1:5000; Sigma-Aldrich, Spain), mouse anti-nNOS (1:100), and mouse anti-GAPDH (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rat (1 : 2500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse or anti-rabbit antibodies (Pierce, Spain; diluted) and visualized by enhanced chemiluminescence detection (SuperSignal, Pierce, Spain). Only immunoblots showing similar amount of GAPDH in all lanes were considered. The relevant immunoreactive bands were quantified after acquisition on a Chemiluminescent Imaging with the Chemi-Doc XRS, controlled by Quantity One software (Bio-Rad, Spain).

Rotarod test

An accelerating rotarod (five-lane accelerating rotarod; LE 8200, Panlab, Spain) was used to measure motor balance and coordination 48 h after the last injection of MDMA. Each mouse was placed in a separate compartment on the rotating rod and the latency to fall was automatically recorded by magnetic trip plates. Recording started when all animals remained in the rod at 4 rpm constant speed for at least 1 min. Then, the speed of the rod accelerated from 4 to 20 rpm (acceleration was 1 rpm/s) and remained constant until the end of the trial. Test consisted of twenty consecutive trials each separated by 1 min, with a maximum cutoff latency of 90 s (Bilkei-Gorzo et al., 2005).

Statistical analysis

Paired two-way ANOVA with treatment as between-subject and time as between subjects' factors and subsequent post hoc analysis (Tukey's test) was used to compare differences between groups in body temperature at 21 and 27 ºC. Differences between genotypes in the latency to fall in the rotarod were compared by two-way ANOVA with THC and genotype treatment as between-subjects factors and subsequent one-way ANOVA. One-way ANOVA was used to compare treatment differences between groups

in the AUC values for body temperature, nNOS, DAT and TH levels, and treatement and genotype differences in microglia and astrocytes staining. In all the experiments, differences were considered significant if the probability of error was less than 5%.

Results

Effects of THC on MDMA effects at room temperature (21 ± 1℃)

THC reverses MDMA induced hyperthermia at room temperature

A baseline body temperature was recorded for all animals housed at 21 ± 1℃. In that time the average core temperature was 37.5 ± 0.08ºC. After baseline measurements, animals were injected with THC (3 mg/kg, i.p) followed by MDMA (20 mg/kg, i.p.) 1 h later. This treatment was repeated for four times every 2 h. Body temperature was measured after each injection to determine the ability of THC to reverse MDMA-induced hyperthermia (Fig. 1). Two-way ANOVA analysis indicated a significant effect of time ($F(8, 288) = 23.454$, $p < 0.001$), treatment ($F(3, 36) = 36.703$, $p < 0.001$), and interaction between these two factors ($F(24, 288) = 12.568$, $p < 0.001$). Subsequent post hoc analysis (Tukey's test) showed a trend of MDMA increase body temperature compared to vehicle-treated group ($p = 0.08$). A significant reduction of body temperature after THC treatment was observed in both saline- ($p < 0.001$) and MDMA- ($p < 0.001$) treated mice. Moreover, THC significantly reduced body temperature in MDMA treated animals when compared to the group treated with MDMA alone.

THC prevents microglia and astrocytes activation in MDMA-treated mice by CB_1 and CB_2 receptor mediated mechanisms

The activation of both microglia and astrocytes in striatum was evaluated in THC (3 mg/kg, i.p. x4) or vehicle-treated mice under a neurotoxic administration of MDMA (20 mg/kg, i.p. x4). Counts of CD11b-positive (Fig. 2 a-c) and GFAP-positive (Fig. 3 a-c) cells indicated that THC completely suppressed activated microglia and astrocytes activation induced by MDMA administration. To determine the mechanism by which THC reverses microglia and astrocytes activation, CB_1 (Fig. 2 and 3 d-f), CB_2 (Fig. 2 and 3 g-i) and double CB_1-CB_2 (Fig. 2 and 3 j-l) knockout mice were treated with THC or vehicle after MDMA regimen. THC was unable to inhibit microglia and astrocytes activation in CB_1 and CB_1-CB_2 mutant mice, indicating that the activation of CB_1 cannabinoid receptor abolished the activation of glial cells involved in MDMA neurotoxicity (Fig. 2 and 3 d-f and j-l). On the other hand, THC partially suppressed microglial activation in CB_2 knockout mice treated with MDMA (Fig. 2 g-i). This result suggests that the activation of CB_2 cannabinoid receptor by THC contributes to the suppression of MDMA-induced microglial activation. In contrast, MDMA-induced astrocytes activation in CB_2 knockout was completely eliminated by THC (Fig. 3 g-i), suggesting that CB_2 receptor is not involved in the THC-induced suppression of astrocytes activation

The selective CB_2 receptor agonist JWH-133 was administered with the same schedule as THC to MDMA-treated mice to determine the participation of this receptor on MDMA-induced microglial and astrocytes activation. Microglia and astrocytes were similarly activated in MDMA-treated animals pretreated with JWH-133 or its corresponding vehicle (Fig. 4). This result indicates that the pretreatment with JWH-133 does not inhibit microglial or astrocytes activation in MDMA-treated mice. However, prolonged treatment with this agonist may have an affect, since microglial activation peak takes place 2 days after MDMA treatment.

THC reduced the expression of nNOS in MDMA-treated animals

Animals receiving a neurotoxic regiment of MDMA (20 mg/kg, x 4) were pretreated with THC (3 mg/kg, x 4) or vehicles for three consecutive days. After the last MDMA injection animals were sacrificed and levels of nNOS in the striatum were evaluated (Fig. 5). Treatment with MDMA induced a moderate but significant increase of nNOS levels (119.2 ± 3.0 compared to control). Most importantly, THC administration reduced nNOS levels to 72.5 ± 5.3 % from control in animals treated with MDMA. THC-induced reduction of nNOS levels indicates a reduction in NO levels, and as a consequence a reduction of MDMA-induced oxidative stress and neurotoxicity.

MDMA does not cause visible damage in striatal DA terminals at room temperature

TH and DAT levels, sensitive markers of DA terminal damage, were evaluated in the striatum of animals administered with THC (3 mg/kg i.p. x4) and MDMA (20 mg/kg, x 4). No significant differences in the content of TH (Fig. 6a) or DAT (Fig. 6b) were observed between MDMA- and saline-treated animals, suggesting that MDMA treatment at room temperature do not cause a significant loss of DA terminals. No differences in MDMA-treated animals receiving THC were observed either.

Effects of THC on MDMA effects at high temperature (27 ± 1ºC)

THC reverses MDMA induced hyperthermia at high temperature

Baseline body temperature was measured in animals housed at 27 ± 1ºC. The average core temperature was 37.8 ± 0.06ºC. Basal body temperature were similar between animals housed at 21 and 27 ºC, suggesting that increased ambient temperature does not affect animals' core temperature. After baseline recordings, animals were injected with MDMA and THC, and body temperature was measured after each injection to determine the ability of THC to reverse MDMA-induced hyperthermia (Fig. 7). Two-way

ANOVA analysis revealed a significant effect of time ($F(8, 224) = 3,443$, $p < 0.001$), treatment ($F(3, 28) = 16.999$, $p < 0.001$), and interaction between these two factors ($F(24, 224) = 6.446$, $p < 0.001$). Subsequent post hoc analysis (Tukey's test) showed that MDMA administration produced a significant hyperthermia when compared to vehicle-treated group ($p < 0.01$), whereas THC alone produced significant hypothermia when compared with control animals ($p < 0.05$). However, when THC was administered to MDMA-treated animals housed at 27ºC, a significant reduction of body temperature was observed when compared to animals treated with MDMA alone ($p < 0.01$), showing similar levels to vehicle treated animals.

AUC values were compared between animals housed at 21ºC and 27ºC (Fig 7b). No significant differences in core temperature were observed in saline-treated animals housed at 21ºC and 27ºC. However, a significant enhancement in body temperature of MDMA-treated mice receiving either THC or vehicle was observed when animals were housed at 27ºC (Fig 7b), indicating that high ambient temperature potentiates the hyperthermic effects of MDMA.

THC protects against DA terminal loss induced by MDMA at high ambient temperature

Integrity of DA terminals was observed in animals housed at 27ºC and treated with THC (3 mg/kg, i.p. x4) and MDMA (20 mg/kg, i.p. x4). TH levels of MDMA-treated animals were reduced to a 39.3 ± 4.8% (Fig. 8a), whereas DAT levels were reduced to a 22.1 ± 4.1% (Fig. 8b) when compared to vehicle treated animals. These results indicate the administration of MDMA at high ambient temperature causes a significant loss of DA terminals. Conversely, MDMA-treated animals receiving THC showed similar levels of TH and DAT than control animals. TH levels in these animals were 92.4 ± 4.2% (Fig. 8a), and DAT levels reached 49.5± 11.2% (Fig. 8b). These results indicate that THC attenuates DA terminals loss induced by MDMA in striatum.

THC reverses impaired motor coordination in the rotarod induced by MDMA at high ambient temperature

Motor coordination in the rotarod test was evaluated in animals treated with THC (3 mg/kg, i.p. x4) and MDMA (20 mg/kg, i.p. x4) or their corresponding vehicles 48 h after receiving the last injection (Fig. 9). Animals treated with MDMA showed impaired motor coordination through the whole experimental sequence, and average of the last 5 trials indicates a significant decrease in the latency to fall when compared to vehicle-treated group. However, MDMA-treated mice receiving THC showed similar motor coordination

than control animals. Thus, structural damage to the DA nerve terminals in the striatum of MDMA-treated mice was confirmed functionally, with a significant impairment in motor coordination. These results suggest that THC not only protects against DA terminal loss, but also prevents deficits in motor coordination caused by MDMA. No significant effects were observed in animals receiving THC alone.

Discussion

In this study, the neuroprotective effects of THC on MDMA-induced neurotoxicity, and the mechanisms of this neuroprotection were explored. One of the principal adverse effects of MDMA is the alteration of body temperature. These changes in body temperature contribute to important health problems derived from MDMA use, as heat shocks and neurotoxicity (Milroy, 1999). Alterations in body temperature after MDMA treatment have been also reported in animals (Green et al., 2003). The effects of MDMA on body temperature depends on the ambient temperature, dose administered, specie or strain used, but the most frequent result is hyperthermia. MDMA not only alters body temperature but impairs thermoregulation, what makes body temperature dependent on ambient temperature. Consequently, a small increase in ambient temperature result in strong enhancement of core temperature (Malberg and Seiden, 1998). Conversely, hypothermia is one of the most typical effects of cannabinoids administration in animals (Chaperon and Thiebot, 1999). In order to evaluate if THC may reduce MDMA-induced hyperthermia, body termpeature was measured in mice treated with MDMA and THC, and housed at room (21ºC) and at high ambient (27ºC) temperature. MDMA caused a slight increase in body temperature in mice housed at room temperature. However, a strong hyperthermia was observed in mice housed at high ambient temperature, since thermoregulation impairment caused by MDMA made animal's core temperature susceptible to ambient temperature. Hyperthermia observed in MDMA-treated animals housed at room temperature was decreased after THC administration. Moreover, pretreatment with THC also reversed the severe hyperthermia occurring at high ambient temperature. The modulation of body temperature by THC was also reported in rats. Similarly, THC and the synthetic cannabinoid agonist CP-55,940 reduced MDMA-induced hyperthermia in rats (Morley et al., 2004). In this study, the effect of cannabinoids on MDMA-induced hyperthermia was reversed by rimonabant, indicating that CB_1 receptors mediate the effects of THC in this response. However, CB_1 cannabinoid receptor might play a role by itself in the effects of MDMA on body temperature, since the involvement of CB_1 receptors on MDMA-induced hyperthermia has been reported (Touriño et al., 2008).

The main adverse consequence related to MDMA-induced hyperthermia is the enhancement of neurotoxicity. High ambient temperature increases MDMA-induced hyperthermia, and exacerbates MDMA-induced neurotoxicity (Malberg and Seiden, 1998). MDMA is frequently consumed in night clubs with elevated ambient temperature, where neurotoxicity may be potentiated. Interestingly, most of MDMA users smoke cannabis, which not only reduces body temperature, but also exerts neuroprotective effects on neurodegenerative or neuroinflammatory diseases (Grundy et al., 2001; Sarne

and Mechoulam, 2005). Thus, the frequent consume of cannabis observed among MDMA users might have interactive rather than additive effects, so THC may prevent not only hyperthermia-related adverse effects, but most importantly MDMA neurotoxic effects. Therefore, reduction of body temperature exerted by THC would protect against MDMA neurotoxicity. In agreement, cold ambient temperature, as well as drugs that lower body temperature has been shown to protect against neurotoxicity (Ali et al., 1994; Farfel and Seiden, 1995; Green et al., 2005). Furthermore, the formation of MDMA-derived neurotoxic metabolites and its uptake into the cell is promoted at high ambient temperature (Malberg et al., 1996; Goñi-Allo et al., 2008). Thus, THC may prevent MDMA-treated animals from hyperthermia, reducing both the formation of neurotoxic metabolites and its internalization in the neuron. As a consequence, MDMA-induced neurotoxicity would be reduced. In order to evaluate the neuroprotective effects of THC against MDMA-induced neurotoxicity several parameters that evaluate neuronal damage were assessed.

The activation of glial cells is a sensitive marker to detect brain damage. Brain injury is usually followed by the activation of brain immune cells, namely microglia. Once MDMA-induced neuronal injury has occurred, microglial cells are activated to repair damage and eliminate cell debris. When activated, microglia releases proinflammatory cytokines such as IL1-β or TNF-α and additional immune cells are recruited to repair the damaged tissue. In case of prolonged or massive neuronal damage, microglial cells might become chronically activated, and harm is exacerbated rather that ameliorated. After MDMA exposure, microglia becomes activated to repair neuronal damage, but prolonged exposure to the drug may cause a sustained microglial activation that would worsen cell damage. In contrast, THC and other cannabinoid drugs have been reported to inhibit microglial activation in neurodegenerative disorders such as multiple sclerosis Alzheimer's disease, HIV encephalopathy, ischemia and traumatic brain injury (Walter and Stella, 2004). The neuroprotective effects of cannabinoids in these neurodegenerative disorders is caused by the activation of CB_2 receptor in microglia (Cabral et al., 2008). Consequently, THC exerts an anti-inflammatory effects that inhibits the release of proinflammatory mediators (Puffenbarger et al., 2000) by microglial cells. Therefore we observed that pretreatment with THC completely abolished MDMA-induced microglial activation. However, the mechanisms by which THC inhibits MDMA-induced microglial activation remain unclear. The activation of CB_1 receptor by THC reduces body temperature, and would attenuate the formation of toxic metabolites. Consequently, tissue damage would be decrease and microglia would not become activated. On the other hand, THC may directly inhibit microglia through CB_2 receptor activation. Hence, to

elucidate the specific mechanism of THC neuroprotection, we studied the involvement of CB_1, and CB_2 receptors on the reduction of MDMA-induced microglial activation by THC. With that purpose, microglial activation of mice lacking CB_1, CB_2, and both cannabinoid receptors, and treated with THC and MDMA was explored. We observed that THC did not prevent MDMA-induced microglial activation in CB_1 or double knockout mice, indicating a main role of this receptor in the inhibitory effects of THC on MDMA-induced microglial activation. This result suggests that the ability of THC to reduce MDMA-induced hyperthermia prevents cell damage and microglial activation. Nevertheless, the involvement of CB_2 receptors can not be fully discarded. Indeed, THC significantly did not completely reverse MDMA-induced microglial activation in CB_2 knockout mice. This result indicates that the activation of CB_2 receptors contributes to inhibit microglial activation, preventing the release of proinflammatory cytokines, reducing the expression of iNOS, and contributing to the reduction of cell damage. Surprisingly, pretreatment with the CB_2 receptor agonist JWH-133 did not attenuate MDMA-induced microglial activation. This result is not necessarily contradictory to the one observed with CB_2 receptor knockout mice. JWH-133, as well as THC, was administered before MDMA. Indeed, maximum microglial activation takes place two days after MDMA treatment. At that time JWH-133 had already been cleared from the organism, and can not activate CB_2 receptors and inhibit microglial activation. Thus, we can not rule out that a prolonged treatment with JWH-133 may attenuate MDMA-induced microglial activation.

Not only microglia but also astrocytes are activated after MDMA treatment. In our studies we observed that THC also prevented MDMA-induced astrocytes activation. THC reduction of MDMA-induced hyperthermia may attenuate cell damage, and consequently reduce astrocytes activation. However, astrocytes express CB_1 receptor (Sanchez et al., 1998), so a direct effect of THC on astrocytes activation should not be ruled out. The involvement of CB_1 and CB_2 receptors on THC reduction of MDMA-induced astrocytes activation was also explored. With that purpose, astrocytes were evaluated in CB_1, CB_2 and double knockout mice after treatment with MDMA and THC. THC was unable to protect CB_1 and CB_1/CB_2 knockout mice against MDMA-induced astrocytes activation. These data indicates that CB_1 receptors mediate THC-induced inhibition of astrocytes in MDMA-treated mice, probably by preventing hyperthermia. However, the activation of CB_1 receptor directly inhibits the activation of astrocytes (Molina-Holgado et al., 2002; Sheng et al., 2005). Additionally, excessive glutamatergic transmission also contributes to MDMA neurotoxicity (Cadet et al., 2007; Quinton and Yamamoto, 2006). Astrocytes are involved in the modulation of glutamatergic transmission, and CB_1 receptors play an important role in this process (Navarrete and Araque, 2008). Thus, excess of glutamate

transmission caused by MDMA might contribute to activate astrocytes, but THC might attenuate their activation through CB_1 receptor. On the other hand, astrocytes activation was evaluated in CB_2 knockout mice. Surprisingly, astrocytes activation in CB_2 knockout animals was just partially reversed by THC. After THC and MDMA treatment, microglia remained partially activated in these animals, as well. Cytokines released by microglia promote astrocytes activation. Therefore, activated microglia in CB_2 knockout mice after THC and MDMA treatment might induce astrocytes activation. Altogether, these data suggest that the reduction of MDMA-induced hyperthermia by THC is the main cause of the reduction in microglia and astrocytes activation. However, the direct activation of CB_2 receptors in microglia and CB_1 receptors in astrocytes may directly contribute to the inhibition of these cells.

The previous data suggest that THC prevents MDMA-induced cell damage mainly by reducing body temperature. Nevertheless, antioxidant effects of THC may contribute to reduce neuronal damage. In addition, attenuation of body temperature does not completely suppress the formation of neurotoxic metabolites derived from MDMA, and free radicals, and reactive oxygen and nitrogen species are still generated. The main enzyme involved in the formation of reactive oxygen and nitrogen species is NOS. Several reports describe an important contribution of NOS to the neurotoxic effects of MDMA and other amphetamine derivatives (Colado et al., 2001; Darvesh et al., 2005). On the other hand, THC attenuates the expression of iNOS (Jeon et al., 1996), suggesting a putative effect of THC reducing MDMA-induced formation of reactive oxygen and nitrogen species. For that reason, we evaluated the action of MDMA on NOS expression, and the effect of THC on this expression. We observed that MDMA-induced NOS expression was slightly increased, and THC significantly attenuated this expression. THC may reduce oxidative stress not only by reducing NOS expression, but also by its receptor-independent antioxidant properties due to its phenolic structure (Chen and Buck, 2000). Together, the antioxidant properties of THC may contribute to attenuate the oxidative stress, and the reduction of cell damage generated by MDMA metabolites.

Hyperthermia, NOS induction, or glial activation, are indirect indicators of MDMA-induced neurotoxicity. The presence of these markers after MDMA treatment is associated with brain damage. However, the presence of these markers would not have any functional effect if they are not together with a nerve terminal decrease. In fact, the principal negative consequences of the prolongued consumtion of MDMA in human subjects, such as cognitive impairment or emotional disorders, are associated with nerve terminal degeneration. To confirm that brain damage revealed by glial activation was associated with DA terminal loss, TH and DAT protein levels were quantified in MDMA-treated

animals at both room temperature and high ambient temperature. TH and DAT are proteins constitutively expressed in DA terminals, and its reduction indicates DA terminal loss. Animals treated with MDMA at room temperature showed hyperthermia and a significant increase in glial activation, but no significant reduction in TH and DAT levels. In contrast, animals treated with MDMA at high ambient temperature (27ºC) displayed a severe reduction in the levels of TH and DAT. Together, these results indicate that the administration of MDMA at room temperature, caused a modest hyperthermia, slight neuronal damage, but not detectable DA terminals loss. On the contrary, animals treated with MDMA at high ambient temperature showed an intense hyperthermia, and a strong decrease in TH and DAT levels. In agreement, previous reports described that MDMA-induced terminal damage was not observed at room temperature, whereas severe damage occurred at high ambient temperature (Malberg and Seiden, 1998). After that, ability of THC to reduce MDMA-induced DA terminal loss was evaluated. MDMA-treated animals housed at 27ºC, and administered with THC showed a complete recovery of TH levels, but only a partial recovery of DAT levels. DAT is placed in the extracellular membrane, so that damage in the cell membrane might reduce DAT levels. On the contrary, TH is only expressed at the intracellular level. Therefore, complete recovery of TH indicates that THC prevents the complete destruction of DA terminals observed in MDMA-treated animals.

MDMA-induced nerve terminals degeneration is usually translated into functional and behavioral deficits. MDMA-induced 5-HT terminal loss is related to mood and cognitive disorders (Morgan, 2000). Nevertheless, MDMA-induced 5-HT terminal damage is not clearly associated with impairment in any specific animal behavior. Conversely, DA neuronal damage occurring in MDMA-treated mice causes DA terminals degeneration in the striatum. Similarly to the MPTP model of Parkinson's disease, DA terminal degeneration induced by MDMA results in motor coordination impairment (Jeng et al., 2006). Motor coordination is easily evaluated with the rotarod test, a rotating rod, whose speed of rotation is gradually increased. The rodent's ability to remain on the rotating rod is recorded, and sensorimotor coordination assessed. This test is sensitive to damage in the basal ganglia and cerebellum, and to drugs that affect motor function. We observed that animals treated with MDMA at high ambient temperature, suffering from strong hyperthermia and severe DA nerve terminal damage, also showed impaired motor coordination in the rotarod. Conversely, rotarod performance of MDMA-treated animals pretreated with THC was similar to saline-treated animals. This result indicates that THC not only reduces nerve terminals damage, but also improves the behavioral deficits derived from this damage.

Thus, THC and other cannabinoid derivatives showed neuroprotective effects in several neuroinflammatory and neurodegenerative diseases (Pacher et al., 2006). The mechanisms of neuroprotection imparted by cannabinoids include the reduction of neuroinflammation (Walter and Stella, 2004), oxidative stress (Chen and Buck, 2000; Marsicano et al., 2002), and excitotoxicity (Chen and Buck, 2000; Marsicano et al., 2003). Nevertheless, we report for the first time that THC might exert a neuroprotective action against amphetamine derivatives neurotoxicity, specifically MDMA. THC neuroprotection occurred mainly through the reduction of MDMA-induced hyperthermia. Interestingly, hyperthermia is the main factor contributing to aggravate the neurotoxicity of this drug, and its attenuation prevents the consequent oxidative stress, glial activation, terminal loss and behavioral impairment. These two drugs are frequently consumed in combination, therefore, the present study may help to clarify the reason why polydrug users show improved neurological parameters when compared to pure MDMA users.

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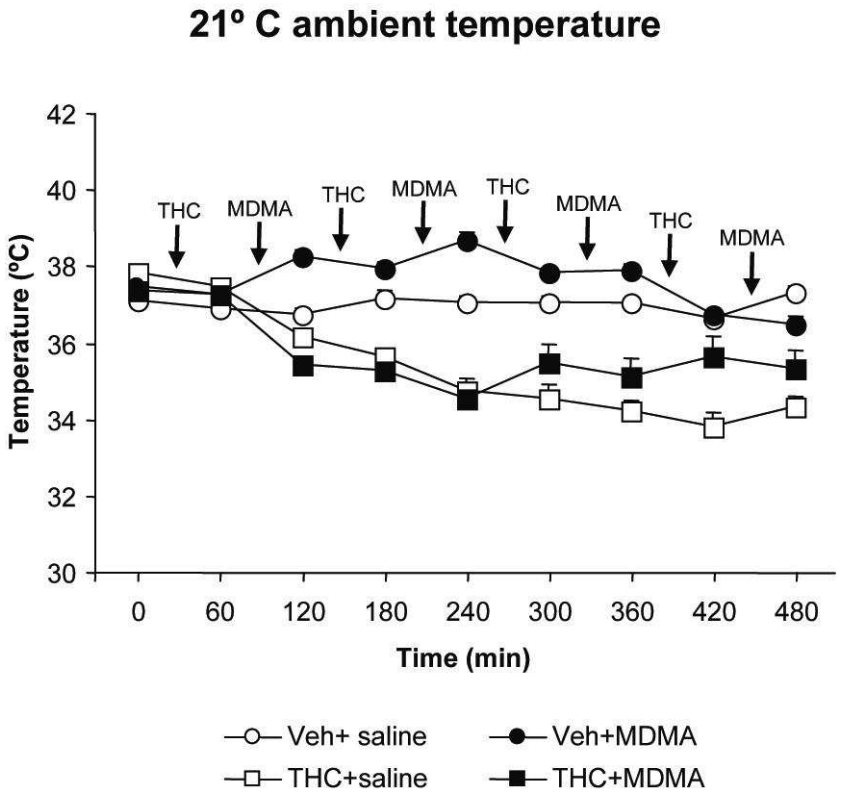
Figure 1


21º C ambient temperature
Temperature (ºC)
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34
32
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MDMA
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Time (min)
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Veh+MDMA
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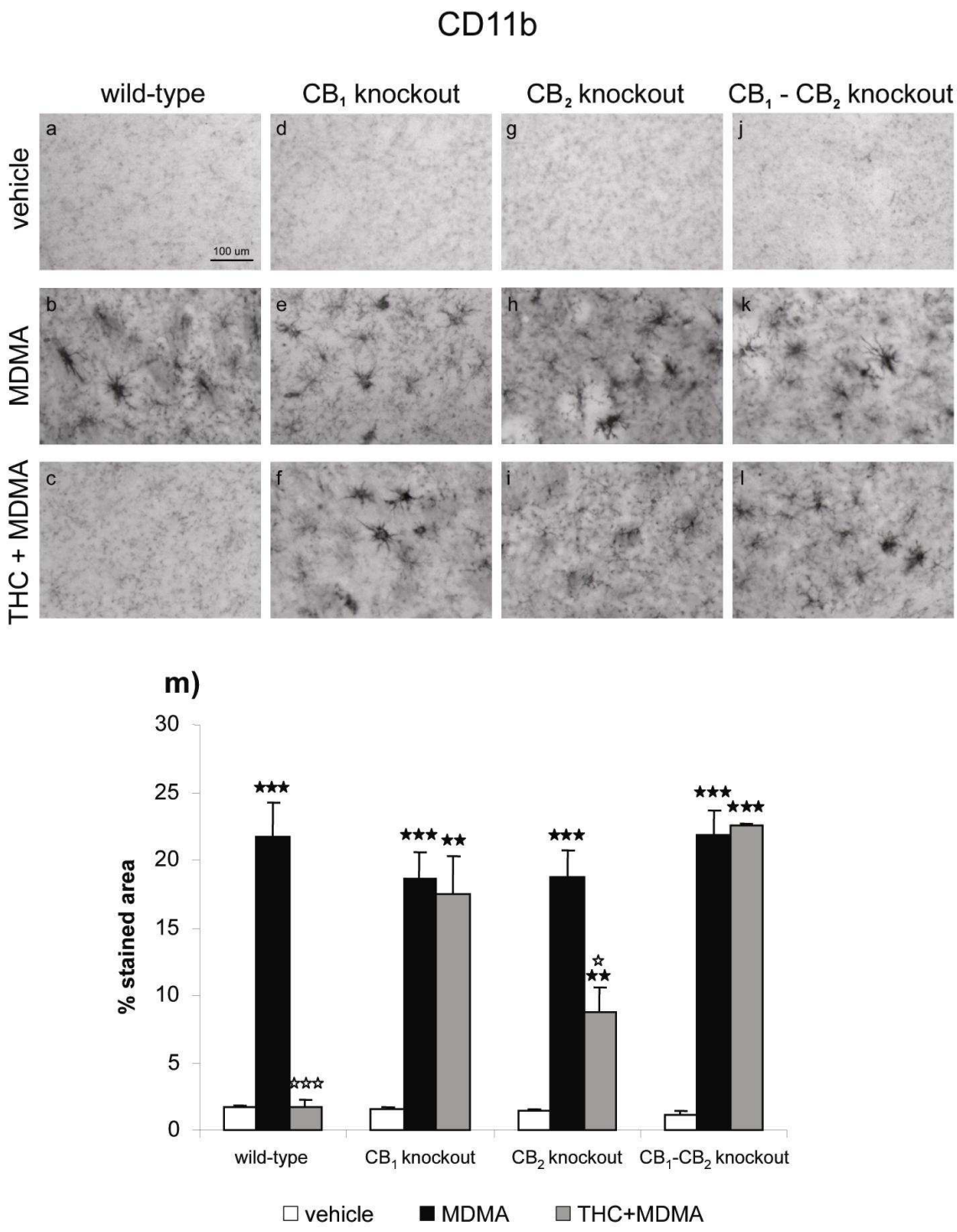
THC+MDMA

Figure 2


CD11b
wild-type
CB1 knockout
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MDMA
THC + MDMA
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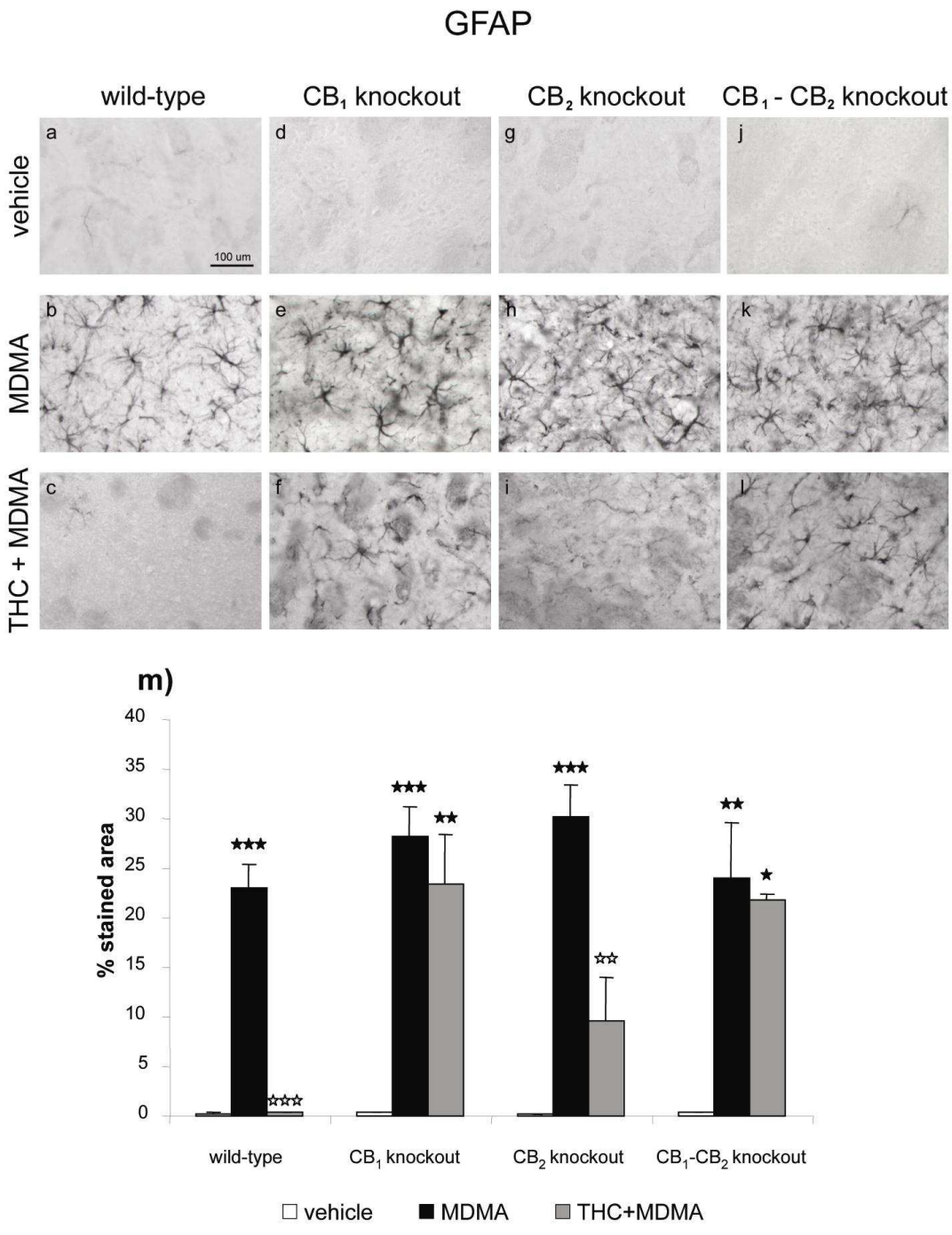
THC+MDMA

Figure 3


GFAP
wild-type
CB1 knockout
CB2 knockout
CB1 - CB2 knockout
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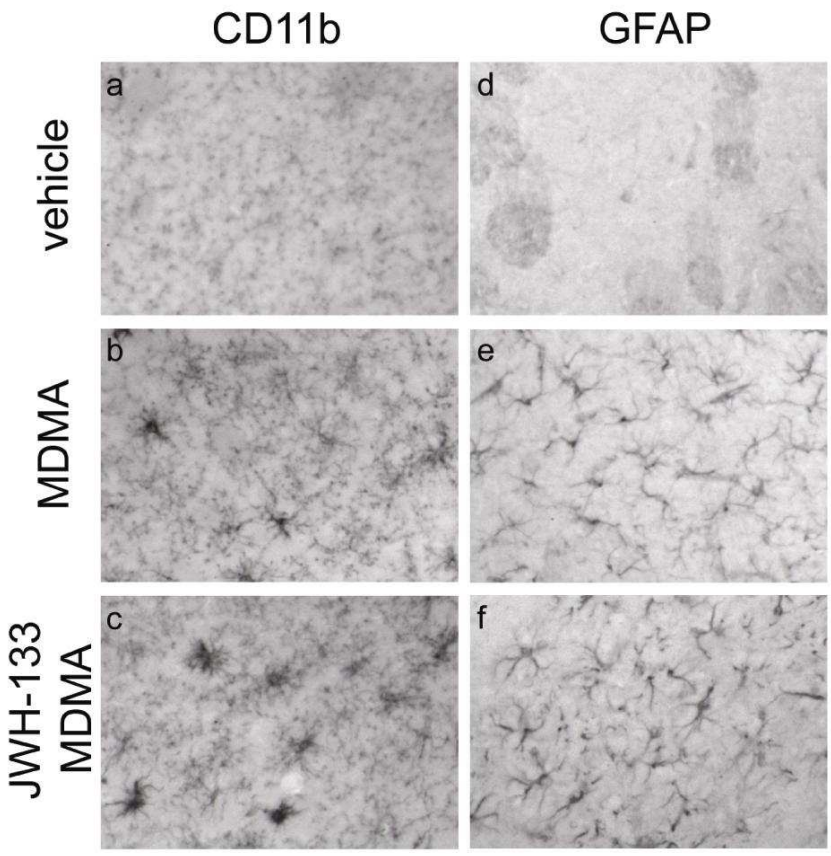
wild-type
CB1 knockout
CB2 knockout
CB1-CB2 knockout
vehicle
MDMA
THC+MDMA

Figure 4


CD11b
GFAP
vehicle
MDMA
JWH-133
MDMA
a
b
c
d
e
f

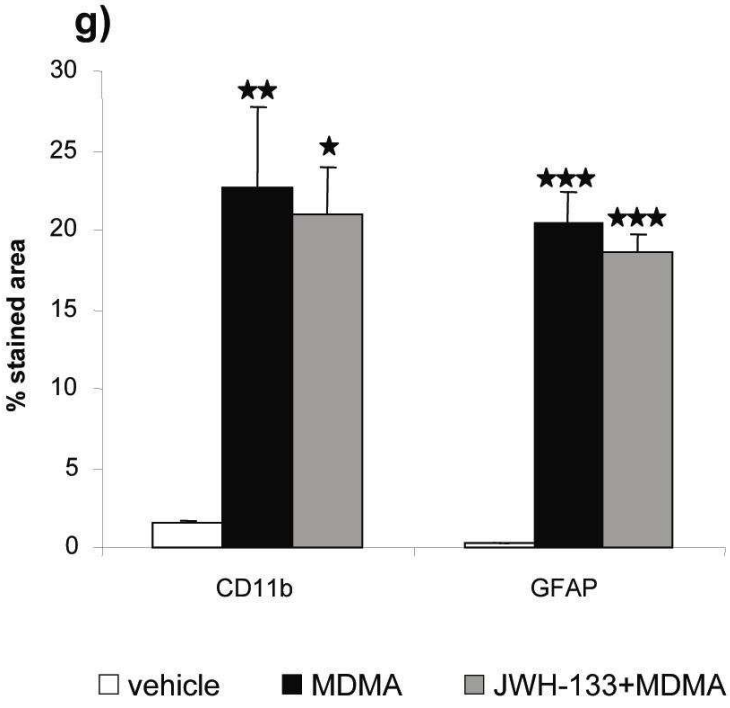

g)
% stained area
30
25
20
15
10
5
0
CD11b
GFAP
vehicle
MDMA
JWH-133+MDMA

Figure 5

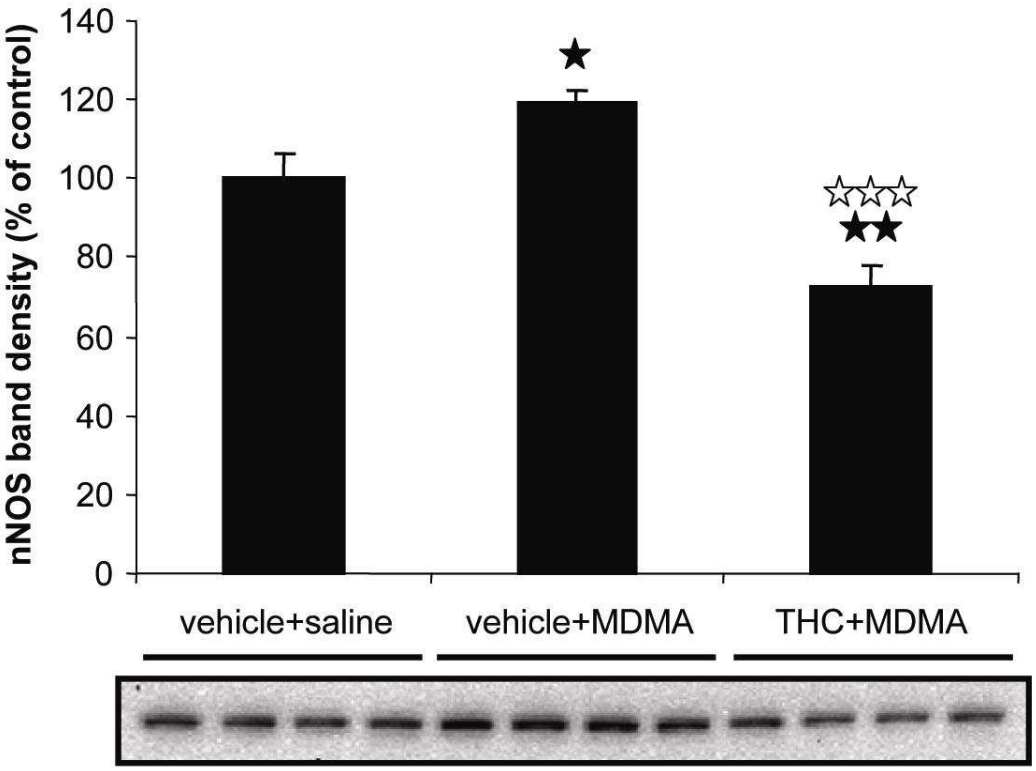

nNOS band density (% of control)
140
120
100
80
60
40
20
0
vehicle+saline
vehicle+MDMA
THC+MDMA

Figure 6

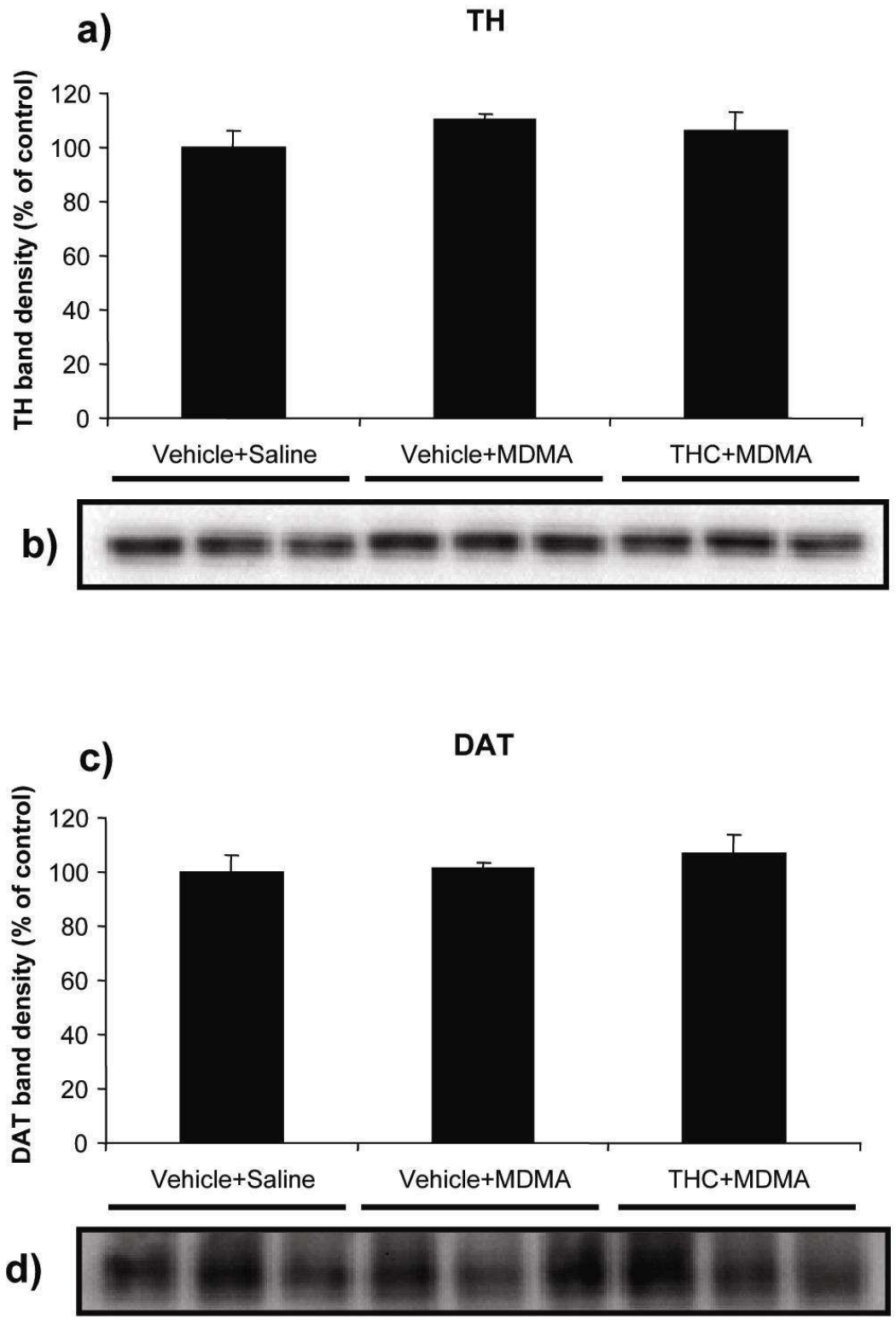

a)
TH
TH band density (% of control)
120
100
80
60
40
20
0
Vehicle+Saline
Vehicle+MDMA
THC+MDMA
b)
c)
DAT
DAT band density (% of control)
120
100
80
60
40
20
0
Vehicle+Saline
Vehicle+MDMA
THC+MDMA
d)

Figure 7

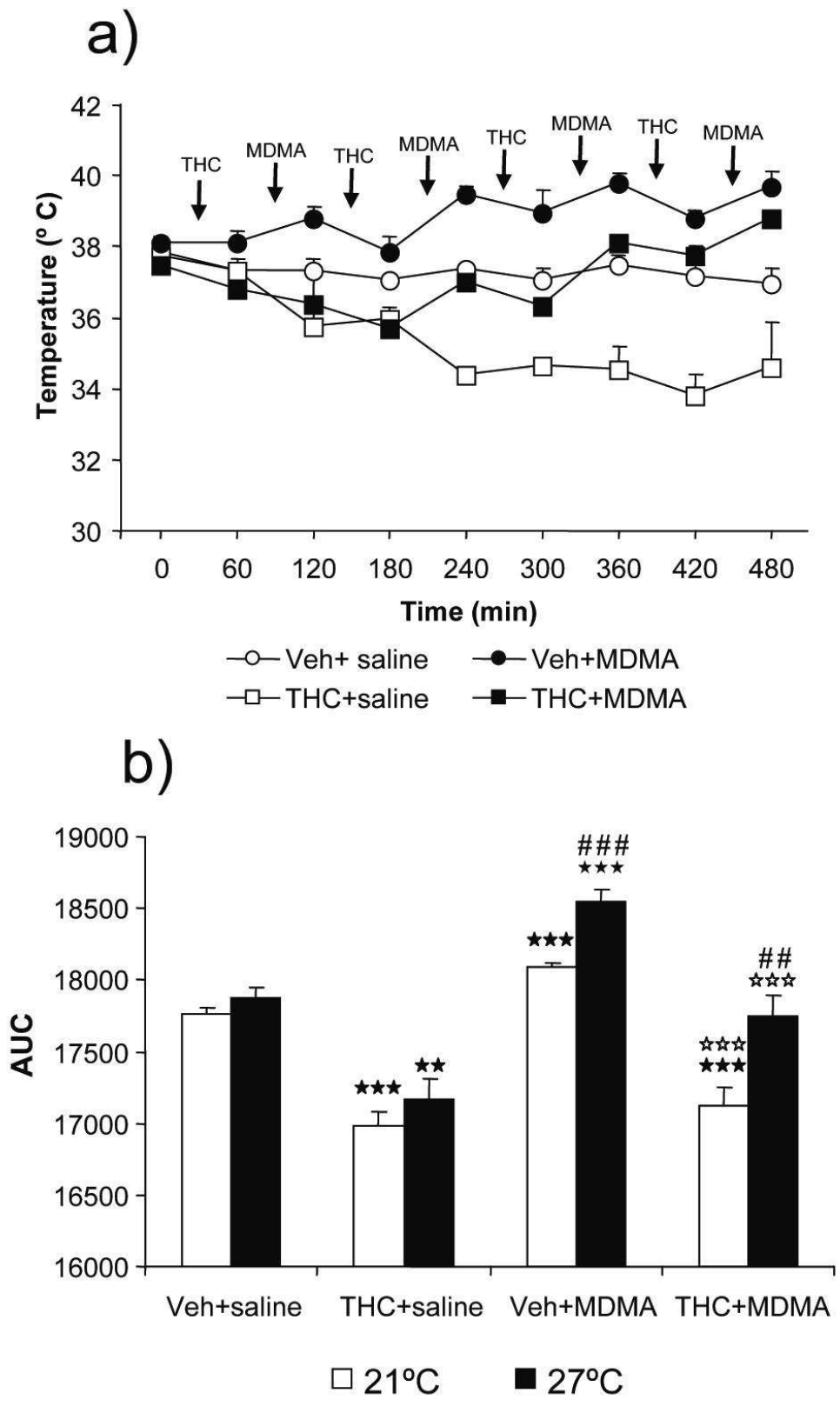

a)
Temperature (° C)
42
40
38
36
34
32
30
THC
MDMA
THC
MDMA
THC
MDMA
THC
MDMA
0
60
120
180
240
300
360
420
480
Time (min)
Veh+ saline
Veh+MDMA
THC+saline
THC+MDMA
b)
AUC
19000
18500
18000
17500
17000
16500
16000
###
##
Veh+saline
THC+saline
Veh+MDMA
THC+MDMA
21ºC
27ºC

Figure 8

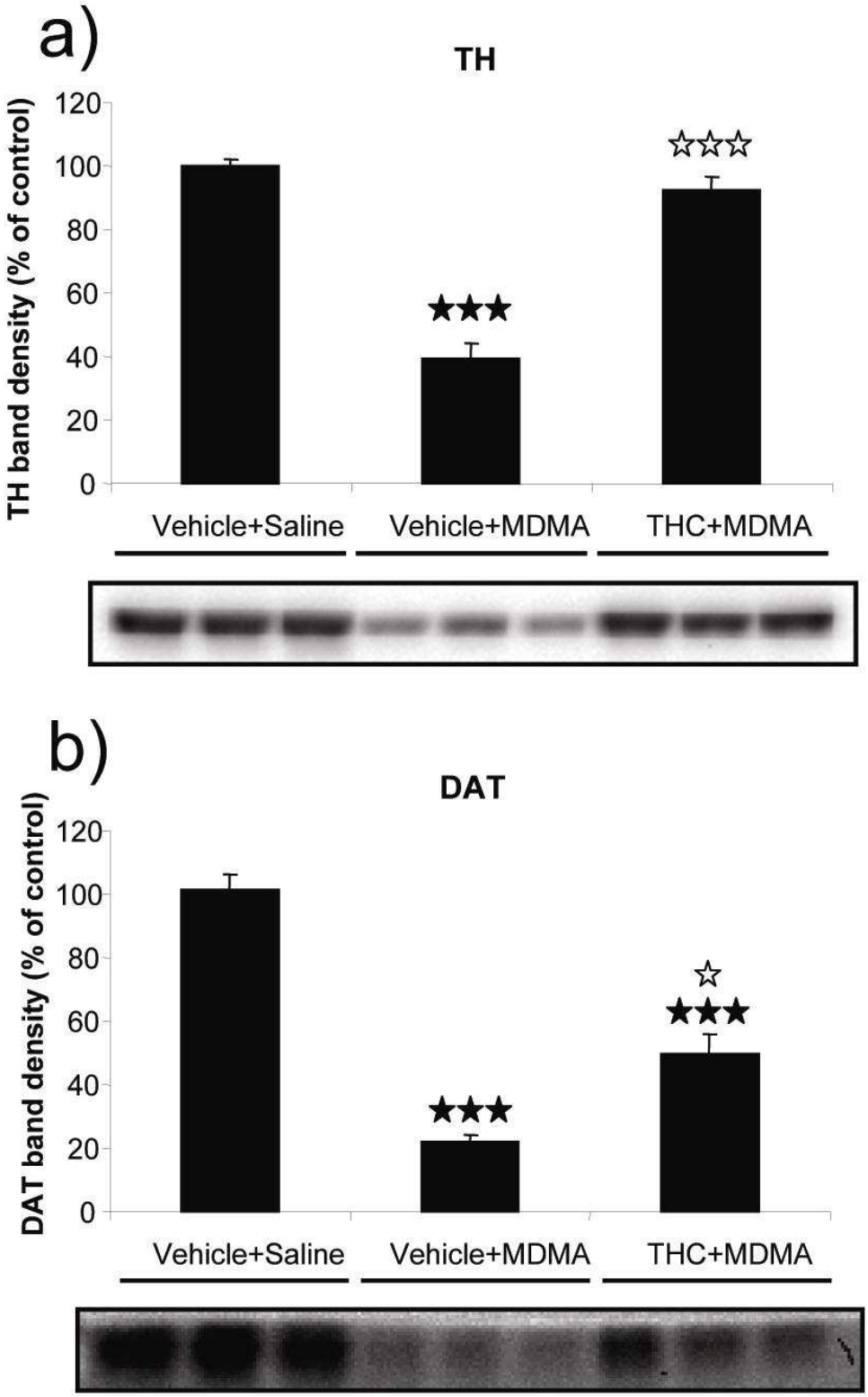
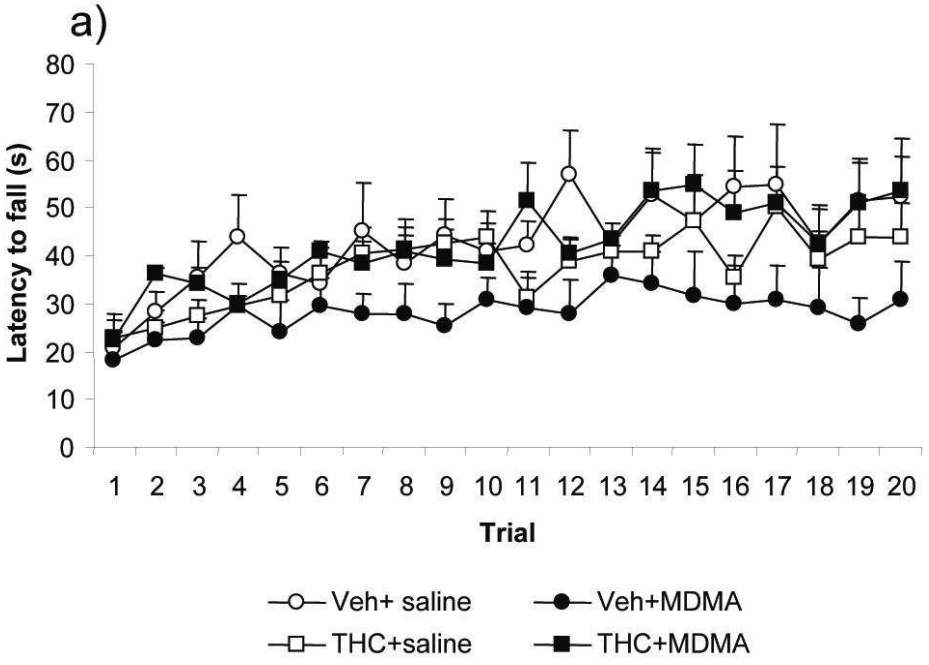
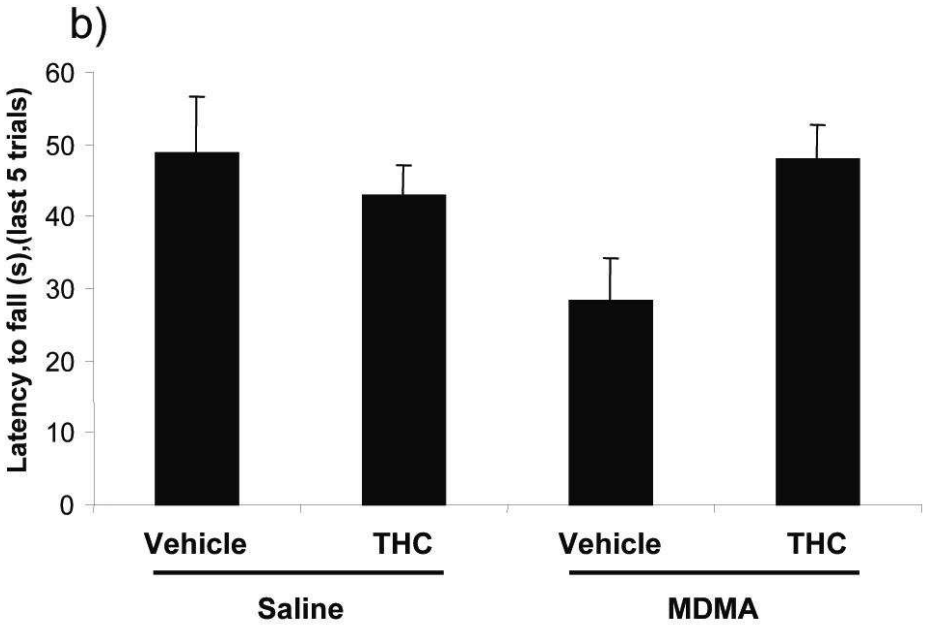

a)
TH
TH band density (% of control)
120
100
80
60
40
20
0
☆☆☆
★★★
Vehicle+Saline
Vehicle+MDMA
THC+MDMA
b)
DAT
DAT band density (% of control)
120
100
80
60
40
20
0
☆
★★★
★★★
Vehicle+Saline
Vehicle+MDMA
THC+MDMA

Figure 9


a)
Latency to fall (s)
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Trial
Veh+ saline
Veh+MDMA
THC+saline
THC+MDMA


b)
Latency to fall (s),(last 5 trials)
60
50
40
30
20
10
0
Vehicle
THC
Vehicle
THC
Saline
MDMA

.

Table 1. One-way ANOVA calculated for CD11b and GFAP staining, nNOS expression, DAT and TH levels at 21 and 27ºC, and latency to fall in the rotarod test.

			One-way ANOVA	
			Treatment	
		Factor	F-value	p-value
CD11b	vehicle	**Genotype**	$F_{(3, 21)} = 0.959$	n.s.
	MDMA		$F_{(3, 27)} = 0.959$	n.s.
	THC+MDMA		$F_{(3, 15)} = 49.549$	< 0.001
	WT	**Treatment**	$F_{(2,26)} = 38.656$	< 0.001
	CB_1 KO		$F_{(2, 12)} = 25.853$	< 0.001
	CB_2 KO		$F_{(2, 12)} = 32.976$	< 0.001
	CB_1-CB_2 KO		$F_{(2, 13)} = 55.084$	< 0.001
GFAP	vehicle	**Genotype**	$F_{(3, 19)} = 0.959$	n.s.
	MDMA		$F_{(3, 26)} = 1.028$	n.s.
	THC+MDMA		$F_{(3, 14)} = 17.375$	< 0.001
	WT	**Treatment**	$F_{(2, 21)} = 51.671$	< 0.001
	CB_1 KO		$F_{(2, 13)} = 23.213$	< 0.001
	CB_2 KO		$F_{(2, 13)} = 25.361$	< 0.001
	CB_1-CB_2 KO		$F_{(11, 187)} = 121.025$	< 0.01
nNOS		**Treatement**	$F_{(2, 12)} = 23.425$	< 0.001
TH (21ºC)		**Treatement**	$F_{(2, 9)} = 4.434$	n.s.
DAT (21ºC)		**Treatement**	$F_{(2, 9)} = 0,056$	n.s.
TH (27ºC)		**Treatement**	$F_{(2, 9)} = 72.558$	< 0.001
DAT (27ºC)		**Treatement**	$F_{(2, 9)} = 65.132$	< 0.001
Latency to fall		**Treatement**	$F_{(3, 418)} = 37.544$	< 0.001

One-way ANOVA for genotype and treatment as between-subject factors. See Materials and methods for details. WT: wild-type; KO: knockout; n.s.: non-significant.

Discussion

Ecstasy and cannabis are two drugs of abuse, which are often consumed in combination (Parrott et al., 2007). Ecstasy users usually report pleasurable effects after consuming the drug (Baylen and Rosenberg, 2006), and rewarding and reinforcing effects of MDMA have also been reported in animals. Thus, MDMA established CPP in rats and mice (Bilsky et al., 1990; Robledo et al., 2004), and was self-administered by primates, rats and mice (Beardsley et al., 1986; Lamb and Griffiths, 1987; Schenk et al., 2003; Trigo et al., 2006). Hence, the reinforcing properties of MDMA are a consequence of its ability to increase extracellular DA levels in the NAc (White et al., 1994; Colado et al., 2004). However, MDMA-induced release has also been proposed to contribute to MDMA reinforcing effects (Trigo et al., 2007). On the other hand, the pleasurable effects of cannabis have been known for centuries. THC, the major psychoactive constituent of cannabis, also showed rewarding effects in animals. THC established CPP in both rats (White et al., 1994; Lepore et al., 1995) and mice (White et al., 1994; Valjent and Maldonado, 2000), and induced intravenous self-administration in squirrel monkeys (White et al., 1994; Tanda et al., 2000). The peculiar pharmacokinetic properties of THC complicate the acquisition of a self-administration in rodents (Gardner and Lowinson, 1991; Martellotta et al., 1998). Indeed, the long half-life of THC complicates the association between stimuli and response required for an operant behavior paradigm such as self-administration. However, WIN-55,212-2, a synthetic cannabinoid agonist with shorter half-life, was intravenously self-administered in mice (Martellotta et al., 1998; Mendizabal et al., 2006). Acute administration of THC and other cannabinoid agonists enhance DA release in the NAc (Tanda et al., 1999) and increase neuronal firing rates in VTA DA neurons, supporting the fact that cannabinoid drugs have rewarding effects. As many other drugs of abuse, MDMA and THC may be frequently consumed together to enhance their pleasurable effects. This is supported by animal studies showing that subeffective doses of THC (0.3 mg/kg) enhance the rewarding effects of subeffective doses of MDMA (3 mg/kg) in the CPP, and the reinforcing effects of MDMA (0.06 mg/kg/infusion) in the self-administration paradigm (Robledo et al., 2007).

THC rewarding properties and its effects on DA release in the NAc are mediated by the activation of CB_1 cannabinoid receptors. Thus, mice lacking CB_1 cannabinoid receptors do not show THC reinforcing properties (Ledent et al., 1999). Interestingly, genetic deletion of CB_1 receptor also impairs rewarding and reinforcing effects of opioids (Ledent et al., 1999), nicotine (Castañé et al., 2002) and ethanol (Thanos et al., 2005). Surprisingly, neither cocaine (Martin et al., 2000) nor MDMA (Touriño et al., 2008) CPP was impaired in these animals. Likewise, opioids (Caille and Parsons, 2003), nicotine (Cohen et al., 2002a) and alcohol (Hungund et al., 2003) were unable to enhance

extracellular DA levels in the NAc in CB_1 knockout mice or after the pharmacological blockade of CB_1 receptor, whereas cocaine (Soria et al., 2005) or MDMA (Touriño et al., 2008) similarly induced DA release in the NAc in CB_1 knockout mice and their wild-type littermates. This finding might be due to the different site of action of psychostimulant drugs. Nicotine, opioids, ethanol and cannabinoids enhance the firing of VTA DA neurons increasing DA release in the NAc (Fig. 12). Cannabinoids enhance DA levels in the NAc by activating presynaptic CB_1 receptors in VTA γ-aminobutiric acid-A ($GABA_A$) neurons. Opioids enhance DA extracellular levels in the NAc by activating mu-opioid receptors in VTA GABAergic outputs to DA neurons (Bergevin et al., 2002). Ethanol enhances DA release by activating $GABA_A$ receptors in GABA neurons, and facilitating the release of endogenous opioid in the VTA (Samson et al., 1987). Nicotine increases DA release by activating acetylcholine receptors expressed in glutamatergic terminals (Pidoplichko et al., 2004) and opioid receptors in GABAergic neurons (Berrendero et al., 2005) in the VTA Eventually, all these drugs facilitate the release of DA in the NAc by activating or disinhibiting DA neurons in the VTA. Conversely, cocaine and MDMA directly increase DA release by inhibiting DAT in the NAc. The genetic deletion or blocking of CB_1 receptors just affects the rewarding properties and DA release of drugs acting on the VTA and depolarizing DA neurons. The prolonged depolarization of DA neurons could result in transient reduction of $GABA_A$ receptor mediated inhibitory postsynaptic currents. This effect known as depolarization-induced suppression of inhibition (DSI) (Pitler and Alger, 1992) was determined to be mediated by endocannabinoids. After depolarization of DA neurons, endocannabinoids are synthesized from the DA neuron and bind to presynaptic CB_1 receptors in the GABAergic neuron. DSI facilitates the depolarization of DA neurons, prolonging DA release in the NAc. As DSI does not occur in the absence of CB_1 receptors, the rewarding properties of opioids, cannabinoids, nicotine or ethanol are abolished in CB_1 knockout mice. On the contrary, as rewarding effects of psychostimulants are not mediated by DSI, their rewarding effects are preserved in CB_1 mutant animals.

Cocaine (Soria et al., 2005) and MDMA (Touriño et al., 2008) intravenous self-administration was impaired in CB_1 knockout mice. Operant self-administration paradigm evaluates the reinforcing properties of drugs of abuse. Therefore, this procedure evaluates not only the pleasurable effects of a drug, but also the motivation for obtaining it. Whereas primary reward evaluated by CPP is under control of mesolimbic DA pathway, drug-seeking behavior also involves other brain circuits such as PFC, hippocampus and amygdala (Kalivas and Volkow, 2005; Everitt and Robbins, 2005). DA neurons in PFC do not seem to play an important role in drug-seeking behavior (Martin-

Iverson et al., 1986). However, glutamate release into the NAc core from PFC pyramidal neurons is essential for drug-seeking behavior (McFarland et al., 2003; LaLumiere and Kalivas, 2008). These glutamatergic neurons are modulated by presynaptic CB_1 cannabinoid receptors (Robbe et al., 2002). Thus, CB_1 receptors in PFC glutamatergic projections to NAc may play a role in drug-seeking behavior, including psychostimulant drugs. For that reason, lack of CB_1 receptor impairs psychostimulant-seeking behavior, but not psychostimulants primary reward. This hypothesis is supported by other studies revealing the absence of cocaine seeking behavior after the blockade of CB_1 receptor (De Vries et al., 2001) and describing the importance of PFC-NAc glutamatergic projections in this process (Xi et al., 2006).

It is worth mentioning that CB_1 cannabinoid receptor deletion produced a more severe impairment of MDMA self-administration than cocaine self-administration. While MDMA self-administration was completely abolished in CB_1 null mice, 25% of mutants still acquired a cocaine self-administration behavior. Both, MDMA and cocaine enhance DA release in the NAc, but MDMA and not cocaine exerts a strong increase in 5-HT release. Several studies indicate the relevance of 5-HT in the rewarding and reinforcing properties of MDMA. For instance, $5\text{-}HT_3$ receptor antagonists block the ability of MDMA to establish CPP (Bilsky and Reid, 1991), a recent study in our laboratory described that MDMA self-administration was impaired in mice lacking SERT (Trigo et al., 2007). Pyramidal cortical neurons, which send glutamatergic projections to the NAc, express several types of 5-HT receptors (Martin-Ruiz et al., 2001). Moreover, glutamate release of these pyramidal neurons is modulated by CB_1 cannabinoid receptors. Thus, MDMA-induced 5-HT release may enhance the activity of these pyramidal neurons mediating MDMA seeking-behavior. Consequently, the lack of CB_1 receptors may cause a stronger impairment in MDMA than in cocaine seeking behavior.

Despite the fact that both THC and MDMA are drugs of abuse with rewarding properties, the pharmacological effects of THC markedly contrast with those of MDMA. Relaxant, hypothermic and anti-inflammatory effects of cannabinoids (Halikas et al., 1971; Croxford, 2003) are opposite to the stimulatory, anxiogenic, hyperthermic and neurotoxic effects of ecstasy (Martin-Ruiz et al., 2001; Zhou et al., 2003; Baylen and Rosenberg, 2006; Quednow et al., 2007). Similarly, MDMA caused hyperactivity, hyperthermia, anxiogenic-like effects and neurotoxicity in animals (Martin-Ruiz et al., 2001; Green et al., 2003), whereas THC and other cannabinoid agonists showed hypolocomotion, hypothermia, anxiolytic, anti-oxidant and anti-inflammatory effects (Dewey, 1986; Grundy et al., 2001). Therefore, the combination of MDMA and THC may result in compensatory rather than

additive effects, being cannabinoids able to counterbalance some of the negative effects of MDMA.

Anxiety is a widely reported consequence of MDMA administration in both humans and animals (Green et al., 2003; Baylen and Rosenberg, 2006). Nevertheless, animal studies report heterogeneous results depending on the dose, frequency of administration, behavioral test and animal species used (Green et al., 2003). On the other hand, cannabinoid drugs showed biphasic effects on anxiety. Anxiolytic or anxiogenic properties of cannabinoid agonists depend on the dose, the behavioral model, the context, the animal species, and the genetic strain (Valverde, 2005). Hence, it is not surprising that animals receiving THC together with MDMA showed lower anxiety levels in an emergence test than animals treated just with MDMA (Morley et al., 2004; Valverde, 2005). The effects of cannabinoids on anxiety are mediated by CB_1 receptors. The high levels of CB_1 receptors in the hippocampus, amygdala, prefrontal and anterior cingular cortex, which are key regions in the regulation of anxiety, suggest that the endocannabinoid system plays an important role in the control of anxiety (Tzavara et al., 2003). Further support of this theory came from studies using CB_1 receptor antagonists or CB_1 receptor knockout mice in a wide variety of tests (defensive withdrawal test, the elevated plus-maze, open-field test or light-dark box). Most of the studies describe anxiogenic-like properties of rimonabant (Navarro et al., 1997; Arévalo et al., 2001), although anxiolytic-like effects were also reported (Haller et al., 2002). In agreement, CB_1 knockout mice displayed increased anxiogenic-like responses and marked alterations in the hypothalamic-pituitary-adrenal (HPA) axis (Martin et al., 2002; Haller et al., 2004), what suggests the existence of an anxiolytic-like endocannabinoid tone. In agreement with this data, we observed both, an increase anxiogenic-like response in mice lacking CB_1 receptors and an anxiogenic effect of high doses of MDMA in the elevated-plus maze. Surprisingly, when CB_1 knockout mice were administered with high doses of MDMA, the anxiogenic effects caused by the drug were completely absent. These results indicate the participation of endocannabinoid system on the anxiogenic effects of MDMA. It is widely accepted that 5-HT system plays an essential role in emotional behavior, including anxiety responses. Thus, CB_1 receptor may facilitate the effects of 5-HT released by MDMA on brain areas that control emotional responses, such as hippocampus, amygdala, PFC, and anterior cingular cortex. Therefore, CB_1 receptors would promote the anxiogenic effects of MDMA. The fact that the anxiolytic-like effect of 5-HT_{1A} agonist buspirone was lower in CB_1 knockout mice (Uriguen et al., 2004) supports this hypothesis.

One of the most characteristic effects of MDMA, as well as other psychostimulant drugs, is the increase on locomotor activity (Green et al., 2003). In contrast, reduction of locomotor activity is a prototypical feature of cannabinoids (Ameri et al., 1999). Then, it is not surprising that the administration of the cannabinoid agonists THC or CP-55,940 significantly attenuated MDMA-induced hyperlocomotion (Morley et al., 2004). This reduction was prevented by the administration of rimonabant, indicating the participation of CB_1 cannabinoid receptors in the hyperlocomotor effects of MDMA (Morley et al., 2004). To confirm this hypothesis, the effects of both, pharmacological blockade with rimonabant and genetic ablation of CB_1 receptors on MDMA-induced hyperlocomotion were explored. Surprisingly, the administration of rimonabant did not modify the increase in locomotor activity produced by MDMA (Touriño et al., 2007). However, this hyperlocomotion was attenuated in mice lacking CB_1 cannabinoid receptor (Touriño et al., 2008). This result indicates that the acute blockade of CB_1 receptors does not affect MDMA-induced hyperlocomotion, whereas the lack of this receptor may produce neuroadaptations in the circuits mediating MDMA hyperactivity. Both DA and 5-HT are involved in the hyperlocomotion produced by MDMA (Scearce-Levie et al., 1999; Benturquia et al., 2008). Hyperlocomotor effects of cocaine, which predominantly induces DA release, were not affected in CB_1 knockout mice. These data indicate that the neuroadaptations in CB_1 knockout mice affecting MDMA-induced hyperlocomotion would take place in 5-HT rather than in DA system. Supporting this hypothesis, cross-interactions between endocannabinoid and 5-HT system have been previously described (Cheer et al., 1999), particularly in the cerebellum (Devlin and Christopoulos, 2002) and the caudate-putamen (Oliva et al., 2005), two essential brain areas in the control of locomotor activity. Nevertheless, the specific mechanism of this interaction remains to be clarified.

An interaction between MDMA and cannabinoids were observed in THC physical dependence (Touriño et al., 2007). Thus, MDMA produced a dose-dependent decrease in THC withdrawal syndrome when administered both acutely and chronically. Physical dependence is originated by the chronic administration of a drug resulting in adaptive changes occuring at different brain areas. Some of the relevant adaptations inducing cannabinoids physical dependence take place in the cerebellum, a brain region involved in motor control. Thus, adenylate cyclase was increased in the cerebellum, but not in other regions after the administration of rimonabant to mice chronically treated with THC (Hutcheson et al., 1998). In addition, a withdrawal syndrome was triggered by directly administering rimonabant into this area (Castañé et al., 2002). Furthermore, many of the signs observed in cannabinoid withdrawal syndrome comprise locomotor and postural

alterations under cerebellum control such as, body tremor, paw tremor, mastication, genital licks and sniffing. On the other hand, MDMA also affects locomotor activity, as mentioned above. However, motor signs of THC abstinence were not reduced by a masking effect of MDMA-induced hyperlocomotion. In fact, cannabinoid withdrawal syndrome was strongly attenuated at doses where MDMA did not significantly enhance locomotor activity. MDMA should attenuate THC abstinence by a modulation of DA or 5-HT system, both involved in locomotion. Therefore, the release of both DA in the NAc and 5-HT in the PFC was measured in animals chronically treated with THC after the administration of MDMA and the precipitation of a withdrawal syndrome with rimonabant. DA release into the NAc was not altered after MDMA or rimonabant administration, indicating that this neurotransmitter does not play a crucial role in the attenuation of THC abstinence by MDMA. This result is in agreement with the lack of changes in striatum adenylate cyclase activity of animals on THC withdrawal syndrome (Hutcheson et al., 1998). Moreover, no somatic signs of withdrawal were observed after the administration of rimonabant directly into the striatum, which primarily contains DA terminals (Castañé et al., 2002). Nevertheless, MDMA administration increased 5-HT release in the PFC of THC dependent animals before the precipitation of withdrawal syndrome with rimonabant. This result suggests that MDMA attenuates THC withdrawal syndrome by increasing 5-HT. PFC is not directly involved in cannabinoids physical dependence or the control of motility. However, these data clearly reveal an enhancement of 5-HT release that may be taking place in other brain areas. Indeed, there is wide evidence of 5-HT activity in the cerebellum (Ghetti et al., 1988). 5-HT and CB_1 cannabinoid receptors are also expressed in other areas involved in motor control, such as motor cortex or basal ganglia, which in a lesser extent could be contributing to the attenuation of cannabinoids abstinence by MDMA. It was reported that MDMA reduced dyskinesias in a marmoset model of Parkinson's disease through a 5-HT mediated mechanism (Iravani et al., 2003). Hence, MDMA may attenuate THC withdrawal syndrome by compensating an abnormal 5-HT transmission that could be affecting directly or indirectly brain regions involved in the appearance of motor somatic signs revealed during cannabinoid abstinence. The alteration in 5-HT transmission might be occurring during the development of cannabinoid dependence, since rimonabant administration did not alter 5-HT levels in THC dependent animals.

Non-motor signs of withdrawal, such as diarrhea, ptosis, wet dog shakes or tremor were also significantly attenuated by MDMA. Some of these signs are related with hypothermia, including tremor, piloerection or wet dog shakes. MDMA alters body temperature with a wide variety of effects, depending on the dose, treatment schedule or

animal species (Green et al., 2003). In our experimental conditions, THC-abstinent mice exhibited a significant decrease in body temperature that may contribute to the appearance of some somatic signs such as tremor, wet dog shakes or piloerection, and MDMA administration reduced this hypothermia. Thus, the action of MDMA on body temperature might contribute to the reduction of the temperature-dependent somatic signs of THC abstinence. 5-HT release mediates MDMA effects on body temperature. Thus, MDMA-induced 5-HT release would ameliorate the severity of THC abstinence somatic signs related not only to motor impairment, but also to hypothermia.

One of the principal adverse effects of MDMA is the enhancement of body temperature. These changes in body temperature contribute to important health problems derived from MDMA use, as heat shocks and neurotoxicity (Milroy, 1999). Alterations in body temperature after MDMA treatment have also been reported in animals, usually resulting in hyperthermia (Green et al., 2003). MDMA impairs thermoregulation, what makes body temperature dependent on ambient temperature. Thus, small increases in ambient temperature produce strong increases in body temperature (Malberg and Seiden, 1998). Conversely, one of the most characteristic effects of cannabinoids in animals is hypothermia (Chaperon and Thiebot, 1999), and they also have the ability to reduce pyrogen-induced hyperthermia (Paton, 1973). For that reason, we tested the effects of THC on MDMA-induced hyperthermia in mice housed at room (21ºC) and at high ambient (27ºC) temperature. We observed that MDMA caused a slight increase in body temperature in mice housed at room temperature, but a strong hyperthermia in mice housed at high ambient temperature. Then, pretreatment with THC reversed not only the slight hyperthermia produced by MDMA at room temperature, but also the severe hyperthermia occurring at high ambient temperature. Similarly, THC and the synthetic cannabinoid agonist CP-55,940 reduced MDMA-induced hyperthermia in rats, and the effect of cannabinoids on MDMA-induced hyperthermia was reversed by rimonabant, indicating a selective role of CB_1 receptors (Morley et al., 2004). Therefore, the hypothermic properties of THC seem to compensate the hyperthermic effects caused by MDMA. However, CB_1 cannabinoid receptor might play a role by itself in the effects of MDMA on body temperature. Indeed, the involvement of CB_1 receptors on MDMA-induced hyperthermia was directly demonstrated in one of our studies, where MDMA-induced hyperthermia was significantly reduced in CB_1 knockout mice (Touriño et al., 2008). CB_1 knockout mice exhibit similar basal body temperature than wild-type mice, which reveals that the differences between genotypes after MDMA are treatment-specific. Then, CB_1 receptors seem to be specifically involved in the mechanisms mediating MDMA-induced hyperthermia that involve an increase of 5-HT release (Shankaran and

Gudelsky, 1999). 5-HT released by MDMA activates 5-HT receptors in preoptic nucleus of the hypothalamus (Stephenson et al., 1999), a key brain area in the control of thermoregulation. Preoptic nucleus of the hypothalamus also expresses CB_1 receptors (Cota et al., 2003), which have been involved in control of body temperature (Ameri, 1999). Thus, CB_1 receptors in the preoptic nucleus of the hypothalamus may facilitate the body temperature increase caused by MDMA-induced 5-HT release. The interaction between 5-HT and endocannabinoid system in the regulation of body temperature is also supported by other studies. Hence, THC-induced hypothermia was modified by the administration of the 5-HT uptake inhibitor fluoxetin (Malone and Taylor, 1998). On the other hand, temperature changes induced by a 5-HT_{1A} agonist are reduced in CB_1 receptor knockout mice indicating an impaired functionality of 5-HT_{1A} receptor, which is directly involved in the regulation of body temperature (Mato et al., 2007). Therefore, the impaired functionality of 5-HT_{1A} receptor in CB_1 knockout mice may explain the reduced effect of MDMA on body temperature in these mutant mice.

The main adverse consequence related to MDMA-induced hyperthermia is the enhancement of neurotoxicity. High ambient temperature increases MDMA-induced hyperthermia, which exacerbates MDMA neurotoxicity (Malberg and Seiden, 1998). MDMA is a drug frequently consumed in night clubs with elevated ambient temperature, where neurotoxicity may be potentiated. Interestingly, most of MDMA users smoke cannabis, which not only reduces body temperature, but also exerts neuroprotective effects on neurodegenerative and neuroinflammatory diseases (Grundy et al., 2001; Sarne and Mechoulam, 2005). Thus, we proposed that THC may prevent not only hyperthermia-related adverse effects of MDMA, but most importantly MDMA neurotoxic effects. MDMA metabolism results in several compounds with neurotoxic potential (Monks et al., 2004; Capela et al., 2006). However, these neurotoxic compounds may be different between species and affect diverse neuronal types, because MDMA metabolic pathways are different between species (de la Torre and Farré, 2004). MDMA metabolites generated in humans, primates and rats affect 5-HT nerve terminals, whereas metabolites generated in mice mainly damage DA terminals, as occurs with methamphetamine (Stone et al., 1987). Nevertheless, the mechanisms of neurotoxicity are similar between species. Neurotoxic MDMA metabolites are translocated into the cell via the DA or 5-HT uptake carrier, generating DA quinones, reactive oxygen and nitrogen species and leading to oxidative stress and free radicals formation (Colado et al., 2004; Quinton and Yamamoto, 2006). Free radicals damage proteins and induce lipid peroxidation in nerve terminals, causing severe neuronal damage. This damage causes the activation of microglial cells, the main immune cells in the brain that protects brain

against external threatens. Under normal conditions, microglial cells play trophic functions, but they may exacerbate brain damage when chronically activated. Activated microglia releases a bunch of proinflammatory cytokines such as IL-1β or TNF-α. Hence, the chronic consumption of MDMA may lead to chronic microglial activation, aggravating neuronal damage (Orio et al., 2004; Thomas et al., 2004a). Likewise, astrocytes are activated after MDMA-induced brain injury. Astrocytes are activated after brain injury to provide neurons metabolic support, protect the integrity of blood-brain barrier, phagocyte cell debris, repair and replace cells that could not regenerate, and modulate enhanced glutamatergic transmission (Chen and Swanson, 2003; Santello and Volterra, 2008). Finally, this process ends up in an important nerve terminal loss, which is aggravated with higher doses of MDMA, longer exposure to the drug, and high ambient temperatures. MDMA-induced terminal loss takes place in the forebrain 5-HT neurons of humans, primates and rats (O'Hearn et al., 1988) and in the striatum DA neurons of mice (Logan et al., 1988). MDMA-induced 5-HT terminal loss may lead to long-term alterations in mood and behavior such as depression, anxiety, impulsivity or aggressiveness. Indeed, there is a growing body of evidence to suggest an association between MDMA consumption and neuropsychiatric disorders (Morgan, 2000). Anxiogenic-like effects were observed in rats chronically treated with MDMA even months after administration (Morley et al., 2001). A number of studies suggest cognitive impairment and memory deficits in MDMA users (Parrott et al., 1998; Bhattachary and Powell, 2001). These deficits include reduced memory for new information, impaired higher executive process, or heightened impulsivity (Parrott, 2000). MDMA also caused cognitive deficits in monkeys (Taffe et al., 2002) and rats (Morley et al., 2001) even months after treatment. Mood and cognitive alterations induced by MDMA in humans, primates and rats are directly related to damage in PFC and hippocampus 5-HT terminals (Colado and Green, 1994; Parrott, 2000). However, MDMA neurotoxicity mostly affects striatum DA terminal in mice. As a consequence, mice under a neurotoxic regimen of MDMA might show impaired motor coordination (Jeng et al., 2006), as occurs with other DA neurotoxins such as MPTP (Sedelis et al., 2000). Motor coordination deficits as a consequence of MDMA-induced DA terminals degeneration can be easily evaluated in the rotarod test, a rotating rod used to assess sensorimotor coordination (Petzinger et al., 2007). Thus, although the neurotoxic effects of MDMA taking place in humans are not exactly reproduced in mice, MDMA neurotoxicity is easily detectable with both neurochemical and behavioral methods.

A number of evidence suggests that THC may protect against MDMA neurotoxicity (Parrott et al., 2007), since THC reversed MDMA-induced hyperthermia not only at room temperature, but also at high temperature. Due to the fact that MDMA-induced

hyperthermia aggravates its neurotoxic effects, the reduction of body temperature exerted by THC would protect against this MDMA neurotoxicity. In agreement, cold ambient temperature, as well as drugs that lower body temperature has been shown to protect against neurotoxicity (Ali et al., 1994; Farfel and Seiden, 1995; Green et al., 2005). Furthermore, the formation of MDMA-derived neurotoxic metabolites and its uptake into the cell is promoted at high ambient temperature (Malberg et al., 1996; Goñi-Allo et al., 2008). Thus, THC may prevent MDMA-treated animals from hyperthermia, reducing both the formation of neurotoxic metabolites and its internalization in the neuron. As a consequence, MDMA-induced neurotoxicity would be reduced.

Attenuation of body temperature does not completely suppress the formation of neurotoxic metabolites derived from MDMA. Therefore, free radicals, and reactive oxygen and nitrogen species are still generated. The main enzyme involved in the formation of reactive oxygen and nitrogen species is NOS. Several reports describe an important contribution of NOS to the neurotoxic effects of MDMA and other amphetamine derivatives (Colado et al., 2001; Darvesh et al., 2005). On the other hand, THC attenuates the expression of iNOS (Jeon et al., 1996), suggesting a putative effect of THC reducing MDMA-induced formation of reactive oxygen and nitrogen species. Consequently, we evaluated the action of MDMA on NOS expression, and the effect of THC on this expression. We observed that MDMA-induced NOS expression was slightly increased, and THC significantly attenuated this expression. THC may reduce oxidative stress not only by reducing NOS expression, but also by its receptor-independent antioxidant properties due to its phenolic structure (Chen and Buck, 2000). Together, the antioxidant properties of THC may contribute to attenuate the oxidative stress, and the reduction of cell damage generated by MDMA metabolites.

Brain injury is usually followed by the activation of brain immune cells, namely microglia. Once MDMA-induced neuronal injury has occurred, microglial cells are activated to repair damage and eliminate cell debris. When activated, microglia releases proinflammatory cytokines such as IL1-β or TNF-α and additional immune cells are recruited to repair the damaged tissue. In case of prolonged or massive neuronal damage, microglial cells might become chronically activated, exacerbating rather that ameliorating harm. After MDMA exposure, microglia becomes activated to repair neuronal damage. However, prolonged exposure to the drug may cause a sustained microglial activation that would enhance cell damage rather than decreasing it. In addition, activated microglia also expresses iNOS, which may contribute to aggravate oxidative stress. In contrast, THC and other cannabinoid drugs have been reported to inhibit microglial activation in

neurodegenerative disorders such as multiple sclerosis Alzheimer's disease, HIV encephalopathy, ischemia, traumatic brain injury and neuropathic pain (Walter and Stella, 2004; Racz et al., 2008). This inhibition is mediated by CB_2 cannabinoid receptor, which is expressed in microglial cells (Cabral et al., 2008). Consequently, THC also inhibits the release of proinflammatory mediators (Puffenbarger et al., 2000) and the expression of iNOS (Jeon et al., 1996) by microglial cells. We observed that pretreatment with THC completely abolished MDMA-induced microglial activation. However, the mechanisms by which THC inhibits MDMA-induced microglial activation remain unclear. The activation of CB_1 receptor by THC reduces body temperature, and would attenuate the formation of toxic metabolites and tissue damage. Therefore, in absence of neuronal damage microglia would not become activated. Nonetheless, THC may directly inhibit microglia activating CB_2 receptors. Hence, we studied the involvement of CB_1, and CB_2 receptors on the reduction of MDMA-induced microglial activation by THC. With that purpose, mice lacking CB_1, CB_2, and both cannabinoid receptors were treated with THC and MDMA, and microglial activation was analyzed. We observed that THC did not prevent MDMA-induced microglial activation in CB_1 or double knockout mice, indicating that this receptor is responsible for the inhibitory effects of THC on MDMA-induced microglial activation. This result suggests that THC activates CB_1 receptor and reduces MDMA-induced hyperthermia. Consequently, cell damage is prevented and microglia is not activated. However, the involvement of CB_2 receptors can not be fully discarded. Indeed, THC significantly reduced but did not completely reverse MDMA-induced microglial activation in CB_2 knockout mice. This result indicates that THC also exerts its neuroprotective effects through the activation of CB_2 receptors. The activation of this receptor inhibits microglial cells activation, preventing the release of proinflammatory cytokines, reducing the expression of iNOS, and contributing to the reduction of cell damage. Surprisingly, pretreatment with the CB_2 receptor agonist JWH-133 did not attenuate MDMA-induced microglial activation. This result is not necessarily contradictory to the one observed with CB_2 receptor knockout mice. JWH-133, as well as THC, was administered before MDMA. Indeed, maximum microglial activation takes place two days after MDMA treatment. At that time JWH-133 had already been cleared from the organism, and can not activate CB_2 receptors and inhibit microglial activation. Thus, we can not rule out that a prolonged treatment with JWH-133 may attenuate MDMA-induced microglial activation.

In our studies we observed that THC also prevented MDMA-induced astrocytes activation. Both microglia and astrocytes are activated after MDMA treatment, as a consequence of cell damage and excessive glutamatergic transmission. Several studies suggest that MDMA neurotoxicity is caused not only by oxidative stress, but by an

excessive glutamatergic transmission (Quinton and Yamamoto, 2006; Cadet et al., 2007), As in the case of microglia, THC might reduce MDMA-induced hyperthermia attenuating cell damage, and as a consequence, reduce astrocytes activation. However, a direct effect of THC on astrocytes should not be ruled out since these cells express CB_1 receptor (Sanchez et al., 1998). Then, we explored the involvement of CB_1 and CB_2 receptors on THC reduction of MDMA-induced astrocytes activation. With that purpose, astrocytes activation was evaluated in CB_1, CB_2 and double knockout mice after treatment with MDMA and THC. THC was unable to protect CB_1 and CB_1/CB_2 knockout mice against MDMA-induced astrocytes activation. These data indicate that CB_1 receptors mediate THC-induced inhibition of astrocytes in MDMA-treated mice. As mentioned before, hypothermic effects of THC, which prevent neuronal damage, are mediated by CB_1 receptors. Moreover, astroglial cells express CB_1 receptors, which mediates astrocytes inhibition by cannabinoid agonist (Molina-Holgado et al., 2002; Sheng et al., 2005). Additionally, excessive glutamatergic transmission also contributes to MDMA neurotoxicity (Quinton and Yamamoto, 2006; Cadet et al., 2007). Astrocytes are involved in the modulation of glutamatergic transmission, and CB_1 receptors play an important role in this process (Navarrete and Araque, 2008). Thus, excess of glutamate transmission caused by MDMA might contribute to activate astrocytes, and THC might attenuate their activation through CB_1 receptor. Surprisingly, astrocytes activation in CB_2 knockout animals was just partially reversed by THC, such as MDMA-induced microglial activation. There is wide evidence that cytokines released by microglia promote astrocytes activation. Therefore, microglia that remains activated in CB_2 knockout mice even after THC treatment might be inducing the activation of astrocytes. Altogether, these data suggest that the reduction of MDMA-induced hyperthermia by THC is the main responsible for the observed attenuation in microglia and astrocytes activation. However, the activation of CB_2 receptors in microglia and CB_1 receptors in astrocytes may directly contribute to this neuroprotective effect by inhibiting the overactivation of these cells and the release of proinflammatory mediators.

Hyperthermia, NOS overexpression or microglia and astrocytes activation are just indirect indicators of MDMA-induced neurotoxicity. The presence of these markers after MDMA treatment is associated with brain damage. However, we need to confirm that MDMA administration induced nerve terminal loss, and if high ambient temperature may aggravate this loss. To confirm that brain damage revealed by glial activation was associated with DA terminal loss, TH and DAT protein levels were assessed in MDMA-treated animals at both room temperature and high ambient temperature. TH and DAT are proteins constitutively expressed in DA terminals, and its reduction indicates DA

terminal loss. Animals treated with for one day MDMA at room temperature showed a significant increase in astrocytes and microglia activation, which was reversed by THC administration. However, no significant reduction in TH and DAT levels was observed. Likewise, animals treated with MDMA for three days at room temperature showed a significant increase in NOS levels, which was also reversed by THC treatment. Nevertheless, no significant reduction in TH and DAT levels was observed in these animals either. In contrast, animals treated with MDMA at high ambient temperature (27ºC) displayed a severe reduction in the levels of TH and DAT. Together, these results indicate that the administration of MDMA at room temperature, even repeatedly, caused a modest hyperthermia, and damage produced was not severe enough to cause detectable DA terminals loss. Conversely, different studies showed that MDMA caused DA loss in mice after administration at room temperature. For instance, some studies used different experimental conditions, such as female subjects (Miller and O'Callaghan, 1995) and Swiss mice (Colado et al., 2001), which may be more sensitive to MDMA. Aditionally, other studies used or higher doses of MDMA (O'Shea et al., 2001), which can cause damage not observed with lower doses. On the contrary, animals treated with MDMA at high ambient temperature showed an intense hyperthermia, causing a strong decrease in TH and DAT levels. Previous reports already described that MDMA-induced terminal damage was not observed at room temperature, whereas severe damage occurred at high ambient temperature (Malberg and Seiden, 1998). After that, the neuroprotective effects of THC on MDMA-induced DA terminal loss were evaluated. Hence, MDMA-treated animals housed at 27ºC and administered with THC showed a complete recovery of TH levels but only a partial recovery of DAT levels. DAT is a protein located in the extracellular membrane, so that damage in the cell membrane might reduce DAT levels. On the contrary, TH is an intracellular protein, so that its complete recovery after THC treatment indicates that the integrity of the intracellular space in DA terminals is maintained in MDMA-treated animals. Therefore, although THC does not completely reverse MDMA-induced DA damage at high ambient temperature, it strongly reduces this harm, and preserves the integrity of DA terminals.

In this study, we showed that THC administration reduced hyperthermia, NOS expression, glial activation, and DA terminal loss induced by MDMA. However, the ability of THC to reduce hyperthermia seems to be the main mechanism to protect against MDMA neurotoxicity. To confirm this hypothesis, we evaluated DA terminal degeneration in MPTP-treated mice. Unlike MDMA, MPTP-induced neurotoxicity is not enhanced by core or ambient temperature. According to our hypothesis, we observed that THC

exclusively prevented MDMA- but not MPTP-induced DA terminal loss (data not shown), confirming that THC prevents MDMA-induced neurotoxicity by reducing hyperthermia.

Harmful effects of MDMA on nerve terminals are usually translated into functional and behavioral deficits. As discussed before, MDMA-induced 5-HT terminal damage causes mood and cognitive disorders (Morgan, 2000). Although an increase in the anxiogenic-like responses have been observed in rats long time after MDMA treatment, MDMA-induced 5-HT terminal loss in cortex and hippocampus is not clearly associated with impairment in a specific animal behavior. However, neuronal damage occurring in MDMA-treated mice mainly causes DA terminals degeneration in the striatum, and as a consequence motor coordination impairment. Motor coordination is easily evaluated with the rotarod test, which is sensitive to damage in the basal ganglia and cerebellum, and to drugs that affect motor function. Indeed, other studies describe motor coordination impairment in the rotarod after MDMA administration (Jeng et al., 2006). We observed that animals treated with MDMA at high ambient temperature, and suffering severe DA nerve terminal damage, also showed impaired motor coordination in the rotarod. Conversely, rotarod performance of MDMA-treated animals pretreated with THC was similar to saline-treated animals. This result indicates that THC neuroprotective effects not only reduce damage in nerve terminals, but also improves the behavioral deficits derived from this damage.

THC and other cannabinoid derivatives showed neuroprotective effects in several neuroinflammatory and neurodegenerative diseases, including Alzheimer (Ramirez et al., 2005) and multiple sclerosis (Arévalo-Martin et al., 2003), usually by its anti-inflammatory properties. However, we report for the first time that THC exerts a neuroprotective action against amphetamine derivatives neurotoxicity, specifically MDMA. THC neuroprotection occurred mainly through the reduction of MDMA-induced hyperthermia. Interestingly, hyperthermia is the main factor contributing to aggravate the neurotoxicity of this drug, and its attenuation prevents the consequent oxidative stress, glial activation, terminal loss and behavioral impairment. These two drugs are frequently consumed in combination, therefore, the present study may help to clarify the reason why polydrug users show improved neurological parameters when compared to pure MDMA users.

Conclusions

The work developed in the current thesis project allows us to draw the following conclusions:

1. CB_1 cannabinoid receptor is involved in the reinforcing but not the rewarding effects of MDMA. CB_1 receptors are not expressed in DA terminals in the NAc and do not modify either MDMA-induced DA release induced in this area or reward. However, CB_1 receptors are expressed in pyramidal neurons projecting from the PFC to the NAC, and mediating motivation required for MDMA reinforcement. Thus, CB_1 receptor may be a pharmacological target for the treatment of addiction, since is a common neurobiological substrate for all drugs of abuse.

2. CB_1 cannabinoid receptor participates in the hyperlocomotion induced by MDMA. The acute pharmacological blockade of this receptor does not affect MDMA-induced hyperlocomotion, but the genetic ablation of this receptor reduces this effect. This result indicates that the permanent lack of CB_1 receptor produce neuroadaptations that affect the circuits involved in MDMA induced hyperlocomotion.

3. CB_1 cannabinoid receptor participates in the hyperthermia induced by MDMA. 5-HT receptors that mediate MDMA-induced hyperthermia might be altered in mice lacking CB_1 receptor.

4. CB_1 cannabinoid receptors play a crucial role in the anxiogenic-like effects induced by MDMA. Alterations in 5-HT receptors that mediate anxiety and other emotional behaviors are modified in CB_1 receptor deficient mice, and may impair the expression of MDMA-induced anxiogenic-like effects.

5. Both acute and chronic treatment with MDMA inhibits THC withdrawal syndrome. Enhancement of 5-HT release, but not DA release, induced by MDMA seems to be responsible for this inhibition, as shown by *in vivo* microdialysis studies. To reduce withdrawal syndrome, 5-HT system is compensating neuroadaptations produced by the chronic administration of MDMA, and not intercept the effect of rimonabant triggering withdrawal syndrome. These results suggest that combination of THC with MDMA may

ameliorate the symptoms produced by a prolonged exposure to cannabinoids. This fact may explain the frequent combination of cannabis and MDMA observed among users.

6. THC prevents MDMA induced DA terminal loss in mice. The principal mechanism of THC neuroprotective effects is the reduction of MDMA-induced hyperthermia through the activation of CB_1 receptor. However, the inhibition of microglial through the activation of CB_2 receptors, or the inhibition of NOS expression carried out by THC may also contribute to these neuroprotective effects. Moreover, THC not only prevents MDMA-induced loss of striatum DA terminals, but also motor coordination impairment due to the degeneration of these nerve terminals. Thus, the frequent use of THC observed among MDMA users may be beneficial rather than deleterious, and may protect heavy MDMA users against neuronal damage and its functional consequences.

7. Taken together, these results suggest that the negative effects cannabis and MDMA use are ameliorated when these drugs are consumed together.

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Appendix

Soria G, Mendizábal V, Touriño C, Robledo P, Ledent C, Parmentier M, Maldonado R, Valverde O.
Lack of CB1 cannabinoid receptor impairs cocaine self-administration.
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Lack of FAAH promotes energy storage and enhances the motivation for food.

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Abstract

FAAH is the main degrading enzyme of the fatty acid ethanolamides anandamide (AEA) and oleoylethanolamide (OEA), which have opposite effects on food intake and energy balance. AEA is an endogenous ligand of CB_1 cannabinoid receptors that enhances food intake and energy storage, whereas OEA binds to PPAR-α receptors, reducing food intake and promoting lipolysis. To elucidate the role of FAAH in food intake and energy balance, we have evaluated different metabolic and behavioral responses related to feeding in mutant mice deficient in FAAH and their wild-type littermates. Total daily food intake was similar in both genotypes, but the circadian oscillations in food consumption were enhanced in FAAH knockout mice. The reinforcing and motivational effects of food were also enhanced in FAAH mutant mice as revealed by operant behavioral paradigms. These behavioral responses were reversed by the administration of the selective CB_1 cannabinoid antagonist rimonabant. Further, body weight, total amount of adipose tissue, and triglyceride content in the liver and adipose tissue were increased in FAAH knockout mice. In addition, both AEA and OEA levels were increased in hypothalamus, small intestine and liver in mutants. These results indicate that the lack of FAAH predominantly promotes energy storage by food intake-independent mechanisms, through the enhancement of AEA levels rather than promoting the anorexic effects of OEA.

Introduction

The orexigenic properties of Cannabis sativa derivatives have been known for centuries, but their psychoactive side effects represent a serious limitation for the clinical use. The orexigenic effects of cannabinoids are mediated by CB1 cannabinoid receptors, which are involved in the regulation of food intake and energy balance (Cota, 2007). Thus, the CB1 receptor selective antagonist rimonabant reduces body weight and improves several metabolic parameters in animals (Colombo et al., 1998) and humans (Van Gaal et al., 2005). CB1 receptors are expressed in central and peripheral tissues involved in the control of food intake and metabolism (Cota et al., 2003). Thus, activation of CB1 receptors in the paraventricular nucleus of the hypothalamus (PVH) increases appetite (Jamshidi & Taylor, 2001), and in the limbic system enhances the incentive value of food (Kirkham et al., 2002; Thornton-Jones et al., 2005). Conversely, the stimulation of CB1 receptor in the small intestine inhibits the peripheral satiety signals transmitted through vagal sensory fibers to the PVH (Burdyga et al., 2004; Pertwee, 2001). CB1 receptor activation in peripheral tissues also promotes energy storage by food intake-independent mechanisms. Hence, the activation of CB1 receptors facilitates fatty acid storage in adipocytes (Bensaid et al., 2003; Cota et al., 2003), and liponeogenesis in the liver (Osei-Hyiaman et al., 2005b).

One of the endogenous ligands of CB1 receptors is anandamide (AEA). AEA stimulates appetite when administered systemically (Williams & Kirkham, 1999) and locally in the hypothalamus (Jamshidi & Taylor, 2001), and enhances the incentive value of food when administered into the nucleus accumbens (Mahler et al., 2007). AEA is degraded by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), which is widely distributed in the organs involved in food intake and energy balance such as brain, liver and small intestine (Ueda & Yamamoto, 2000). The endogenous levels of AEA are regulated by food intake. Thus, feeding decreases FAAH activity, enhancing AEA levels in the small intestine (Fu et al., 2007), adipose tissue, pancreas (Matias et al., 2006) and

liver (Osei-Hyiaman et al., 2005b). FAAH also metabolizes another fatty acid ethanolamide with opposite effects to AEA, namely oleoylethanolamide (OEA). OEA is a feeding-controlled signal, which activates peroxisome proliferator-activated receptor-α (PPAR-α). OEA is mainly synthesized in the small intestine. Peripheral OEA produces anorexic effects by activating the satiety signals forwarded from the vagal afferent neurons to the nucleus of the PVH (Lo Verme et al., 2005), and stimulates peripheral lipolysis by activating PPAR-α in adipocytes (Guzman et al., 2004). However, OEA was also detected in hypothalamus (Murillo-Rodriguez et al., 2006), where might modulate food intake (Chakravarthy et al., 2007).

Previous studies have reported the specific role of AEA and OEA in food intake and metabolism (Kunos, 2007; Lo Verme et al., 2005). However, the role of FAAH in the regulation of feeding and energy expenditure remains uncertain. In the present study, mutant mice deficient in FAAH have been used to investigate the role of this enzyme in the control of body weight, feeding behavior, motivation for food, and lipid turnover.

Materials and Methods

Animals

FAAH knockout mice were generated from 129/SvJ embryonic stem cells, and obtained by intercrossing 129SvJ-C57BL/6 FAAH heterozygous mice, as described previously (Cravatt et al., 2001). FAAH knockout mice and their wild-type littermates were backcrossed into the C57BL/6J//Nnt (Nicotinamide nucleotide transhydrogenase) substrain for at least five generations to limit potential strain-dependent allelic variations that might contribute to behavioral and physiological differences. The backcrossing procedure was continued, being all experimental subjects derived from a backcross generation. Wild-type littermates were used as controls. The number of litters per breeding pair was 4.00 ± 0.43 in wild-type and 4.29 ± 0.42 in FAAH knockout animals, and the number of pups per litter was 7.24 ± 0.72 in wild-type and 8.00 ± 0.41 in FAAH knockout mice. Thus, differences in the breeding capacity were not statistically significant between wild-type and mutants. Male FAAH knockout and wild-type mice weighing 18–25 g at the beginning of the experiment were used in this study. They were individually housed in a controlled temperature (22 ± 2℃) and h umidity (55% to 65%) room with a 12 h light/dark cycle (lights on at 7:00 AM and off at 7:00 PM). They were kept on a standard chow diet (Pro-lab RMH 2500; PMI Nutrition International, Brentwood, MO) or a high-fat diet (60 kcal % fat; D12492; Research Diets, New Brunswick, NJ) for 11 weeks. Body weight and food intake were measured weekly. Water and food were available ad libitum except for the operant self-administration studies. In operant studies consisted food access was restricted to 3.5 g per day during the acquisition period, which usually reduced schedule reduced body weight to 85% from the original. In case of overweight (more than 90% of the original weight) 0.5 g of food was subtracted and in case of underweight (less than 80% of the original weight) 0.5 g of food was added until obtaining the appropriate reduction of body weight. All experimental procedures were approved by the local ethical committees (Institutional Animal Care and Use Committee of the

University of California, Irvine and CEEA-IMAS-UPF) and carried out in strict accordance with the National Institutes of Health and the European Communities Directive 86/609/EEC guidelines for care and use of experimental animals.

Drugs

The selective CB1 receptor antagonist rimonabant was a kind gift from Sanofi-Aventis (Montpellier, France). Rimonabant was administered at the dose of 5 mg/kg and prepared in a solution containing 5% polyethylene glycol (PEG-400) (Sigma-Aldrich, Spain), 5% Tween-80 (Sigma-Aldrich, Spain) and 90% saline (0.9%). The same solution without rimonabant was used as vehicle. It was injected by the intraperitoneal route (i.p.) in a volume of injection of 0.1 ml/10 g body weight.

Analysis of Feeding Behavior

Apparatus. Food intake parameters and locomotor activity were recorded with an automated system (Scipro Inc, NY, USA), as previously described (Gaetani et al., 2003). The system consisted of 24 cages equipped with baskets connected to weight sensors. The baskets contained standard or high-fat pellets and were accessible to the mice through a hole in the wire lid of the cage. Each time food was removed from the basket, the computer recorded the duration of the event, the amount of food retrieved, and the time at which the event occurred. Weight variations were monitored every second and detection limit was set at 0.5 g of food and 1 min between eating episodes.

Feeding behavior. Mice were habituated to the test cages for 3 days and average feeding behavior was calculated. Food intake was recorded for 20 h and the following parameters were measured: locomotor activity, dark and light period average food intake (g/100 g BW/h), first meal latency, first and average meal size (g/100 g BW) expressed as the amount of food consumed during a meal, first and average post-meal interval (min) measured as the time interval separating two consecutive meals, first and average satiety

ratio (min/g/100 g BW) measured as the ratio between post-meal interval and meal size, and number of meals consumed during the test period with a minimum inter-response interval separating two meals of 10 min.

Tissue dissection

After 18 h of food deprivation, mice exposed to a high-fat diet for 12 weeks were slightly anesthetized with halothane and decapitated. Hypothalamus, liver, duodenum and jejunum were removed within approximately 30 sec from decapitation, frozen in dry ice, and stored at -80℃ until analyses. Retroperitoneal fat tissue was removed and weighed to determine differences in adipose tissue accumulation.

Lipid quantification

Total triacylglycerols (TAGs) levels were measured in retroperitoneal adipose tissue and liver homogenates. Tissue TAGs were extracted with chloroform:methanol:NaCl (1 M) (1:1:0.5), suspended in a solvent of tert-butanol:methanol:Triton X-114 (3:1:1), and measured using the Infinity TAG kit (Thermo Electron Corporation, Australia). Frozen liver, hypothalamus, duodenum and jejunum were weighed and homogenized in methanol (1 ml/100 mg of tissue) containing 2-[2H8]AG (Cayman Chemical, Ann Arbor, MI) and [2H4] fatty acid ethanolamides (FAEs) (prepared in the lab) as internal standards. Endocannabinoids and related lipids were extracted with methanol-chloroform (1:2, v/v). The chloroform phase was recovered, evaporated to dryness under a stream of N2, reconstituted in 1 ml of chloroform, and passed through silica Gel G columns. Briefly, columns were prepared by adding 1 ml of a chloroform silica Gel G (60-Å 230-400 Mesh ASTM; Whatman, Clifton, NJ) mixture (1:1, v/v) to 5’ Pasteur pipets, plugged with glass wool. The samples were loaded onto the columns and washed with 1 ml of chloroform. FAEs and N-acyl phosphatidylethanolamines (NAPEs) were eluted with 2 ml of chloroform/methanol mixture (9:1, v/v). The eluate was recovered and evaporated to

dryness under N2, reconstituted in a mixture of chloroform/methanol (1:3 v/v) and transferred to 2.0 ml screw top vials with 0.1 ml glass inserts to be injected into the HPLC/MS. FAEs and 2-AG were quantified using an isotope dilution liquid chromatography/mass spectrometry (LC/MS) assay in positive ionization mode, as previously reported (Giuffrida et al., 2000).

Operant Food Self-Administration

Apparatus. The food self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT, Medical Associates, GA, USA) equipped with two levers that were counterbalanced in the different chambers as active and inactive lever. Responding on the active lever resulted in a food pellet delivery, while responding on the inactive lever had no consequences. The chambers were placed in sound and light-attenuated boxes equipped with fans to provide ventilation and ambient noise. A food dispenser between the two levers permitted food pellets delivery when required. A stimulus light, located above the active lever, was paired contingently with the delivery of the food.

Food-maintained behavior. First, FAAH knockout mice and wild-type littermates were food restricted (3.5 ± 0.5 g of food were provided daily) for the whole acquisition period of food-maintained operant behavior. Water was available ad libitum during all the experiment. Four days after starting food restriction, mice were trained in the operant chambers to respond for food pellets (Noyes Precision Pellets, Research Diets Inc, USA). Self-administration sessions (1 h daily) were conducted every day. The house light was on at the beginning of the session for 3 s and off during the remaining period of the session. First, mice were trained under a fixed ratio 1 (FR1) schedule of reinforcement. Each reinforcement was followed by a 10 s time-out period. During this 10 s period, the cue light was off and no reward was provided on the active lever. Responses on the inactive lever and all the responses during the 10 s time-out period were also recorded.

The session was terminated after 100 reinforcers were delivered or after 1 h, whichever occurred first. After each session, mice were returned to their homecages. The criteria for the acquisition were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability), with at least 75% responding on the active lever, and a minimum of 10 reinforcers per session. Once all these acquisition criteria were achieved, food restriction was ended and standard chow was available ad libitum in the homecage. Animals that acquired were distributed in 3 different counterbalanced groups that were trained to obtain chocolate, fat or standard, pellets under an FR1 schedule of reinforcement. Animals were then exposed to chocolate and fat pellets for the first time. When animals achieved the acquisition criteria as above, the reinforcement schedule was changed to FR5. The same criteria were used to move mice from FR5 to the progressive ratio (PR) schedule in which the response requirement to earn a pellet escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The PR session lasted for 4 h or until mice did not complete the ratio for delivery of one reinforcer within 1 h, and was performed only once. The breaking point to extinguish self-administration behavior was determined in each animal. After animals finished the PR with the first assigned type of pellets, another FR1-FR5-PR experimental sequence was started to train the animals to obtain a second type of pellets, and a last FR1-FR5-PR experimental sequence was then completed to test the third type of pellets. The sequence order to evaluate the different kinds of food in the three counterbalanced groups followed a Latin square design. The first group followed a standard-chocolate-high-fat food sequence, the second group followed a chocolate-high-fat-standard food sequence, and the third group followed a high-fat-standard-chocolate sequence. The previous exposure to one type of food did not interfere on the performance with the other types of food, as revealed by the absence of a significant effect in the order of food exposure on the one-way ANOVA performed with the data obtained using the Latin

square design (data not shown). Mice were stabilized on FR1 and on FR5 before moving to the corresponding PR schedule.

In a second experiment, the effects of the CB_1 receptor antagonist rimonabant on the reinforcing properties and motivational strength of chocolate pellets were evaluated in FAAH knockout mice and wild-type littermates. After the acquisition of food-maintained self-administration under an FR5 schedule, as in the previous experiment, animals were pretreated with vehicle 30 min before a new FR5 self-administration session. This procedure was repeated for 2 consecutive days to habituate animals to the injection. Only values obtained on the second day of vehicle administration were considered for the statistical analysis. On the next day, animals received an acute injection of rimonabant (5 mg/kg, i.p.) 30 min before starting the FR5 session. The PR sessions were performed after the FR5 determinations in 2 consecutive days. On the first day, mice received vehicle, whereas the following day rimonabant (5 mg/kg, i.p.) was acutely administered both 30 min before the session.

Statistical analysis

Body weight and food intake between FAAH knockout and wild-type animals under standard or high fat diet were compared by within-subjects three-way ANOVA followed by subsequent two-way ANOVAs. Feeding behavior, incentive values of the different types of food, and rimonabant effects on the reinforcing and motivational effects of chocolate pellets between FAAH knockout and wild-type littermates were compared by a within-subjects two-way ANOVA, followed by one-way ANOVA or post hoc comparisons (Dunnett's test) for individual differences when required. Unpaired two-tailed Student t-test was used to evaluate differences between genotypes in the initial body weight, meal parameters, locomotor activity, fat mass, adipose tissue and liver TAG levels, and AEA, OEA and 2-AG levels. In all the experiments, differences were considered significant if the probability of error was less than 5%.

Results

FAAH knockout mice show similar food intake but enhanced body weight compared to wild-type littermates

FAAH knockout mice and their wild-type littermates were fed ad libitum on a standard or high-fat diet for 7 weeks, and body weights were recorded weekly. FAAH knockout mice showed higher body weight than wild-types from the beginning of the experiment ($p < 0.001$), and maintained a significantly enhanced body weight throughout the experimental sequence (Figure 1a). Differences in body weight were compared between FAAH knockout and wild-type animals under standard and high-fat diet. Three-way ANOVA revealed a significant effect of the interaction between age, diet and genotype ($F(6, 216) = 2.740$, $p < 0.05$). Then, differences between diet and genotype were calculated by subsequent two-way ANOVA (Table 1). A significant difference in the body weight of mice under standard diet or high-fat diet was observed between genotypes. In animals under standard diet this difference in body weigh was similar from the beginning to the end of the experiment. In contrast, body weight differences between FAAH knockouts and wild-types under high fat diet were increasing with time. Furthermore, the body weight increase resulting from the exposure to high-fat diet is significantly enhanced in FAAH knockout mice than in wild-type animals, when compared to the same genotype under a standard diet. This result suggests a higher sensitivity of FAAH knockout mice to gain weight under a high-fat diet when compared to their wild-type littermates.

Food intake of FAAH knockout and wild-type animals under standard and high-fat diet was also observed. Three-way ANOVA revealed no significant interaction between week, diet and genotype. Thus, no significant differences between genotypes were observed under either a standard diet or a high-fat diet (Figure 1b). To determine whether differences in physical activity were contributing to the enhanced body weight of FAAH knockout mice, locomotor activity was measured in both genotypes. FAAH knockout and wild-type mice showed circadian variations in locomotor activity, both increasing the

activity during the dark period. A similar locomotor activity pattern was observed in FAAH knockout and wild-type littermates during the whole circadian period, and no differences between genotypes were revealed in any of the locomotor measurements (Table 1).

FAAH knockout mice show increased food intake during the dark period and decreased food intake during the light period

Hourly food intake and differences in feeding behavior were evaluated in FAAH knockout and wild-type mice. Both, genotypes consumed a higher amount of standard food during the dark period than during the light period, but no differences between genotypes were observed (Figure 2a & c; Table 1). However, significant differences between genotypes were observed when animals were fed with high-fat diet (Figure 2 b & d; Table 1). Wild-type mice consumed a similar amount of high-fat food during the dark and light cycles ($F(1, 24) = 0.019$, n.s.). However, FAAH knockout mice consumed higher amounts of food during the light than during the dark cycle ($F(1, 24) = 153.082$, $p < 0.001$), as occurred with standard diet. Thus, a significant increase in the consumption of high-fat food was observed during the dark cycle in FAAH knockout animals ($F(1, 24) = 8.631$, $p < 0.01$), and this difference was progressively reduced through the light cycle ($F(1, 24) = 21.704$, $p < 0.001$). Eventually, total amount of food intake at the end of the day was similar between wild-type and knockouts.

Analysis of first meal parameters revealed a significant decrease of the first satiety ratio in FAAH knockout animals under a high-fat diet ($p < 0.05$) when compared to wild-type littermates, but no genotype differences were observed in the first meal latency, first and average meal size, first and average post-meal interval, average satiety ratio, and total number of meals. In animals exposed to a standard diet no differences between genotypes were revealed in any of these parameters (data not shown).

Enhanced operant performance and motivation for food in FAAH knockout mice

FAAH knockouts and their wild-type littermates were kept under food restriction and trained to self-administer food under an FR1 schedule of reinforcement for 20 days. The reinforcing effects and the motivation to obtain different types of food (standard pellets, fat pellets or chocolate pellets) were evaluated under FR1, FR5 and PR schedules of reinforcement in mice that had previously achieved the self-administration criteria (Figure 3; Table 1). The time of re-acquisition was similar for the different types of food in both genotypes. In wild-type animals, the number of responses during the achievement of the FR1 criteria was similar for standard, chocolate and fat pellets ($F(2, 36) = 1.763$, n.s.). When wild-type animals were trained under an FR5 schedule, the number of active responses during the achievement of the acquisition criteria was different depending on the type of food ($F(2, 36) = 6.846$, $p < 0.01$). Thus, post hoc analysis showed that the number of responses for chocolate was significantly higher than for standard pellets ($p < 0.01$). In wild-type animals, significant differences were also revealed in the breaking point for the different types of food obtained in the PR schedule ($F(2, 36) = 3.738$, $p < 0.05$). Subsequent post hoc analysis showed that the breaking point for chocolate was significantly higher than for standard pellets ($p < 0.05$).

In FAAH knockout animals, the number of responses during the achievement of the FR1 criteria was different for standard, chocolate and fat pellets ($F(2, 45) = 3.745$, $p < 0.05$). Subsequent post hoc analysis indicated that the number of active responses for chocolate was significantly enhanced compared to standard pellets ($p < 0.05$). When FAAH knockout mice were trained under an FR5 schedule, the number of active responses during the achievement of the acquisition criteria was also different depending on the type of food ($F(2, 45) = 15.182$, $p < 0.001$). Thus, subsequent post hoc analysis also showed that the number of responses to obtain chocolate was significantly higher than for standard pellets ($p < 0.001$). In FAAH deficient animals significant differences were also revealed in the breaking point for the different types of food during the PR

schedule ($F(2, 45) = 14.083$, $p < 0.001$). Subsequent post hoc analysis showed that the breaking point for chocolate was significantly higher than for standard pellets ($p < 0.001$). Comparisons between genotypes revealed that the number of active responses to obtain chocolate ($F(1, 27) = 20.564$, $p < 0.001$) and standard ($F(1, 27) = 11.592$, $p < 0.01$) pellets was significantly higher in FAAH knockout mice in comparison to wild-type littermates under an FR1 schedule (Figure 3a). No differences between genotypes were revealed under an FR1 schedule in the number of active responses to obtain fat pellets. Similar differences between genotypes were revealed under an FR5 schedule, where FAAH knockout mice also showed a higher responding for standard ($F(1, 27) = 7.469$, $p < 0.05$) and chocolate ($F(1, 27) = 6.794$, $p < 0.05$) pellets, whereas no differences were shown when responding for fat pellets (Figure 3b). Under a PR schedule, significantly higher breaking points were observed in FAAH knockout mice than in wild-type littermates, when trained to obtain chocolate ($F(1, 27) = 18.598$, $p < 0.001$), high-fat ($F(1, 27) = 4.745$, $p < 0.05$) or standard ($F(1, 27) = 11.058$, $p < 0.01$) pellets (Figure 3c).

Rimonabant decreases operant performance and motivation for food in FAAH knockouts and wild-type littermates

The reinforcing properties of chocolate pellets were evaluated in FAAH knockouts and wild-type littermates under FR5 and PR schedules after vehicle and rimonabant acute administration (Figure 4). The administration of CB1 antagonist rimonabant reduced the performance of FAAH knockouts in the food-rewarding operant tasks to levels comparable to those of wild-types, as indicated by the significant interaction of treatment and genotype (Table 1). As in the previous experiment, FAAH knockout mice showed a higher number of responses under an FR5 schedule and a higher breaking point under a PR schedule than wild-type littermates after vehicle administration. Rimonabant administration significantly decreased the number of active responses in both wild-type ($F(1, 11) = 58.010$, $p < 0.001$) and FAAH knockout mice ($F(1, 14) = 157.073$, $p < 0.001$)

under an FR5 schedule (Figure 4a). Rimonabant also reduced the breaking point obtained under PR schedule in FAAH deficient mice ($F(1, 13) = 44.822$, $p < 0.001$) and wild-type littermates ($F(1, 10) = 24.777$, $p < 0.001$) (Figure 4b). No significant differences between genotypes were observed after the administration of rimonabant.

FAAH knockout mice have increased fat mass and lipid content

The total amount of retroperitoneal fat mass and TAGs content in adipose tissue and liver were measured in FAAH knockouts and wild-type littermates exposed to a high-fat diet (Figure 5). Mice lacking FAAH exhibited a higher amount of total visceral fat mass when compared to wild-type mice ($p < 0.001$) (Figure 5a). FAAH knockout animals also showed significantly higher levels of TAGs in fat tissue ($p < 0.001$) (Figure 5b) and liver ($p < 0.001$) than did wild-type littermates (Figure 5c).

FAAH knockout mice have enhanced levels of AEA and OEA, but not 2-AG

The levels of the two main endocannabinoids AEA and 2-AG, and OEA were analyzed in different tissues involved in feeding behavior and metabolism (hypothalamus, duodenum, jejunum and liver) from FAAH knockout mice and wild-type littermates exposed to a high-fat diet. AEA and OEA, which are degraded by FAAH, showed significant enhanced levels in the hypothalamus ($p < 0.001$) (Figure 6 a & c), duodenum ($p < 0.001$) (Figure 6 d & f), jejunum ($p < 0.001$) (Figure 6 g & i) and liver ($p < 0.001$) (Figure 6 j & l) in FAAH knockout mice when compared to wild-type littermates. However, 2-AG, which is not metabolized by FAAH, showed similar levels in all the central and peripheral tissues evaluated in both FAAH knockout and wild-type mice (Figure 6 b, e, h & k).

Discussion

In the present study knockout mice were used to determine the involvement of FAAH in the regulation of feeding behavior and energy balance. Pharmacological inhibition or genetic deletion of FAAH was reported to enhance the levels of two fatty acid ethanolamides, AEA and OEA (Cravatt et al., 2001; Fegley et al., 2005), which play an opposite role in the control of food intake and metabolism. Thus, AEA promotes food intake (Jamshidi & Taylor, 2001; Williams & Kirkham, 1999) and energy storage (Cota, 2008), whereas OEA exerts anorexic effects (Fu et al., 2003; Rodriguez de et al., 2001). The levels of both fatty acid ethanolamides were enhanced in the hypothalamus and small intestine of FAAH knockout animals. These mutants and their wild-type littermates showed similar food intake. AEA enhances feeding through the activation of CB1 receptors in the PVH, lateral hypothalamus, nucleus accumbens, brainstem, vagus nerve and gastrointestinal tract (Cota et al., 2003; Matias et al., 2006). On the other hand, OEA released in the small intestine activates PPAR-α (Fu, et al 2003), and transmits through the vagus nerve a satiety signal to the nucleus of the solitary tract and then to the PVH (Lo Verme et al., 2005). Our results suggest that the effects of high levels of AEA and OEA on food intake compensate each other in FAAH knockout mice, resulting in similar food consumption when compared to wild-type animals. Despite the similar food intake observed in both genotypes, a decrease in the first satiety ratio was revealed in FAAH knockout mice under a high-fat diet, but no differences were revealed in the other feeding parameters evaluated. In addition, while the pattern of standard food consumption was similar, significant differences in the circadian pattern of high-fat food consumption were observed between genotypes. Animals exposed to a high-fat diet exhibit a disruption in circadian regulation of feeding, leading to a consumption of extra calories during the light period, the usual restcaloric intake period, in agreement with previous studies (Kohsaka et al., 2007). Interestingly, the high-fat diet-induced disruption of the circadian feeding behavior was prevented in FAAH deficient animals. Several studies have reported a

circadian regulation of the endocannabinoid system with enhanced levels of AEA and decreased FAAH activity during the dark period (Valenti et al., 2004). Moreover, CB1 receptor knockout mice showed circadian alterations in the hypothalamic-pituitary-adrenal axis (Cota et al., 2007), which plays an important role in the regulation of food intake (Mastorakos & Zapanti, 2004). The endocannabinoid system modulates several orexigenic and anorexic hypothalamic peptides that are under a circadian control such as CRH (Cota et al., 2003), neuropeptide Y (Gamber et al., 2005), hypocretin, melanin-concentrating hormone (Huang et al., 2007), and cocaine- and amphetamine-regulated transcript (CART) (Osei-Hyiaman et al., 2005a). Interestingly, the circadian levels of these neuropeptides are altered by the exposure to a high-fat diet (Kohsaka et al., 2007). Together, these results suggest that AEA plays an important role in the circadian modulation of food intake, probably through the control of feeding-regulating neuropeptides.

Interestingly, FAAH-deficient mice showed an enhanced body weight in comparison to wild-type littermates from early age. Furthermore, FAAH knockout animals are more sensitive than wild-types to gain weight when fed with high-fat diet. The effects of high-fat diet exposure were mild in wild-type animals (129SvJ-C57BL/6), since 129 strain has been reported to be resistant to diet-induced obesity (Kokkotou et al., 2005). However, exposure to a high-fat diet caused a marked body weight enhancement in FAAH knockout mice. Both genotypes showed equivalent caloric intake, body temperature (Cravatt et al., 2001) and spontaneous locomotor activity. Hence, FAAH seems to enhance energy expenditure by food intake-independent mechanisms. FAAH-deficient mice showed the opposite phenotype than CB1 knockouts, which are leaner than wild-types under both standard and high-fat diet by food intake-independent mechanism (Cota et al., 2003; Ravinet et al., 2004). By contrast, PPAR-α null mice were heavier than wild-types only when exposed to high-fat diet (Fu et al., 2003). Then, OEA administration reduced body weight by a mechanism directly involving a reduction of caloric intake

(Rodriguez de et al., 2001). Therefore, FAAH may play an important role in the control of metabolism by mechanisms independent from food intake, where AEA exerts a predominant effect, leading to an enhanced body weight in FAAH deficient mice. In agreement, the amount of adipose tissue and the triglycerides levels in adipose tissue and liver were higher in FAAH knockout mice than in wild-types. The enhanced weight of FAAH knockout animals suggests an increased lipogenesis in these peripheral organs. Both, endocannabinoids and OEA modulate lipid turnover (Cota et al., 2003; Guzman et al., 2004), while CB1 receptors (Osei-Hyiaman et al., 2005b) and PPAR-α (Lefebvre et al., 2006) participate in liver fatty acid metabolism. AEA levels are increased in adipose tissue (Matias et al., 2006) and liver (Osei-Hyiaman et al., 2005b) of animals under a high-fat diet, whereas OEA levels in visceral adipose tissue were similar in obese and lean mice (Matias et al., 2007). Interestingly, animals lacking FAAH showed enhanced levels of both AEA and OEA in the liver. Therefore, AEA seems to play a predominant role over OEA in the control of liver lipogenesis, as revealed in FAAH knockout mice.

The reinforcing and motivational properties of food were studied in FAAH knockout mice by using an operant paradigm. The hypothalamus and the limbic system are two brain structures that are involved in the control of food intake. Central and peripheral signals are integrated in the hypothalamus to modulate appetite and satiety responses (Grossman, 1975). The limbic system provides the incentive value of food, and promotes food intake by enhancing motivation to eat palatable foods (Berthoud, 2004). In this study, the use of an operant paradigm allowed to evaluate the incentive value of a standard food, a highly palatable food (chocolate pellets), and a high-caloric food (fat pellets) in FAAH knockout and wild-type animals. The better performance of animals to obtain chocolate pellets rather than other types of food indicates a predominant effect of palatability over the caloric value in maintaining operant behavior in both FAAH knockout and wild-type mice. Genetic deletion of FAAH enhanced the performance of an operant behavior to obtain standard and chocolate pellets under different effort requirements (FR1

and FR5), which suggests an enhancement of the reinforcing properties of these types of food. However, the performance on the operant behavior to obtain high-caloric fat pellets was not modified in FAAH knockouts. On the other hand, the motivational strength for standard, chocolate and high-fat food evaluated as the breaking point obtained in PR sessions was enhanced in FAAH knockout mice. In spite of the increased motivation for food, the total food intake was not modified in FAAH knockouts, suggesting a predominant effect of energy signals from both central and peripheral tissues over the motivational signals leading to food intake in these animals.

The specific involvement of CB1 receptors in the enhanced motivation for food in FAAH knockouts was also investigated. Thus, the selective CB1 antagonist rimonabant was injected into animals trained to self-administer chocolate pellets, the type of food showing the highest reinforcing effect in the previous experiment. Rimonabant strongly reduced the number of responses on FR5 or PR schedules in both FAAH knockouts and wild-type mice. CB1 receptors play a crucial role in the reinforcing effects of palatable food and in the behavioral phenotype of FAAH knockouts. This involvement was indicated by a significant interaction between rimonabant treatment and genotype in the chocolate food self-administration and by the similar performance of both genotypes after rimonabant administration observed in the same paradigm. In agreement, CB1 receptor deletion and rimonabant administration were reported to markedly reduce the reinforcing effects of sweet, but not fat food (Thornton-Jones et al., 2005; Ward & Dykstra, 2005). Reinforcing effects of palatable food are mediated by the nucleus accumbens, which contains high levels of CB1 receptors (Matyas et al., 2006). Local administration of AEA in the nucleus accumbens enhances food rewarding effects (Mahler et al., 2007). Thus, the increased levels of AEA could be responsible for the enhanced motivation for palatable food of FAAH knockout animals through the activation of CB1 receptors in the nucleus accumbens.

In conclusion, the present results suggest that FAAH plays an important role in the control of energy balance. Targeted deletion of this enzyme enhances the endogenous levels of AEA and OEA in central and peripheral organs involved in food intake and energy metabolism. The effects of AEA and OEA on food intake appear to compensate each other, leading to a normal food intake in FAAH-deficient mutants. However, these mutants showed a significant alteration in the circadian properties of feeding behavior as well as an enhancement in the reinforcing effects and motivation to obtain food. In spite of the similar caloric intake, FAAH mutants have an enhanced body weight and fat content, which is probably due to a predominant effect of AEA over OEA on lipogenesis in peripheral organs. The strong ability of OEA to induce satiety and lipolysis suggest that additional metabolizing enzymes such as NAPE-PLD (Fu et al., 2008) may regulate the action of OEA levels on feeding and lipid metabolism. Sound evidence supports the predominant role of CB1 receptors in the overweight, enhanced motivation for food, and increased lipogenesis of FAAH knockout mice. Nevertheless, the OEA receptor PPAR-α and the transient receptor potential vanilloid type 1 (TRPV1) activated by AEA also participate in the control of energy balance (Fu et al., 2003; Motter & Ahern, 2008) Therefore, the contribution of these receptors to the phenotype of FAAH knockout animals can not be completely excluded, and further studies using TRPV1 and PPAR-α antagonists are required in FAAH knockout mice to elucidate this issue.

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Figure Legends

Figure 1. Body weight (a) and food intake (b) in FAAH knockout mice (black squares) (n = 10 to 12) and wild-type littermates (white circles) (n = 9 to 12) under standard and high-fat diet. Body weight and food intake were determined once a week during 7 and 9 consecutive weeks respectively. Body weight data are expressed as mean ± SEM of grams of body weight, and food intake data are expressed as mean ± SEM of grams of food consumed per 100 g of body weight.

Figure 2. Food intake in FAAH knockout mice (black bars) (n = 10 to 12) and wild-type littermates (white bars) (n = 9 to 12) hourly (a and c) and during the dark and light periods (b and d) under standard and high-fat diet. Food intake was measured once per hour during 20 consecutive hours. Data are expressed as mean ± SEM of average grams of food consumed per 100 g of body weight per hour. □□□ $p < 0.001$, when compared with the dark cycle of the same genotype. ***** $p < 0.05$, ****** $p < 0.01$, ******* $p < 0.001$, comparisons between genotypes (one-way ANOVA).

Figure 3. Operant self-administration of standard, chocolate and high-fat pellets in FAAH knockout mice (black bars) (n = 14) and wild-type littermates (white bars) (n = 15) under a fixed ratio 1 (FR1) (a), fixed ratio 5 (FR5) (b) and progressive ratio (PR) (c) schedules of reinforcement. FR1 and FR5 data are expressed as mean ± SEM of the average number of pellets obtained during the 3 days of the acquisition criteria. PR data are expressed as mean ± SEM of the breaking point achieved. □ $p < 0.05$, □□ $p < 0.01$, □□□ $p < 0.001$, when compared with the standard pellets of the same genotype (Dunnett's test).***** $p < 0.05$, ****** $p < 0.01$, ******* $p < 0.001$, comparisons between genotypes (one-way ANOVA).

Figure 4. Effects of acute rimonabant (5 mg/kg, i.p.) on operant self-administration of chocolate pellets in FAAH knockout mice (black bars) (n = 15) and wild-type littermates (white bars) (n = 12) under FR5 (a) and PR (b) schedules of reinforcement. Data are expressed as mean ± SEM of reinforcers obtained during 1 h in animals under FR5, and the breaking point achieved under PR. □□□ $p < 0.001$, when compared with the vehicle group of the same genotype, ** $p < 0.01$, comparisons between genotypes (one-way ANOVA).

Figure 5. Total amount of fat content (a) and analysis of triglycerides (TAG) levels in adipose tissue (b) and liver (c) in FAAH knockout mice (black bars) (n = 12) and wild-type littermates (white bars) (n = 12) under high-fat diet. Amount of adipose tissue data are expressed as mean ± SEM of grams of fat per kg of body weight, and TAG levels data are expressed as mean ± SEM of µg of TAG per grams of tissue. *** $p < 0.001$, comparisons between genotypes (Student t-test).

Figure 6. Anandamide (AEA), 2-arachidonoylglycerol (2-AG) and oleoylethanolamide (OEA) levels in hypothalamus, duodenum, jejunum and liver in FAAH knockout mice (black bars) (n = 12) and wild-type littermates (white bars) (n = 12) under a high-fat diet. Data are expressed as mean ± SEM of picomols of AEA, 2-AG or OEA per gram of tissue. *** $p < 0.001$, comparisons between genotypes (Student t-test).

Figure 1

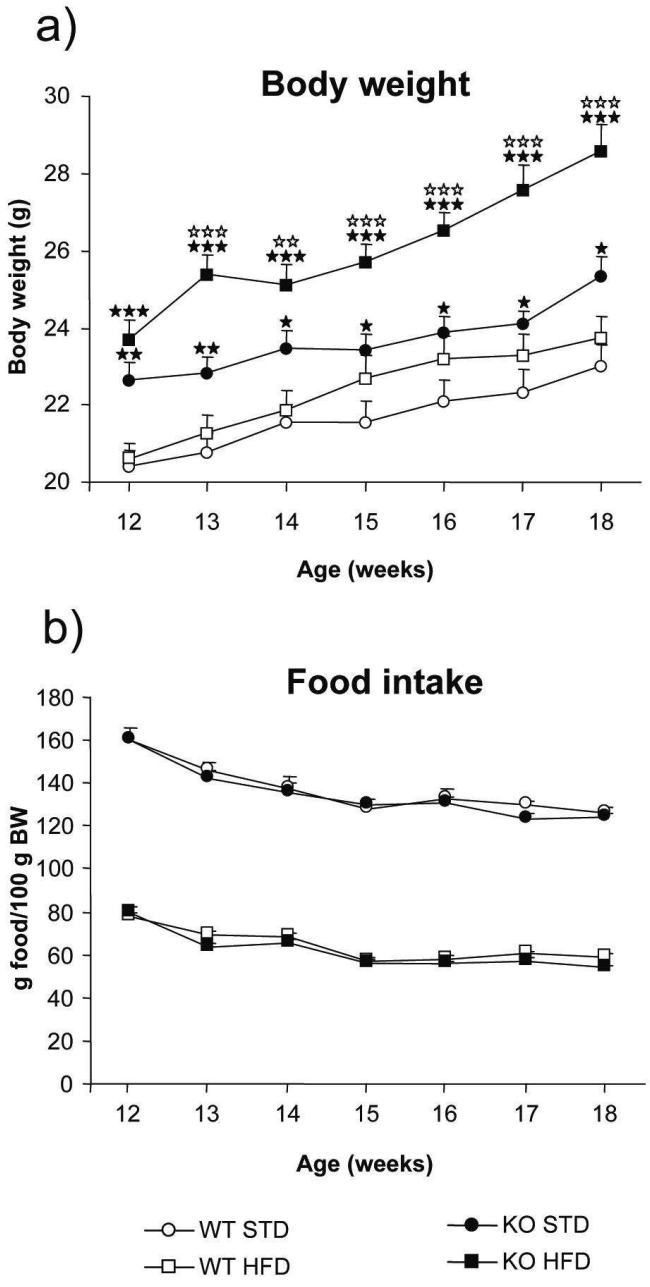
a)
Body weight
Body weight (g)
30
28
26
24
22
20
12
13
14
15
16
17
18
Age (weeks)
b)
Food intake
g food/100 g BW
180
160
140
120
100
80
60
40
20
0
12
13
14
15
16
17
18
Age (weeks)
WT STD
WT HFD
KO STD
KO HFD

Figure 2

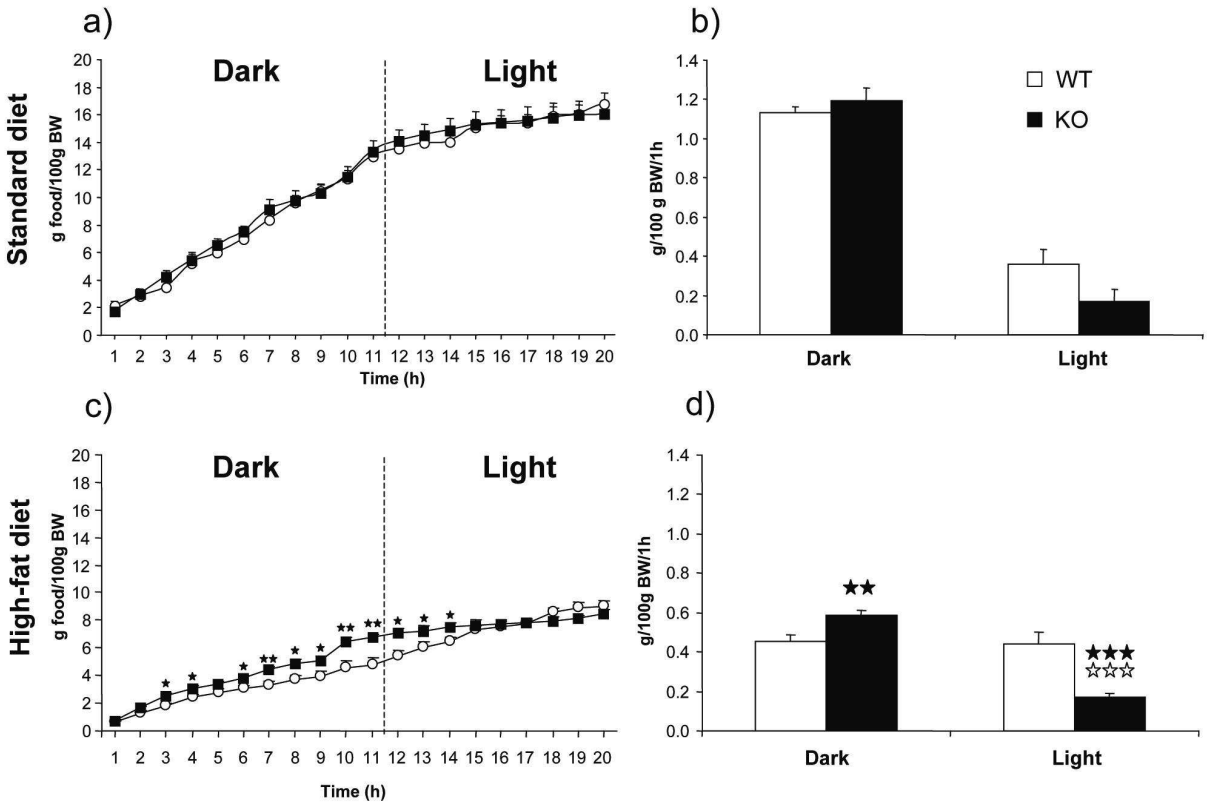
a)
Dark
Light
Standard diet
g food/100g BW
Time (h)
b)
WT
KO
g/100 g BW/1h
Dark
Light
c)
Dark
Light
High-fat diet
g food/100g BW
Time (h)
d)
g/100g BW/1h
Dark
Light

Figure 3

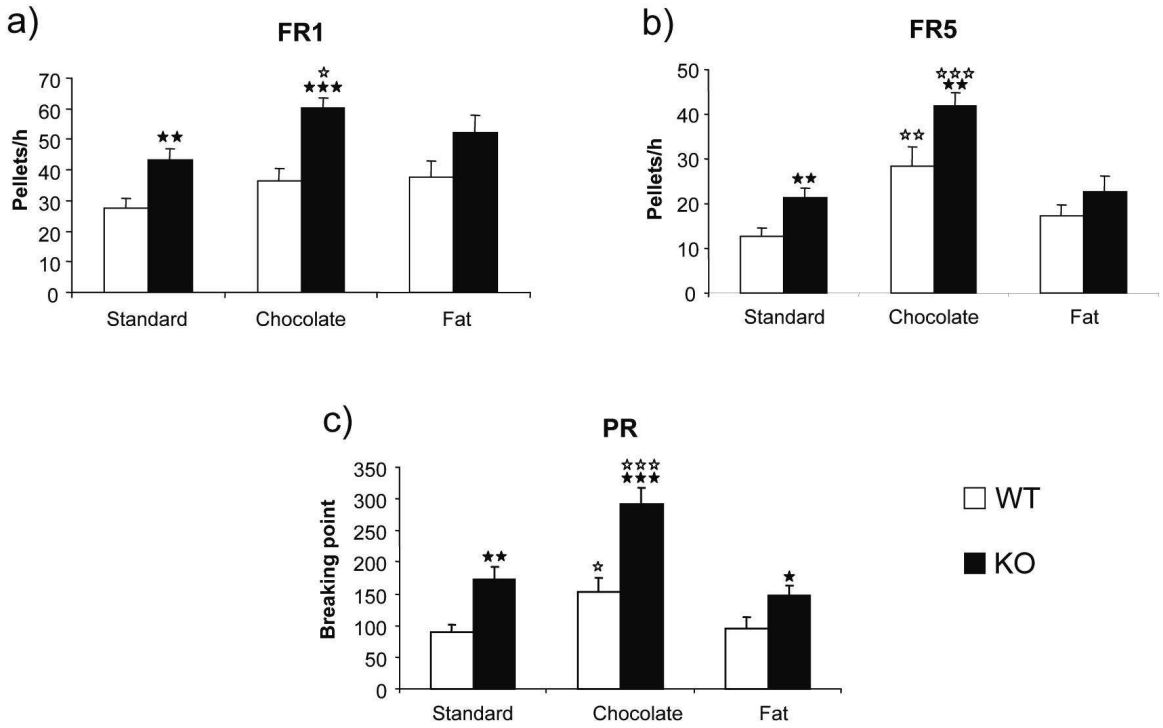
a)
FR1
Pellets/h
70
60
50
40
30
20
10
0
Standard
Chocolate
Fat
b)
FR5
Pellets/h
50
40
30
20
10
0
Standard
Chocolate
Fat
c)
PR
Breaking point
350
300
250
200
150
100
50
0
Standard
Chocolate
Fat
WT
KO

Figure 4

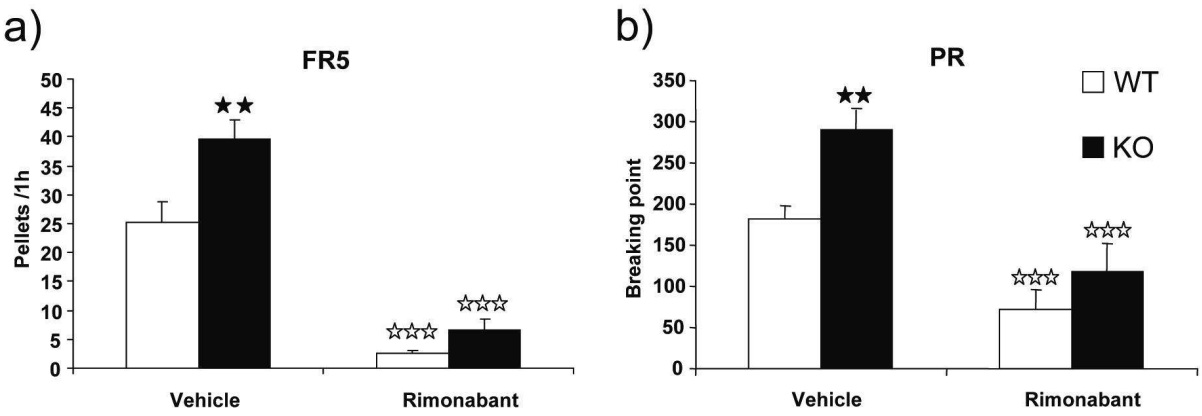
a)
FR5
Pellets /1h
50
45
40
35
30
25
20
15
10
5
0
Vehicle
Rimonabant
b)
PR
Breaking point
350
300
250
200
150
100
50
0
WT
KO
Vehicle
Rimonabant

Figure 5

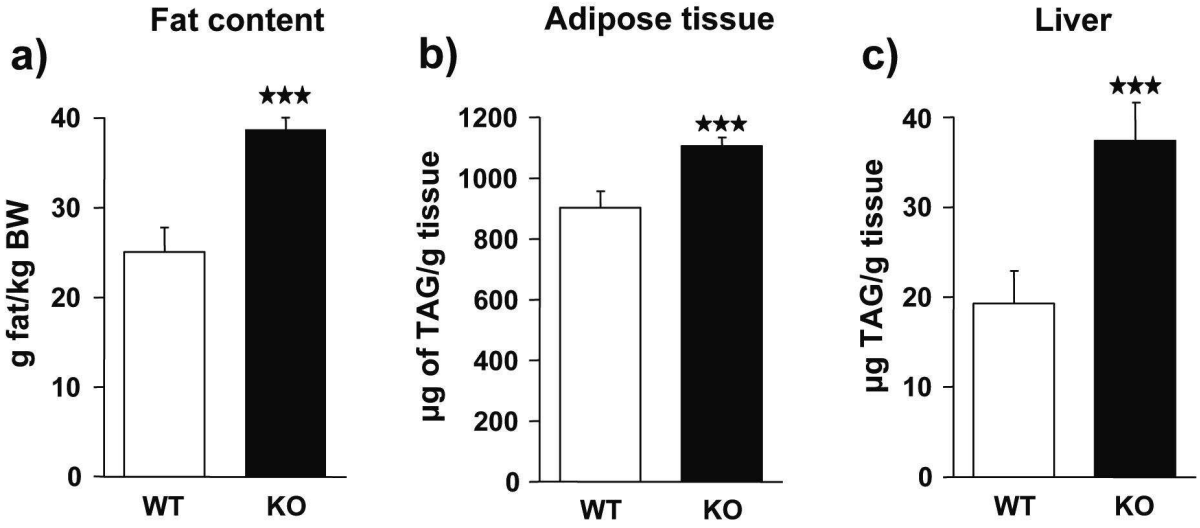
Fat content
a)
g fat/kg BW
40
30
20
10
0
★★★
WT
KO
Adipose tissue
b)
µg of TAG/g tissue
1200
1000
800
600
400
200
0
★★★
WT
KO
Liver
c)
µg TAG/g tissue
40
30
20
10
0
★★★
WT
KO

Figure 6

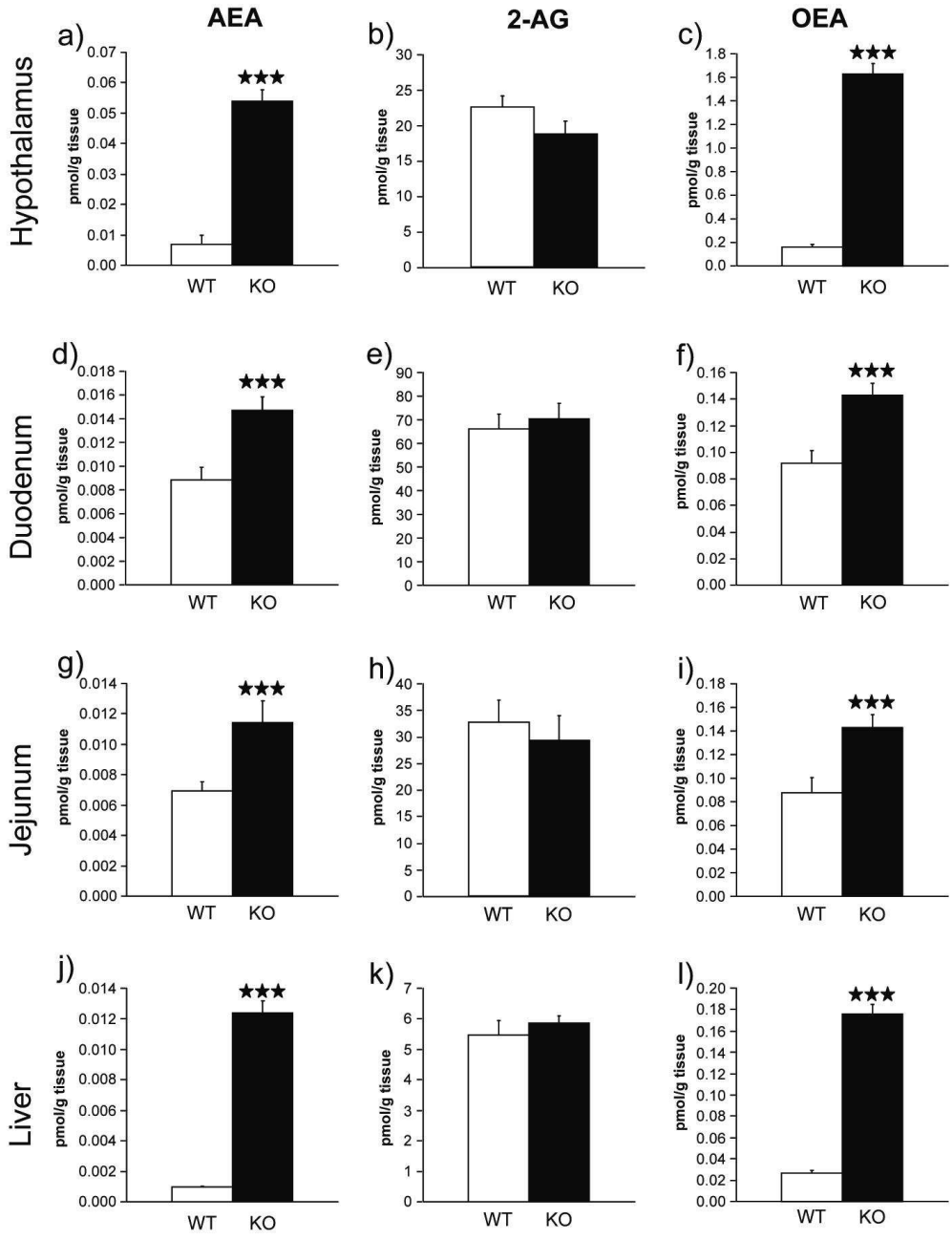
AEA
2-AG
OEA
Hypothalamus
Duodenum
Jejunum
Liver
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
pmol/g tissue
WT
KO
★★★

Table 1. Two-way ANOVA calculated for body weight and food intake, locomotor activity, feeding behavior and effects of rimonabant on chocolate pellets self-administration in FAAH knockout and wild-type mice.

		Two-way ANOVA					
		Age		Genotype		Interaction	
		F-value	p-value	F-value	p-value	F-value	p-value
Body weigh	Regular diet (WT vs KO)	$F_{(6, 90)} = 94.974$	< 0.001	$F_{(1, 15)} = 9.734$	< 0.01	$F_{(6, 90)} = 1.667$	n.s.
	High-fat diet (WT vs KO)	$F_{(6, 126)} = 82.260$	< 0.001	$F_{(1, 21)} = 27.534$	< 0.001	$F_{(6, 126)} = 5.294$	< 0.001
	WT (regular vs high-fat diet)	$F_{(6, 108)} = 71.441$	< 0.001	$F_{(1, 18)} = 0.892$	n.s.	$F_{(, 108)} = 2.398$	< 0.05
	KO (regular vs high-fat diet)	$F_{(6, 108)} = 68.844$	< 0.001	$F_{(1, 18)} = 1.689$	< 0.01	$F_{(6, 108)} = 7.697$	< 0.001
		Time		Genotype		Interaction	
		F-value	p-value	F-value	p-value	F-value	p-value
Feeding behavior (hour)	Regular diet	$F_{(19, 323)} = 280.471$	< 0.001	$F_{(1, 17)} = 0,093$	n.s.	$F_{(19, 323)} = 0,484$	n.s.
	High-fat diet	$F_{(19, 418)} = 293.304$	< 0.001	$F_{(1, 22)} = 3.599$	n.s.	$F_{(19, 418)} = 6.945$	< 0.001
Locomotor activity		$F_{(19, 418)} = 37.544$	< 0.001	$F_{(1, 22)} = 0.02$	n.s.	$F_{(19, 418)} = 0.034$	n.s.
		Cycle		Genotype		Interaction	
		F-value	p-value	F-value	p-value	F-value	p-value
Feeding behavior (cycle)	Regular diet	$F_{(1, 38)} = 4.311$	< 0.001	$F_{(1, 38)} = 0,220$	n.s.	$F_{(1, 38)} = 1.791$	n.s.
	High-fat diet	$F_{(1, 48)} = 32.721$	< 0.001	$F_{(1, 48)} = 3.218$	n.s.	$F_{(1, 48)} = 14.834$	< 0.001
		Food type		Genotype		Interaction	
		F-value	p-value	F-value	p-value	F-value	p-value
Chocolate self-administration	FR1	$F_{(2, 81)} = 4.762$	< 0.05	$F_{(1, 81)} = 26.551$	< 0.001	$F_{(2, 81)} = 0.621$	n.s.
	FR5	$F_{(2, 81)} = 20.446$	< 0.001	$F_{(1, 81)} = 13.236$	< 0.001	$F_{(2, 81)} = 0.898$	n.s.
	PR	$F_{(2, 81)} = 20.446$	< 0.001	$F_{(1, 81)} = 33.827$	< 0.001	$F_{(2, 81)} = 2.875$	n.s.
		Treatment		Genotype		Interaction	
		F-value	p-value	F-value	p-value	F-value	p-value
Chocolate self-administration	FR5	$F_{(1, 25)} = 178.864$	< 0.001	$F_{(1, 25)} = 9.358$	< 0.01	$F_{(1, 25)} = 6.175$	< 0.05
	PR	$F_{(1, 23)} = 63.844$	< 0.001	$F_{(1, 23)} = 4.523$	< 0.05	$F_{(1, 23)} = 4.306$	< 0.05

Two-way ANOVA with time (body weight, food intake and locomotor activity), cycle (feeding behavior) and treatment (chocolate self-administration) as within-subject and and genotype as between-subject factors. See Materials and methods for details. n.s.: non-significant; WT: wild-type; KO: knockout.