Lack of CB₁ receptor activity impairs serotonergic negative feedback

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Abbreviations: 5-hydroxytryptamine or serotonin (5-HT), 5-hydroxytryptamine transporter (5-HTT), cannabinoid receptor 1 (CB₁), dorsal raphe nucleus (DRN), prefrontal cortex (PFC), hypothalamic pituitary adrenal axis (HPA).

Abstract

Serotonergic and endocannabinoid systems are important substrates for the control of emotional behavior and growing evidence show an involvement in the pathophysiology of mood disorders. In the present study, the absence of the activity of the CB₁ cannabinoid receptor impaired serotonergic negative feedback in mice. Thus, *in vivo* microdialysis experiments revealed increased basal 5-HT extracellular levels and attenuated fluoxetine-induced increase of 5-HT extracellular levels in the prefrontal cortex of CB₁ knockout compared to wild-type mice. These observations could be related to the significant reduction in the 5-HT transporter binding site density detected in frontal cortex and hippocampus of CB₁ knockout mice. The lack of CB₁ receptor also altered some 5-HT receptors related to the 5-HT feedback. Extracellular recordings in the dorsal raphe nucleus revealed that the genetic and pharmacological blockade of CB₁ receptor induced a 5-HT_{1A} autoreceptor functional desensitization. *In situ* hybridization studies showed a reduction in the expression of the 5-HT_{2C} receptor within several brain areas related to the control of the emotional responses, such as the dorsal raphe nucleus, the nucleus *accumbens* and the paraventricular nucleus of the hypothalamus, whereas an overexpression was observed in the CA3 area of the ventral hippocampus. These results reveal that the lack of CB₁ receptor induces a facilitation of the activity of serotonergic neurons in the dorsal raphe nucleus by altering different components of the 5-HT feedback as well as an increase in 5-HT extracellular levels in the prefrontal cortex in mice.

Keywords: 5-HT, CB₁ cannabinoid receptor, 5-HTT, 5-HT_{1A}, 5-HT_{2C}

Running title: 5-HT feedback in CB₁ KO mice

Introduction

The serotonergic system has been related to the control of emotional responses and the impairment of serotonin (5-HT) neurotransmission has been classically associated to the pathophysiology of mood disorders. Thus, some pharmacological agents that increase 5-HT synaptic levels exhibit antidepressant properties (Artigas et al, 2002; Nutt et al, 2002). 5-HT neurons are mainly located within the raphe nuclei. 5-HT is released from the terminals that project to multiple brain regions to regulate several behaviours, including cognition and mood. The removal of 5-HT from the synaptic cleft is carried out by a single protein, the 5-HT transporter (5-HTT). The 5-HTT determines the size and duration of the serotonergic responses, playing a key role in serotonergic neurotransmission (Lesch and Mössner, 2006). Up to 14 pre- and post-synaptic 5-HT receptors have been identified, which mediate 5-HT responses (Nichols and Nichols, 2008). 5-HT pre- and post-synaptic receptors also display a regulatory action on the serotonergic activity. Accordingly, the somatodendritic 5-HT_{1A} autoreceptor negatively controls cell firing and impulse-dependent 5-HT release of serotonergic neurons whereas the axonal 5-HT_{1B} receptor controls 5-HT synthesis and release at terminal level (Barnes and Sharp, 1999). Similarly, some post-synaptic receptors such as 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, also participate in the inhibition of 5-HT neurons activity (Hajós et al, 1999; Boothman et al, 2003). Among all these post-synaptic receptors, growing attention has been addressed toward understanding the role of 5-HT_{2C} receptor in the regulation of emotional state beyond the involvement in 5-HT feedback regulation. This receptor plays an essential role in the 5-HT-induced hypothalamic pituitary adrenal (HPA) axis activation (Heisler et al, 2007) and also mediates the

inhibition of dopaminergic system, a key component for hedonia and motivation (Dremencov et al, 2006).

Endocannabinoid system is one of the major neuromodulatory systems in the brain and previous studies revealed its contribution on maintaining the homeostasis of various states including mood and emotion (Valverde, 2005; Moreira and Lutz, 2008). In fact, the pharmacological and genetic blockade of the cannabinoid CB₁ receptor induces a phenotypic state analogous to depression in experimental animals (Hill and Gorzalka, 2005; Aso et al, 2008). Therefore, CB₁ knockout mice exhibit depressive-like symptoms such as reduced responsiveness to reward stimuli (Sanchís-Segura et al, 2004; Maldonado et al, 2006), altered neurovegetative functions (Cota et al, 2003), deficits in extinction of aversive memories (Marsicano et al, 2002) and higher anxiety levels and sensitivity to stress than control animals (Martín et al, 2002; Aso et al, 2008). The role of endocannabinoid system in regulating emotions and mood could be provided by the modulation of neurotransmitter release, including 5-HT, exerted by CB₁ receptors (Tzavara et al, 2003; Szabo and Schlicker, 2005; Bambico et al, 2007). The interaction between 5-HT and endocannabinoid systems is also sustained by the fact that 5-HT is able to evoke endocannabinoid release (Parrish and Nichols, 2006; Best and Regehr, 2008). However, further investigations are needed to better understand the interaction between the endocannabinoid system and the different components of the serotonergic system in the modulation of mood and emotions. In this study, we therefore aimed to investigate the role of endocannabinoid system in the regulation of 5-HT neurotransmission by assessing the functionality of some serotonergic components involved in 5-HT feedback in the absence of CB₁ receptor.

Materials and methods

Animals

The experiments were carried out in male CB₁ knockout mice and wild-type littermates 8-12 weeks old at the beginning of the experiments. The generation of mice lacking CB₁ cannabinoid receptor was described previously (Ledent et al, 1999). In order to homogenize the genetic background of mice, the first generation heterozygous was bred for 30 generations on a CD1 background, with selection for the mutant CB₁ gene at each generation. After the 30th generation of backcross, heterozygote-heterozygote mating of CB₁ knockout mice produced wild-type and knockout littermates for subsequent experiments. All animals used in a given experiment were matched for age and weight. Mice were housed five per cage in a temperature ($21 \pm 1^{\circ}\text{C}$) and humidity-controlled ($55 \pm 10\%$) room with a 12:12-h light/dark cycle (light between 08:00 and 20:00 h) with food and water *ad libitum*. Animal procedures were conducted according to ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Local Ethical Committee (PRBB).

Drugs

The selective 5-HT reuptake inhibitor (SSRI) fluoxetine hydrochloride was supplied by Sigma[®] Chemical Co (Madrid, Spain). Fluoxetine was dissolved in distilled water and injected intraperitoneally (i.p.) in a volume of 10 mL/kg body weight. The CB₁ receptor antagonist, rimonabant, was kindly provided by Sanofi-Aventis (Bagneux, France). Rimonabant was emulsified in two drops of Tween 80 and then suspended in saline for i.p. administration in a volume of 10 mL/kg body weight. The 5-HT_{1A} receptor agonist ipsapirone was purchased by Bayer-Troponwerke (Cologne, Germany).

***In vivo* microdialysis procedure**

Mice were anesthetized with a ketamine/xylazine mixture (0.2 mL/10 g body weight i.p.) and placed in a stereotaxic apparatus with a flat skull (Paxinos and Franklin, 1997). A small hole was drilled on the skull and a 2 mm analytical probe (CMA/7/2 mm, CMA Microdialysis, Stockholm, Sweden) was implanted directly in the prefrontal cortex (PFC) (AP, +2.1 mm; ML, ± 0.5 mm; DV, -3.0 mm from bregma) and then fixed to the skull with dental cement, as described (Trigo et al, 2007). One day after probe implantation, animals were habituated to the experimental environment overnight. The following morning, a Ringer solution was pumped through the dialysis probe (NaCl, 148 mM; KCl, 2.7 mM; CaCl₂, 1.2 mM and MgCl₂, 0.8 mM, pH 6.0) at a constant rate of 1 μ L/min. Five consecutive 18-min dialysis samples were collected for the determination of baseline 5-HT levels. Then, mice were injected with saline or fluoxetine (10 mg/kg, i.p.) and collection of samples was continued for 4 h following injection. Dialysates (15 μ L) were injected without any purification into a HPLC system that consisted of a pump linked to an automatic injector (Agilent 1100, Palo Alto, USA), a reverse-phase column (Zorbax SB C18, 3.5 μ m, 150 $\times$ 4.6 mm, Agilent Technologies, Palo Alto, USA) and a coulometric detector (Coulochem II, ESA, Chelmsford, USA) with a 5011A analytical cell. 5-HT was quantified as described (Trigo et al, 2007). Briefly, the first electrode was fixed at + 50 mV and the second electrode at +300 mV. The gain of the detector was set at 10 nA. The composition of the mobile phase was 50 mM sodium acetate, 0.1 mM Na₂EDTA, 0.65 mM octane sulfonic acid sodium salt and 24% (vol/vol) methanol, pH 5.0. The flow rate was set at 0.8 mL/min and the sensitivity of the assay for 5-HT was 0.3 pg/15 μ L. Percentage of 5-HT concentrations after either fluoxetine or saline challenge respect to basal concentrations

were converted to area under the curve (AUC) using a standard trapezoid method (Gibaldi and Perrier, 1975). The following equation was used: $AUC = [0.5*(B + C_1)*d + 0.5*(C_1 + C_2)*d + 0.5*(C_2 + C_3)*d + \dots + 0.5*(C_{n-1} + C_n)*d]$, where B is the average basal value, C_1 to C_n are values during drug challenge, and d is the duration of the sample (18 min in this case). At the end of the experiments, animals were sacrificed, and brains were quickly removed and stored at -80°C . To check the position of the probe, 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 with correct probe placements were used in the study.

[^3H]Citalopram binding onto brain membranes

Dissected tissues (anterior raphe area, hippocampus, striatum and frontal cortex) were homogenized in 40 vol (w/v) of ice-cold Tris-salt buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) using a Polytron. Homogenates were centrifuged at 40,000 g for 20 min, the pellets were suspended in 40 vol of the same buffer and incubated at 37°C for 10 min to remove endogenous 5-HT. The membranes were spun down at 40,000 g for 20 min and washed three times by suspension/centrifugation as before. The final pellet was suspended in 10 vol of the same buffer and 25 μL aliquots were mixed with 225 μL of fresh Tris-salt buffer, pH 7.4, containing [^3H]citalopram (83.0 Ci/mmol; 2 nM final concentration) in an Optiplate 96-well apparatus (Perkin Elmer, Wellesley, MA) for 1 h incubation at room temperature. Nonspecific binding was determined in the presence of 10 μM fluoxetine. After the incubation, the unifilter GF/B plate was drained with a FilterMate harvester (Perkin Elmer) and each well was washed three times with 500 μL of ice-cold buffer. Microscint-40 scintillation cocktail (30 μL) was added to each well of the plate, and radioactivity was counted using a TopCount scintillation

counter (Perkin Elmer). The specific binding (expressed as cpm/10 μ g protein) was obtained by subtracting non-specific binding from total binding. Proteins were quantified using the method of Lowry et al (1951) with bovine serum albumin as standard.

Electrophysiological experiments

Immediately after removal from the skull, brains were immersed in an ice-cold artificial cerebrospinal fluid (aCSF) (NaCl 126 mM, KCl 3.5 mM, NaH_2PO_4 1.2 mM, MgCl_2 1.3 mM, CaCl_2 2.0 mM, NaHCO_3 25 mM, D-glucose 11 mM), continuously bubbled with carbogen (95% O_2 , 5% CO_2) to maintain pH value at 7.3. A block of tissue containing the dorsal raphe nucleus (DRN) was cut into sections (400 μ m thick) in the same aCSF using a vibratome. Brainstem slices were immediately immersed in oxygenated aCSF at room temperature (22 $^{\circ}\text{C}$). A single slice was then placed on a nylon mesh, completely submerged in the recording chamber and continuously superfused with oxygenated aCSF (34 $^{\circ}\text{C}$) at a constant flow rate of 2–3 mL/min. Extracellular recordings of the firing of DRN serotonergic neurons were made using glass microelectrodes filled with 2 M NaCl (12–15 M Ω). Cells were identified as 5-HT neurons according to previously described criteria (Lanfumeey et al, 1999). Firing was evoked in the otherwise silent neurons by adding the α_1 adrenoceptor agonist phenylephrine (3 μ M) into the superfusing aCSF (VanderMaelen and Aghajanian 1983). Electrical signals were fed into a high-input impedance amplifier (VF 180, BioLogic, Claix, France), an oscilloscope and an electronic ratemeter triggered by individual action potentials, connected to an A/D converter and a personal computer. Using dedicated software, the integrated firing rate was recorded, computed, and displayed on a chart recorder as consecutive 10-s samples. Baseline activity was recorded for at least 10 min prior to

perfusion of drugs into the chamber, via a three-way tap system. Because complete exchange of fluids occurred within 2 min following the arrival of a new solution into the chamber, the duration of each drug application was 3 min. The effects of the 5-HT_{1A} receptor agonist ipsapirone were evaluated by comparing the mean discharge frequency during the 2 min prior to its application with that recorded at the peak action of the drug, i.e. 2–3 min after its removal from the perfusing aCSF. Data are expressed as percentages of the baseline firing rates.

Pharmacological treatment

The CB₁ receptor antagonist, rimonabant, and its vehicle were administered i.p. (3 mg/kg) once a day (6:00 p.m.) during 2 weeks to two different groups of wild-type mice. Animals were sacrificed and brains removed for the electrophysiological experiments 18 h after the last rimonabant injection.

***In situ* hybridization**

The protocols for *in situ* hybridization were based on previously described procedures (López-Giménez et al, 2002; Serrats et al, 2005). Briefly, an oligonucleotide complementary to 1100–1147 bases of the rat 5-HT_{2C} receptor cDNA (GenBank acc. no. U35315) was labeled at its 3'-end with terminal deoxynucleotidyltransferase (TdT, Oncogene Research Products, San Diego, CA, USA) and [³³P] α-dATP (3000 Ci mmol⁻¹, New England Nuclear, Boston, MA, USA). Labeled probes were purified through ProbeQuant G-50 microcolumns (GE Healthcare, Little Chalfont, UK). The specificity of the probe has been previously established and published elsewhere (Pompeiano et al. 1994)

Frozen tissue sections were brought to room temperature, air-dried and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS: 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 136 mM NaCl, 2.6 mM KCl), washed once in 3xPBS, twice in 1xPBS, 5 min each, and incubated in a freshly prepared solution of predigested pronase (Calbiochem, San Diego, USA) at a final concentration of 12 U/mL in 50 mM Tris-HCl pH 7.5 and 5 mM EDTA for 2 min at room temperature. The enzymatic activity was stopped by immersion for 30 s in 2 mg/mL glycine in PBS. Tissues were finally rinsed in PBS and dehydrated. The radioactively labeled probe (1.5 nM) was dissolved in the hybridization buffer (50% formamide, 4xSSC, 1x Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 20 mM phosphate buffer, pH 7, 250 µg /ml yeast tRNA and 500 µg/ml salmon sperm DNA), Tissue sections were covered with hybridization buffer, overlaid with Nescofilm coverslips (Bando Chemical Ind., Kobe, Japan) and incubated overnight at 42 °C in humid boxes. The sections were then washed in a buffer, in 600 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1mM EDTA at 60 °C. For film autoradiography, hybridized sections were exposed to Biomax-MR (Kodak) films for 66h at -70°C with intensifying screens. Film autoradiograms were scanned with the GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA). The average density of 5-HT_{2C} mRNA in different brain regions was evaluated semiquantitatively with Quantity One 4.6.3 software (Bio-Rad Laboratories, Hercules, USA).

Statistical analyses

All data are given as means ± SEM. *In vivo* microdialysis data were analyzed using three-way ANOVA with treatment (saline and fluoxetine) and genotype (wild-type and CB₁ knockout) as between-subjects factors and time after fluoxetine injection as within-subjects factor and subsequent two-way ANOVA with genotype and treatment as

between-subjects factors, followed by one-way ANOVA, when required. AUC values were compared by using a between-subjects two-way ANOVA (genotype and treatment), followed by one-way ANOVA for individual differences when required. Extracellular recordings were analyzed by between-subjects two-way ANOVA (ipsapirone concentration and genotype/chronic treatment) followed by Bonferroni's post hoc *t* test. Nonlinear regression was carried out using Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) for the calculation of EC₅₀ values of the drugs. Finally, 5-HTT binding and 5-HT_{2C} receptor expression quantification were analyzed by one-way ANOVA (genotype). In all the experiments, the significance level was set at $p < 0.05$.

Results

5-HT extracellular levels in the PFC under basal conditions and after acute fluoxetine administration

In vivo microdialysis procedure revealed a significant increase of basal 5-HT extracellular levels in the PFC of CB₁ knockout compared to wild-type mice ($F_{(1, 50)} = 4.225$, $p < 0.05$) (Fig 1A). Consequently, the further 5-HT extracellular levels measure in response to fluoxetine acute administration was expressed as the percentage respect to the basal values for each genotype.

Fluoxetine (10 mg/kg, i.p.) induced an increase in 5-HT extracellular levels in the PFC in both genotypes. However, the CB₁ knockout mice response was lower than wild-type animals (Fig 1B). Three-way ANOVA showed a significant main effect of time ($F_{(11, 132)} = 1.975$, $p < 0.05$) and treatment ($F_{(1, 12)} = 26.668$, $p < 0.001$), without genotype effect after fluoxetine or saline injection. A significant interaction between time and treatment ($F_{(11, 132)} = 2.102$, $p < 0.05$) was revealed, but no other two- or three-way interactions between factors were observed. Two-way ANOVA (genotype and treatment) indicated a significant genotype effect 18 min and 144 min after fluoxetine or saline administration ($F_{(1, 22)} = 4.808$, $p < 0.05$; $F_{(1, 22)} = 4.376$, $p < 0.05$, respectively) and treatment effect 36 min ($F_{(1, 21)} = 15.071$, $p < 0.001$), 54 min ($F_{(1, 22)} = 26.213$, $p < 0.001$), 72 min ($F_{(1, 20)} = 36.753$, $p < 0.001$), 90 min ($F_{(1, 23)} = 41.090$, $p < 0.001$), 108 min ($F_{(1, 21)} = 27.412$, $p < 0.001$), 126 min ($F_{(1, 19)} = 25.518$, $p < 0.001$), 144 min ($F_{(1, 22)} = 28.262$, $p < 0.001$), 162 min ($F_{(1, 21)} = 36.343$, $p < 0.001$), 180 min ($F_{(1, 20)} = 38.684$, $p < 0.001$), 198 min ($F_{(1, 22)} = 32.420$, $p < 0.001$) and 216 min ($F_{(1, 20)} = 24.619$, $p < 0.001$) after fluoxetine or saline administration. Significant interaction between both genotype and treatment was observed 108 min and 144 min after fluoxetine or saline

administration ($F_{(1, 21)} = 7.545$, $p < 0.05$; $F_{(1, 22)} = 4.660$, $p < 0.05$, respectively). Subsequent one-way ANOVA (genotype) showed a reduction in the percentage of increase of the 5-HT extracellular levels in CB₁ knockout mice respect to wild-type littermates 108 min, 126 min and 144 min after the fluoxetine administration ($F_{(1, 14)} = 10.877$, $p < 0.01$; $F_{(1, 13)} = 8.885$, $p < 0.05$; $F_{(1, 14)} = 8.052$, $p < 0.05$, respectively). Figure 1C represents the AUC values for percentage of 5-HT extracellular levels after fluoxetine or saline administration respect to basal values. Two-way ANOVA revealed treatment effect ($F_{(1, 23)} = 64.719$, $p < 0.001$), no genotype effect, and a significant interaction between these two factors ($F_{(1, 23)} = 7.270$, $p < 0.05$). Subsequent one-way ANOVA (treatment) revealed a significant effect of fluoxetine in wild-type mice ($F_{(1, 12)} = 41.417$, $p < 0.001$) and in CB₁ knockout mice ($F_{(1, 11)} = 27.998$, $p < 0.001$). Comparisons between genotypes indicated no differences after saline treatment but lower AUC value in mutant than in wild-type mice after fluoxetine treatment ($F_{(1, 14)} = 7.575$, $p < 0.05$).

Decreased 5-HTT binding site density in frontal cortex and hippocampus of CB₁ knockout mice

One-way ANOVA revealed a significant decrease of the 5-HTT binding site density in the frontal cortex ($F_{(1, 19)} = 5.960$, $p < 0.05$) and in the hippocampus ($F_{(1, 19)} = 18.906$, $p < 0.001$) of CB₁ knockout compared to wild-type. No differences between genotypes were observed either in the striatum or in the anterior raphe (Fig 2).

5-HT_{1A} autoreceptor functional desensitization in the absence of CB₁ receptor activity

Extracellular recordings were made from serotonergic neurons of the DRN in slices preparations of brains from CB₁ knockout and wild-type mice. Two-way ANOVA revealed a significant ipsapirone concentration-dependent inhibition ($F_{(7, 140)} = 119.980$, $p < 0.001$) and a significant genotype effect ($F_{(1, 140)} = 7.120$, $p < 0.01$) on the firing of serotonergic neurons, without interaction between ipsapirone and genotype (Fig 3A). However, Bonferroni's post hoc t test indicated a significant difference between genotypes at the ipsapirone 60 nM and 100 nM concentrations ($p < 0.05$, respectively). Indeed, the concentration–response curve of ipsapirone in CB₁ mutants was shifted to the right as compared with that for wild-type mice (Fig 3B). Thus, the EC₅₀ value of ipsapirone was higher in CB₁ knockout mice (EC₅₀: 58.04 nM; 95% CI: 50.66 – 66.50 nM) than in wild-type mice (EC₅₀: 37.36 nM; 95% CI: 33.39 – 41.79 nM), indicating a functional desensitization of the 5-HT_{1A} autoreceptor in the DRN of CB₁ knockout mice.

The electrophysiological data obtained from the CB₁ knockout mice were in the same line than the results found by the pharmacological blockade of the CB₁ receptor with a chronic treatment with rimonabant (3mg/kg/day, 14 days) (Fig 4A and 4B). Two-way ANOVA revealed a significant ipsapirone concentration-dependent inhibition ($F_{(6, 96)} = 85.250$, $p < 0.001$), a significant effect of rimonabant treatment ($F_{(1, 96)} = 12.610$, $p < 0.001$) and a significant interaction between ipsapirone and rimonabant ($F_{(6, 76)} = 2.960$, $p < 0.05$) on the firing of serotonergic neurons (Fig 4A). Bonferroni's post hoc t test indicated a significant difference between vehicle- and rimonabant-treated mice at the ipsapirone 60 nM concentration ($p < 0.001$). Indeed, the concentration–response curve of ipsapirone in rimonabant chronically treated animals was significantly shifted to the right as compared to vehicle treated mice (Fig 4B). Thus, the EC₅₀ value of ipsapirone was higher in rimonabant-treated (EC₅₀: 53.99 nM; 95% CI: 43.88– 66.45 nM) than in

vehicle-treated mice (EC_{50} : 30.13 nM; 95% CI: 23.16 – 34.76 nM), confirming a functional desensitization of the 5-HT_{1A} autoreceptor in the DRN in the absence of CB₁ receptor activity.

Altered 5-HT_{2C} receptor expression in CB₁ knockout mice

In situ hybridization experiments were carried out to study the distribution of the mRNA coding for 5-HT_{2C} receptor in the brain of CB₁ receptor knockout and wild-type mice (Fig 5). Brain sections from both genotypes were included in the same hybridization experiment, and regions to be compared from both groups were exposed to the same β -max film. After the appropriate exposure time, optical densities over the regions of interest were determined for both groups and the values statistically analyzed by one-way ANOVA. A significant decrease in the expression of 5-HT_{2C} receptor was observed in most of the brain areas examined, including the nucleus *accumbens* (NAcc) ($F_{(1, 9)} = 9.069$, $p < 0.05$), caudate putamen nucleus (CPu) ($F_{(1, 10)} = 6.408$, $p < 0.05$), piriform cortex (Pir) ($F_{(1, 10)} = 17.264$, $p < 0.01$), paraventricular nucleus of the hypothalamus (PVN) ($F_{(1, 9)} = 7.078$, $p < 0.05$), retrosplenial granular cortex (RSG) ($F_{(1, 10)} = 12.371$, $p < 0.01$), choroid plexus (ChP) ($F_{(1, 10)} = 59.036$, $p < 0.001$), habenula (Hab) ($F_{(1, 9)} = 6.855$, $p < 0.05$) and DRN ($F_{(1, 10)} = 5.682$, $p < 0.05$). In contrast, a significant increase in the 5-HT_{2C} receptor expression was observed in the CA3 area of the ventral hippocampus ($F_{(1, 10)} = 9.959$, $p < 0.05$). No significant differences were observed in the PFC, lateral amygdala (Amy) or periaqueductal gray (PAG) brain areas.

Discussion

The present study demonstrates that endocannabinoid system through CB₁ receptor modulates the activity of the serotonergic system. In particular, mice lacking CB₁ receptor exhibited increased 5-HT extracellular levels in the PFC that could be related to alterations of components involved in 5-HT negative feedback, such as 5-HTT, 5-HT_{1A} and 5-HT_{2C} receptors.

In vivo microdialysis experiments demonstrated that CB₁ knockout mice display a slight elevation in the basal extracellular 5-HT levels in the PFC. Our observation is in agreement with the moderately increased 5-HT efflux reported in the rat frontal cortex after the administration of the CB₁ antagonist rimonabant (Tzavara et al, 2003). However, the peripheral administration of fluoxetine resulted on lower increase of 5-HT extracellular levels in the PFC of the CB₁ knockout mice compared to wild-type animals, demonstrating that the blockade of CB₁ receptor activity deregulates serotonergic function. In order to raise this issue, we analysed some of the most important mechanisms that control serotonergic feedback in the absence of CB₁ receptor activity.

5-HTT mediates the 5-HT reuptake from the synaptic terminal and determines the size and duration of the serotonergic responses (Lesch and Mössner, 2006). Radioligand binding approaches revealed that CB₁ knockout mice exhibited a significantly lower 5-HTT binding site density in the frontal cortex and the hippocampus than wild-type mice. This finding could explain in part the decreased response to the fluoxetine treatment observed in mutants in the *in vivo* microdialysis experiments, given that 5-HTT is the target for the 5-HT reuptake-inhibiting antidepressant compounds such as fluoxetine. In addition, our observation also supports the increased basal 5-HT

extracellular levels in the PFC as seems to indicate a reduction in the 5-HT clearance of the synaptic cleft in the absence of CB₁ receptor activity. However, the use of a method such as the no-net-flux technique should be used to confirm a possible reduction of the 5-HT clearance *in vivo* (Shippenberg et al, 1999).

The activity of the somatodendritic 5-HT_{1A} autoreceptor, which negatively controls the firing rate of serotonergic neurons located in the DRN (Barnes and Sharp, 1999), was evaluated by using an *in vitro* electrophysiological approach. Extracellular recordings were performed in brain slices containing the DRN in the presence of increasing concentration of 5-HT_{1A} receptor agonist ipsapirone. Ipsapirone concentration required to obtain a reduction in the serotonergic firing was significantly higher in the CB₁ knockout mice than in wild-type mice, revealing a functional desensitization of 5-HT_{1A} autoreceptors in those mutants. Similar results were obtained in wild-type animals receiving a chronic treatment with the CB₁ receptor antagonist rimonabant. The functional desensitization of the 5-HT_{1A} autoreceptor demonstrated in this study through a genetic and a pharmacological blockade of CB₁ receptor is supported by the reduced hypothermia induced by the 5-HT_{1A} receptor agonist 8-OH-DPAT in CB₁ knockout mice previously reported (Mato et al, 2007). This finding could be linked to the increased basal 5-HT extracellular levels observed in the PFC of mutant animals since 5-HT_{1A} receptors were less efficient in inhibiting 5-HT neurons activity. In contrast to this assumption, the genetic deletion of the 5-HT_{1A} receptors has not been reported to modify basal 5-HT extracellular levels in different brain areas including PFC (He et al, 2001; Guilloux et al, 2006).

Although 5-HTT and 5-HT autoreceptors exert a clear-cut role within the regulation of 5-HT system, emerging findings indicate additional complexity of 5-HT feedback control involving post-synaptic 5-HT receptors (Sharp et al, 2007). Among all the post-

synaptic receptors, we focused on 5-HT_{2C} receptor because of its participation on the regulation of emotional state beyond the involvement in 5-HT feedback regulation. This receptor plays an essential role in the 5-HT-induced HPA axis activation (Heisler et al, 2007) and also mediates the inhibition of dopaminergic system, a key component for hedonia and motivation (Dremencov et al, 2006). Then, we assessed the expression of 5-HT_{2C} receptors mRNA in different brain areas related to motivation, HPA axis and 5-HT feedback regulation by *in situ* hybridization. CB₁ knockout mice exhibited a reduction in the expression of the 5-HT_{2C} receptor in the DRN, the NAcc and the PVN, among other brain areas. Interestingly, 5-HT_{2C} receptor was overexpressed in the CA3 area of the ventral hippocampus of CB₁ knockout mice, suggesting a differential role of this receptor in this discrete brain area, which would deserve additional attention. The decreased expression of 5-HT_{2C} receptor in the DRN of CB₁ mutant mice could lead to a reduction in the inhibitory effect exerted by 5-HT_{2C} receptor on 5-HT neurons through GABAergic mechanism (Boothman et al, 2006). This assumption could support the increased 5-HT extracellular levels in the brain areas receiving projections from the DRN in CB₁ knockout mice revealed by the present results. Nevertheless, the genetic or pharmacological blockade of 5-HT_{2C} receptors seems not to affect the basal 5-HT extracellular levels (Cremers et al, 2004). In a similar way than in DRN, the decreased levels of 5-HT_{2C} mRNA in mutant mice within the NAcc and the PVN could indicate a diminished capacity of this receptor to inhibit dopamine activity (Dremencov et al, 2006) and to stimulate corticotrophin-releasing factor release (Heisler et al, 2007), respectively.

In conclusion, the present results indicate that the lack of CB₁ receptor activity alters 5-HT_{1A} and 5-HT_{2C} receptors as well as the 5-HTT density, leading to increased 5-HT extracellular levels as we summarize in Fig 6. Classically, mood disorders have been

associated to reduced 5-HT activity basing on the mechanism of action of antidepressant compounds (Charney, 1998; Artigas et al, 2002). In contrast to what we could expect considering this classical hypothesis, the genetic and pharmacological blockade of the CB₁ receptor induces a depressive-like phenotype (Hill and Gorzalka, 2005; Mangieri and Piomelli, 2007; Aso et al, 2008). From our point of view, this apparent controversy points out that the chronic absence of CB₁ receptor could impair mechanisms controlling emotional homeostasis beyond 5-HT neurotransmission. Thus, the absence of CB₁ receptor alters the HPA axis activity (Cota, 2008) and neural plasticity that are likely to contribute to emotional disturbances (Berton and Nestler, 2006). In this sense, we have previously demonstrated that the enhanced glucocorticoids levels in CB₁ knockout mice could produce hippocampal BDNF impairment, which plays a crucial role on the increased despair behavior exhibited by those mutants (Aso et al, 2008). The alterations on 5-HT neurotransmission reported in the present study are supposed to be a substrate for counteracting the stress-induced emotional impairments in mice lacking CB₁ receptor, since similar alterations have been described after antidepressant treatments (Le Poul et al, 2000; Lanfumey et al, 2000; Gould et al, 2006). Nevertheless, it has been reported an altered post-synaptic serotonergic signalling in CB₁ mutant mice (Mato et al, 2007) that could lead to a reduction in the efficacy of 5-HT for ameliorating their depressive-like phenotype. As a result, the deregulation of glucocorticoid release and the deficiency of neurotrophic mechanisms could prevail upon the alterations on 5-HT neurotransmission, leading to the depressive-like phenotype exhibited by CB₁ mutant mice. However, we can not rule out the possibility that the reduced 5-HTT levels and the 5-HT_{1A} desensitization reported in the present study could be *per se* a possible substrate for the depressive-like phenotype exhibited by CB₁ knockout mice. Thus, an impaired 5-HTT function (Caspi et al, 2003; Olivier et al, 2008) and a 5-HT_{1A}

desensitization (Lesch et al, 1990; Cowen et al, 1994; Bambico et al, 2009) have been associated to an increased vulnerability to depressive symptoms in animal models as well as in humans.

In summary, we can conclude that the lack of CB₁ receptor induces an increase in 5-HT extracellular levels by modulating different components of the serotonergic feedback. These alterations could account for the depressive-like phenotype exhibited by CB₁ knockout mice.

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Figure 1. *In vivo* microdialysis experiments in the PFC of CB₁ knockout mice. (A) Increased basal 5-HT extracellular levels in the PFC of CB₁ knockout (filled bar) compared to wild-type (open bar) mice (n = 26). (B) Decreased percentage of increase of the 5-HT extracellular levels in the CB₁ knockout mice (filled squares) respect to the wild-type littermates (open squares) after the fluoxetine administration (n = 8-10). (C) AUC values for the percentage of increase of the 5-HT concentration in dialysates after fluoxetine or saline administration in CB₁ knockout (filled bars) and wild-type (open bars) mice respect to basal values. Data are expressed as mean $\pm$ SEM. ★ p < 0.05, ★★ p < 0.01 comparison between genotypes; ☆☆☆ p < 0.001, when compared with the saline group of the same genotype (one-way ANOVA). Significant comparisons between the fluoxetine-treated (squares) and the saline-treated (triangles) groups are not included in order to clarify the figure.

Figure 2. 5-HTT quantification by [³H]Citalopram binding onto brain membranes revealed decreased 5-HTT levels in the frontal cortex and hippocampus of CB₁ knockout (filled bar) compared to wild-type (open bar) mice at basal conditions. Data are expressed as mean $\pm$ SEM (n = 10-11). ★ p < 0.05, ★★★ p < 0.001 compared to wild-type mice (one-way ANOVA).

Figure 3. Effect of ipsapirone on the firing of DRN 5-HT neurons in CB₁ mutants compared with wild-type mice. (A) Integrated firing rate histograms (in spikes per 10 sec) show the effect of increasing doses of the 5-HT_{1A} receptor agonist ipsapirone on the electrical activity of a DRN 5-HT neuron in a control animal (upper) compared with a CB₁ mutant (down). (B) Dose–response curves of ipsapirone-induced inhibition of the firing of DRN 5-HT neurons in brainstem slices from CB₁ knockout compared with

wild-type mice. Ipsapirone-induced inhibition is expressed as percentage of the baseline firing rate. Each point is the mean $\pm$ SEM of data obtained from 15–21 individual cells. The dotted lines illustrate the EC₅₀ values of ipsapirone (abscissa) in CB₁ mutants and wild-type mice. ★ $p < 0.05$ as compared with the respective inhibition in wild-type mice.

Figure 4. Effect of ipsapirone on the firing of DRN 5-HT neurons in CD1 mice chronically treated with rimonabant (3 mg/kg/day, 14 days) or vehicle. (A) Integrated firing rate histograms (in spikes per 10 sec) show the effect of increasing doses of the 5-HT_{1A} receptor agonist ipsapirone on the electrical activity of a DRN 5-HT neuron in a vehicle treated (upper) compared with a rimonabant chronically treated animal (down). (B) Dose–response curves of ipsapirone-induced inhibition of the firing of DRN 5-HT neurons in brainstem slices from rimonabant chronically treated animals compared with vehicle treated mice. Ipsapirone-induced inhibition is expressed as percentage of the baseline firing rate. Each point is the mean $\pm$ SEM of data obtained from 13–16 individual cells. The dotted lines illustrate the EC₅₀ values of ipsapirone (abscissa) in rimonabant chronically treated animals and vehicle treated mice. ★★★ $p < 0.001$ as compared with the respective inhibition in wild-type mice.

Figure 5. *In situ* hybridization of 5-HT_{2C} receptor in CB₁ knockout mice. (A) Representative images of mRNA encoding 5-HT_{2C} in different coronal levels of wild-type (A1 to A5) and CB₁ knockout mice (B1 to B5). (B) Quantification of the optical density in each brain area examined. CB₁ knockout mice (filled bars) exhibited decreased 5-HT_{2C} receptor expression in almost all the brain areas studied, excepting the CA3 area of the ventral hippocampus in which a significant increase was observed. Data are expressed as mean $\pm$ SEM ($n = 5-6$). ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$

compared to wild-type mice (one-way ANOVA). *PFC*, prefrontal cortex; *NAcc*, nucleus accumbens; *CPu*, caudate-putamen nucleus; *Pir*, piriform cortex; *PVN*, paraventricular nucleus of the hypothalamus; *Amy*, lateral amygdala; *RSG*, retrosplenial granular cortex; *ChP*, choroid plexus; *Hab*, habenula; *CA3*, CA3 area of the ventral hippocampus; *PAG*, periaqueductal gray; *DR*, dorsal raphe nuclei.

Figure 6. Schematic representation of the mechanisms involved in serotonergic feedback that are altered in the absence of CB₁ receptor activity. A 5-HT projection from the DRN to the PFC is represented in wild-type mice (upper panel) and CB₁ knockout mice (lower panel). 5-HT (red circles) is released by 5-HT neurons (in red) located in the DRN, binds to 5-HT post-synaptic receptors (in dark blue) and is selectively reuptaken by 5-HTT (in light blue) located in 5-HT terminals. 5-HT_{1A} autoreceptors (in light green) situated in the soma of 5-HT neurons are able to inhibit the 5-HT firing. In the DRN, CB₁ cannabinoid receptors (in purple) and 5-HT_{2C} receptors (in orange) are supposed to be mainly located on GABAergic interneurons (in dark green). Activation of CB₁ receptors in the DRN produces inhibition of GABA release, thus removing the inhibitory effect of these GABAergic cells on 5-HT neurons. In contrast, activation of 5-HT_{2C} receptors induces GABA release, promoting the inhibitory effect of these GABAergic cells on 5-HT neurons. Thus, the balance between the activity of 5-HT_{2C} and CB₁ receptors could determine the net effect of the GABAergic modulation on 5-HT neurons. The absence of CB₁ receptor activity (lower panel) alters different serotonergic components leading to increased 5-HT extracellular levels by decreasing the activity of the receptors that directly or indirectly produce an inhibitory effect on 5-HT neurons and reducing the 5-HT clearance of the synaptic cleft. Hence, CB₁ knockout mice exhibited a significant desensitization of 5-HT_{1A}

autoreceptor, decreased 5-HTT levels in the PFC and diminished expression of 5-HT_{2C} receptor in the DRN.

A **B** **C**

Fig 1 Aso et al

Fig 2 Aso et al

Fig 3 Aso et al

A **B**

Fig 5 Aso et al