CB_1 knockout mice display impaired functionality of $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A/C}$ receptors

Susana Mato,*[,1] Ester Aso,† Elena Castro,*[,‡] Miquel Martín,† Olga Valverde,† Rafael Maldonado† and Ángel Pazos*[,‡]

*Department of Physiology and Pharmacology, University of Cantabria, Cantabria, Spain

†Laboratori de Neurofarmacologia, Departament de Cienciès Experimentals i de la Salut. Universitat Pompeu Fabra, Barcelona, Spain

‡Instituto de Biomedicina y Biotecnología de Cantabria IBBTEC (Universidad de Cantabria-CSIC-IDICAN), Cantabria, Spain

Abstract

Interaction between brain endocannabinoid (EC) and serotonin (5-HT) systems was investigated by examining 5-HT-dependent behavioral and biochemical responses in CB_1 receptor knockout mice. CB_1 knockout animals exhibited a significant reduction in the induction of head twitches and paw tremor by the $5\text{-}HT_{2A/C}$ receptor selective agonist (±) DOI, as well as a reduced hypothermic response following administration of the $5\text{-}HT_{1A}$ receptor agonist (±)-8-OH-DPAT. Additionally, exposure to the tail suspension test induced enhanced despair responses in CB_1 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 CB_1 receptor knockout and wild-type mice. No differences were found between both genotypes with regard to $5\text{-}HT_{2A}$ receptor and $5\text{-}HT_{1A}$ receptors levels, measured by autoradiography in different brain areas. However, a significant decrease in the ability of both, the $5\text{-}HT_{1A}$ receptor agonist (±)-8-OH-DPAT and the $5\text{-}HT_{2A/C}$ receptor agonist (–)DOI, to stimulate $[^{35}S]GTP\gamma S$ binding was detected in the hippocampal CA_1 area and fronto-parietal cortex of CB_1 receptor knockout mice, respectively. This study provides evidence that CB_1 receptors are involved in the regulation of serotonergic responses mediated by $5\text{-}HT_{2A/C}$ and $5\text{-}HT_{1A}$ receptors, and suggests that a reduced coupling of $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptors to G proteins might be involved in these effects.

Keywords: $5\text{-}HT_{1A}$ receptors, $5\text{-}HT_{2A/2C}$ receptors, antidepressants, CB_1 receptors, hypothermia, $[^{35}S]GTP\gamma S$ labelling, tail suspension test.

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Cannabis sativa derivatives have been used for over 4000 years and remain the most widely consumed illicit drugs nowadays because of their mood-altering properties (Lichtman and Martin 2005). The pleasurable subjective effects associated with *Cannabis* use include an initial period of euphoria and relaxation that can be associated with perceptive distortions depending on the dose and individual susceptibility. Nevertheless, the use of *Cannabis* preparations, generally at higher doses, has also been associated with unpleasant mind-disturbing effects, such as anxiety and panic attacks, acute psychosis and paranoia (Ameri 1999). The main psychoactive compound of *Cannabis* preparations, Δ^9-tetrahydrocannabinol (Δ^9-THC), modulates brain function mainly through the activation of a $G_{i/o}$ protein-coupled receptor known as CB_1 (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). CB_1 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 CB_1 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.*

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Address correspondence and reprint requests to Dr Ángel Pazos, Department of Physiology and Pharmacology, University of Cantabria, Santander 39011, Cantabria, Spain. E-mail: pazosa@unican.es

[1]The present address of Susana Mato is the Department of Neuroscience, University of the Basque Country, 48940 Leioa, Bizkaia, Spain.

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

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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-HT_{1A} and 5-HT_{2A} receptor subtypes because of 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 (Stockmeier *et al.* 1998). Furthermore, the antagonism of pre-synaptic 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.* 2004).

Experimental data of different kind support the existence of interaction mechanisms between brain EC and 5-HT systems. Concerning the role of CB_1 receptors in 5-HT_{2A} receptor-mediated responses, conflicting data have been reported, as cannabinoid agonists have been proposed to potentiate and/or decrease the behavioral effects of the $5\text{-HT}_{2A/C}$ receptor agonist DOI in rats and mice (Cheer *et al.* 1999; Darmani and Pandya 2000; Darmani *et al.* 2003; Gorzalka *et al.* 2005). 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), and consistently, pharmacological blockade of CB_1 receptors has been reported to increase 5-HT efflux in the rat and shrew forebrain (Darmani *et al.* 2003; Tzavara *et al.* 2003). Nevertheless, a recent study showed antidepressant-like activity and 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 both the anxiogenic and the anxiolytic effect of cannabinoid agonists (Marco *et al.* 2004; Braida *et al.* 2007). 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 CB_1 gene (Ledent *et al.* 1999). Taking into account the experimental evidence abovementioned, as well as the fact that CB_1 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 CB_1-5-HT interactions. The specific objective of this study was to examine the state of 5-HT functionality in CB_1 receptor knockout mice, in an attempt to clarify the interaction mechanisms between EC and 5-HT brain systems. We evaluated the behavioral responses elicited by the selective 5-HT_{1A} and $5\text{-HT}_{2A/C}$ receptor agonists, as well as by several antidepressant drugs, in mice lacking the CB_1 receptor. 5-HT_{1A} and $5\text{-HT}_{2A/C}$ density and their functional coupling to G proteins were also examined in the brain of CB_1 receptor knockout mice using $[^{35}S]$GTP γS procedures.

Materials and methods

Animals

Male CB_1 receptor knockout mice and wild-type littermates (30–32 g) were used in this study. CB_1 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 CB_1 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°C) 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 $5\text{-HT}_{2A/C}$ receptor agonist, 1-(2, 5-dimethoxy-4-odophenyl)-2-aminopropane ((±)DOI), the 5-HT_{1A} 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.

Behavioral observations

Behavioral responses to (±)DOI

Mice were administered with saline or the $5\text{-HT}_{2A/C}$ 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 s to record the temperature. Basal body temperature was recorded

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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 behaviors 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 use. For autoradiographic studies, 20 μm thick-coronal sections were cut using a microtome cryostat, thaw-mounted in gelatinised slides and stored at −20°C until use.

For binding assays, samples from the anterior fronto-parietal cortex (2–2.5 mm; about 30 mg) were homogenized in 100 volumes of cold buffer containing 50 mmol/L Tris–HCl, 1 mmol/L EGTA and 100 mmol/L NaCl (pH 7.5). The homogenate was first centrifuged at 900 *g* for 5 min at 4°C and the resultant supernatant was centrifuged at 40 000 *g* for 20 min at 4°C. The pellet was resuspended in the same buffer, incubated at 30°C for 10 min and centrifuged again (40 000 *g* for 20 min at 4°C). A second incubation and final centrifugation (40 000 *g* for 20 min at 4°C) were performed to obtain the final pellet that was stored at −80°C until assay.

5-HT_{2A} receptor autoradiography
Sections were pre-incubated at 25°C for 30 min in 170 mmol/L Tris–Cl (pH 7.6), and then incubated at 25°C for 60 min in the same buffer containing 2 nmol/L [^{3}H]ketanserin. Non-specific binding was determined using 10 μmol/L mianserin. Sections were washed twice for 10 min in ice-cold 170 mmol/L Tris–Cl (pH 7.4), dipped in cold distilled water and cold air-dried. Slides were exposed to Hyperfilm™-^{3}H along with ^{3}H-labeled standards for 8 weeks at 4°C.

5-HT_{1A} receptor autoradiography
Sections were pre-incubated at 25°C for 30 min in a 170 mmol/L Tris–HCl buffer (pH 7.5) containing 4 mmol/L $CaCl_2$ and 0.01% ascorbic acid, and subsequently incubated for 60 min in the same buffer containing 10 μmol/L pargyline and 2 nmol/L [^{3}H]8-OH-DPAT. Non-specific binding was determined using 10 μmol/L 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™- ^{3}H, Amersham, Switzerland) along with ^{3}H-labeled standards (Amersham) for 2 months at 4°C.

DOI-stimulated [^{35}S]GTPγS binding
The 5-HT_{2A} receptor-induced [^{35}S]GTPγS binding was carried out according to Adlersberg *et al.* (2000) with slight modifications. The membranes were resuspended (20–30 μg protein/tube) in assay buffer (50 mmol/L Tris–HCl, 1 mmol/L EGTA, 5 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L dl-dithiothreitol, 100 μmol/L guanosine 5′-[γ-thio]triphosphate tetralithium salt, pH 7.7) containing 10 mU/mL adenosine deaminase and 0.1% bovine serum albumin. Then, the membranes were pre-incubated with the agonist (−) DOI (5 and 10) for 20 min at 30°C. The incubation was continued in the presence of 0.2 nmol/L [^{35}S]GTPγS for 2 h and terminated by rapid filtration through presoaked Whatman GF/B filters with ice-cold Tris–HCl buffer (0.1% bovine serum albumin) following by three washes. Basal binding was determined in the absence of the agonist and non-specific binding was determined in the presence of unlabeled 10 μmol/L of guanosine-5-*O*-(3-thio) triphosphate (GTPγS).

5-HT_{1A} receptor-stimulated [^{35}S]GTPγS autoradiography
Sections were pre-incubated for 30 min at 25°C in a buffer containing 50 mmol/L Tris–HCl, 0.2 mmol/L EGTA, 3 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L dl-dithiothreitol and 2 mmol/L guanosine 5′-[γ-thio]triphosphate tetralithium salt (pH 7.7), and subsequently incubated for 120 min in the same buffer containing 3 mU/mL adenosine deaminase and 0.04 nmol/L [^{35}S]GTPγS. Consecutive sections were incubated with 100 μmol/L (±)-8-OH-DPAT alone or in the presence of 10 μmol/L WAY100635. Non-specific binding was determined in the presence of 10 μmol/L guanosine-5-*O*-(3-thio) triphosphate (GTPγS). After the incubation, the sections were washed twice for 15 min in 50 mmol/L Tris–HCl buffer (pH 7.4) at 4°C, rinsed in distilled cold water and cold air dried. Sections were exposed to β radiation-sensitive films (Hyperfilm™-βmax, Amersham, Switzerland) together with ^{14}C-polymer standards (Amersham, Switzerland) for 2 days at 4°C.

Data analysis

All data were expressed as mean ± SEM. Behavioral 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 *t*-test). Autoradiograms were scanned and images analyzed by using the National Institute of Health IMAGE program (Bethesda, MD, USA). 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 ^{3}H and ^{14}C microscales, respectively, and compared using a two-tail Student's *t*-test. For

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[^{35}S]GTPγS membranes assays, specific (−)DOI-stimulated binding was expressed as the percentage over the basal binding (100%).

Results

Behavioral changes induced by the 5-HT$_{2A/C}$ receptor agonist DOI were attenuated in CB$_1$ knockout mice

The administration of 5-HT$_{2A/C}$ 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 (±)-8-OH-DPAT 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

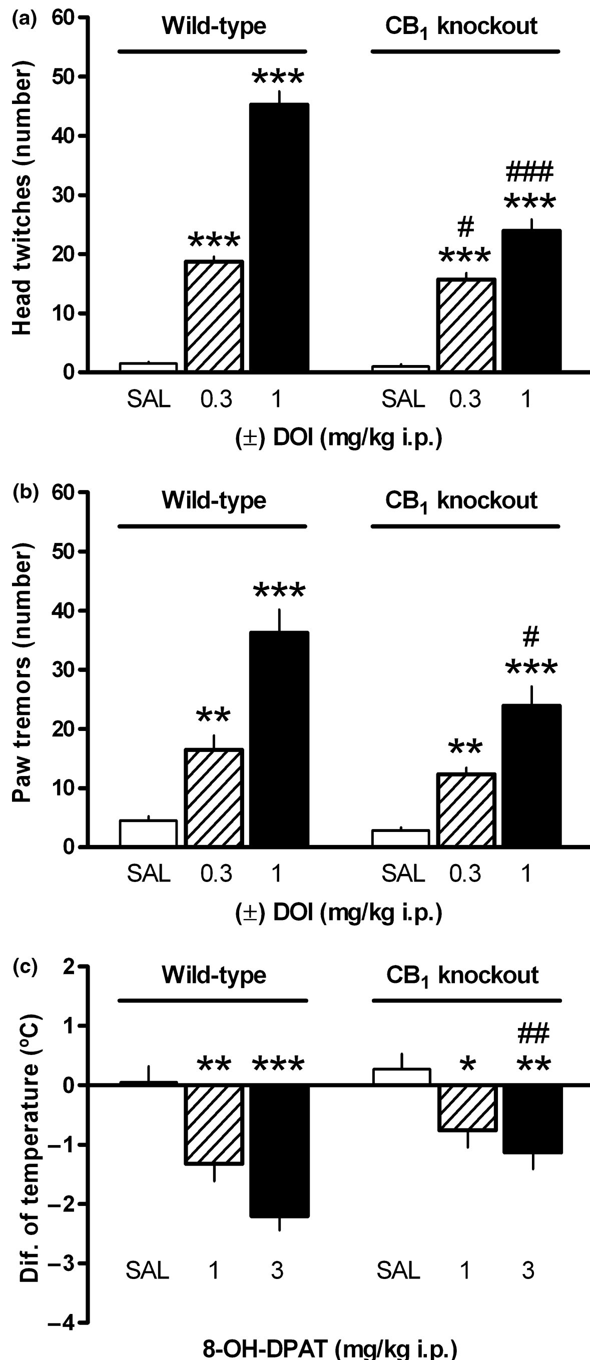

Fig. 1 Behavioral responses to 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptor agonists in wild-type and CB$_1$ receptor knockout mice. Effects of the 5-HT$_{2A}$ receptor agonist (±)DOI on head twitches (a) and paw tremor (b) behaviors. (±)DOI (0.3 and 1 mg/kg, i.p.) induced both responses in wild-type and CB$_1$ receptor knockout mice. When compared with wild-type animals, CB$_1$ receptor knockout mice exhibited a significant reduction in head twitches and paw tremor behavior. (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 ± SEM *$p < 0.05$,**$p < 0.01$ 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. $n = 13–15$.

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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, ($F_{1,17} = 43.60$, $p < 0.001$); fluoxetine, ($F_{1,18} = 5.67$, $p < 0.05$)], as well as significant differences were observed between wild-type and knockout antidepressant-treated groups ($F_{1,17} = 5.43$, $p < 0.05$, imipramine experiment; $F_{1,18} = 5.80$, $p < 0.05$, 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).

$5\text{-}HT_{2A}$ receptor autoradiography and (−) DOI stimulation of $[^{35}S]GTP\gamma S$ binding

High densities of $[^3H]$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 CB_1 receptor knockout mice revealed no significant changes in the density of $5\text{-}HT_{2A}$ receptors in any of the brain areas analyzed (Fig. 3a).

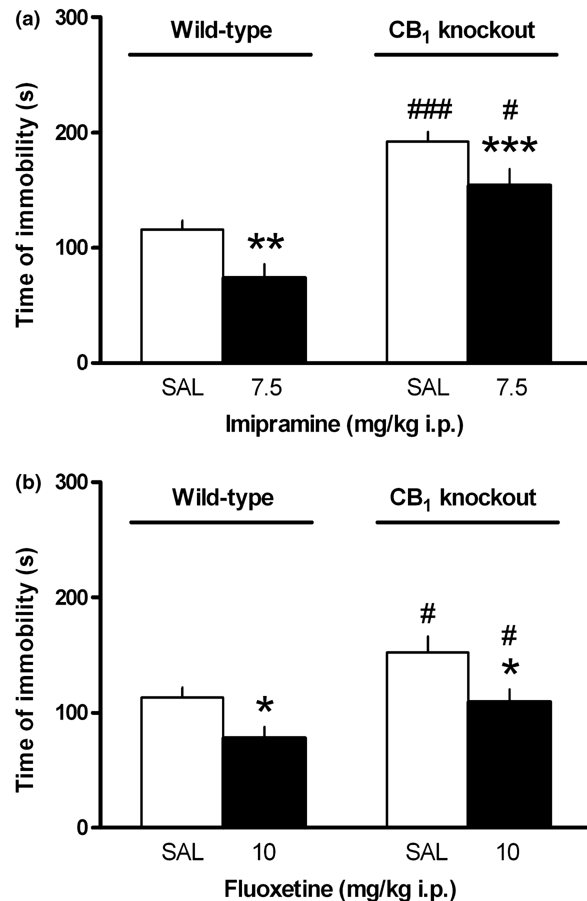

Fig. 2 Effects of the tricyclic antidepressant imipramine (7.5 mg/kg, i.p.) (a) and the SSRI fluoxetine (10 mg/kg, i.p.) (b) in the tail suspension test. Both antidepressant compounds induced a decrease in immobility time of wild-type and CB_1 receptor knockout mice. Data are presented as mean ± SEM $^*p < 0.05$, $^{**}p < 0.01$ 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.

In wild-type mice, a specific (−)DOI-induced stimulation of $[^{35}S]GTP\gamma S$ binding was obtained in homogenates from the anterior frontoparietal cortex (Fig. 4). In knockout animals, a significant reduction of the $5\text{-}HT_{2A/C}$ agonist-induced stimulation of $[^{35}S]GTP\gamma S$ binding was observed for both concentrations of DOI (131.4 ± 6.1% vs. 112.7 ± 6.6%; 130.2 ± 5.9 vs. 107.8 ± 6.8%; $p < 0.05$) (Fig. 4).

$5\text{-}HT_{1A}$ receptor and 8-OH-DPAT-stimulated $[^{35}S]GTP\gamma S$ autoradiography

The hippocampal formation (dentate gyrus and CA_1 field), cortical areas, including the entorhinal cortex, and raphe nuclei showed the highest levels of $[^3H]$8-OH-DPAT specific binding in the mice brain. Similar $[^3H]$8-OH-DPAT binding levels were measured in the different brain areas of wild-type and CB_1 receptor knockout mice (Fig. 3b).

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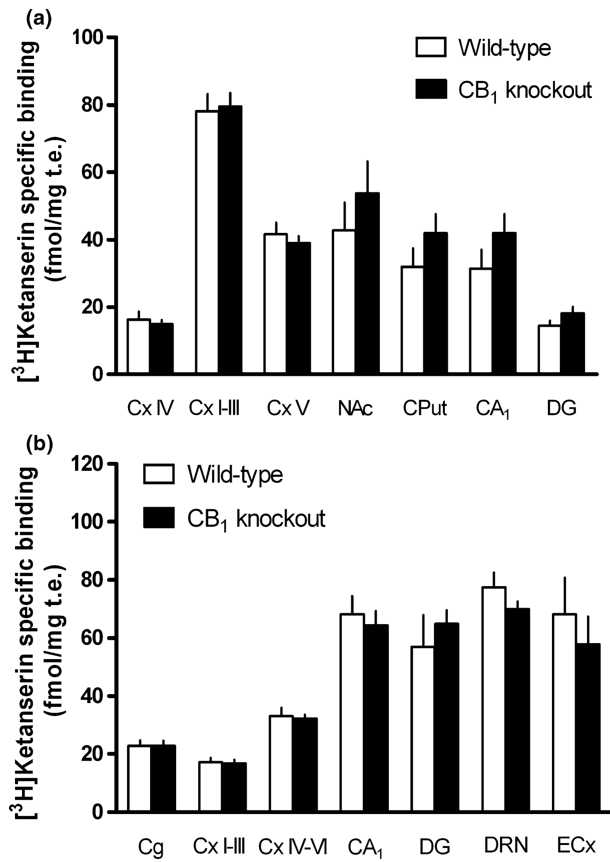

Fig. 3 Autoradiography of 5-HT_{1A} and 5-$HT_{2A/C}$ receptors in wild-type and CB_1 receptor knockout mice. Quantitative values of (a) [^{3}H]ketanserin and (b) [^{3}H](±)-8-OH-DPAT binding sites throughout the brain from wild-type (white bars) and CB_1 receptor knockout (black bars) mice. Cg, cingulate cortex; Cx, fronto-parietal cortex; CPut, caudate-putamen; ECx, entorhinal cortex; NAc, nucleus accumbens; CA_1, CA_1 field of the hippocampus; DG, dentate gyrus; DNR, dorsal raphe nucleus. Data are shown as mean ± SEM n = 6–11.

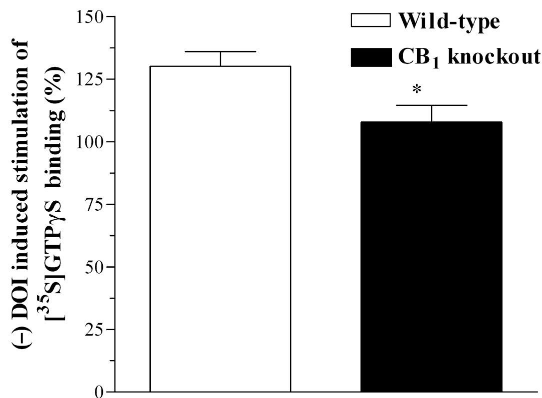

Fig. 4 Stimulation induced by 10 nmol/L (–) DOI on [^{35}S]GTPγS binding in fronto-parietal cortex membranes. The data represent the percentage of basal binding (100%) and are expressed as the mean ± SEM from five separate experiments (*$p < 0.05$ Student's t-test unpaired data; n = 5 per group).

The range of (±)8-OH-DPAT-stimulated [^{35}S]GTPγS binding in brain sections from both wild-type and CB_1 receptor knockout mice revealed a pattern of localization in good match with the anatomical distribution of 5-HT_{1A} receptors. The selective 5-HT_{1A} antagonist WAY100635 (10 μmol/L) blocked the effect of (±)8-OH-DPAT in all brain areas (Fig. 5). Basal [^{35}S]GTPγS 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}S]GTPγS 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. 5).

Discussion

This study provides evidence suggesting that lifelong deletion of the CB_1 receptor reduces the functionality of brain 5-HT system. In this regard, three behavioral responses associated with the activation of 5-HT receptors were attenuated in the CB_1 knockout mice: the head twitches and paw tremors elicited by the 5-$HT_{2A/C}$ receptor agonist DOI, as well as the decrease in body temperature induced by the 5-HT_{1A} agonist 8-OH-DPAT.

Regarding the role of the EC system in the effects elicited by activation of 5-$HT_{2A/C}$ receptors, the present data strengthen the idea that certain 5-$HT_{2A/C}$ receptors-mediated behaviors involve the activation of CB_1 receptors, previously suggested by Cheer *et al.* (1999), who reported that the administration of cannabinoid agonists potentiates DOI-induced rat back muscle contractions in a CB_1 receptor-dependent manner. Also, the present report is consistent with a recent work showing that chronic exposure to the cannabinoid agonist HU210 reduces DOI-induced back muscle contractions in rats (Hill *et al.* 2006a), suggesting that CB_1 receptor-activation contributes to this 5-$HT_{2A/C}$ receptors-mediated response (Cheer *et al.* 1999). Indeed, chronic administration of cannabinoid agonists induces desensitization and/or down-regulation of CB_1 receptors (Sim-Selley 2003), and this fact could contribute to the reduction in DOI-induced back muscle contractions following a long-term cannabinoid agonist exposure (Hill *et al.* 2006a). Nevertheless, the data currently available in the literature indicate that the regulation of 5-$HT_{2A/C}$ receptor-mediated responses by the EC system is a complex issue. For instance, both a potentiation of DOI-induced back muscle contractions and a reduction of DOI-induced wet-dog shakes in the rat following the administration of the cannabinoid agonist HU-210 (Cheer *et al.* 1999) or the EC uptake inhibitor AM404 (Gorzalka *et al.* 2005) have been described. Furthermore, chronic HU-210 administration enhances the wet-dog shakes induced by DOI in rats at the same time that

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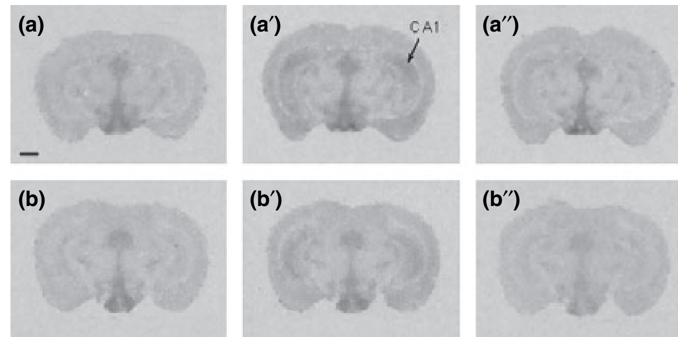

Fig. 5 $5\text{-}HT_{1A}$ receptor-stimulated [^{35}S]GTPγS autoradiography. Brain sections at the level of the hippocampus showing basal (a and b), (±)-8-OH-DPAT (100 μmol/L)-stimulated [^{35}S]GTPγS binding (a′ and b′) and [^{35}S]GTPγS binding in the presence of (±)-8-OH-DPAT (100 μmol/L) and WAY10635 (10 μmol/L) (a″and b″) in wild-type (a) and CB_1 receptor knockout (b) mice. CA_1, CA_1 field of the hippocampus. Scale bar: 0.1 mm.

Table 1 $5\text{-}HT_{1A}$ receptor-stimulated [^{35}S] GTPγS binding in brain sections of CB_1 receptor knockout mice

	Basal binding (nCi/g te)		% Stimulation (±)-8-OH-DPAT	
Brain area	Wild-type	CB_1 knockout	Wild-type	CB_1 knockout
Cingulate cortex	64.2 ± 5.9	67.2 ± 13.2	72.3 ± 19.7	59.7 ± 21.8
Fronto-parietal cortex				
Layers I–III	53.9 ± 5.6	56.2 ± 10.2	107.4 ± 55.4	86.9 ± 47.0
Layers IV–VI	69.1 ± 6.6	68.0 ± 11.6	106.5 ± 49.2	68.6 ± 28.5
Entorhinal cortex	106.3 ± 11.9	99.2 ± 8.8	77.9 ± 16.6	42.7 ± 8.3
CA_1	89.9 ± 8.7	94.0 ± 6.2	128.7 ± 32.6	69.7 ± 10.2*
Dentate gyrus	117.2 ± 16.9	89.1 ± 6.0	58.0 ± 18.3	53.3 ± 12.6
DNR	173.7 ± 26.2	176.9 ± 20	31.3 ± 9.3	37.4 ± 11.0

Data are shown as mean ± SEM 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.

reduces the DOI-induced back muscle contractions (Hill *et al.* 2006a). Taking into account that both DOI-induced behaviors are mediated by $5\text{-}HT_{2A/C}$ receptors (Fone *et al.* 1991), these data strongly indicate that distinct $5\text{-}HT_{2A/C}$ receptors-behaviors are differently regulated by the EC system. In contrast with the idea that activation of CB_1 receptors contributes to $5\text{-}HT_{2A/C}$ receptor-mediated responses, it has also been reported that cannabinoid agonists reduce DOI-induced head twitches in mice (Darmani 2001; Egashira *et al.* 2004), and that the administration of CB_1 receptor antagonists mimics or potentiates $5\text{-}HT_{2A/C}$ receptors-related behaviors in both rats and mice (Darmani and Pandya 2000; Gorzalka *et al.* 2005). The apparent discrepancy between some of these data and the present report is difficult to interpret, but might reflect species and/or strain differences (CD1 vs. C57BL6 mice) or differences between the effects of genetic deletion and pharmacological blockade of CB_1 receptors. The behavioral data reported here cannot be associated with reduced levels of brain $5\text{-}HT_{2A/C}$ receptors in CB_1 receptor knockout mice, since no significant change in receptor density was observed in our autoradiographic studies. They could also be the consequence of a reduction in the functionality of $5\text{-}HT_{2A/C}$ receptors. In this regard, we have carried out [^{35}S]GTPγS studies following stimulation with DOI. It is noteworthy that previous studies have shown that specific $5\text{-}HT_2$ labeling is almost restricted cortical regions, i.e., frontoparietal motor and somatosensory and anterior cingulate cortex (Gresch *et al.* 2005). This evidence led us to perform these [^{35}S]GTPγS studies in cortical (frontoparietal) homogenates. Our results show that CB_1 receptor knockout mice present a significant decrease in the ability of DOI to stimulate [^{35}S]GTPγS binding, which could be in support of the existence of a functional deficit for $5\text{-}HT_{2A/C}$ receptors. However, caution is needed when interpreting these results: the involvement of other mechanisms cannot be discarded and, in addition, head twitches behavior appears to depend partially on striatum, an area where the labeling of specific $5\text{-}HT_2$ stimulation of [^{35}S]GTPγS binding is not yet feasible (Adlersberg *et al.* 2000; Gresch *et al.* 2005).

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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 genetic inactivation of the CB_1 receptor might alter the expression and/or functionality of 5-HT_{1A} receptors. Our findings reveal that the lack of the CB_1 receptors induces a decrease in the ability of 8-OH-DPAT to stimulate [^{35}S]GTPγS binding in the CA_1 field of the hippocampus, whereas no difference between genotypes was found concerning the coupling ability of 5-HT_{1A} autoreceptors in the dorsal raphe nucleus to $G_{i/o}$ proteins. Regarding these results, chronic administration of cannabinoid agonists has been shown to reduce 5-HT_{1A} receptor-mediated stimulation of [^{35}S]GTPγS binding in the hippocampus (Kelai *et al.* 2006), as well as the hypothermic response elicited by 8-OH-DPAT (Hill *et al.* 2006a). When interpreting these data, it should be taken into account that the anatomical basis of 8-OH-DPAT-induced hypothermia is controversial. This response has been proposed to be pre-synaptically mediated in mice (Goodwin *et al.* 1985). Nevertheless, 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. Furthermore, mice selectively over-expressing 5-HT_{1A} receptors in cortex and dentate gyrus exhibit enhanced hypothermic response to 8-OH-DPAT, suggesting that forebrain 5-HT_{1A} receptors are involved in thermoregulation (Bert *et al.* 2006). At the light of these data, it can be suggested that the reduced coupling ability of 5-HT_{1A} receptors in the hippocampus of CB_1 knockout mice may contribute to the reduced hypothermic response to 8-OH-DPAT observed in these mutants, in concordance with an involvement of post-synaptic 5-HT_{1A} receptors in this effect (Blier *et al.* 2006). In the same sense, the desensitization of CB_1 receptors in the hippocampus induced by its chronic stimulation (Sim-Selley 2003) would contribute to an attenuated 5-HT_{1A} receptor-mediated response (Hill *et al.* 2006a; Kelai *et al.* 2006). Noteworthy, previous data indicate that the 5-HT system is involved in the hypothermia induced by the activation of CB_1 receptors, with increased 5-HT firing activity resulting in a potentiation of Δ^9-THC-induced hypothermia (Malone and Taylor 2001). Indeed, Gobbi *et al.* (2005) have recently shown that the inhibition of anandamide hydrolysis increases firing activity of 5-HT neurons, suggesting that activation of post-synaptic 5-HT_{1A} receptors may contribute to the hypothermia induced by CB_1 receptor-stimulation. Along with these data, the present report strengthens the idea that EC and 5-HT systems use common pathways for the regulation of body temperature.

The precise mechanism by which genetic deletion of CB_1 receptors reduces 5-HT_{1A} and 5-$HT_{2A/C}$ receptors-mediated behaviors remains to be established. A number of studies have shown that CB_1 receptors are expressed in the developing nervous system, and there is some evidence from human and animal studies that suggest that prenatal exposure to cannabinoid affects neurobehavioral development (Fernández-Ruiz *et al.* 2000; Jin *et al.* 2004). Thus, it is conceivable that mice develop subtle brain defects in the absence of CB_1 receptors, and consequently affect the functionality of monoamine transmission (neurotransmitter levels, receptorial, and transductional mechanisms). Additional research further exploring the consequences of CB_1 receptor-genetic deletion on 5-HT neurotransmission may help clarify the exact mechanisms mediating the reduced 5-HT_{1A} and 5-$HT_{2A/C}$ receptors-behaviors observed in these animals.

Previous data suggest that relationship between CB_1 and 5-HT_{1A} receptors may also participate in the control of emotional behavior. Mice deficient in 5-HT_{1A} receptors exhibit heightened anxiety-like behaviors (Parks *et al.* 1998), whereas agonist activation of these receptors results in anxiolytic-like effects (De Vry 1995). Reminiscent of the 5-HT_{1A} receptor knockout phenotype, CB_1 receptor knockout mice also display increased levels of anxiety in different behavioral paradigms (Martín *et al.* 2002; Haller *et al.* 2004; Urigüen *et al.* 2004). Consistently, activation of CB_1 receptors induces anxiolytic-like responses (Kathuria *et al.* 2003; Patel and Hillard 2006; Braida *et al.* 2007), whereas the CB_1 receptor antagonist rimonabant induces anxiety-like effects in the rat (Navarro *et al.* 1997). Noteworthy, activation of 5-HT_{1A} receptors has been recently involved in the anxiolytic effects elicited by CB_1 receptor activation (Braida *et al.* 2007). In agreement with the reduced hypothermic response to 8-OH-DPAT in CB_1 deficient mice reported here, the anxiolytic-like effect of the partial 5-HT_{1A} agonist buspirone was impaired in the same strain of CB_1 knockout mice (Urigüen *et al.* 2004). Although studies of the mechanisms underlying the anxiolytic properties of 5-HT_{1A} receptor agonists tend to favor a pre-synaptic action, experimental data also indicate the possible involvement of post-synaptic mechanisms (Barnes and Sharp 1999). Altogether, these data suggest that the reduced functional coupling of 5-HT_{1A} receptors to $G_{i/o}$ proteins in the brain of CB_1 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 behavior regulation is further supported by additional findings. Thus, post-mortem studies report increased coupling ability of CB_1 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 CB_1 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.* 2006b). Furthermore, the inhibitor of anandamide enzymatic hydrolysis URB597 enhances the firing activity of 5-HT neurons in anesthetized rats through CB_1 receptor-

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dependent mechanisms, and exerts antidepressant-like effects in acute predictive antidepressant tests, such as the mouse tail suspension test and the rat forced swimming test (Gobbi *et al.* 2005). Paradoxically, it has also been suggested that CB_1 receptor antagonists could also induce a suppression of the immobility in both behavioral models (Shearman *et al.* 2003; Tzavara *et al.* 2003). In the present study, we report that CB_1 receptor knockout mice exhibit higher despair behavior in the tail suspension test compared to wild-type littermates. These data are consistent with those reported by Martín *et al.* (2002), showing that CB_1 receptor knockout mice present a higher sensitivity to exhibit anhedonia after the exposure to the chronic unpredictable mild stress procedure, supporting a CB_1 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 also observed after the administration of these compounds in the forced swimming test. Overall, these data indicate that CB_1 receptors do not contribute to the acute behavioral 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.* 2006b), further studies using transgenic animals should be carried out in order to clarify the role of CB_1 receptors in the long-term effects of antidepressant compounds.

In conclusion, the results of the present study indicate that the integrity of CB_1 receptor-dependent EC system is necessary for the correct activation of brain $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptors, further demonstrating the existence of crosstalk mechanisms between brain EC and 5-HT systems. Our data suggest that the activation of CB_1 receptors contributes to certain $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A/C}$ receptor-mediated behavioral responses and strengthen the idea that pharmacological manipulation of the EC system could be useful for the management of anxiety and depressive disorders.

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