Long-Term Fluoxetine Treatment Modulates Cannabinoid Type 1 Receptor-Mediated Inhibition of Adenylyl Cyclase in the Rat Prefrontal Cortex through 5-Hydroxytryptamine<sub>1A</sub> Receptor-Dependent Mechanisms

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ABSTRACT
Increasing data indicate that brain endocannabinoid system plays a role in the effects of antidepressant medications. Here we examined the effect of in vivo exposure to the selective serotonin uptake inhibitor fluoxetine on cannabinoid type 1 (CB<sub>1</sub>) receptor density and functionality in the rat prefrontal cortex (PFC) and cerebellum. Long-term treatment with fluoxetine (10 mg/kg/day) enhanced CB<sub>1</sub> receptor inhibition of adenylyl cyclase (AC) in the PFC and reduced it in the cerebellum without altering receptor density and agonist stimulation of guanosine 5'-O-(3-thio)triphosphate ([<sup>35</sup>S]GTP<sub>S</sub>) in either area. Analysis of [<sup>35</sup>S]GTP<sub>S</sub>-labeled G<sub>x</sub> subunits allowed for the detection of up-regulated CB<sub>1</sub> receptor coupling to G<sub>x1</sub>, G<sub>x3</sub> in the PFC, and reduced coupling to G<sub>x3</sub> in the cerebellum of fluoxetine-treated rats. Concomitant administration of the 5-HT<sub>1A</sub> receptor antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl][ethyl]-N-2-pyridinlycyclohexanecarboxamide maleate (WAY100635; 0.1 mg/kg/day) reduced fluoxetine-induced modulation of CB<sub>1</sub> receptor coupling to G<sub>x</sub> subunits and AC in the PFC but not in the cerebellum. These results indicate that increased CB<sub>1</sub> receptor signaling at the G<sub>x</sub>-AC transduction level is a long-term adaptation induced by fluoxetine in the PFC and point to a role for 5-HT<sub>1A</sub> receptors in this effect. Basal AC activity, protein kinase A (PKA) catalytic subunit expression, and phospho-CREB response element-binding protein (pCREB)/CREB ratio were also up-regulated in the PFC of fluoxetine-treated animals, whereas no differences were detected in the cerebellum. It is interesting that long-term Δ<sub>4</sub>-tetrahydrocannabinol treatment did not elicit antidepressant-like effects or modulated behavioral responses of fluoxetine in an animal model of depression (olfactory bulbectomy). These data suggest that altered signal transduction through CB<sub>1</sub> receptors in the PFC may participate in the regulation of the AC-PKA-CREB cascade induced by fluoxetine in this brain area.

Depression is a debilitating disease with a high prevalence and societal cost. Among antidepressant drugs (ADs), selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine are the most widely prescribed. Despite their well-established efficacy, the molecular mechanisms underlying the therapeutic action of SSRIs remain unclear. In the short term, SSRIs enhance the efficacy of 5-HT neurotransmission via the inhibition of 5-HT uptake. Nevertheless, clinical improvement results evident only after 2 to 3 weeks of treatment, suggesting a key role for long-term adaptations induced by these compounds (i.e., desensitization of presynaptic inhibitory 5-HT<sub>1A</sub> receptors) (Blier and de Montigny 1994; Elena Castro et al., 2003). Other neurobiological theories propose that the efficacy of SSRIs is due to alterations in various signaling pathways regulating cellular functions.

ABBREVIATIONS: AD, antidepressant drug; AC, adenylyl cyclase; BSA, bovine serum albumin; CB<sub>1</sub>, cannabinoid type 1; [<sup>3</sup>H]CP55,940, [<sup>3</sup>H](1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; 8-OH-DPAT, 8-hydroxydi-9-tetrahydrocannabinol; WAY100635, (piperidin-1-yl)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[(3-[35S]thio)triptophan]-[trans]-[2-

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plasticity and survival. It has been demonstrated that long-term treatment with ADs up-regulates the cAMP-protein kinase A (PKA) cascade and increases neurogenesis in the hippocampus and prefrontal cortex (PFC) (Malberg and Blendy, 2005). 5-HT1A receptors could be involved in the latest effect, because both proliferative and behavioral effects of fluoxetine are abolished by genetic deletion of the 5-HT1A gene (Santarelli et al., 2003).

Cannabinoid CB1 receptors, negatively coupled to adenylyl cyclase (AC) via G\textsubscript{i/o} proteins, mediate most of the actions of exogenous and endogenous cannabinoids in the central nervous system (Howlett et al., 2002). CB1 receptor knockout mice exhibit enhanced “depressive-like” behaviors in animal models (Martin et al., 2002; Mato et al., 2007). Pharmacological modulation of CB1 receptors has led to more conflicting results, because both CB1 receptor activation and blockade induce antidepressant-like responses in the same tasks (Griebel et al., 2005; Hill and Gorzalka, 2005; Witkin et al., 2005; Bambico et al., 2007; McLaughlin et al., 2007; Steiner et al., 2008; Morrish et al., 2009). These evidences strongly support the involvement of the brain endocannabinoid (EC) system in the modulation of mood, suggesting that CB1 receptors could play a role in the cause of major depression (Vinod and Hungund, 2006). In favor of this hypothesis, CB1 receptor mRNA (Bortolato et al., 2007), protein expression (Hill et al., 2008a), and signaling through G\textsubscript{i/o} proteins (Rodriguez-Gaztelumendi et al., 2009) have been shown to be up-regulated in the PFC of rats subjected to different models of depression and in PFC samples from victims of major depression (Hungund et al., 2004).

In addition, evidence for the contribution of brain EC system to the long-term effects of ADs mainly comes from the fact that their long-term administration modulates CB1 receptor expression (Hill et al., 2008b) and coupling to G\textsubscript{i/o} proteins (Rodriguez-Gaztelumendi et al., 2009), as well as the levels of the ECs anandamide and 2-arachidonylglycerol (Hill et al., 2008b), in the PFC and/or hippocampus. It is noteworthy that information about the putative adaptations of brain CB1 receptors at the cAMP signaling level in response to long-term antidepressants treatment is lacking, despite the proposed importance of this transduction pathway for the long-term adaptations underlying antidepressants efficacy (Malberg and Blendy, 2005). Taking into account that 5-HT receptors are the primary targets of SSRI
dors in these effects, strengthening the hypothesis that the EC system may play a role in the long-term adaptations induced by this SSRI.

Materials and Methods

Materials. [3H]CP55,940 (165 Ci/mmol) and [35S]GTP\textsubscript{S} (1250 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). cAMP \textsuperscript{3}H assay kit (1028 GBq/mmol) was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). WAY100635, DTT, GDP, GTP, GTP\textsubscript{S}, and Sigmacote were from Sigma-Aldrich (Madrid, Spain). WIN55,212-2 was obtained from Tocris Cookson (Bristol, UK). SR141716A was a generous gift from Sanofi Recherche (Montpellier, France). Δ\textsuperscript{2}-Tetrahydrocannabinol (Δ\textsuperscript{2}-THC) was purchased from THC Pharm GMBH The Health Concept (Frankfurt, Germany). Rabbit anti-G\textsubscript{i1}, anti-G\textsubscript{i2}, anti-G\textsubscript{a\textsubscript{2}}, anti-G\textsubscript{a\textsubscript{1}} and anti-extracellular signal-regulated-kinase (ERK) 1/2, and mouse anti-glycerolaldehyde-3-phosphate dehydrogenase and anti-histone H1 were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Mouse anti-\textsuperscript{p}ERK1/2 and goat anti-rabbit and anti-mouse peroxidase-conjugated antibodies were purchased from Sigma-Aldrich. Rabbit anti-CREB and anti-pCREB (Ser133) were from Millipore (Billerica, MA). Fluoxetine-HCL was kindly donated by Eli Lilly and Co. (Barcelona, Spain). All other chemicals used were from the highest commercial grade available.

Animals. Male Wistar rats (200–250 g) were maintained on a 12-h light/dark cycle, with access to food and water ad libitum. All experimental procedures were done in accordance with the Declaration of Helsinki, the Spanish legislation, and the European Communities Council Directive on “Protection of Animals Used in Experimental and Other Scientific Purposes” (86/609/EEC).

Drug Treatment and Tissue Preparation. Rats were randomly assigned to one of the treatment groups, anesthetized with ether, and implanted the same day with an osmotic minipump Alzet 2002 (Alza Corporation, Palo Alto, CA), which delivered 0.5 μl/h. Animals (n = 9 per experimental group) were treated with fluoxetine HCl (10 mg/kg/day), WAY100635 (0.1 mg/kg/day), the combination of both drugs, or with vehicle (50% propylene glycol, 10% ethanol, and 40% distilled water), and minipumps were removed after 14 days of treatment. Rats were killed by decapitation 24 h after removing the minipumps. Two additional groups of rats (n = 4 rats randomly assigned to each experimental group) were treated with a single intraperitoneal injection of fluoxetine HCl (10 mg/kg) or vehicle and were killed 24 h later. In all cases, rat brains were rapidly removed and brain areas were dissected, frozen immediately in isopentane, and stored at −80°C until assay. For behavioral studies (olfactory bulbectomy), animals were treated with vehicle (5% ethanol, 5% Emulphor, and 90% saline), fluoxetine HCl (10 mg/kg/day s.c., 14 days, osmotic minipump), Δ\textsuperscript{2}-THC (10 mg/kg/day i.p., 14 days), and the combination thereof, as described below.

Bilateral Olfactory Bulbectomy and Open Field Test. The bilateral olfactory bulbectomy (OBX) procedure was performed as described previously (Rodriguez-Gaztelumendi et al., 2009). Animals (sham = 8, OBX = 32) were anesthetized, and the bulbs were excised and removed under stereotactic surgery. Sham-operated animals underwent the same procedure except for excision and aspiration of the olfactory bulbs. An open field test was conducted after a 15-day post-surgery recovery period, as described previously, recording for a
5-min period the number of ambulations, rearings, grooming episodes, and defections. The next day, OBX animals were randomly divided into five subgroups to receive vehicle-treated sham-operated (n = 8), vehicle-treated OBX (n = 8), fluorescent-treated OBX (n = 8), Δ^2-THC-treated OBX (n = 8), or fluorescent plus Δ^2-THC-treated OBX (n = 8). An open field session was performed at the end of the treatment. At the end of the experiments, animals were killed, and the success of the operation was confirmed anatomically.

**Protein Content Determination.** Membrane protein content was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Munich, Germany), using γ-globulin as standard.

cAMP Assays. For quantification of endogenous cAMP levels, brain samples were homogenized [1:30 (w/v)] in ice-cold buffer (20 mM Tris-HCl, 300 mM sucrose, 1 mM EGTA, 5 mM EDTA, 5 mM DTT, and 25 μM leupeptin, pH 7.4) using a Teflon tissue grinder (10 strokes, 800 rpm) and centrifuged (1500 rpm, 5 min, 4°C). The resulting supernatants were incubated during 5 min at 100°C and then centrifuged (14,000 rpm, 5 min). cAMP accumulation was quantified in 50-, 100-, and 200-ng aliquots by using a [³²⁵]cAMP commercial kit (Amersham Signal Transduction Assays; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

To analyze AC activity, brain samples were homogenized [1:100 (w/v)] in ice-cold buffer and centrifuged (1500 rpm, 5 min, 4°C) as described above, and the resulting supernatants were centrifuged again (14,000 rpm, 15 min, 4°C). Pellets were resuspended (150 μg protein/ml) in ice-cold assay buffer (80 mM Tris-HCl, 60 mM sucrose, 2 mM MgCl₂, 0.2 mM EGTA, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 10 μM GTP, 0.5% mg/ml BSA, 0.5 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 5 U/ml myokinase, and 5 μM forskolin, pH 7.4) and incubated (5 min at 37°C) in the absence or presence of WIN55,212-2 (10 nM to 100 μM). The specificity of the cannabinoid agonist was verified by incubation of 10 μM WIN55,212-2 with 10 μM SR141716A. Nonspecific binding was determined in the presence of 10 μM GTP·S. Membrane suspensions were then solubilized on ice with 1% IGEPS, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM CHAPS, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 M aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μl/ml antipain, and 10 μg/ml chymostatin for 30 min. Solubilized membranes were incubated for 3 h at room temperature with 15 μl of specific rabbit anti-G_{α_s}, anti-G_{α_i}, anti-G_{α_o}, and anti-G_{α_o} antibodies immobilized to superparamagnetic Dynabeads Protein A (overnight, 4°C). After three washes with 1 ml of phosphate-buffered saline, the beads were pelleted, and the entrapped radioactivity was counted in 4 ml of Ecolite scintillation cocktail. Antibody specificity was confirmed in our experimental conditions by Western blot as reported previously (Valdizán et al., 2009).

**Western Blot.** Brain samples were homogenized [1:15 (w/v)] by using a Potter homogenizer provided with a loosely fitting Teflon pestle in ice-cold buffer (50 mM Tris-HCl, 250 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, and 1 mM DTT, pH 7.4) containing the following protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml antipain, 10 μg/ml chymostatin, 5 μg/ml trypsin inhibitor, 1 mM NaV, 1 mM NaF, 1 mM cantharidin, and 10 μM E-64. After homogenization, 100 μl of homogenate was lysed in homogenization buffer containing 1% IGEPS, 0.1% sodium deoxycholate, 0.2% SDS, and 0.1% Triton X-100 for 30 min on ice for the total cell lysate (TCL). The remaining homogenate was centrifuged at 1500 rpm (5 min, 4°C), and the resulting pellets were used to obtain the nuclear fraction by homogenization in 20 mM HEPES, 0.45 M NaCl, and 1 mM EDTA, pH 7.9, containing protease and phosphatase inhibitors and incubation on ice for 30 min. Solubilized proteins were recovered in the supernatant after centrifugation (14,000 rpm, 10 min, 4°C). Protein preparations (30–50 μg per lane) were resolved on 12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (nonphosphorylated proteins) or to nitrocellulose (phosphorylated proteins) membranes. Membranes were incubated in the following primary antibodies: rabbit anti-ERK1/2 (1:2000), mouse anti-pERK1/2 (1:10,000), rabbit anti-CREB (1:1000), and rabbit anti-pCREB (1:1000). After extensive washings in Tris-buffered saline 0.05% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Secondary antibodies were detected with ECL Advance kit (GE Healthcare). Blot quantifications were performed by densitometric scanning using Scion Image software (Scion Corporation, Frederick, MD). The densitometric values were normalized with respect to the values obtained with anti-glyceralddehyde-3-phosphate dehydrogenase (TCL) and/or anti-histone H1 (nuclear fraction) antibodies. Data for every sample were the mean of at least two independent experiments.
Data Analysis. [3H]CP55,940 saturation binding data were transformed using the method described by Scatchard (1949), and GraphPad Prism computer software for Windows (GraphPad Software Inc., San Diego, CA) was used to estimate maximal [3H]CP55,940 binding sites (B_max) and dissociation constants (K_d). K_d values were normalized for comparison as \(-\log K_d\) (pK_d). The effect of each concentration of cannabinoid agonist was expressed as a percentage of stimulation: [% = (agonist effect \times 100)/basal activity] in [35S]GTP \gamma S assays and the percentage of inhibition in cAMP assays [% = (agonist effect \times 100)/forskolin effect \times 100]. Analysis of concentration-effect curves was conducted by nonlinear regression using Prism software to estimate the theoretical maximal effect (E_{max}) and the potency (EC_{50}) in [35S]GTP \gamma S binding assays and the (I_{max}) and (IC_{50}) values in cAMP assays of the cannabinoid agonist in each assay. EC_{50} and IC_{50} values were normalized as \(-\log EC_{50}\) and \(-\log IC_{50}\) (pEC_{50} and pIC_{50}) for comparison. Levels of coupling of CB_1 receptors by WIN55,212-2 (10^{-6} M) to the diverse G protein subunits were obtained as the percentage over the value in the absence of agonist. The efficiency of coupling of CB_1 receptors was obtained from the control group. Statistical comparison of experimental groups was made using one-way ANOVA with Newman-Keuls post hoc tests. Differences were taken as statistically significant when \(p < 0.05\). Data are presented as mean \pm S.E.M.

Results

Long-Term Fluoxetine Treatment Modulates Basal and CB_1 Receptor-Dependent Inhibition of Adenylyl Cyclase Activity but Not Endogenous cAMP Levels. Endogenous cAMP levels were unchanged in the PFC or in the cerebellum after long-term treatment with fluoxetine, the combination of fluoxetine plus WAY100635, or WAY100635 alone (in picomoles per milligram of protein; vehicle: PFC, 1.76 \pm 0.43, cerebellum, 3.47 \pm 0.59; fluoxetine: PFC, 1.78 \pm 0.20, cerebellum, 3.57 \pm 0.46; fluoxetine + WAY100635: PFC, 1.79 \pm 0.47, cerebellum, 3.23 \pm 0.18; WAY100635: PFC, 1.81 \pm 0.42, cerebellum, 3.38 \pm 0.23).

The effects of long-term treatment with fluoxetine, the combination of fluoxetine plus WAY100635, or WAY100635 alone on basal, forskolin-stimulated, and CB_1 receptor-mediated inhibition of AC in the PFC and cerebellum are depicted in Fig. 1. Long-term fluoxetine treatment significantly increased basal AC activity in the PFC, and this effect was not observed in the animals treated with fluoxetine + WAY100635 or with WAY100635 alone (Fig. 1A). One-way ANOVA for independent measures revealed an effect of the group (\(F = 3.2, p < 0.05\); Fig. 1A) with significant differences between the vehicle and fluoxetine groups (Fig. 1A; *; \(p < 0.05\)) and between the fluoxetine versus WAY100635 alone groups (Fig. 1A, **; \(p < 0.05\)). Differences in the ability of the AC activator forskolin to increase cAMP levels were not detected in any of the treated groups (vehicle, 164 \pm 54; fluoxetine, 207 \pm 42; fluoxetine + WAY100635, 181 \pm 36; and WAY100635, 172 \pm 22 pmol/min/mg protein). Incubation with the cannabinoid agonist WIN55,212-2 induced a concentration-dependent decrease of forskolin-stimulated AC in the PFC of vehicle-treated rats, with a maximal inhibition (I_{max}) of 30.0 \pm 3.0\% and a pIC_{50} of 5.7 \pm 0.08. Long-term treatment with fluoxetine induced a significant increase in the maximal ability of WIN55,212-2 to inhibit AC in the PFC (I_{max} = 41.8 \pm 3.7\%) that was absent in those rats treated with fluoxetine plus the 5-HT_1A receptor antagonist WAY100635 (I_{max} = 25.5 \pm 3.2\%) or WAY100635 alone (I_{max} = 25.3 \pm 2.5\%) (Fig. 1B). One-way ANOVA revealed a significant effect of the group (\(F = 6.11, p < 0.01\); Fig. 1B), with significant differences between the vehicle and fluoxetine groups (Fig. 1B; *; \(p < 0.05\)) and between the fluoxetine versus fluoxetine plus WAY100635 (Fig. 1B; **; \(p < 0.01\)).

Fig. 1. Long-term fluoxetine treatment modulates basal and/or CB_1 receptor-mediated inhibition of AC activity in the rat brain. A, basal AC activity in the rat PFC and cerebellum after 14 days of treatment with vehicle, fluoxetine (10 mg/kg/day), WAY100635 (0.1 mg/kg/day), or the combination of both compounds. B and C, inhibition of forskolin-stimulated AC activity in the rat brain. B, forskolin-stimulated AC activity in the PFC and cerebellum after long-term treatment with fluoxetine, WAY100635, or fluoxetine + WAY100635. Data are mean \pm S.E.M., \(n = 8\) to 9 animals per group. *; \(p < 0.05\); **; \(p < 0.01\) versus vehicle; $; \(p < 0.05\); $$; \(p < 0.01\) versus fluoxetine (Newman-Keuls post-ANOVA).
and WAY100635 alone groups (Fig. 1B; §§, p < 0.01). The potency of the cannabinoid agonist to inhibit AC was not altered by long-term treatment with fluoxetine, fluoxetine plus WAY100635, or WAY100635 alone (Fig. 1B). It is noteworthy that the observed modifications in basal AC activity and WIN55,212-2 efficacy to inhibit AC in the PFC of rats continuously treated with fluoxetine were not detected after a single exposure to the SSRI (Table 1). In the cerebellum of the same groups of rats, we did not observe any modifications of basal (Fig. 1A) or forskolin-stimulated AC activity (vehicle, 76 ± 9; fluoxetine, 94 ± 21; fluoxetine + WAY100635, 78 ± 11; and WAY100635, 88 ± 14 pmol/min/mg protein). In contrast to the observed up-regulation of CB1 receptor coupling to AC in the PFC of fluoxetine-treated rats, the ability of WIN55,212-2 to inhibit cAMP production was reduced in the cerebellum after long-term forskolin-stimulated treatment (I_{max}^F = 23.5 ± 0.9% in fluoxetine-treated rats versus 38.7 ± 2.1% in vehicle-treated rats). This reduced coupling ability of CB1 receptors to AC in the cerebellum of cerebellum-treated rats was not prevented by cotreatment with the 5-HT1A receptor antagonist WAY100635 (I_{max}^F = 18.1 ± 1.9%), which when administered alone did not modify WIN55,212-2 efficacy in cAMP assays (I_{max}^F = 39.6 ± 2.5%) (Fig. 1C). One-way ANOVA indicated a significant effect of the group (F = 25.2, p < 0.001; Fig. 1C), with significant differences between the vehicle versus fluoxetine (Fig. 1C; **, p < 0.01) and fluoxetine + WAY100635 groups (Fig. 1C; **, p < 0.01) and between the fluoxetine versus WAY100635 alone groups (Fig. 1C; p < 0.01). None of the treatments altered the potency of the cannabinoid agonist to inhibit AC in the cerebellum (Fig. 1C).

To determine the specificity of fluoxetine effect on CB1 receptor coupling to AC, we analyzed the inhibition of forskolin-stimulated cAMP levels by a G_{i/o} protein-coupled receptor with a well-known implication in mood disorders, as is the case of 5-HT_{1A} receptors. Inhibition of AC by 5-HT_{1A} receptors was studied in the hippocampus of the same treated rats, given the high expression of 5-HT_{1A} protein in this brain area, and its involvement in the long-term effects of ADs. As reported above for the PFC, long-term fluoxetine treatment increased basal AC activity in the hippocampus, and this effect was not observed in the animals treated with fluoxetine + WAY100635 or with WAY100635 alone (Table 2). Statistical comparison using one-way ANOVA indicated an effect of the group (Table 2; F = 4.1; p < 0.05), with significant differences between the vehicle and fluoxetine groups (Table 2; *, p < 0.05) and between the fluoxetine versus fluoxetine + WAY100635 and WAY100635 alone groups (Table 2; #, p < 0.05 in both cases). Differences in the stimulatory effect of forskolin on cAMP levels were not detected in any of the treatment groups (Table 2). In contrast to the reported effect of fluoxetine on CB1 receptor coupling to AC in the PFC, the I_{max} for AC inhibition by the 5-HT_{1A} receptor agonist 8-OH-DPAT was reduced in the hippocampus of the same treated rats. One-way ANOVA revealed a significant effect of the group (Table 2; F = 4; *, p < 0.05), with significant differences between the vehicle and fluoxetine groups (Table 2; **, p < 0.01). The potency of 8-OH-DPAT to inhibit AC was not altered by any of the treatments (Table 2).

**Long-Term Fluoxetine Treatment Does Not Alter CB1 Receptor Density or Agonist-Stimulated \[^{35}S\]GTP\_S Binding.** The observed alterations in the efficacy of WIN55,212-2 to inhibit AC in the PFC and cerebellum of fluoxetine-treated rats could reflect changes in CB1 receptor expression and/or coupling to G_{i/o} proteins. To test this hypothesis, we carried out saturation binding experiments with the cannabinoid agonist \[^{3}H\]CP55,940 and with WIN55,212-2-stimulated \[^{35}S\]GTP\_S binding assays after long-term treatment with fluoxetine, fluoxetine + WAY100635, or WAY100635 alone. \[^{3}H\]CP55,940 binding to rat PFC and cerebellum membranes was saturated at 3 nM, with the nonspecific binding being approximately 30% of the total radioligand binding at a concentration close to the estimated apparent dissociation constant (K_{d}). Long-term in vivo treatment with fluoxetine, WAY100635, or the combination of both did not modify CB1 receptor density or agonist affinity in either brain area (Table 3). In addition, none of treatments altered the maximal effect or the potency of WIN55,212-2 to stimulate \[^{35}S\]GTP\_S binding (Table 3).

**Long-Term Fluoxetine Treatment Modulates CB1 Receptor Coupling to Specific Ga Protein Subunits.** Results from \[^{35}S\]GTP\_S binding experiments suggest that the observed changes in CB1 receptor signaling at the AC level induced by long-term fluoxetine treatment in the PFC

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**TABLE 1**

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<th>Effect of short-term fluoxetine exposure on CB1 receptor-mediated inhibition of AC in the rat prefrontal cortex</th>
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<td>Basal AC activity (pmol cAMP/min/mg protein)</td>
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<td>Forskolin-stimulated AC activity (pmol cAMP/min/mg protein)</td>
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<td>WIN55,212-2 inhibition of forskolin-stimulated AC activity</td>
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<td>I_{max}^F (%)</td>
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**TABLE 2**

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<td>pEC_{50}</td>
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* p < 0.05 versus vehicle.  
** P < 0.05 versus fluoxetine (Newman-Keuls post-ANOVA).
** p < 0.01 versus vehicle.
and cerebellum take place in the absence of any modification in the receptor coupling to Go proteins. Nevertheless, the possibility also exists that the administration of this SSRI affects the coupling ability of CB1 receptors to specific Go subunits in a complex manner, so that these modifications are overlooked when assessing global coupling to Go proteins. Post hoc test revealed that the level of Go12 and Go13 subunits activation by WIN55,212-2 was significantly increased in the PFC of fluoxetine-treated rats (Fig. 2A; p < 0.05 versus vehicle). As reported above for the fluoxetine effect on CB1 receptor coupling to AC in this brain area, the 5-HT1A receptor antagonist WAY100635 did not alter the profile of Go protein activation by WIN55,212-2 when administered alone but prevented the increased stimulation of Go12 protein subunits when coadministered with the SSRI (Fig. 2A; $, p < 0.05 versus fluoxetine).

In the cerebellum of fluoxetine-treated rats, we detected a selective down-regulation of CB1 receptor coupling to Go12 subunits. This effect of the SSRI in the cerebellum was not prevented by concomitant administration of WAY100635; in the PFC, one-way ANOVA indicated a significant difference in the efficacy of the cannabinoid agonist to activate Go12 and Go13 subunits among groups (Fig. 2A; F = 3.5, *p < 0.05; and F = 3.03, *p < 0.05, respectively), whereas no difference was detected regarding stimulation of Go0 and Go1 proteins. Post hoc test revealed that the level of Go0 and Go1 proteins. Post hoc test revealed that the level of Go0 and Go1 proteins of Go protein subunits when coadministered with the SSRI (Fig. 2A; $, p < 0.05 versus fluoxetine).

In the cerebellum of fluoxetine-treated rats, we detected a selective down-regulation of CB1 receptor coupling to Go12 subunits. This effect of the SSRI in the cerebellum was not prevented by concomitant administration of WAY100635; in the PFC, one-way ANOVA indicated a significant difference in the efficacy of the cannabinoid agonist to activate Go12 and Go13 subunits among groups (Fig. 2A; F = 3.5, *p < 0.05; and F = 3.03, *p < 0.05, respectively), whereas no difference was detected regarding stimulation of Go0 and Go1 proteins. Post hoc test revealed that the level of Go0 and Go1 proteins. Post hoc test revealed that the level of Go0 and Go1 proteins.

### Table 3

CB1 receptor density and agonist-stimulated [35S]GTPgammaS binding in rat prefrontal cortex and cerebellum after long-term treatment with fluoxetine and/or WAY100635

<table>
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<tr>
<td>[35S]GTPgammaS binding</td>
<td>140 ± 3.4</td>
<td>139 ± 2.8</td>
<td>144 ± 5.6</td>
<td>138 ± 6.7</td>
</tr>
<tr>
<td>Emax (%)</td>
<td>5.7 ± 0.25</td>
<td>5.7 ± 0.16</td>
<td>5.7 ± 0.25</td>
<td>5.8 ± 0.28</td>
</tr>
<tr>
<td><strong>Cerebellum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]CP55,940 binding</td>
<td>2021 ± 184</td>
<td>1750 ± 132</td>
<td>2115 ± 154</td>
<td>1470 ± 118</td>
</tr>
<tr>
<td>pKd (fmol/mg protein)</td>
<td>9.1 ± 0.2</td>
<td>9.2 ± 0.1</td>
<td>9.1 ± 0.1</td>
<td>9.4 ± 0.2</td>
</tr>
<tr>
<td>[35S]GTPgammaS binding</td>
<td>530 ± 70</td>
<td>579 ± 39</td>
<td>656 ± 27</td>
<td>737 ± 70</td>
</tr>
<tr>
<td>Emax (%)</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td>5.0 ± 0.3</td>
<td>4.98 ± 0.1</td>
</tr>
</tbody>
</table>

Bmax, estimated maximal [3H]CP55,940 binding sites; pKd, estimated dissociation constants normalized as −logKd.

**Fig. 2.** CB1 receptor-mediated activation of Go subunits in the rat brain is modulated by long-term fluoxetine: stimulation of Go0, Go1, Go12, Go13, and Go3 protein subunits by the cannabinoid agonist WIN55,212-2 (10 μM) in the PFC (A) and cerebellum (B) of rats continuously treated with vehicle, fluoxetine, WAY100635, or the combination of both compounds. Activation of G protein subunits was determined with anti-Go0, Go1, Go12, Go13, and Go3 antibodies immobilized to superparamagnetic Dynabeads as described under Materials and Methods. Data are mean ± S.E.M. of stimulation percentages relative of basal binding. Two independent experiments performed in brain samples from five animals for each condition. *p < 0.05; ***p < 0.001 versus vehicle; $, p < 0.05 versus fluoxetine (Newman-Keuls post-ANOVA).
that by itself did not modify WIN55,212-2 activation of Ga subunits (Fig. 2B). One-way ANOVA indicated a significant effect of the group (F = 21.9, p < 0.001; Fig. 2B), with significant differences between the vehicle versus fluoxetine (Fig. 2B; ***p < 0.001) and fluoxetine + WAY100635 groups (Fig. 2B; **p < 0.01).

**Long-Term Fluoxetine Treatment Modulates PKA Expression and CREB Phosphorylation in the PFC.** Long-term administration of ADs, including SSRIs, has been consistently shown to up-regulate different components of the cAMP pathway, including PKA and CREB (Malberg and Blendy, 2005). On the other hand, CB1 receptors are coupled to the activation of the ERK-CREB transduction cascade in several brain areas, including the PFC and the cerebellum (Rubino et al., 2004). In an attempt to elucidate the functional consequences of the observed modulation of CB1 receptor coupling to Ga proteins-AC by long-term fluoxetine treatment, we analyzed the effects of the SSRI on the expression levels of PKA catalytic and regulatory 1α domains (PKA_c and PKA_r1α) and of pCREB/CREB, ERK1/2, and pERK1/2. One-way ANOVA indicated that PKA_c levels in TCLs of PFC varied among treated groups (F = 3.2; *p < 0.05), whereas PKA_r1α expression remained unaltered. Post hoc tests revealed increased PKA_c levels in fluoxetine-treated rats (*p < 0.05 versus vehicle) that were not detected in rats treated with the combination of fluoxetine + WAY100635 or with the 5-HT1A receptor antagonist alone (Fig. 3, A and B).

Long-term fluoxetine treatment also modulated CREB and pCREB expression in TCL of rat PFC (F = 2.8, p < 0.05 and F = 22.2, p < 0.001, respectively; Fig. 3) without altering ERK1/2 and pERK1/2 levels in the same area (Fig. 3A and Table 4). The expression of CREB was significantly enhanced in PFC lysates from fluoxetine-treated rats (Table 4; *, p < 0.05 versus vehicle), and this effect was not observed in the fluoxetine + WAY100635 group. Increased pCREB levels were also detected in the TCL of rat PFC after long-term fluoxetine treatment (Table 4; ***p < 0.001 versus vehicle) and after fluoxetine + WAY100635 (Table 4; ***p < 0.001 versus vehicle). Consistently, pCREB/CREB ratio was found to be increased in PFC lysates from rats continuously treated with fluoxetine (Fig. 3C; F = 4.7, p < 0.01; *, p < 0.05 versus vehicle) and fluoxetine + WAY100635 (Fig. 3C; *, p < 0.05 versus vehicle). Consistently, we detected enhanced pCREB expression in nuclear fractions of the same treated rats (Fig. 3D; F = 6.5; ***, p < 0.01). Post hoc analysis indicated that long-term treatment with fluoxetine and with fluoxetine + WAY100635 enhanced pCREB levels in nuclear fractions of PFC (Fig. 3D; ***, p < 0.01; *, p < 0.05 versus vehicle, respectively) (Fig. 3D). Treatment with WAY100635 alone did not modulate CREB, pCREB, ERK1/2, or pERK1/2 levels in the TCL and/or nuclear fractions of rat PFC (Fig. 3 and Table 4). In contrast to this observed up-regulation of the PKA-CREB transduction cascade in the PFC by long-term fluoxetine treatment, no modifications in PKA_c, PKA_r1α, pCREB/CREB, or pERK/ERK levels were detected in the cerebellum of treated rats (Table 5).

**Role of CB1 Receptors in the Antidepressant Efficacy of Long-Term Fluoxetine Treatment in the OBX Depression Model.** To test whether the observed modulation of CB1 receptor coupling to specific Ga protein sub-

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**Fig. 3.** Long-term fluoxetine modulates the expression of PKA catalytic subunit, CREB, and pCREB in the rat PFC. A, representative Western blot analyses of PKA catalytic subunit (PKA cat), CREB, and pCREB levels in TCLs from vehicle, fluoxetine, fluoxetine + WAY100635, or WAY100635 alone-treated rats. B, increased PKA cat levels were detected in TCL after long-term fluoxetine, but not in the fluoxetine + WAY100635 or WAY100635-treated groups. None of the treatments modulated the expression of PKA regulatory 1α subunit (PKA 1α reg). C, long-term fluoxetine treatment and fluoxetine + WAY100635 up-regulated pCREB/CREB expression ratio in TCL, and this effect was not observed in rats treated with WAY100635 alone. D, increased pCREB levels were detected in PFC nuclear fractions from rats treated with fluoxetine and fluoxetine + WAY100635. Values are means ± S.E.M. corresponding to densitometry levels of the different proteins in prefrontal cortex TCL or nuclear fractions from rats treated with fluoxetine, fluoxetine + WAY100635, or WAY100635 alone, expressed as the percentage of the same proteins in vehicle-treated animals. Two independent experiments were performed in brain samples from five animals for each condition. *, p < 0.05; **, p < 0.01 versus vehicle; $, p < 0.05; $$, p < 0.01 versus fluoxetine (Newman-Keuls post-ANOVA).
inhibition in a brain area involved the pathogenesis of mood disorders and in the long-term effects of ADs, as is the case with the PFC. Among the few studies that have addressed the effects of long-term antidepressant exposure on the activity of brain EC system, most of them have focused on CB1 receptor density. Some of them suggest an enhancement of CB1 receptor expression in brain areas with a well-established role in depression (Hill et al., 2006a,b; Hill et al., 2007). Increased CB1 receptor density in the rat PFC has been reported after long-term administration of the monoamine oxidase inhibitor tranylcypromine or with fluoxetine (Hill et al., 2008b) but not with the tricyclic desipramine (Hill et al., 2006a). Although the absence of CB1 receptor expression modulation by fluoxetine reported here is in contrast to the findings of Hill et al. (2008b), probably because of differences in the treatment regimen between both studies, our data demonstrating up-regulated CB1 receptor coupling to Gαi proteins/AC strengthen the idea that long-term administration of chemical antidepressants enhance CB1 receptor activity in this brain area. When interpreting these findings, it must also be considered that reduced CB1 receptor density has also been reported in the PFC after electroconvulsive shock (Hill et al., 2007). These data suggest that forebrain EC system may be important for the efficacy of antidepressant treatments, reinforcing the interest of simultaneously analyzing the effects of AD administration on the different levels of the CB1 receptor transduction cascade.

Stimulation of CB1 receptors by WIN 55212-2 resulted in the activation of at least five different Gαi subunits in PFC and cerebellum, with slight differences in the efficacy of subunit activation across brain regions, as described previously (Prather et al., 2000). Our results also indicate increased CB1 receptor coupling to the activation of Gαi3 and Gαi1 subunits, and not to Gαi5 subunits, in the PFC of fluoxetine-treated rats. The absence of fluoxetine effect on CB1 receptor-stimulated [35S]GTPγS binding strengthens this idea, because these assays mainly detect the activation of Gαi3 and Gαi5, or Gαi1, subunits (Jiang et al., 2001), which might be related to the fact that Gαi are in significant excess over Gβi3 in the brain (Sternweis and Robishaw, 1984; Spicher et al., 1992). It is noteworthy that both CB1 receptor stimulation of Gαi3 and AC inhibition were significantly reduced in the cerebellum of the same fluoxetine-treated rats. Altogether, these data strongly suggest that fluoxetine-induced modulation of CB1 receptor coupling to Gαi proteins may contribute to the observed changes in the level of CB1 receptor-dependent modulation of AC activity. The fact that opposite modulation of CB1 receptor coupling to Gαi proteins/AC by long-term flu-
oxetine treatment was detected in the PFC and cerebellum of the same animals strengthens the emerging idea that the effects of long-term AD treatment on the activity of EC system differ among brain structures (Hill et al., 2008b).

A major finding of the present study is that pharmacological blockade of 5-HT$_{1A}$ receptors with WAY100635 suppresses fluoxetine-induced up-regulation of CB$_1$ receptor coupling to $G_{\alpha_{12}}$ proteins and to AC in the PFC. It is noteworthy that WAY100635 did not prevent desensitization of CB$_1$ receptor signaling at the $G_{\alpha_{12}}$ protein/AC level in the cerebellum, a brain area devoid of 5-HT$_{1A}$ receptors (Pazos and Palacios, 1985; Pompeiano et al., 1992). These results reinforce the hypothesis that fluoxetine-induced modulation of CB$_1$ receptor signaling through $G_{\alpha_{12}}$ proteins underlies the observed changes at the AC level. In addition, these findings strongly suggest that the enhanced CB$_1$ signaling at the $G_{\alpha_{12}}$ protein/AC in the PFC by long-term fluoxetine treatment results from the activation of 5-HT$_{1A}$ receptors. The efficacy of our WAY100635 administration protocol to block 5-HT$_{1A}$ receptors during fluoxetine administration is supported by the ability of this compound to prevent the desensitization of 5-HT$_{1A}$ receptor-mediated responses in the dorsal raphe nucleus (DRN) (Castro et al., 2008) and in the hippocampus (present study). Another important question is whether long-term administration of other antidepressant compounds that inhibit 5-HT uptake induces a similar effect in brain areas expressing 5-HT$_{1A}$ receptors. In this regard, data from our laboratory suggest that this is indeed the case, because administration of the dual 5-HT and noradrenaline inhibitor venlafaxine (40 mg/kg/day s.c., 14 days, minipumps) also enhances CB$_1$ receptor-mediated inhibition of AC in the rat PFC (E. M. Valdizán, E. Castro, and A. Pazos, unpublished observations). Although the molecular mechanisms by which fluoxetine up-regulates CB$_1$ receptor functionality remain to be elucidated, a growing body of evidence supports the existence of interactions between brain EC and 5-HT systems, with our results pointing out to a special involvement of 5-HT$_{1A}$ receptors. It is noteworthy that adequate CB$_1$ receptor functionality seems to be important for 5-HT$_{1A}$ receptor-mediated biochemical and behavioral responses, because decreased efficacy of the 8-OH-DPAT to activate $G_{\alpha_{12}}$ proteins (Mato et al., 2007), impaired anxiolytic (Urigüen et al., 2004) and hypothermic (Mato et al., 2007) effects of 5-HT$_{1A}$ agonists and reduced functionality of 5-HT$_{1A}$ autoreceptors in the DRN (Aso et al., 2009) have been detected in CB$_1$ knockout animals. In this context, the present report uncovers the possibility that modulation of CB$_1$ receptors participates in those effects of long-term fluoxetine treatment that are triggered by 5-HT$_{1A}$ receptors.

Regarding the observed increase in basal AC activity (without modification of endogenous cAMP levels), PKA$_c$ subunit expression and pCREB/CREB ratio in the PFC by long-term fluoxetine treatment, our findings are in good agreement with research carried out during the past decade, which provides strong support for the up-regulation of the cAMP pathway after long-term antidepressant administration (Malberg and Blendy, 2005). Results include increased coupling of stimulatory G protein ($G_{s}$) to AC (Donati and Rasenick, 2003) in the presence of persistent activation of various $G_{\alpha_{12}}$-coupled receptors, consistent with the partial reversion of the increase of basal AC activity and PKA$_c$ subunit observed after association of fluoxetine with the 5-HT$_{1A}$ receptor antagonist in the PFC and/or hippocampus. Although the modulation of CB$_1$ receptor functionality could be involved in the activation of the cAMP-CREB cascade by long-term fluoxetine treatment, our methodological approach does not allow for the clarification of this issue. It is noteworthy that WAY100635 completely prevented a fluoxetine-induced increase in CB$_1$ receptor coupling to $G_{\alpha_{12}}$ protein/AC and partially reversed the up-regulation of both basal AC activity and PKA-c subunit expression induced by the SSRI in the PFC. These findings suggest that up-regulated CB$_1$ receptor signaling contributes to some extent to 5-HT$_{1A}$ receptor-mediated modulation of the cAMP cascade after fluoxetine administration, although the involvement of other mechanisms cannot be discarded. In this scenario, the lack of ability of WAY100635 to prevent fluoxetine-induced increase in pCREB/CREB ratio would indicate that the up-regulation of CB$_1$ receptor signaling does not contribute to this effect.

Indeed, although studies addressing the effects of CB$_1$ receptor antagonists on the modulation of CAMP-CREB cascade by long-term AD administration are lacking, we have observed no significant differences between control and CB$_1$ knockout animals with respect to pCREB up-regulation by long-term fluoxetine treatment (E. M. Valdizán, A. Ozaita, E. Aso, R. Maldonado, A. Pazos, and O. Valverde, unpublished data). On the other hand, we found no significant modification in pERK/ERK expression in the PFC after long-term fluoxetine treatment.

The role of the observed up-regulation of CB$_1$ receptor coupling to AC in the behavioral adaptations induced by long-term fluoxetine treatment is also difficult to interpret at the present time. In addition to the already mentioned possibility that CB$_1$ receptors participate in these effects via the modulation of fluoxetine-induced adaptations in cAMP cascade, recent data also suggest that up-regulated CB$_1$ receptor signaling in the PFC could also elicit antidepressant effects by enhancing the activity of 5-HT neurons in the DRN (Gobbi et al., 2005; Bambico et al., 2007). Consistent with the idea that enhanced signaling through CB$_1$ receptors may result in antidepressant effects, CB$_1$ receptor knockout mice exhibit enhanced depressive-like behaviors (Martin et al., 2002; Mato et al., 2007), and low doses of cannabinoid agonists (Bambico et al., 2007; McLaughlin et al., 2007; Morrish et al., 2009) or inhibitors of EC degradation (Gobbi et al., 2005; Bortolato et al., 2007) produce antidepressant-like effects in rodents. Nevertheless, it should be noted that CB$_1$ receptor antagonists also behave as antidepressants in behavioral models (Griebel et al., 2005; Witkin et al., 2005; Steiner et al., 2008). When interpreting these controversial data, an important consideration is that, although studies specifically ad-
Fluoxetine Modulates CB1 Coupling to Adenylyl Cyclase


dressing the antidepressant effects of CB1 cannabinoid agonists and antagonists in humans are lacking, the high incidence of depression and anxiety in clinical trials with the CB1 receptor antagonist rimobanfor the treatment of obesity, despite depressed mood being an exclusion criterion in these trials (Mitchell and Morris, 2007), has motivated its recent withdrawal from use or lack of approval. Along with preclinical data, these clinical findings strongly suggest that CB1 receptors may represent a novel target for the treatment of mood disorders. In support of this, we have recently demonstrated in the PFC of OBX rats, an animal model of depression, a significant increase in CB1 receptor density and coupling to G proteins in the PFC, together with the characteristic hyperactivity in the open field test: both neurochemical and behavioral responses were absent after fluoxetine (Rodríguez-Gaztelumendi et al., 2009). It is noteworthy that exposure to a single Δ9-THC injection before the initiation of long-term treatment with fluoxetine significantly decreased hyperactivity in OBX rats, indicating that direct activation of CB1 receptors elicits antidepressant-like effects in this depression model (Rodríguez-Gaztelumendi et al., 2009). In contrast, here we report that long-term Δ9-THC administration neither elicits antidepressant-like effects nor modulates the behavioral efficacy of long-term fluoxetine treatment in this model. It could be hypothesized that sustained administration of Δ9-THC is expected to induce adaptive changes in the CB1 receptor signaling axis, including down-regulation in many brain areas (Breivogel et al., 1999). These changes could strongly modify those reported here induced by fluoxetine, resulting in the lack of influence of Δ9-THC on the antidepressant responses. The full clarification of this issue goes beyond the scope of the present study.

In summary, this study demonstrates that long-term fluoxetine treatment modulates the functionality of CB1 receptors in the rat brain in a region-specific manner. Up-regulated CB1 receptor signaling at the Gαi protein-AC transduction level in the PFC, even in the absence of significant changes in receptor density, is one of the long-term molecular adaptations triggered by long-term fluoxetine treatment. This response is mediated through the activation of 5-HT circuits implicating 5-HT1A receptors, because it is prevented by co-administration of WAY106035 and does not take place in the cerebellum, a brain area devoid of these receptors. These results unveil the possible relevance of the EC/5-HT interactions for the therapeutic responses of ADs that inhibit the uptake of this monoamine. Further research is necessary to fully clarify the extent to which modulation of the EC system may play a role in the therapeutic efficacy of these compounds.

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References


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