CB₁ CANNABINOID RECEPTOR MODULATES MDMA ACUTE RESPONSES AND REINFORCEMENT

Abbreviated title: CB₁ receptor and MDMA reward

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Abbreviations: 3, 4 - methylenedioxymethamphetamine: MDMA; Dopamine: DA; Serotonin: 5-HT; Δ^9 -tetrahydrocannabinol: THC; Conditioned place preference (CPP); Area under the curve (AUC).

ABSTRACT

Background: 3, 4-methylenedioxymethamphetamine (MDMA) is a popular recreational drug **widely abused by young people**. The endocannabinoid system is involved in the addictive processes induced by different drugs of abuse. However, the role of this system in the pharmacological effects of MDMA has not been yet clarified.

Methods: Locomotion, body temperature and anxiogenic-like responses were evaluated after acute MDMA administration in CB₁ knockout mice. Additionally, MDMA rewarding properties were investigated in the place conditioning and the intravenous self-administration paradigms. Extracellular levels of DA in the nucleus accumbens were also analyzed after a single administration of MDMA by in vivo microdialysis.

Results: Acute MDMA administration increased locomotor activity, body temperature and anxiogenic-like responses in wild type mice, but these responses were **lower** or abolished in knockout animals. MDMA produced similar conditioned place preference and increased dopamine extracellular levels in the nucleus accumbens in both genotypes. Nevertheless, CB₁ knockout mice failed to self-administer MDMA at any of the doses used.

Conclusions: These results indicate that CB₁ cannabinoid receptors play an important role in the acute prototypical effects of MDMA, and are essential in the acquisition of an operant behavior to self-administer this drug.

INTRODUCTION

The amphetamine derivative 3, 4-methylenedioxymethamphetamine (MDMA or ecstasy) is a popular recreational drug with a particular mechanism of action, different from classical psychostimulants since mainly acts on both dopamine (DA) and serotonin (5-HT) re-uptake systems (1). In humans, MDMA produces euphoria, empathy and increased energy, often accompanied by **post-drug** anxiety and agitation (2, 3). The prototypical pharmacological responses induced by MDMA in animals include hyperthermia (4), hyperlocomotion (5) and anxiety-like responses (6, 7). In addition, MDMA induces rewarding effects in different experimental models. Thus, this drug establishes conditioned place preference (CPP) in rats (8) and mice (9), and maintains operant intravenous self-administration in rats (10), mice (11) and monkeys (12, 13). In agreement, MDMA administration increases DA extracellular levels in the nucleus accumbens, as revealed by *in vivo* microdialysis experiments (14).

In humans, MDMA is frequently consumed in combination with cannabis (15). Several interactions between MDMA and Δ^9 -tetrahydrocannabinol (THC), the *Cannabis sativa* main psychoactive compound have been reported. Some animal studies showed MDMA-THC interactions in pharmacological responses such as locomotor activity, body temperature, anxiety-like effects and withdrawal syndrome (16, 17). THC produces its psychoactive effects activating the CB₁ cannabinoid receptor, which is mainly expressed in the central nervous system (18). Several physiological responses that are mediated by MDMA administration such as locomotor activity, body temperature, mood, learning and reward are also modulated by the endocannabinoid system (19, 20). This system interacts with a variety of neurotransmitters, including DA and 5-HT (20, 21), that are key components in the mechanism of action of MDMA. The endocannabinoid system also represents a common neurobiological substrate for the addictive properties of different drugs of abuse (22), including opiates (23), nicotine (24), ethanol (25) and cocaine (26).

In the present study, CB₁ receptor knockout mice were used to evaluate the role of this receptor in the acute pharmacological responses and reinforcing properties of MDMA.

MATERIAL AND METHODS

Animals

Male CB₁ knockout mice on a CD1 background and their wild type littermates, **and male CD1 mice (Charles River, France)** weighing 30–35 g at the beginning of the experiments were used in this study (23). Mice were housed in a temperature (21 ± 1 °C), humidity ($55 \pm 10\%$) and light-cycle controlled room. Food and water were available *ad libitum*. For the pharmacological studies, the light was on between 08:00 AM and 08:00 PM, and the experiments took place during the light phase. For the self-administration studies, mice were exposed to a reversed cycle, and the experiments took place during the dark phase. **A different cohort of animals was used in each experiment.** 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 (CEE-IMAS-UPF). All experiments were performed under blind conditions.

Drugs

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland), dissolved in 0.9% physiological saline and injected by intraperitoneal route (i.p.) in a volume of injection of 0.1 ml/10g body weight. 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 absolute ethanol and distilled water (1:9). This anesthetic mixture was administered in an injection volume of 0.2 ml/10g body weight by i.p. route.

Acute pharmacological responses

Locomotor responses induced by MDMA were evaluated by using locomotor activity boxes (9 x 20 x 11 cm) (Imetronic, Lyon France) in a low luminosity room (5 lux) with white noise, as previously reported (24). Locomotor activity was measured as the number of beam breaks on horizontal and vertical movements. Locomotor activity was recorded during one hour immediately after MDMA (5, 10, 20 and **30 mg/kg i.p.**) or saline injection in CB₁ knockout mice and their wild type littermates. **In order to evaluate MDMA potency**

depending on the route of administration, locomotor activity was also measured under the same experimental conditions in CD1 mice after MDMA (2.5, 5, 10 and 20 mg/kg i.p. or i.v.) or saline administration.

Rectal temperature was measured in each mouse by placing 3 cm of an electronic thermocouple flexible rectal probe in the mice rectum for 10 s (Panlab, Madrid, Spain). Basal temperature was measured 60 min before the injection. Temperature was also evaluated, 30, 60, 90, 120 and 180 min after MDMA (10, 20 and 30 mg/kg) or saline i.p. administration. Data is represented as the temperature increase from the basal temperature. **Area under the curve (AUC) was calculated by using a standard trapezoid method (27). The following equation was used:**

$$AUC = [(0.5 * (T_{30} + T_{60}) * t) + [(0.5 * (T_{60} + T_{90}) * t) + [(0.5 * (T_{90} + T_{120}) * t) + [(0.5 * (T_{120} + T_{180}) * t]$$

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

Elevated plus maze was performed as previously reported (28). During a 5-min observation session. Results were evaluated as percentage of the time spent in the open arms, and percentage of entries in the open arms. The observation sessions started 30 min after MDMA (1, 10 and 20 mg/kg, i.p.) or saline injection.

Place conditioning paradigm

The MDMA CPP was performed using a non-biased procedure, as previously reported (9). The apparatus is composed by two main square conditioning compartments (15 × 15 × 15 cm) separated by a neutral area (29). During the conditioning phase, mice were pre-treated with MDMA 10 mg/kg, i.p. or saline 30 min before being confined into the conditioning compartment for 30 min. Four pairings were carried out with MDMA and four pairings with saline on alternate days. A preference score was calculated for each animal as the difference between the time spent in the drug-paired compartment during the test and pre-conditioning phases.

Microdialysis procedure

Microdialysis studies were performed as previously described (26). Three days after cannula guide implantation 1 mm above the nucleus accumbens (**antero-posterior**, +1.60 mm; **medio-lateral**, -0.9 mm; **dorso-ventral**, -3.60 mm from bregma), the analytical probe was inserted (**Fig 5c**). Two days after probe implantation, animals were habituated to the experimental environment overnight. The following morning, Ringer's solution was pumped through the dialysis probe at a constant rate of 1 µl/min. Four consecutive 20-min dialysis samples were collected for the determination of baseline DA levels. Then, mice were injected with either saline or MDMA (10 or 20 mg/kg) and samples were collected every 20 min for 4 h. Dialysate samples (15 µl) were injected without any purification into a HPLC system to quantify DA as previously reported (30). At the end of the experiments, animals were killed, and brains were quickly removed and stored at -80°C in order to check the probe placement. Brains were cut using a cryostat in 20 µm serial coronal sections, which were then processed with cresyl violet and observed under a microscope. Only those animals that had been implanted correctly were included in the analysis. **To calculate AUC the following equation was used:**

$$AUC = [(0.5 * (St_0 + St_{20}) * t)] + [(0.5 * (St_{20} + St_{40})) * t] + \dots + [(0.5 * (St_m + St_n) * t)]$$

where St_n is the percentage of DA increase compared to basal levels, and t is the time (min) between the consecutive measurements.

Operant self-administration

The self-administration experiments were conducted as previously described (11). Operant responding was maintained by MDMA (0.03; 0.06; 0.125, and 0.25 mg/kg/infusion) delivered in 23.5 µl over 2 s. Mice were trained to nose-poke in order to receive a MDMA injection under a FR1 schedule of reinforcement for 10 days. Self-administration sessions (2 h daily) started with a priming injection of the appropriate dose of the drug. The number of reinforcers was limited to 100 infusions per session and each reinforcer was followed by a 30 s time-out period where active nose-poking had no consequences. The

stimulus light signaled delivery of the reinforcer. **A mouse was considered to acquire self-administration when it follows these criteria for at least three consecutive sessions: (a) the number of responses on the active hole exceeded 65 % the inactive hole, (b) the number of responses on the active hole was higher than 5, and (c) the mean deviation of the total number of reinforcers earned in three consecutive sessions was less than 30% (70% stability).**

Statistical analysis

Effects of MDMA on locomotor activity, anxiety-like behavior, CPP and AUC values were compared by using a between-subjects two-way ANOVA (genotype and treatment), followed by one-way ANOVA for individual differences and *post hoc* comparisons (Dunnett's test) when required. Body temperature and microdialysis data were analyzed using three-way ANOVA with treatment, genotype and time factors. Three-way ANOVA with hole and genotype as between group factors, and time as within group factor, was used to analyze the acquisition of MDMA self-administration at the different training doses. Between subjects two-way ANOVA with hole and genotype was calculated to compare the mean of nose-pokes performed during the last 3 acquisition days, followed by one-way ANOVA when required. Unpaired two-tailed Student t-test was used to compare the basal dopamine levels between genotypes. **ED₅₀ values to determine MDMA potency by i.p. or i.v. route were determined by non-linear regression analysis of the dose-response curves using PRISM (GraphPad, San Diego). Paired Student t-test was used to compare the time required to discriminate between active and inactive holes.** In all the experiments, differences were considered significant if the probability of error was less than 5%.

RESULTS

Comparative potency of MDMA administered by i.p. and i.v. route

MDMA (2.5, 5, 10 and 20 mg/kg) similarly increased locomotor activity when administered by i.p. or i.v. route. The ED₅₀ value (95% confidence limits) calculated for i.p. administered MDMA [5.61 mg/kg (4.354 – 6.866)] did not significantly differ from the ED₅₀ value calculated for i.v. administered MDMA [5.98 mg/kg (4.33 – 7.62)].

MDMA effects on locomotor activity

MDMA (5, 10, 20 and 30 mg/kg) increased locomotor activity in a dose-dependent manner in both genotypes as revealed by two-way ANOVA (Table 1). However, this increase was significantly **lower** in CB₁ knockout animals (Fig 1), as indicated by one-way ANOVA (genotype), when MDMA was administered at the dose of 10 ($F_{(1, 23)} = 27.068$, $p < 0.001$) and 20 mg/kg ($F_{(1, 19)} = 15.646$, $p < 0.001$) (Fig 1a). This attenuation was also observed for the vertical activity (rearing) since one-way ANOVA (genotype) revealed significant differences when MDMA was administered at the dose of 5 ($F_{(1, 22)} = 5.595$, $p < 0.05$), 10 ($F_{(1, 23)} = 8.299$, $p < 0.01$), 20 ($F_{(1, 19)} = 4.684$, $p < 0.05$) and 30 mg/kg ($F_{(1, 22)} = 6.478$, $p < 0.05$) (Fig 1b).

MDMA-induced hyperthermia

MDMA (10, 20 and 30 mg/kg) induced hyperthermia in a dose-dependent manner in both genotypes. However, the elevation of body temperature was significantly **lower** in CB₁ knockout animals (Fig 2) (Table 2). Two-way ANOVA calculated for the body temperature of animals treated with MDMA 20 mg/kg showed a significant effect of genotype ($F_{(1, 17)} = 9.577$, $p < 0.01$), time ($F_{(4, 68)} = 21.333$, $p < 0.001$) and interaction between both factors ($F_{(4, 68)} = 2.496$, $p = 0.05$) (Fig 2c). Similarly, two-way ANOVA calculated for the body temperature of mice treated with MDMA 30 mg/kg revealed an effect of genotype ($F_{(1, 17)} = 6.548$, $p < 0.05$), time ($F_{(4, 58)} = 8.877$, $p < 0.001$) and interaction between both factors ($F_{(4, 68)} = 2.519$, $p < 0.05$). No significant differences between genotypes were observed in animals treated with saline or MDMA 10 mg/kg. In order to compare the global effect of MDMA on body temperature, we calculated the AUC values for each dose evaluated (Fig 2e) (Table 1). One-way ANOVA

(genotype) revealed significant differences between genotypes when MDMA was given at the dose of 20 ($F_{(1,20)} = 10.994$, $p < 0.01$) and 30 mg/kg ($F_{(1, 18)} = 11.056$, $p < 0.05$).

MDMA-induced anxiety-like responses

MDMA at the dose of 10 **and 20 mg/kg** induced anxiety-like responses in wild type mice, as revealed by a decrease in the percentage of time spent and entries in the open arms of the elevated-plus maze. This effect was significantly **lower** in CB₁ knockout animals (Fig 3) (Table 1). One-way ANOVA (genotype) indicated a significant decrease in the percentage of time spent in the open arms under basal conditions in CB₁ knockout mice ($F_{(1, 30)} = 4.547$, $p < 0.05$), as reported (31). One-way ANOVA also revealed an effect of MDMA treatment in wild type ($F_{(3, 45)} = 29.287$, $p < 0.001$), but not in CB₁ knockout mice. In agreement, a strong anxiogenic-like response occurred when MDMA was administered at 10 **and 20 mg/kg** in wild type ($p < 0.001$), but not in knockout mice ($F_{(1, 17)} = 7.085$, $p < 0.05$) (Fig 3a). No significant effects on anxiety-like responses were observed either in wild type or mutant mice when administered with MDMA 1 mg/kg.

One-way ANOVA calculated for the percentage of entries in the open arms showed a treatment effect in wild type ($F_{(3, 45)} = 8.709$, $p < 0.001$), **and ($F_{(3, 45)} = 4.234$, $p < 0.05$) knockout animals**. *Post hoc* analysis showed a reduction in the percentage of entries in the open arms in wild type animals when treated with MDMA at the dose of 10 ($p < 0.05$) and 20 ($p < 0.001$) mg/kg. A reduction in the percentage of entries in the open arms was also reduced in knockout animals when treated with MDMA 20 mg/kg ($p < 0.1$). One-way ANOVA to compare both genotypes showed significant differences in the percentage of entries in the open arms between groups treated with saline ($F_{(1, 30)} = 10.445$, $p < 0.01$) and MDMA 1 mg/kg ($F_{(1, 30)} = 7.425$, $p < 0.05$), but not with MDMA 10 **or 20 mg/kg** (Fig 3b).

MDMA-induced Conditioned Place Preference

MDMA induced similar rewarding effects in the CPP paradigm in both genotypes as revealed by two-way ANOVA (Table 1). The time spent in the drug-paired compartment during the preconditioning phase was similar in the

different groups of animals ($F_{(1, 71)} = 0.009$, n.s.), ensuring the use of an unbiased procedure. One-way ANOVA showed a CPP in wild type ($F_{(1, 37)} = 4.319$, $p < 0.05$) and knockout ($F_{(1, 34)} = 10.394$, $p < 0.01$) mice treated with MDMA (10 mg/kg) (Fig 4).

Increase in dopamine extracellular levels in the nucleus accumbens after MDMA administration

Basal extracellular DA levels in nucleus accumbens dialysates were similar in wild type (8.1 ± 1.4 pg/15 μ l) and knockout mice (6.9 ± 1.3 pg/15 μ l) ($p = 0.135$, ns). MDMA induced similar **increase in DA extracellular levels** in the nucleus accumbens in both genotypes as revealed by three-way ANOVA (Table 2). Acute MDMA at the dose of 20 mg/kg, (i.p.) enhanced DA extracellular levels in both genotypes, whereas at the dose of 10 mg/kg did not produce any significant effect (Fig 5a).

Two-way ANOVA for AUC values (from 0 to 240 min) (Fig. 5b) indicated a treatment effect ($F_{(2, 34)} = 31.199$, $p < 0.001$), without genotype effect nor interaction between these two factors. One-way ANOVA (treatment) indicated an effect of MDMA in wild type ($F_{(2, 17)} = 9.247$, $p < 0.01$) and knockout mice ($F_{(2, 17)} = 29.287$, $p < 0.001$) as well as *post hoc* analysis when MDMA was administered at 20 mg/kg (wild type, $p < 0.01$; CB₁ knockout, $p < 0.001$). The maximal MDMA-induced increase of DA levels was similar in both wild type (318.9%) and knockout (391.1%) mice, and occurred in both groups from 40 to 60 min after MDMA administration.

MDMA self-administration

Wild type and knockout mice were trained to self-administer MDMA (0.03, 0.06, 0.12, and 0.25 mg/kg/infusion) during 10 days under a FR1 schedule of reinforcement (Fig 6 and 7). MDMA at the lowest dose (0.03 mg/kg/infusion) did not maintain self-administration behavior in any genotype since no discrimination between the active and the inactive hole was observed (Fig 6a) (Table 2), and none of the animals reached the acquisition criteria. At the doses of 0.06, 0.12 and 0.25 mg/kg/infusion of MDMA only wild type mice maintained a reliable MDMA self-administration behavior (Table 2) (Figure 6b, 6c and 6d). The percentage of wild type mice that reached the acquisition criteria at the

dose of 0.06 mg/kg/infusion was 56%, at the dose of 0.12 mg/kg/infusion was 67 %, and 57 % at the dose of 0.25 mg/kg/infusion. None of the CB₁ knockout mice trained with MDMA achieved the acquisition criteria at these doses. The time required to discriminate the active hole was MDMA dose-dependent. Thus, paired Student t-test revealed that wild type mice trained with MDMA 0.06 mg/kg/infusion discriminated between the holes after 9 days of training ($p < 0.05$) (Figure 6b), animals receiving the dose of 0.12 mg/kg/infusion discriminated after 5 days ($p < 0.05$) (Figure 6c), and animals self-administering MDMA 0.25 mg/kg/infusion after 3 days ($p < 0.05$) (Figure 6d). MDMA dose-response curve was calculated by the mean of infusions received in the last three days of the self-administration training (Figure 7). A bell-shaped curve was observed in wild type mice, whereas no dose-response effect was revealed in knockout mice (Table 1). One-way ANOVA (genotype) showed significant differences between wild type and knockouts when MDMA was given at the dose of 0.06 ($F_{(1, 16)} = 9.307$; $p < 0.01$), 0.12 ($F_{(1, 22)} = 16.086$; $p < 0.001$) and 0.25 ($F_{(1, 22)} = 9.301$; $p < 0.01$) mg/kg/infusion.

DISCUSSION

This study demonstrates that CB₁ cannabinoid receptors modulate several pharmacological responses and play a relevant role in the reinforcing effects of MDMA. Indeed, hyperlocomotion, hyperthermia and anxiety-like behavior produced by MDMA were impaired in mice lacking CB₁ cannabinoid receptors. Furthermore, CB₁ knockout mice did not acquire self-administration behavior at any dose used of MDMA (0.03, 0.06, 0.12 and 0.25 mg/kg/infusion), although this drug induced CPP and enhanced the extracellular levels of DA in the nucleus accumbens of mutant mice.

Hyperthermia is one of the most characteristic pharmacological effects of MDMA in humans and animals (32, 33). MDMA increases temperature by activating 5-HT receptors in the preoptic nucleus of hypothalamus (34), an area known to express CB₁ receptors (35). No pharmacological studies have ever described the interaction between chronic administration of CB₁ antagonist and MDMA. However, it has been reported that CP55,940 prevented MDMA-induced hyperthermia (16), and THC eliminated fluoxetine-induced hyperthermia (36). Then, the increase of 5-HT in the preoptic nucleus of the hypothalamus that induces MDMA hyperthermia seems to be modulated by the cannabinoid system.

Hyperlocomotion is a common response observed in animals after MDMA administration, and both DA and 5-HT systems participate in this effect (37). Previous studies have described a reduction of MDMA-induced hyperlocomotion by cannabinoid agonists in rats (16). CB₁ receptors regulate motor activity, and is highly expressed in brain structures related with motor control (38). Interestingly, the hyperlocomotor effects of cocaine, which acts preferentially on DA reuptake, were not modified in CB₁ knockout mice (39). Therefore, the attenuation of MDMA-induced hyperactivity could be related to an interaction between CB₁ receptors and the 5-HT system rather than changes in DA functionality. Accordingly, different findings support the existence of cross-interactions between endocannabinoid and 5-HT system (40), particularly in the cerebellum (41) and the **caudate-putamen** (42), which are crucial brain areas in the control of motor coordination. However, the detailed nature of these interactions remains unclear.

Changes in anxiety-like responses are another prototypical effects of MDMA, although heterogeneous results have been reported depending on the dose, frequency of administration and behavioral test used (1). This study reveals an anxiety-like effect after acute MDMA (10 mg/kg) administration in wild type mice according with previous studies performed under similar experimental conditions (6, 7, 43). CB₁ knockout mice used in this study presented an anxiety-like phenotype, as previously reported (43, 44, 45). However, MDMA did not modify the anxiety-like behavior of mutant animals in the elevated plus maze. Previous studies have suggested an interaction between MDMA and the cannabinoid system in emotional responses. In this sense, THC reduced the anxiety-like effects of MDMA (5 mg/kg) in the emergence test in rats (16). This interaction could also be related with the modulation of 5-HT transmission by the cannabinoid system since the anxiolytic-like effect of the 5-HT_{1A} agonist buspirone was **lower** in CB₁ knockout mice (45).

MDMA rewarding properties have been widely described in previous studies (9, 10, 11, 12, 13). The blockade of CB₁ receptor with rimonabant abolished MDMA self-administration (46), and MDMA-induced CPP in rats (47). These results are in contrast to our study where no differences between genotypes were revealed in MDMA-induced CPP. Differences in the species, route of administration and experimental conditions used in these studies could explain this differences. In the present study, MDMA administration produced a similar enhancement of DA extracellular levels in the nucleus accumbens in both genotypes. In contrast, MDMA self-administration was abolished in CB₁ mutant mice. A possible learning impairment can be excluded to explain the lack of self-administration in CB₁ knockout mice since these animals exhibited similar operant behavior maintained by natural stimuli such as food and water (26). The absence of MDMA self-administration and the presence of MDMA-induced CPP in these mutant mice can be explained by the different behavioral parameters evaluated in these paradigms and the different neural pathways involved in each task performance. Operant self-administration is a contingent model in which drug is freely self-administered. Nevertheless, drug is passively received by animals in CPP. On a contingent operant paradigm such as self-administration multiple brain

circuits involved in motivation, consciousness and long term learning are also engaged, while CPP mainly evaluates the effect of a drug on the circuits of primary reward, namely, the mesolimbic system. Thus, medio-dorsal thalamus and prefrontal cortex, which participate on consciousness and motivation show increased cerebral metabolic rates in psychostimulant self-administering animals, but not in animals with passive drug administration such as CPP (48). Then, CB₁ receptor seems to play a specific role in these brain circuits that control complex operant responses, but not in the circuits of MDMA primary reward. The CB₁ cannabinoid receptor is essential in the acute rewarding effects of several drugs of abuse that produce their effect on ventral tegmental area such as nicotine ethanol or morphine (22). However, psychostimulant drugs produce their effect directly on the nucleus accumbens DA terminals (22). For that reason, CB₁ cannabinoid receptor does not seem to contribute to their acute rewarding effects evaluated in the CPP. Accordingly, previous studies reported that cocaine-induced CPP but not self-administration was preserved in CB₁ knockout mice (26, 39). Although the reinforcing effects of cocaine and MDMA in CB₁ knockout mice are abolish, they show considerable differences. A low percentage of CB₁ knockout mice (25 %) acquired cocaine self-administration (26), whereas no animal trained with MDMA did. The lower reinforcing efficacy of MDMA with regard to cocaine (49, 50) could justify this difference in acquisition. However, the role that 5-HT system plays in MDMA but not in cocaine reinforcing properties seems to be the key for the complete elimination of MDMA self-administration in CB₁ knockout mice (51, 52, 53). The relevance of 5-HT in MDMA reinforcing properties suggest an involvement of the cortical neurons activated by this neurotransmitter in the abolishment of operant self-administration in CB₁ knockout mice. Glutamatergic projections from prefrontal cortex to the mesolimbic system control motivation and the initiation of the appropriate responses to seek for a rewarding stimulus (54). These glutamatergic neurons express 5-HT receptors in the cell bodies of the prefrontal cortex (55) and CB₁ receptors in the nucleus accumbens terminals (56). In addition, these pyramidal neurons show reduced dendritic arborization in CB₁ mutant mice (57). Thus, the 5-HT activation in glutamatergic projections from prefrontal cortex to **mesolimbic** areas produced by MDMA (58) could be

impaired in CB₁ knockout mice since this pyramidal neurons are modulated by CB₁ receptors (56).

In summary, this study shows that CB₁ receptor mediates the prototypical pharmacological responses induced by acute MDMA. This receptor is also critical for the acquisition of an operant behavior to self-administer MDMA, through the modulation of neuronal circuits related to reward, motivation and long-term learning. However, CB₁ receptor does not participate in the acute rewarding properties of MDMA revealed in a non-contingent paradigm, in which DA transmission would play a predominant role. This study provides additional findings to support the crucial role played by the endocannabinoid system in the control of the addictive behavior. CB₁ receptor directly participate in the rewarding properties of different drugs of abuse including cannabinoids, opioids, alcohol and nicotine (22), but are also required for the acquisition of cocaine (26) and MDMA self-administration. MDMA has a low addictive potential, but important health risks are associated to the consumption of this drug, such as hyperthermia, mood alterations and binge administration, which could also be minimized by the chronic blockade of CB₁ cannabinoid receptors.

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REFERENCES

1. Green AR, Mehan AO, Elliott JM, O'Shea E, Colado MI (2003): The pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"). *Pharmacol Rev* 55: 463-508.
2. **Parrott AC, Gouzoulis-Meyfrank E, Rodgers J, Solowij N (2004): Ecstasy/MDMA and cannabis: the complexities of their interactive neuropsychobiological effects. *J.Psychopharmacol.* 18: 572-575.**
3. Baylen CA, Rosenberg H (2006): A review of the acute subjective effects of MDMA/ecstasy. *Addiction* 101: 933-947.
4. Nash JF, Jr., Meltzer HY, Gudelsky GA (1988): Elevation of serum prolactin and corticosterone concentrations in the rat after the administration of 3,4-methylenedioxymethamphetamine. *J Pharmacol Exp Ther* 245: 873-879.
5. Spanos LJ, Yamamoto BK (1989): Acute and subchronic effects of methylenedioxymethamphetamine [(+/-)MDMA] on locomotion and serotonin syndrome behavior in the rat. *Pharmacol Biochem Behav* 32: 835-840.
6. **Lin HQ, Burden PM, Christie MJ, Johnston GA (1999): The anxiogenic-like and anxiolytic-like effects of MDMA on mice in the elevated plus-maze: a comparison with amphetamine. *Pharmacol.Biochem.Behav.* 62: 403-408.**
7. **Morley KC, McGregor IS (2000): (+/-) - 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') increases social interaction in rats. *Eur.J.Pharmacol.* 408: 41-49.**
8. Bilsky EJ, Hui YZ, Hubbell CL, Reid LD (1990): Methylenedioxymethamphetamine's capacity to establish place preferences and modify intake of an alcoholic beverage. *Pharmacol Biochem Behav* 37: 633-638.

9. Robledo P, Balerio G, Berrendero F, Maldonado R (2004): Study of the behavioural responses related to the potential addictive properties of MDMA in mice. *Naunyn Schmiedebergs Arch Pharmacol* 369: 338-349.
10. Schenk S, Gittings D, Johnstone M, Daniela E (2003): Development, maintenance and temporal pattern of self-administration maintained by ecstasy (MDMA) in rats. *Psychopharmacology (Berl)* 169: 21-27.
11. Trigo JM, Panayi F, Soria G, Maldonado R, Robledo P (2006): A reliable model of intravenous MDMA self-administration in naive mice. *Psychopharmacology (Berl)* 184: 212-220.
12. Beardsley PM, Balster RL, Harris LS (1986): Self-administration of methylenedioxymethamphetamine (MDMA) by rhesus monkeys. *Drug Alcohol Depend* 18: 149-157.
13. Lamb RJ, Griffiths RR (1987): Self-injection of d,1-3,4-methylenedioxymethamphetamine (MDMA) in the baboon. *Psychopharmacology (Berl)* 91: 268-272.
14. White SR, Duffy P, Kalivas PW (1994): Methylenedioxymethamphetamine depresses glutamate-evoked neuronal firing and increases extracellular levels of dopamine and serotonin in the nucleus accumbens in vivo. *Neuroscience* 62: 41-50.
15. Parrott AC, Milani RM, Gouzoulis-Mayfrank E, Daumann J (2007): Cannabis and Ecstasy/MDMA (3,4-methylenedioxymethamphetamine): an analysis of their neuropsychobiological interactions in recreational users. *J. Neural Transm.* 114:959-68
16. Morley KC, Li KM, Hunt GE, Mallet PE, McGregor IS (2004): Cannabinoids prevent the acute hyperthermia and partially protect against the 5-HT depleting effects of MDMA ("Ecstasy") in rats. *Neuropharmacology* 46: 954-965.
17. Touriño C, Maldonado R, Valverde O (2007): MDMA attenuates THC withdrawal syndrome in mice. *Psychopharmacology (Berl)* 193: 75-84.

18. Ameri A (1999): The effects of cannabinoids on the brain. *Prog. Neurobiol.* 58: 315-348.
19. Piomelli D (2005): The endocannabinoid system: a drug discovery perspective. *Curr. Opin. Investig. Drugs* 6: 672-679
20. Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D (1999): Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nat. Neurosci.* 2: 358-363.
21. Nakazi M, Bauer U, Nickel T, Kathmann M, Schlicker E (2000): Inhibition of serotonin release in the mouse brain via presynaptic cannabinoid CB1 receptors. *Naunyn Schmiedeberg's Arch. Pharmacol.* 361: 19-24.
22. Maldonado R, Valverde O, Berrendero F (2006): Involvement of the endocannabinoid system in drug addiction. *Trends Neurosci* 29: 225-232.
23. Ledent C, Valverde O, Cossu G, Petitot F, Aubert JF, Beslot F et al (1999): Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* 283: 401-404.
24. Castañé A, Valjent E, Ledent C, Parmentier M, Maldonado R, Valverde O (2002): Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. *Neuropharmacology* 43: 857-867.
25. Thanos PK, Dimitrakakis ES, Rice O, Gifford A, Volkow ND (2005): Ethanol self-administration and ethanol conditioned place preference are reduced in mice lacking cannabinoid CB1 receptors. *Behav Brain Res* 164: 206-213.
26. Soria G, Mendizabal V, Touriño C, Robledo P, Ledent C, Parmentier M et al (2005): Lack of CB1 Cannabinoid Receptor Impairs Cocaine Self-Administration. *Neuropsychopharmacology* 30:1670-80.
27. Gibaldi M. and Perrier D. (1975) *The trapezoid rule, in Pharmacokinetics*, 2nd ed. New York: Gibaldi M. and Perrier D., eds.
28. Simonin F, Valverde O, Smadja C, Slowe S, Kitchen I, Dierich A et al (1998): Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to

- chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J.* 17: 886-897.
29. Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E (1997): Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* 388: 586-589.
 30. Robledo P, Mendizabal V, Ortuño J, de la Torre R, Kieffer BL, Maldonado R (2004): The rewarding properties of MDMA are preserved in mice lacking mu-opioid receptors. *Eur J Neurosci* 20: 853-858.
 31. Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2002): Involvement of CB1 cannabinoid receptors in emotional behaviour. *Psychopharmacology (Berl)* 159: 379-387.
 32. Chadwick IS, Curry PD, Linsley A, Freemont AJ, Doran B (1991): Ecstasy, 3-4 methylenedioxymethamphetamine (MDMA), a fatality associated with coagulopathy and hyperthermia. *J.R.Soc.Med.* 84: 371.
 33. Shankaran M, Gudelsky GA (1999): A neurotoxic regimen of MDMA suppresses behavioral, thermal and neurochemical responses to subsequent MDMA administration. *Psychopharmacology (Berl)* 147: 66-72.
 34. Stephenson CP, Hunt GE, Topple AN, McGregor IS (1999): The distribution of 3,4-methylenedioxymethamphetamine "Ecstasy"-induced c-fos expression in rat brain. *Neuroscience* 92: 1011-1023.
 35. Cota D, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M et al (2003): The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J.Clin.Invest* 112: 423-431.
 36. Malone DT, Taylor DA (1998): Modulation of delta9-tetrahydrocannabinol-induced hypothermia by fluoxetine in the rat. *Br.J.Pharmacol.* 124: 1419-1424.

37. Searce-Levie K, Viswanathan SS, Hen R (1999): Locomotor response to MDMA is attenuated in knockout mice lacking the 5-HT_{1B} receptor. *Psychopharmacology (Berl)* 141: 154-161.
38. Herkenham M, Lynn AB, de Costa BR, Richfield EK (1991): Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. *Brain Res* 547: 267-274.
39. Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2000): Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. *Eur J Neurosci* 12: 4038-4046.
40. Cheer JF, Cadogan AK, Marsden CA, Fone KC, Kendall DA (1999): Modification of 5-HT₂ receptor mediated behaviour in the rat by oleamide and the role of cannabinoid receptors. *Neuropharmacology* 38: 533-541.
41. Devlin MG, Christopoulos A (2002): Modulation of cannabinoid agonist binding by 5-HT in the rat cerebellum. *J Neurochem* 80: 1095-1102.
- 42. Oliva JM, Uriguen L, Perez-Rial S, Manzanares J (2005): Time course of opioid and cannabinoid gene transcription alterations induced by repeated administration with fluoxetine in the rat brain. *Neuropharmacology* 49: 618-626.**
43. Navarro JF, Maldonado E (2002): Acute and subchronic effects of MDMA ("ecstasy") on anxiety in male mice tested in the elevated plus-maze. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 26: 1151-1154.
44. Haller J, Bakos N, Szirmay M, Ledent C, Freund TF (2002): The effects of genetic and pharmacological blockade of the CB1 cannabinoid receptor on anxiety. *Eur J Neurosci* 16: 1395-1398.
45. Uriguen L, Perez-Rial S, Ledent C, Palomo T, Manzanares J (2004): Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. *Neuropharmacology* 46: 966-973.

46. Braidă D, Sala M (2002): Role of the endocannabinoid system in MDMA intracerebral self-administration in rats. *Br J Pharmacol* 136: 1089-1092.
47. Braidă D, Iosue S, Pegorini S, Sala M (2005): 3,4-Methylenedioxymethamphetamine-induced conditioned place preference (CPP) is mediated by endocannabinoid system. *Pharmacol Res* 51: 177-182.
48. Porrino LJ, Lyons D, Miller MD, Smith HR, Friedman DP, Daunais JB et al (2002): Metabolic mapping of the effects of cocaine during the initial phases of self-administration in the nonhuman primate. *J Neurosci* 22: 7687-7694.
49. Lile JA, Ross JT, Nader MA (2005): A comparison of the reinforcing efficacy of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") with cocaine in rhesus monkeys. *Drug Alcohol Depend* 78: 135-140.
50. Han DD, Gu HH (2006): Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. *BMC Pharmacol* 6: 6.
51. Fantegrossi WE, Ullrich T, Rice KC, Woods JH, Winger G (2002): 3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") and its stereoisomers as reinforcers in rhesus monkeys: serotonergic involvement. *Psychopharmacology (Berl)* 161: 356-364.
52. Trigo JM, Renoir T, Lanfumey L, Hamon M, Lesch KP, Robledo P (2007): 3,4-Methylenedioxymethamphetamine Self-Administration is Abolished in Serotonin Transporter Knockout Mice. *Biol. Psychiatry* . DOI:10.1016.
53. Przegalinski E, Golda A, Frankowska M, Zaniowska M, Filip M (2007): Effects of serotonin 5-HT_{1B} receptor ligands on the cocaine- and food-maintained self-administration in rats. *Eur. J. Pharmacol.* 559: 165-172.
54. Kalivas PW, Volkow ND (2005): The neural basis of addiction: a pathology of motivation and choice. *Am J Psychiatry* 162: 1403-1413.

55. Martin-Ruiz R, Puig MV, Celada P, Shapiro DA, Roth BL, Mengod G et al (2001): Control of serotonergic function in medial prefrontal cortex by serotonin-2A receptors through a glutamate-dependent mechanism. *J. Neurosci.* 21: 9856-9866.
56. Robbe D, Kopf M, Remaury A, Bockaert J, Manzoni OJ (2002): Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc Natl Acad Sci U S A* 99: 8384-8388.
57. Ballesteros-Yañez I, Valverde O, Ledent C, Maldonado R, De Felipe J (2007): Chronic cocaine treatment alters dendritic arborization in the adult motor cortex through a CB1 cannabinoid receptor-dependent mechanism. *Neuroscience* 146:1536-45.
58. Gudelsky GA, Nash JF (1996): Carrier-mediated release of serotonin by 3,4-methylenedioxymethamphetamine: implications for serotonin-dopamine interactions. *J Neurochem* 66: 243-249.

Figure legends

Figure 1. Effects of acute MDMA (5, 10, 20 and **30** mg/kg, i.p.) on locomotion in CB₁ knockout (black bars) and wild type (white bars) mice. Horizontal (a) and vertical (b) locomotion were measured immediately after saline or MDMA administration. Data are expressed as mean $\pm$ SEM of photocell counts during a one hour period in wild type (n = 10 to 12) and CB₁ knockout (n = 9 to 11) mice. $\star\star$ p < 0.01, $\star\star\star$ p < 0.001 when compared with the saline group of the same genotype (Dunnett's test). $\star$ p < 0.05, $\star\star$ p < 0.01, $\star\star\star$ p < 0.001, comparisons between genotypes (one-way ANOVA).

Figure 2. a, b, c and d) Effects of acute MDMA (10, 20, and 30 mg/kg, i.p.) on body temperature in CB₁ knockout (black) and wild type (white) mice. Body temperature was measured 60 min before (basal values), and 30, 60, 120 and

180 min after MDMA or saline injection. Data are expressed as mean $\pm$ SEM of the temperature increase regarding basal values in wild type ($n = 9$ to 10) and CB₁ knockout ($n = 9$ to 10) mice. e) Bar graphs of AUC from 0 to 180 min for the body temperature changes after saline or MDMA (10, 20 and 30 mg/kg, i.p.) administration in CB₁ knockout (black bars) and wild type (white bars) mice. All values are expressed as mean $\pm$ SEM. $\star p < 0.05$, $\star\star p < 0.01$, $\star\star\star p < 0.001$ when compared with the saline group of the same genotype (Dunnett's test). $\star p < 0.05$ $\star\star p < 0.01$, comparisons between genotypes (one-way ANOVA).

Figure 3. Effects of acute MDMA (1, 10 and **20** mg/kg, i.p.) on anxiety-like responses in CB₁ knockout (black bars) and wild type (white bars) mice. The behavioral responses in the elevated-plus maze were measured 30 min after MDMA or saline administration. Data are expressed as mean $\pm$ SEM of the percentage of time spent in the open arms (a) and percentage of entries in the open arms (b) in wild type ($n = 9$ to 13) and CB₁ knockout ($n = 10$ to 14) mice. $\star\star p < 0.01$, $\star\star\star p < 0.001$, when compared with the saline group of the same genotype (Dunnett's test), $\star p < 0.05$, comparisons between genotypes (one-way ANOVA).

Figure 4. Effects of saline and MDMA (10 mg/kg, i.p.) on the CPP paradigm in CB₁ knockout (black bars) and wild type (white bars) mice. Four pairings were carried out with MDMA and four pairings with vehicle on alternate days (see methods section). MDMA and saline were administered 30 min before each conditioning session. Data are expressed as mean $\pm$ SEM of score values in CB₁ knockout (saline, $n = 16$; MDMA 10 mg/kg, $n = 22$) and wild type (saline, $n = 14$; MDMA 10 mg/kg, $n = 23$) mice. $\star p < 0.05$, $\star\star p < 0.01$ when compared with the saline group of the same genotype.

Figure 5. a) Effects of MDMA (10 and 20 mg/kg, i.p.) and saline on DA concentrations in dialysates obtained by *in vivo* microdialysis from the nucleus accumbens of CB₁ knockout (saline, $n = 6$; MDMA 10 mg/kg, $n = 7$; MDMA 20 mg/kg, $n = 7$) and wild type mice (saline, $n = 7$; MDMA 10 mg/kg, $n = 7$; MDMA 20 mg/kg, $n = 6$). The arrow indicates MDMA or saline administration at time 0. Dialysate samples were taken every 20 min during one hour before and 4 hours

after injection. Data are expressed as percentage of the basal values. b) Bars represent the AUC values from 0 to 240 min for the DA concentration in dialysates after MDMA or saline administration in CB₁ knockout (black bars) and wild type (white bars) mice. All values are expressed as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ when compared with the saline group of the same genotype (Dunnett's test). c) Diagram showing the representative probe placements between bregma + 1.94 and + 1.10 mm in all the mice included in the data analysis. Probes were successfully placed in the nucleus accumbens in 87.5 % of the tested animals.

Figure 6. Acquisition of MDMA self-administration behavior in CB₁ knockout (black) and wild type (white) mice. Data are expressed as mean $\pm$ SEM of the number of nose-pokes in the active and the inactive hole in the 2 h sessions performed during 10 days at (a) 0.03 mg/kg/infusion ($n = 4-7$); (b) 0.06 mg/kg/infusion ($n = 9$); (c) 0.12 mg/kg/infusion ($n = 11-13$) and (d) 0.25 mg/kg/infusion ($n = 10-14$). * $p < 0.05$; ** $p < 0.01$ comparison between holes (one-way ANOVA).

Figure 7. Dose-response curve for MDMA self-administration in CB₁ knockout (black) (0.03 mg/kg/infusion, $n = 7$; 0.06 mg/kg/infusion, $n = 9$; 0.12 mg/kg/infusion, $n = 11$; 0.25 mg/kg/infusion, $n = 10$) and wild type (white) (0.03 mg/kg/infusion, $n = 4$; 0.06 mg/kg/infusion, $n = 9$; 0.12 mg/kg/infusion, $n = 13$; 0.25 mg/kg/infusion, $n = 14$) mice. Figure represents the mean of infusions of the last three days of acquisition at the different MDMA doses used. Data are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparisons between genotypes (two-way ANOVA).