CB₁ knockout mice display impaired functionality of 5-HT₁₅ and 5-HT₂₅ receptors

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Abstract

Interaction between brain endocannabinoid (EC) and serotonin (5-HT) systems was investigated by examining 5-HT-dependent behavioural and biochemical responses in CB₁ receptor knockout mice. CB₁ knockout animals exhibited a significant reduction in the induction of head twitches and paw tremor by the 5-HT₂A receptor selective agonist (±)DOI, as well as a reduced hypothermic response following administration of the 5-HT₁A receptor agonist (±)-8-OH-DPAT. Additionally, exposure to the tail suspension test induced enhanced despair responses in CB₁ knockout mice. However, the tricyclic antidepressant imipramine and the 5-HT selective reuptake inhibitor fluoxetine induced similar decreases in the time of immobility in the tail suspension test in CB₁ receptor knockout and wild-type mice. No differences were found between both genotypes with regard to 5-HT₂A receptor and 5-HT₁A receptors levels, measured by autoradiography in different brain areas. However, a significant decrease in the ability of the 5-HT₁A receptor agonist (±)-8-OH-DPAT to stimulate [³⁵S]GTPγS binding was detected in the hippocampal CA₁ area of CB₁ receptor knockout mice. This study provides evidence that CB₁ receptors are involved in the regulation of serotonergic responses mediated by 5-HT₂A and 5-HT₁A receptors, and suggests that a reduced coupling of 5-HT₁A receptors to Gᵢ/o proteins in the hippocampus might be involved in these effects.

Keywords: CB₁ receptors, 5-HT₁A receptors, 5-HT₂A receptors, antidepressants, tail suspension test, hypothermia, autoradiography.

Abbreviations: (+)-2,5-demethoxy-4-iodoamphetamine hydrochloride (DOI); 8-hydroxy-2[di-n-propylamino]tetralin (8-OH-DPAT); endocannabinoid (EC); 5-hydroxytryptamine (5-HT); dorsal raphe nucleus (DRN).

Running title: Reduced serotonin function in CB₁ knockout mice
INTRODUCTION

Cannabis sativa derivatives have been used for over 4000 years and remain the most widely consumed illicit drugs nowadays due to their mood-altering properties (Lichtman and Martin 2005). The pleasurable subjective effects associated to Cannabis use include an initial period of euphoria and relaxation that can be associated to perceptive distortions depending on the dose and individual susceptibility. Nevertheless, the use of Cannabis preparations, generally at higher doses, has also been associated to unpleasant mind-disturbing effects such as anxiety and panic attacks, acute psychosis and paranoia (Ameri 1999). The main psychoactive compound of Cannabis preparations, Δ9-tetrahydrocannabinol (Δ9-THC), modulates brain function mainly through the activation of a G\textit{i}/o protein-coupled receptor known as CB1 (Matsuda et al. 1990) that is expressed at high densities in areas such as hippocampus, frontal cortex, basal ganglia and cerebellum (Ong and Mackie 1999). CB1 receptors have raised a considerable interest during the last few years as modulators of the activity of different neurotransmitter systems. In this sense, it has been consistently shown that on-demand activation of CB1 receptors by their endogenous agonists, endocannabinoids (ECs), negatively modulates the release of different neurotransmitters in many brain areas, including those involved in cognition, memory and maintenance of mood, such as the hippocampus and the prefrontal cortex (Wilson and Nicoll 2002; Freund et al. 2003).

The brain serotonin (5-hydroxytryptamine; 5-HT) plays a relevant role in the regulation of several central activities, including mood and anxiety. The complex actions of 5-HT are mediated by at least 14 receptor subtypes (Hoyer et al. 1994) and the relative contributions of each individual receptor to the regulation of brain functions are not fully understood. Particular attention has focused on 5-HT1A and 5-HT2A receptor subtypes due to their high densities throughout the brain, as well as to their proposed role in both the pathogenesis of mood disorders and the mechanism of action of antidepressant and anxiolytic drugs. In this
regard, major depression has been associated with increased 5-HT$_{2A}$ receptor density in the prefrontal cortex (Yates et al. 1990) as well as augmented density of 5-HT$_{1A}$ in the dorsal raphe nucleus (DRN) (Stockmeier et al. 1998). Furthermore, the antagonism of presynaptic 5-HT$_{1A}$ receptors has been reported to accelerate the therapeutic effects of antidepressant medications (Artigas et al. 1996) whereas partial agonists of the 5-HT$_{1A}$ receptor, such as buspirone, present an anxiolytic profile and have also a potential use as antidepressants (Blier and Ward 2003). Finally, several antidepressant drugs, such as nefazodone or mirtazapine behave as antagonists of the 5-HT$_{2A}$ receptor subtype (Celada et al. 2003).

Experimental data of different kind support the existence of interaction mechanisms between brain cannabinoid and 5-HT systems. Cannabinoid agonists have been proposed to increase the behavioural effects of the 5-HT$_{2A}$ receptor agonist DOI in rats (Cheer et al. 1999), suggesting that an enhanced 5-HT function might result from the stimulation of CB$_1$ receptors. In contrast, several data indicate that the activation of CB$_1$ receptors could lead to a reduced degree of 5-HT activity. Darmani (2001) has shown that cannabinoid agonists reduce DOI induced 5-HT$_{2A}$ receptor-related behaviours in mice. Additionally, both in vitro and in vivo data suggest that activation of CB$_1$ receptors decreases 5-HT release in the hippocampus and prefrontal cortex (Nakazi et al. 2000; Egashira et al. 2002). Consistently, pharmacological blockade of CB$_1$ receptors has been reported to increase 5-HT efflux in the rat and shrew forebrain (Tzavara et al. 2003; Darmani et al. 2003). However, recent studies have reported antidepressant-like activity and an increased firing of 5-HT neurons in DNR by blockade of EC hydrolysis (Gobbi et al. 2005). Finally, it is noteworthy that activation of 5-HT$_{1A}$ receptors has been implicated in the anxiogenic, but not the anxiolytic effect of synthetic cannabinoid agonists (Marco et al. 2004). Overall, these findings support the existence of crosstalk mechanisms between brain EC and 5-HT systems, although the detailed nature of these interactions remains unclear.
A useful tool to study the neurophysiology of the EC system is the invalidation of the CB₁ gene (Ledent et al. 1999). Taking into account the experimental evidence above mentioned, as well as the fact that CB₁ knockout mice exhibit increased anxiogenic- and depressive-like responses (Valverde 2005), it is evident that the use of these animals could provide important information on the nature of CB₁-5-HT interactions. The specific objective of this study was to examine the state of 5-HT functionality in CB₁ receptor knockout mice, in an attempt to clarify the interaction mechanisms between EC and 5-HT brain systems. We evaluated the behavioural responses elicited by the selective 5-HT₁A and 5-HT₂A receptor agonists, as well as by several antidepressant drugs, in mice lacking the CB₁ receptor. 5-HT₁A and 5-HT₂A density and the functional coupling of CB₁ receptors to Gᵢ/o proteins were also examined in the brain of CB₁ receptor knockout mice using autoradiographic procedures.
MATERIALS AND METHODS

Animals

Male CB₁ receptor knockout mice and wild-type littermates (30-32 g) were used in this study. CB₁ receptor null mutant mice were generated by homologous recombination as previously reported (Ledent et al. 1999). In order to homogenize background of the mice, the first generation heterozygotes were backcrossed for 30 generations on a CD1 background (Charles Rivers, France) with selection for the mutant CB₁ gene at each generation. Heterozygote mating produced wild-type and knockout littermates for subsequent experiments. Breeding couples were periodically renovated by crossing heterozygote mice with wild-type CD1 females (Charles River, France) in order to maintain a genetically diverse outbred background. Animals were housed in groups of five per cage and maintained in a temperature (21 ± 1 ºC) and humidity (55 ± 10%) controlled room with a 12 h light-dark cycle. Food and water were available ad libitum. Experiments were carried out in accordance to the guidelines of The European Communities Council Directive 86/609/EEC and were approved by the Animal Research Ethical Committee of our institutions.

Drugs

The 5-HT₂A receptor agonist, 1-(2, 5-dimethoxy-4-odophenyl)-2-aminopropane ((±)DOI), the 5-HT₁A receptor ligands, 8-hydroxy-di-n-propylaminotetralin ((±)-8-OH-DPAT) and [N-[2-(4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide]-maleate (WAY100635), the selective 5-HT reuptake inhibitors, fluoxetine hydrochloride and citalopram hydrobromide, the tricyclic antidepressants, imipramine and desipramine and the monoamine oxidase inhibitor, phenelzine, were supplied by Sigma® Chemical Co (Madrid, Spain). All drugs were dissolved in saline (0.9%) and injected intraperitoneally (i.p.) in a volume of 10 ml/kg body weight.
Behavioural observations

Behavioural responses to (±)DOI

Mice were administered with saline or the 5-HT$_{2A}$ receptor agonist (±)DOI (0.3 and 1 mg/kg, i.p.) and immediately placed inside an observation area. This area consisted in a clear plastic cylinder of 25 cm of diameter and 50 cm height. The number of head twitches and paw tremors was counted for 30 min. The number of animals per group of treatment was 15.

Body temperature measurement

Body temperature was measured by using an electronic thermocouple flexible probe (Panlab, Barcelona, Spain). The probe was lubricated and placed 4 cm into the rectum of the mice for 20 sec to record the temperature. Basal body temperature was recorded immediately before drug injection for each animal. Rectal temperature was again measured 45 min after the administration of 5-HT$_{1A}$ receptor agonist (±)-8-OH-DPAT (1 and 3 mg/kg, i.p.) or saline. The hypothermic effect of (±)-8-OH-DPAT was evaluated by calculating the difference between the basal temperature and the body temperature 45 min after injection. The number of animals per group of treatment was 13-15.

Antidepressant-like responses

The antidepressant effects induced by imipramine (7.5 mg/kg, i.p.), desipramine (7.5 mg/kg, i.p.), phenelzine (40 mg/kg, i.p.) and fluoxetine (10 mg/kg, i.p.) were tested in both the tail suspension and forced swimming test as previously reported (Porsolt et al. 1977). The tail suspension test was a modified version of the previously described (Steru et al. 1985). Mice were left in the experimental room for at least 3 h before the test. After that, mice were individually suspended by the tail to a horizontal ring-star bar (distance from floor was 35 cm) using adhesive tape (distance from tip of tail was 2 cm). Typically, mice demonstrated several escape-oriented behaviours interspersed with temporally increasing bouts of
immobility. The recorded parameter was the number of seconds spent immobile during a total
time of 6 min. For the forced swimming test, animals were individually placed in Plexiglas
cylinders (30 cm in height; 15 cm in diameter), containing 20 cm of water maintained at 21
°C. Animals were placed into the cylinders during 6 min and the immobility time was
measured during the last 4-min period. Test was carried out 30 min after drug administration.
The number of animals per group of treatment was 9-15.

**Autoradiographic studies**

*Tissue preparation*

Mice were killed by decapitation and the brains were rapidly removed, frozen in
isopentane and stored at -80 °C until sectioning. 20 μm thick-coronal sections were cut using
a microtome cryostat, thaw-mounted in gelatinised slides and stored at -20 °C until use.

**5-HT2A receptor autoradiography**

Sections were preincubated at room temperature for 30 min in 170 mM Tris-Cl (pH 7.6),
and then incubated at room temperature for 60 min in the same buffer containing 2 nM
\[^3H\]ketanserin. Non-specific binding was determined using 10 μM mianserin. Sections were
washed twice for 10 min in ice cold 170 mM Tris-Cl (pH 7.4), dipped in cold distilled water
and cold air-dried. Slides were exposed to Hyperfilm™. ³H along with ³H-labelled standards
for 8 weeks at 4°C.

**5-HT1A receptor autoradiography**

Sections were preincubated at room temperature for 30 min in a 170 mM Tris-HCl buffer
(pH 7.5) containing 4 mM CaCl₂ and 0.01% ascorbic acid, and subsequently incubated for 60
min in the same buffer containing 10 μM pargyline and 2 nM \[^3H\]8-OH-DPAT. Non-specific
binding was determined using 10 μM 5-HT. Sections were washed twice for 5 min in ice cold
buffer, dipped in distilled cold water and dried in a cold air stream. Autoradiograms were
generated by apposing the tissues to tritium-sensitive films (Hyperfilm\textsuperscript{TM-3H, Amersham, Switzerland}) along with \textsuperscript{3}H-labelled standards (Amersham) for 15 days at 4\textdegree C.

\textit{5-HT\textsubscript{1A} receptor-stimulated \textsuperscript{35}S\textit{GTP\gamma S} autoradiography}

Sections were preincubated for 30 min at room temperature in a buffer containing 50 mM Tris–HCl, 0.2 mM EGTA, 3 mM MgCl\textsubscript{2}, 100 mM NaCl, 1 mM dl-dithiothreitol and 2 mM GDP (pH 7.7), and subsequently incubated for 120 min in the same buffer containing 3 mU/ml adenosine deaminase and 0.04 nM \textsuperscript{35}S\textit{GTP\gamma S}. Consecutive sections were incubated with 100 \textmu M (\textpm)-8-OH-DPAT alone or in the presence of 10 \textmu M WAY100635. Non-specific binding was determined in the presence of 10 \textmu M guanosine-5-O-(3-thio) triphosphate (GTP\gamma S). After the incubation, the sections were washed twice for 15 min in 50 mM Tris–HCl buffer (pH 7.4) at 4 \textdegree C, rinsed in distilled cold water and cold air dried. Sections were exposed to \textbeta radiation-sensitive films (Hyperfilm\textsuperscript{TM-\beta max, Amersham, Switzerland}) together with \textsuperscript{14}C-polymer standards (Amersham, Switzerland) for 2 days at 4 \textdegree C.

\textbf{Data analysis}

All data were expressed as mean \pm S.E.M. Behavioural data were compared by using a two-way ANOVA (genotype and treatment) between subjects. If significant differences were observed, this analysis was followed by corresponding one-way ANOVA and post hoc comparisons (Dunnett \textit{t} test). Autoradiograms were scanned and images analyzed by using the National Institute of Health IMAGE program (Bethesda, MA). Optical densities were corrected to femtomol/mg tissue equivalent (fmol/mg t.e.) or nanocurie/g tissue equivalent (nCi/g t.e.) by comparison with the \textsuperscript{3}H and \textsuperscript{14}C microscales respectively, and compared using a two-tail Student’s \textit{t}-test.
RESULTS

Behavioural changes induced by 5-HT$_{2A}$ receptor agonist were attenuated in CB$_1$ knockout mice

The administration of 5-HT$_{2A}$ receptor agonist (±)DOI dose-dependently induced head twitches (Fig. 1A) and paw tremor (Fig. 1B) that were reduced in CB$_1$ receptor knockout mice. Two-way ANOVA calculated for (±)DOI-induced head twitches responses revealed a significant effect of treatment (F$_{2, 89} = 330.49$, $p < 0.001$), genotype (F$_{1, 89} = 60.96$, $p < 0.001$), and an interaction between these two factors (F$_{2, 89} = 38.12$, $p < 0.001$). Subsequent one-way ANOVA revealed that (±)DOI induced a significant increase in the number of head twitches in wild-type (F$_{2, 44} = 254.26$, $p < 0.001$) and CB$_1$ receptor knockout mice (F$_{2, 43} = 94.54$, $p < 0.001$). Post hoc analysis demonstrated a significant effect in both genotypes when the drug was given at 0.3 ($p < 0.001$) and 1 mg/kg ($p < 0.001$). The comparison between genotypes revealed that the effect of (±)DOI was significantly decreased in CB$_1$ receptor knockout animals at 0.3 ($p < 0.05$) and 1 mg/kg ($p < 0.001$) (Fig. 1A).

Two-way ANOVA calculated for paw tremors indicated a significant effect of treatment (F$_{2, 89} = 63.22$, $p < 0.001$), and genotype (F$_{1, 89} = 9.91$, $p < 0.01$), without significant interaction between these two factors. Subsequent one-way ANOVA revealed that (±)DOI (0.3 and 1 mg/kg) induced a significant increase in paw tremor in wild-type (F$_{2, 44} = 35.72$, $p < 0.001$) and CB$_1$ receptor knockout mice (F$_{2, 43} = 28.97$, $p < 0.001$). Post hoc analysis demonstrated a significant effect in both genotypes when the drug was given at 0.3 ($p < 0.01$) and 1 mg/kg ($p < 0.001$). The comparison between genotypes revealed that the effect of (±)DOI was decreased in CB$_1$ receptor knockout animals at the highest dose used (1 mg/kg) ($p < 0.05$) (Fig. 1B).
Effect of 5-HT$_{1A}$ receptor agonist on body temperature was reduced in CB$_1$ knockout mice

Mice treated with the 5-HT$_{1A}$ receptor agonist (±)-8-OH-DPAT exhibited a dose-dependent hypothermic response shown by the difference in rectal temperature measured before and after drug administration, that was reduced in CB$_1$ receptor knockout mice (Fig. 1C). Two-way ANOVA revealed a significant effect of treatment ($F_{2, 85} = 22.92$, $p < 0.001$) and genotype ($F_{1, 85} = 7.58$, $p < 0.01$), without interaction between these two factors. Subsequent one-way ANOVA showed a significant decrease in body temperature in wild-type (F$_2$, 42 = 18.28, $p < 0.001$) and knockout mice (F$_2$, 41 = 6.63, $p < 0.01$). Post hoc comparisons indicated a significant effect of (±)-8-OH-DPAT in both genotypes at 1 (wild-type, $p < 0.01$; knockout, $p < 0.05$) and 3 mg/kg (wild-type, $p < 0.001$; knockout, $p < 0.01$). The comparison between genotypes revealed that the effect of (±)-8-OH-DPAT was significantly decreased in CB$_1$ receptor knockout animals at the highest dose used (3 mg/kg) ($p < 0.01$).

Antidepressant-like responses observed in the tail suspension and forced swimming tests in CB$_1$ knockout mice and wild-type littermates.

The administration of the tricyclic antidepressant imipramine (7.5 mg/kg, i.p.) and the SSRI fluoxetine (10 mg/kg, i.p.) induced antidepressant-like effects in both genotypes, shown by a decrease in the time of immobility in the tail suspension test (Fig. 2). Two-way ANOVA revealed a treatment effect [imipramine, ($F_{1, 33} = 13.83$, $p < 0.001$); fluoxetine, ($F_{1, 36} = 12.43$, $p < 0.001$)], with genotype effect [imipramine, ($F_{1, 33} = 52.99$, $p < 0.001$); fluoxetine, ($F_{1, 36} = 10.241$, $p < 0.01$)] without interaction between both factors. One-way ANOVA showed a significant decrease in the immobility time of wild-type mice treated with imipramine (F$_1$, 16 = 9.16, $p < 0.01$) and fluoxetine (F$_1$, 18 = 7.14, $p < 0.05$). For CB$_1$ knockout mice, subsequent one-way ANOVA revealed a decrease in the immobility time of mice treated with imipramine (F$_1$, 16 = 18.97, $p < 0.001$), and fluoxetine (F$_1$, 18 = 4.56, $p < 0.05$). One-way ANOVA for
genotype effect, showed a significant difference between wild-type and knockout saline-treated groups [imipramine, (F1, 17 = 43.60, \( p < 0.001 \)); fluoxetine, (F1, 18 = 5.67, \( p < 0.05 \))], as well as significant differences were observed between wild-type and knockout antidepressant-treated groups (F1, 17 = 5.43, \( p < 0.05 \), imipramine experiment; F1, 18 = 5.80, \( p < 0.05 \), fluoxetine experiment).

Similar antidepressant-like responses were observed when antidepressant drugs were evaluated in the forced swimming test. Thus, the administration of the imipramine (7.5 mg/kg, i.p.), and fluoxetine (10 mg/kg, i.p.) induced antidepressant-like effects, shown by a decrease in the time of immobility in the forced swimming test (data not shown).

**5-HT2A receptor autoradiography**

High densities of \([^{3}H]\)ketanserin specific binding autoradiographic grains were found over the cortical areas, and intermediate to high levels of specific binding were observed in other anatomical areas such as caudate-putamen and nucleus accumbens. Comparison between wild-type and CB1 receptor knockout mice revealed no significant changes in the density of 5-HT2A receptors in any of the brain areas analyzed (Fig. 3A).

**5-HT1A receptor autoradiography**

The hippocampal formation (dentate gyrus and CA1 field), cortical areas, including the entorhinal cortex, and raphe nuclei showed the highest levels of \([^{3}H]\)8-OH-DPAT specific binding in the mice brain. Similar \([^{3}H]\)8-OH-DPAT binding levels were measured in the different brain areas of wild-type and CB1 receptor knockout mice (Fig. 3B).

**5-HT1A receptor-stimulated \([^{35}S]GTP\gamma S\) autoradiography**

The range of (±)8-OH-DPAT-stimulated \([^{35}S]GTP\gamma S\) binding in brain sections from both wild-type and CB1 receptor knockout mice revealed a pattern of localization in good match with the anatomical distribution of 5-HT1A receptors. The selective 5-HT1A antagonist
WAY100635 (10 μM) blocked the effect of (±)8-OH-DPAT in all brain areas (Fig. 4). Basal $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding levels were similar between wild-type and CB$_1$ receptor knockout animals in all the brain areas analyzed (Table 1). In contrast, a general tendency to the reduction in the level of (±)8-OH-DPAT-induced stimulation of specific $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding was observed in CB$_1$ receptor knockout mice. This decrease reached statistical significance ($p < 0.05$, Student’s t test) in the CA$_1$ field of the hippocampus (Table 1 and Fig. 4).
DISCUSSION

This study provides evidence suggesting that lifelong deletion of the CB₁ receptor reduces the functionality of brain 5-HT system. In this regard, three behavioural responses associated to the activation of 5-HT receptors were attenuated in the CB₁ knockout mice. Thus, the head twitches and paw tremors elicited by the 5-HT₂A receptor agonist DOI, as well as the decrease in body temperature induced by the 5-HT₁A agonist 8-OH-DPAT, were attenuated in mutant mice.

Studies addressing the role of CB₁ receptors in the activity of 5-HT system are of special interest given the importance of brain 5-HT in the pathogenesis of mood disorders. Within brain 5-HT system, 5-HT₁A and 5-HT₂A receptors have been consistently involved in mood regulation (Nestler et al. 2002), and presynaptic 5-HT₁A receptors are specifically involved in the control of 5-HT neuronal firing rate (Blier et al. 1998). Postmortem brain studies in depressive suicide victims indicate a crucial role of 5-HT₁A and 5-HT₂A receptors in the pathogenesis of major depression (Yates et al. 1990; Stockmeier et al. 1998). Additionally, alterations in the expression and/or functionality of both 5-HT₁A and 5-HT₂A receptors have been reported following chronic antidepressant treatment (Blier and de Montigny 1998, Santarelli et al. 2003).

Behavioural and neurochemical evidence indicate that activation of CB₁ receptors modulates the responses elicited by the stimulation of 5-HT₁A and 5-HT₂A receptors. Cannabinoid agonists acting through CB₁ receptors have been shown to potentiate the expression of behavioural responses related to the activation of 5-HT₂A receptors, as DOI-induced back muscle contractions in rats (Cheer et al. 1999). Nevertheless, stimulation of CB₁ receptors has also been reported to reduce DOI-induced wet dog shakes in rats and head twitches in mice (Cheer et al. 1999; Darmani et al. 2003), whereas acute injection of the CB₁ receptor antagonist SR141716A (rimonabant) mimics 5-HT₂A receptor-related behaviours in
both rats and mice (Rubino et al. 2000; Darmani and Pandya 2000). Furthermore, in vitro administration of cannabinoid agonists reduces both electrically and Ca\(^{2+}\)-induced 5-HT release from cortical slices via presynaptic CB\(_1\) receptors (Nazaki et al. 2000), whereas the cannabinoid antagonist rimonabant produces a slight facilitation of in vivo 5-HT release in the prefrontal cortex (Tzavara et al. 2003).

Regarding the role of CB\(_1\) receptors in the effects elicited by activation of 5-HT\(_{2A}\) receptors, here we report that two behavioural responses elicited by DOI, head twitches (Heal et al., 1992) and paw tremors, are reduced in CB\(_1\) receptor knockout mice, supporting the idea that activation of CB\(_1\) receptors may contribute to these effects. This apparent contrast between our behavioural data and those reported by Darmani and Pandya (2000) is difficult to interpret, but might reflect strain differences (CD\(_1\) vs C57BL\(_6\) mice) or differences between the effects of genetic deletion and pharmacological blockade of CB\(_1\) receptors. The behavioural data reported here cannot be associated to a a reduced expression of 5-HT\(_{2A}\) receptors in the brain of CB\(_1\) receptor knockout mice, as no modification in receptor density was observed in our autoradiographic studies. Nevertheless, the possibility that genetic deletion of CB\(_1\) receptors would be accompanied by a reduced transductional ability of 5-HT\(_{2A}\) receptors cannot be completely excluded. Additional research will be needed in order to clarify the exact mechanisms of interaction between EC system and 5-HT\(_{2A}\) receptor-related responses.

A notable finding in the present study is that deletion of CB\(_1\) receptors reduced the hypothermic response induced by the 5-HT\(_{1A}\) agonist 8-OH-DPAT. This result suggests that inactivation of the CB\(_1\) receptor might alter the expression and/or functionality of 5-HT\(_{1A}\) receptors. Our present findings reveal that the lack of the CB\(_1\) receptors did not modify the density of the 5-HT\(_{1A}\) receptors but induced a tendency to the decrease in the ability of 8-OH-DPAT to stimulate \([^{35}S]\)GTP\(_{\gamma}\)S binding in forebrain areas, this decrease being significant in
the CA1 field of the hippocampus. By contrast, no difference between genotypes was found regarding the coupling ability of 5-HT1A autoreceptors in the DRN to G_i/o proteins. When interpreting these data, it should be taken into account that the anatomical basis of 8-OH-DPAT-induced hypothermia is controversial. In rats and humans, this hypothermic response is widely accepted to be mediated by activation of postsynaptic 5-HT1A receptors (Blier et al. 2006). In mice, although this response has been proposed to be presynaptically-mediated (Goodwin et al. 1985), Meller et al. (1992) did not observe any attenuation of 8-OH-DPAT-induced hypothermia in mice following depletion of central 5-HT by para-chlorophenylalanine (PCPA). Furthermore, mice selectively overexpressing 5-HT1A receptors in cortex and dentate gyrus exhibit enhanced hypothermic response to 8-OH-DPAT, suggesting that forebrain 5-HT1A receptors may also be involved in thermoregulation (Bert et al. 2006). At the light of these data, it can be suggested that the reduced coupling ability of 5-HT1A receptors in the hippocampus of CB1 knockout mice might contribute, at least in part, to the reduced hypothermic response to 8-OH-DPAT observed in these mutant.

Previous data suggest that relationship between CB1 and 5-HT1A receptors may also participate in the control of emotional behaviour. Mice deficient in 5-HT1A receptors exhibit heightened anxiety-like behaviours (Parks et al. 1998), whereas agonist activation of these receptors results in anxiolytic-like effects (De Vry 1995). Regarding the role of CB1 receptors in mood regulation, agonist activation of CB1 receptors or blockade of anandamide hydrolysis results in anxiolytic-like responses (Kathuria et al. 2003; Patel and Hillard 2003), whereas the CB1 receptor antagonist rimonabant induces anxiety-like effects in the rat (Navarro et al. 1997). Consistently, genetic ablation of CB1 receptors exacerbates normal reactions to acute stress, presumably by disabling the EC modulation of these reactions. Thus, CB1 knockout mice also display increased levels of anxiety in different behavioural paradigms (Martín et al. 2002; Haller et al. 2004; Urigüen et al. 2004). In agreement with the
reduced hypothermic response to 8-OH-DPAT in CB₁ deficient mice reported in this study, the anxiolytic-like effect of the partial 5-HT₁₅ agonist buspirone was impaired in the same strain of CB₁ knockout mice (Urigüen et al. 2004). Although studies of the mechanisms underlying the anxiolytic properties of 5-HT₁₅ receptor agonists tend to favour a presynaptic action, experimental data also indicate the possible involvement of postsynaptic mechanisms (Barnes and Sharp 1999). Altogether, these data suggest that the reduced functional coupling of 5-HT₁₅ receptors to Gᵢ/o proteins in the brain of CB₁ knockout mice might also contribute to the reduced anxiolytic efficacy of buspirone reported in these animals.

The possible role of EC-5-HT interactions in mood behaviour regulation is further supported by additional findings. Thus, postmortem studies report increased coupling ability of CB₁ receptors in the prefrontal cortex of depressed suicides (Mato et al. 2001; Hungund et al. 2004), suggesting the involvement of the EC system in the development of depressive disorders. Chronic antidepressant treatment modifies the expression of CB₁ receptors in the rat brain, indicating a possible role of these receptors in the mechanisms of action of antidepressants (Oliva et al. 2005; Hill et al. 2006). Furthermore, the inhibitor of anandamide enzymatic hydrolysis URB597 enhances the firing activity of 5-HT neurons in the DNR of anesthetized rats through CB₁ receptor-dependent mechanisms, and exerts antidepressant-like effects in acute predictive antidepressant tests, such us the mouse tail suspension test and the rat forced swimming test (Gobbi et al. 2005). Paradoxically, it has also been suggested that CB₁ receptor antagonists could also induce a suppression of the immobility in both behavioural models (Tzavara et al. 2003, Shearman et al. 2003). In the present study, we report that CB₁ receptor knockout mice exhibit higher despair behaviour in the tail suspension test compared to wild-type littermates. These data are consistent with those reported by Martin et al. (2002), showing that CB₁ receptor knockout mice present a higher sensitivity to
exhibit anhedonia after the exposure to the chronic unpredictable mild stress procedure, supporting a CB₁ receptor-induced antidepressant-like effect (Gobbi et al. 2005).

Our data show no significant differences between genotypes regarding the responses induced by the antidepressants imipramine and fluoxetine, in the tail suspension test. Similar responses were observed after the administration of these compounds in the forced swimming test. In addition, preliminary experiments have revealed that the antidepressant-like responses induced by two other antidepressant drugs, desipramine (7.5 mg/kg, i.p.) and phenelzine (40 mg/kg, i.p.) in the forced swimming test were also similar in both genotypes (data not shown). Overall, these data indicate that CB₁ receptors do not contribute to the acute behavioural effects of antidepressant drugs. These later results must be considered within the complexity of the depressive disorders and the limitations in the interpretation of the data obtained by using acute animal models in order to elucidate the mechanisms of action of antidepressants. At the light of previous data (Oliva et al. 2005; Hill et al. 2006), further studies using transgenic animals should be carried out in order to clarify the role of CB₁ receptors in the long-term effects of antidepressant compounds.

In conclusion, the results of the present study indicate that the integrity of CB₁ receptor-dependent EC system is necessary for the full activation of brain 5-HT₁A and 5-HT$_{2A}$ receptors, further demonstrating the existence of crosstalk mechanisms between brain EC and 5-HT systems. Our data suggest that activation of CB₁ receptors contributes to certain 5-HT₁A and 5-HT$_{2A}$ receptor-mediated behavioural responses and strengthen the idea that pharmacological manipulation of the EC system could be useful to the management of anxiety and depressive disorders.
Acknowledgements

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### Table 1. 5-HT$_{1A}$ receptor-stimulated [35S]GTP$_{\gamma}$S binding in brain sections of CB$_1$ receptor knockout mice

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Basal binding (nCi/g te)</th>
<th>% Stimulation (±)-8-OH-DPAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>CB$_1$ knockout</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>64.2 ± 5.9</td>
<td>67.2 ± 13.2</td>
</tr>
<tr>
<td>Fronto-parietal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layers I-III</td>
<td>53.9 ± 5.6</td>
<td>56.2 ± 10.2</td>
</tr>
<tr>
<td>Layers IV-VI</td>
<td>69.1 ± 6.6</td>
<td>68.0 ± 11.6</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>106.3 ± 11.9</td>
<td>99.2 ± 8.8</td>
</tr>
<tr>
<td>CA$_1$</td>
<td>89.9 ± 8.7</td>
<td>94.0 ± 6.2</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>117.2 ± 16.9</td>
<td>89.1 ± 6.0</td>
</tr>
<tr>
<td>DNR</td>
<td>173.7 ± 26.2</td>
<td>176.9 ± 20</td>
</tr>
</tbody>
</table>

Data are shown as mean ± S.E.M. of basal activity or of percentage of effect over basal activity. N= 6-10 mice per group. CA$_1$, CA$_1$ field of the hippocampus. DRN, dorsal raphe nucleus. p < 0.05 when compared with wild-type animals using a two-tail Student’s $t$-test.
LEGEND OF THE FIGURES

Figure 1. Behavioural responses to 5-HT_{1A} and 5-HT_{2A} receptor agonists in wild-type and CB_{1} receptor knockout mice. Effects of the 5-HT_{2A} receptor agonist (±)DOI on head twitches (A) and paw tremor (B) behaviours. (±)DOI (0.3 and 1 mg/kg, i.p.) induced both responses in wild-type and CB_{1} receptor knockout mice. When compared with wild-type animals, CB_{1} receptor knockout mice exhibited a significant reduction in head twitches and paw tremor behaviour. (C) Effect of the 5-HT_{1A} receptor agonist (±)-8-OH-DPAT on rectal temperature. 8-OH-DPAT (1 and 3 mg/kg) induced hypothermia in both genotypes. CB_{1} receptor knockout mice (KO) showed a significant reduction of the hypothermic effect at the highest dose used (3 mg/kg) compared with wild-type littermates. Data are shown as mean ± S.E.M. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared with saline group of the same genotype (SAL). # p < 0.05, ## p < 0.01 and ### p < 0.001 when compared with wild-type animals receiving the same treatment. N = 13-15.

Figure 2. Effects of the tricyclic antidepressant imipramine (7.5 mg/kg, i.p.) (A) and the SSRI fluoxetine (10 mg/kg, i.p.) (B) in the tail suspension test. Both antidepressant compounds induced a decrease in immobility time of wild-type and CB_{1} receptor knockout mice. Data are presented as mean ± S.E.M. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared with saline group of the same genotype (SAL). # p < 0.05 and ### p < 0.001 when compared with wild-type animals receiving the same treatment. N = 9-14.

Figure 3. Autoradiography of 5-HT_{1A} and 5-HT_{2A} receptors in wild-type and CB_{1} receptor knockout mice. Quantitative values of (A) [^3H]ketanserin and (B) [^3H](±)-8-OH-DPAT binding sites throughout the brain from wild-type (white bars) and CB_{1} receptor knockout (black bars) mice. Cg, cingulate cortex; Cx, fronto-parietal cortex; CPut, caudate-putamen;
ECx, entorhinal cortex; NAc, nucleus accumbens; CA1, CA1 field of the hippocampus; DG, dentate gyrus; DNR, dorsal raphe nucleus. Data are shown as mean ± S.E.M. N = 6-11.

**Figure 4.** 5-HT$_{1A}$ receptor-stimulated $[^{35}S]$GTP$\gamma$S autoradiography. Brain sections at the level of the hippocampus showing basal (A, B), (±)-8-OH-DPAT (100 μM)-stimulated $[^{35}S]$GTP$\gamma$S binding (A’, B’) and $[^{35}S]$GTP$\gamma$S binding in the presence of (±)-8-OH-DPAT (100 μM) and WAY10635 (10 μM) (A”, B”’) in wild-type (A) and CB$_1$ receptor knockout (B) mice. CA1, CA1 field of the hippocampus. Scale bar = 0.1 mm.
Figure 1
Figure 2
Figure 3
Figure 4