0026-895X/10/7703-424–434$20.00
MOLECULAR PHARMACOLOGY
Vol. 77, No. 3
60079/3560881
Mol Pharmacol 77:424–434, 2010
Printed in U.S.A.

Long-Term Fluoxetine Treatment Modulates Cannabinoid Type 1 Receptor-Mediated Inhibition of Adenylyl Cyclase in the Rat Prefrontal Cortex through 5-Hydroxytryptamine$_{1A}$ Receptor-Dependent Mechanisms

Susana Mato,[1] Rebeca Vidal, Elena Castro, Álvaro Díaz, Ángel Pazos, and Elsa M. Valdizán

Departamento de Fisiología y Farmacología, Universidad de Cantabria, Santander, Spain (S.M., R.V., E.C., A.D., A.P., E.M.V.); Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain (R.V., E.C., A.D., A.P., E.M.V.); and Centro de Investigación Biomédica en Red de Salud Mental, Instituto de Salud Carlos III, Madrid, Spain (R.V., E.C., A.D., A.P., E.M.V.)

Received August 4, 2009; accepted December 7, 2009

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_1) receptor density and functionality in the rat prefrontal cortex (PFC) and cerebellum. Long-term treatment with fluoxetine (10 mg/kg/day) enhanced CB_1 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-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) in either area. Analysis of [^{35}S]GTPγS-labeled Gα subunits allowed for the detection of up-regulated CB_1 receptor coupling to $G\alpha_{i2}$, $G\alpha_{i3}$ in the PFC, and reduced coupling to $G\alpha_{i3}$ in the cerebellum of fluoxetine-treated rats. Concomitant administration of the 5-HT_{1A} receptor antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate (WAY100635; 0.1 mg/kg/day) reduced fluoxetine-induced modulation of CB_1 receptor coupling to Gα subunits and AC in the PFC but not in the cerebellum. These results indicate that increased CB_1 receptor signaling at the $G\alpha_i$-AC transduction level is a long-term adaptation induced by fluoxetine in the PFC and point to a role for 5-HT_{1A} receptors in this effect. Basal AC activity, protein kinase A (PKA) catalytic subunit expression, and phospho-cAMP 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^9-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_1 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_{1A} 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

This work was supported by the Ministerio de Education y Ciencia [Grants CICYT SAF04-00941, SAF07-61862]; Plan Nacional sobre Drogas; and Fundación Marqués de Valdecilla [Grant API 05/23].

Presented in part at the 35th Annual Meeting of the Society for Neuroscience, Washington DC, 2005 Nov 12–16.

[1] Current affiliation: Department of Neuroscience, University of the Basque Country, Bizkaia, Spain.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.109.060079.

ABBREVIATIONS: AD, antidepressant drug; AC, adenylyl cyclase; BSA, bovine serum albumin; CB_1, cannabinoid type 1; [^{3}H]CP55,940, [^{3}H](1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; 8-OH-DPAT, 8-hydroxydi-*n*-propylaminotetralin; DTT, *dl*-dithiothreitol; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; PFC, prefrontal cortex; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SSRI, selective serotonin reuptake inhibitor; Δ^9-THC, Δ^9-tetrahydrocannabinol; WAY100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate; WIN55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanonemesylate; PKA, protein kinase A; EC, endocannabinoid; ERK, extracellular signal regulated-kinase; OBX, olfactory bulbectomy; TCL, total cell lysate; ANOVA, analysis of variance; DRN, dorsal raphe nucleus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CREB, cAMP response element-binding protein; 5-HT, 5-hydroxytryptamine; E-64, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide.

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-HT_{1A} receptors could be involved in the latest effect, because both proliferative and behavioral effects of fluoxetine are abolished by genetic deletion of the 5-HT_{1A} gene (Santarelli et al., 2003).

Cannabinoid CB_1 receptors, negatively coupled to adenylyl cyclase (AC) via $G_{i/o}$ proteins, mediate most of the actions of exogenous and endogenous cannabinoids in the central nervous system (Howlett et al., 2002). CB_1 receptor knockout mice exhibit enhanced "depressive-like" behaviors in animal models (Martin et al., 2002; Mato et al., 2007). Pharmacological modulation of CB_1 receptors has led to more conflicting results, because both CB_1 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 CB_1 receptors could play a role in the cause of major depression (Vinod and Hungund, 2006). In favor of this hypothesis, CB_1 receptor mRNA (Bortolato et al., 2007), protein expression (Hill et al., 2008a), and signaling through $G_{i/o}$ proteins (Rodríguez-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 CB_1 receptor expression (Hill et al., 2008b) and coupling to $G_{i/o}$ proteins (Rodríguez-Gaztelumendi et al., 2009), as well as the levels of the ECs anandamide and 2-arachidonoylglycerol (Hill et al., 2008b), in the PFC and/or hippocampus. It is noteworthy that information about the putative adaptations of brain CB_1 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 SSRIs and the fact that experimental data support the existence of cross-talk mechanisms between brain EC and 5-HT systems (Marco et al., 2004; Gobbi et al., 2005; Hill et al., 2006b; Mato et al., 2007; Aso et al., 2009), it is noteworthy that the possible implication of 5-HT receptors in the adaptations of the EC system induced by long-term AD treatment has not been addressed.

Thus, the main objective of this study was to investigate the possible contribution of 5-HT_{1A} receptors to the modulation of CB_1 receptor activity by long-term fluoxetine treatment. We carried out 2-week treatments with fluoxetine and/or the 5-HT_{1A} receptor antagonist WAY100635, analyzing several steps of CB_1-associated signaling pathway (receptor expression, coupling to $G_{i/o}$ protein subunits, and modulation of AC activity) in the PFC, an area that contains important levels of both CB_1 and 5-HT_{1A} receptors, and in the cerebellum, which contains high levels of CB_1 receptors but lacks 5-HT_{1A} receptors (Pazos and Palacios, 1985; Pompeiano et al., 1992). We also analyzed the effect of the different treatments on the expression of several components of the cAMP-dependent pathway. Finally, we studied the influence of long-term Δ^9-THC administration on the behavioral responses induced by fluoxetine in an animal model of depression (olfactory bulbectomy). Our data demonstrate that CB_1 receptor coupling to specific $G\alpha_i$ proteins and to the inhibition of AC are regionally modulated by long-term fluoxetine administration and indicate a role for 5-HT_{1A} receptors 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. [^{3}H]CP55,940 (165 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). cAMP ^{3}H assay kit (1028 GBq/mmol) was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). WAY100635, DTT, GDP, GTP, GTPγS, 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^9-Tetrahydrocannabinol (Δ^9-THC) was purchased from THC Pharm GMBH The Health Concept (Frankfurt, Germany). Rabbit anti-$G\alpha_{i1}$, anti-$G\alpha_{i2}$, anti-$G\alpha_{i3}$, anti-$G\alpha_z$, anti-$G\alpha_o$, and anti-extracellular signal regulated-kinase (ERK) 1/2, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase and anti-histone H1 were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Mouse anti-pERK1/2 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^9-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 (Rodríguez-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 postsurgery recovery period, as described previously, recording for a

5-min period the number of ambulations, rearings, grooming episodes, and defecations. The next day, OBX animals were randomly divided into five subgroups to receive vehicle-treated sham-operated ($n = 8$), vehicle-treated OBX ($n = 8$), fluoxetine-treated OBX ($n = 8$), Δ^9-THC-treated OBX ($n = 8$), or fluoxetine plus Δ^9-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 quantitation 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-μl supernatant aliquots by using a [^{3}H]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 $MgCl_2$, 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. Experiments assessing 5-HT_{1A} receptor-mediated inhibition of AC in the hippocampus were performed using 8-OH-DPAT (10 nM to 100 μM) as specific agonist and WAY100635 (10 μM) as antagonist. The enzymatic reaction was started by the addition of ATP to a final concentration of 200 μM. The mixture was then incubated (10 min at 37°C), and the reaction was rapidly terminated by 5-min incubation at 100°C. The samples were centrifuged (14,000 rpm, 5 min), and cAMP accumulation was quantified in 50-μl supernatant aliquots by using the [^{3}H]cAMP commercial kit described above. The assays were performed in triplicate Sigmacote-treated borosilicate tubes, and the results were confirmed in two independent experiments.

[^{3}H]CP55,940 Saturation Binding Assay. Frozen brain samples were homogenized [1:100 (w/v] in ice-cold buffer (50 mM Tris-HCl, 250 mM sucrose, 3 mM $MgCl_2$, and 1 mM EGTA, pH 7.4) using a motor-driven Teflon and glass tissue grinder (10 strokes, 1500 rpm). Homogenates were then centrifuged (1500 rpm, 5 min, 4°C), with the resulting supernatants centrifuged again (14,000 rpm, 15 min, 4°C). The obtained pellets were resuspended in assay buffer (50 mM Tris-HCl, 1 mM EDTA, and 3 mM $MgCl_2$, pH 7.4) and centrifuged again (14,000*g*, 15 min, 4°C). Pellets were resuspended in assay buffer (50 μg protein/ml in the assay) containing 1 mg/ml BSA and incubated for 60 min at 30°C in the presence of nine different concentrations of the cannabinoid agonist [^{3}H]CP55,940 (0.0125–3.2 nM). Nonspecific binding was determined with 1 μM WIN55,212-2. Bound radioactivity was determined using a Beckman LS6000 liquid scintillation counter (Beckman Coulter, Fullerton, CA) after overnight extraction in 5-ml Ecolite scintillation fluid. All assays were performed in duplicate Sigmacote-treated borosilicate tubes, and the results were confirmed in two independent experiments.

Agonist-Stimulated [^{35}S]GTPγS Binding. Tissue samples were homogenized [1:100 (w/v)] in ice-cold buffer (50 mM Tris-HCl, 250 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT, pH 7.4) and processed as reported above for [^{3}H]CP55,940 binding assays. Membrane aliquots (200 μg protein/ml in the assay) were incubated for 120 min at 30°C in assay buffer (100 mM NaCl, 50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 μM GDP, and 1 mg/ml BSA, pH 7.4) containing 0.1 nM [^{35}S]GTPγS. Cannabinoid agonist stimulation of [^{35}S]GTPγS binding was determined using 1 nM to 100 μM WIN55,212-2. The specificity of the CB_1 receptor-mediated stimulation was verified by coincubation of 10 μM WIN55,212-2 with 1 μM SR141716A. Nonspecific binding was determined in the presence of 10 μM GTPγS. Experiments were terminated by sample dilution in ice-cold buffer (50 mM Tris-HCl and 1 mg/ml BSA, pH 7.4) and rapid filtration under vacuum (Cell Harvester M-12R; Brandel Inc., Gaithersburg, MD) through GF/C glass fiber filters. [^{35}S]GTPγS binding assays were terminated and measured as described for [^{3}H]CP55,940 experiments. All assays were performed in duplicate Sigmacote-treated borosilicate tubes, and the results were confirmed in two independent experiments.

Immunoprecipitation of [^{35}S]GTPγS-Labeled Gα Subunits. Membrane homogenates were obtained as reported for agonist-stimulated [^{35}S]GTPγS binding assays. Resuspended pellets (500 μg protein/ml in the assay) were incubated with 2 nM [^{35}S]GTPγS and 10 μM WIN55,212-2 in a final 100-μl assay volume for 30 min at 30°C. Nonspecific binding was determined in the presence of 10 μM GTPγS. Membrane suspensions were then solubilized on ice with 1% IGEPAL, 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-$G\alpha_{i1}$, anti-$G\alpha_{i2}$, anti-$G\alpha_{i3}$, anti-$G\alpha_z$, and anti-$G\alpha_o$ 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 $MgCl_2$, 1 mM EGTA, and 1 mM DTT, pH 7.4) containing the following protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μl/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% IGEPAL, 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 quantitations were performed by densitometric scanning using Scion Image software (Scion Corporation, Frederick, MD). The densitometry values were normalized with respect to the values obtained with anti-glyceraldehyde-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. [^{3}H]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 [^{3}H]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 × 100)/basal activity] in [^{35}S]GTPγS assays and the percentage of inhibition in cAMP assays [% = (agonist effect × 100)/(forskolin effect − 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 [^{35}S]GTPγS 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^{-5} 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 ± 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 ± 0.43, cerebellum, 3.34 ± 0.44; fluoxetine: PFC, 1.78 ± 0.20, cerebellum, 3.57 ± 0.46; fluoxetine + WAY100635: PFC, 1.79 ± 0.47, cerebellum, 3.23 ± 0.18; WAY100635: PFC, 1.81 ± 0.42, cerebellum, 3.38 ± 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 no detected in any of the treated groups (vehicle, 164 ± 34; fluoxetine, 207 ± 42; fluoxetine + WAY100635, 181 ± 36; and WAY100635, 172 ± 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 ± 3.0% and a pIC_{50} of 5.7 ± 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 ± 3.7%) that was absent in those rats treated with fluoxetine plus the 5-HT_{1A} receptor antagonist WAY100635 (I_{max} = 25.5 ± 3.2%) or WAY100635 alone (I_{max} = 25.3 ± 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; \$\$, $p < 0.01$)

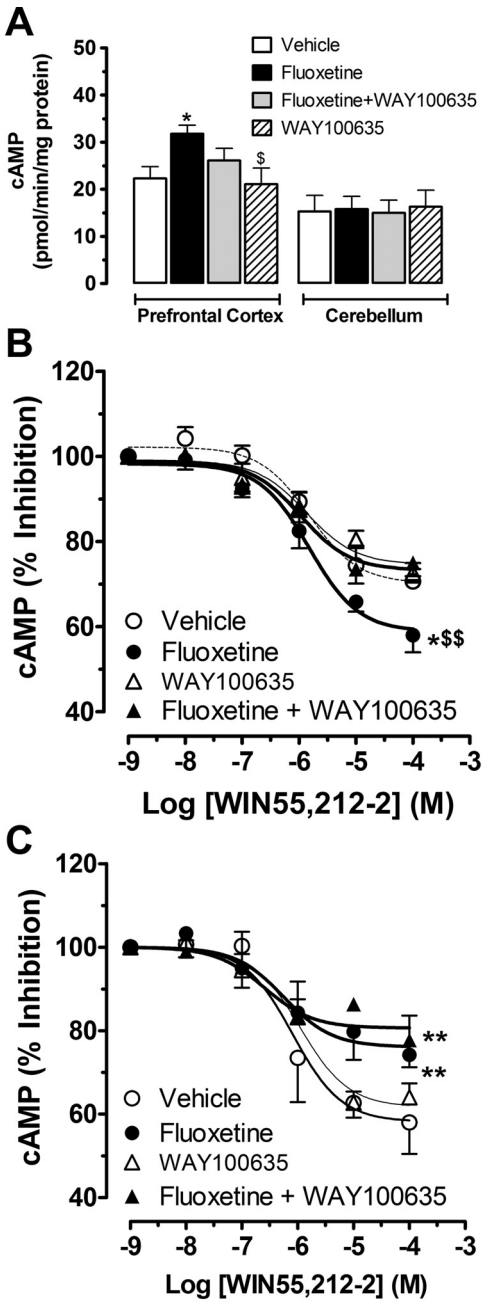

Fig. 1. Long-term fluoxetine treatment modulates basal and/or CB_1 receptor-mediated inhibition of AC activity in the rat brain. A, basal AC activity in the rat PFC and cerebellum after 14 days of treatment with vehicle, fluoxetine (10 mg/kg/day), WAY100635 (0.1 mg/kg/day), or the combination of both compounds. B and C, inhibition of forskolin-stimulated AC by the cannabinoid agonist WIN55,212-2 in the PFC (B) and cerebellum (C) of treated rats. Percentages of inhibition were calculated for each agonist concentration relative to forskolin-stimulated cAMP levels. Prism software was used to generate concentration-response curves and estimate pEC_{50} values in the PFC (vehicle, 5.7 ± 0.1; fluoxetine, 5.9 ± 0.2; fluoxetine + WAY100635, 6 ± 0.1; WAY100635, 5.9 ± 0.5) and cerebellum (vehicle, 5.7 ± 0.08; fluoxetine, 5.9 ± 0.21; fluoxetine + WAY100635, 6.2 ± 0.27; WAY100635, 5.7 ± 0.33). Data are mean ± 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 CB_1 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 fluoxetine treatment (I_{max} = 23.5 ± 0.9% in fluoxetine-treated rats versus 38.7 ± 2.1% in vehicle-treated rats). This reduced coupling ability of CB_1 receptors to AC in the cerebellum of fluoxetine-treated rats was not prevented by cotreatment with the 5-HT_{1A} receptor antagonist WAY100635 (I_{max} = 18.1 ± 1.9%), which when administered alone did not modify WIN55,212-2 efficacy in cAMP assays (I_{max} = 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 CB_1 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 CB_1 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 CB_1 Receptor Density or Agonist-Stimulated [^{35}S]GTPγS 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 CB_1 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]GTPγS 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 CB_1 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]GTPγS binding (Table 3).

Long-Term Fluoxetine Treatment Modulates CB_1 Receptor Coupling to Specific Gα Protein Subunits. Results from [^{35}S]GTPγS binding experiments suggest that the observed changes in CB_1 receptor signaling at the AC level induced by long-term fluoxetine treatment in the PFC

TABLE 1

Effect of short-term fluoxetine exposure on CB_1 receptor-mediated inhibition of AC in the rat prefrontal cortex

I_{max} and pEC_{50} values correspond to the estimated maximal effect and half-maximal effect of the cannabinoid agonist WIN55,212-2 in AC assays. Values are means ± S.E.M. of two independent experiments performed in brain samples from four animals for each condition.

	Vehicle	Fluoxetine
Basal AC activity (pmol cAMP/min/mg protein)	17.5 ± 2.4	18.6 ± 3.1
Forskolin-stimulated AC activity (pmol cAMP/min/mg protein)	160.5 ± 28.4	169.3 ± 50.1
WIN55,212-2 inhibition of forskolin-stimulated AC activity		
I_{max} (%)	33.9 ± 0.9	31.2 ± 0.8
pEC_{50}	6.1 ± 0.3	5.9 ± 0.3

TABLE 2

Long-term fluoxetine modulates basal AC activity and 5-HT_{1A} receptor mediated inhibition of AC in the hippocampus

I_{max} and pEC_{50} values correspond to the estimated maximal effect and half-maximal effect of the 5-HT_{1A} receptor agonist 8-OH-DPAT in AC assays. Values are means ± S.E.M. of two independent experiments performed in brain samples from eight to nine animals for each condition.

	Vehicle	Fluoxetine	Fluoxetine + WAY100635	WAY100635
Basal AC activity (pmol cAMP/min/mg protein)	11.5 ± 0.6	16.8 ± 1.2*	12.4 ± 1.3#	11.3 ± 1.8#
Forskolin-stimulated AC activity (pmol cAMP/min/mg protein)	92 ± 18	113 ± 15	101 ± 6	96 ± 10
WIN55,212–2 inhibition of forskolin-stimulated AC activity				
I_{max} (%)	27.1 ± 1.9	15.6 ± 1.9**	21.8 ± 3.1	22.1 ± 2.4
pEC_{50}	7.8 ± 0.3	7.4 ± 0.4	7.5 ± 0.3	8.1 ± 0.4

* $p < 0.05$ 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 $G_{i/o}$ proteins. Nevertheless, the possibility also exists that the administration of this SSRI affects the coupling ability of CB_1 receptors to specific $G\alpha$ subunits in a complex manner, so that these modifications are overlooked when assessing global coupling to $G_{i/o}$ proteins in [^{35}S]GTPγS binding assays. To further clarify this point, we performed immunoprecipitation of WIN55,212-2-stimulated [^{35}S]GTPγS-labeled $G\alpha$ protein subunits after long-term treatment with fluoxetine, WAY100635, or the combination of both compounds. The coupling efficiency of CB_1 receptors to $G\alpha$ protein subunits induced by the agonist WIN55,212-2 in PFC was $G\alpha_{i3}$ (173 ± 18%) ≅ $G\alpha_{i2}$ (169 ± 6%) > $G\alpha_o$ (145 ± 8%) > $G\alpha_z$ (131 ± 14%). In the cerebellum, coupling efficiency values were $G\alpha_{i3}$ (335 ± 28%) > $G\alpha_o$ (210 ± 36%) ≅ $G\alpha_z$ (210 ± 20%) > $G\alpha_{i2}$ (203 ± 3%) ≅ $G\alpha_1$ (202 ± 15%) (Fig. 2). No significant coupling of CB_1 receptors to $G\alpha_{i1}$ was observed in PFC after activation with WIN55, 212-2.

In the PFC, one-way ANOVA indicated a significant difference in the efficacy of the cannabinoid agonist to activate $G\alpha_{i2}$ and $G\alpha_{i3}$ 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 $G\alpha_0$ and $G\alpha_z$ proteins. Post hoc test revealed that the level of $G\alpha_{i2}$ and $G\alpha_{i3}$ 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 CB_1 receptor coupling to AC in this brain area, the 5-HT_{1A} receptor antagonist WAY100635 did not alter the profile of $G\alpha$ protein activation by WIN55,212-2 when administered alone but prevented the increased stimulation of $G\alpha_{i2}$ 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 CB_1 receptor coupling to $G\alpha_{i2}$ subunits. This effect of the SSRI in the cerebellum was not prevented by concomitant administration of WAY100635;

TABLE 3

CB_1 receptor density and agonist-stimulated [^{35}S]GTPγS binding in rat prefrontal cortex and cerebellum after long-term treatment with fluoxetine and/or WAY100635

The E_{max} and pEC_{50} values correspond to the estimated maximal effect and half-maximal effect of the cannabinoid agonist WIN55,212-2 in [^{35}S]GTPγS binding assays. Values are means ± S.E.M. of two independent experiments performed in brain samples from eight to nine animals for each condition.

	Vehicle	Fluoxetine	Fluoxetine + WAY100635	WAY100635
Prefrontal cortex				
[^{3}H]CP55,940 binding				
B_{max} (fmol/mg protein)	580 ± 41	524 ± 37	463 ± 36	486 ± 37
pK_d	9.6 ± 0.06	9.6 ± 0.04	9.7 ± 0.05	9.6 ± 0.01
[^{35}S]GTPγS binding				
E_{max} (%)	140 ± 3.4	139 ± 2.8	144 ± 5.6	138 ± 6.7
pEC_{50}	5.7 ± 0.25	5.7 ± 0.16	5.7 ± 0.25	5.8 ± 0.28
Cerebellum				
[^{3}H]CP55,940 binding				
B_{max} (fmol/mg protein)	2021 ± 184	1750 ± 132	2115 ± 154	1470 ± 118
pK_d	9.1 ± 0.2	9.2 ± 0.1	9.1 ± 0.1	9.4 ± 0.2
[^{35}S]GTPγS binding				
E_{max} (%)	530 ± 70	579 ± 39	656 ± 27	737 ± 70
pEC_{50}	4.9 ± 0.1	4.9 ± 0.2	5.0 ± 0.3	4.98 ± 0.1

B_{max}, estimated maximal [^{3}H]CP55,940 binding sites; pK_d, estimated dissociation constants normalized as $-\log K_d$.

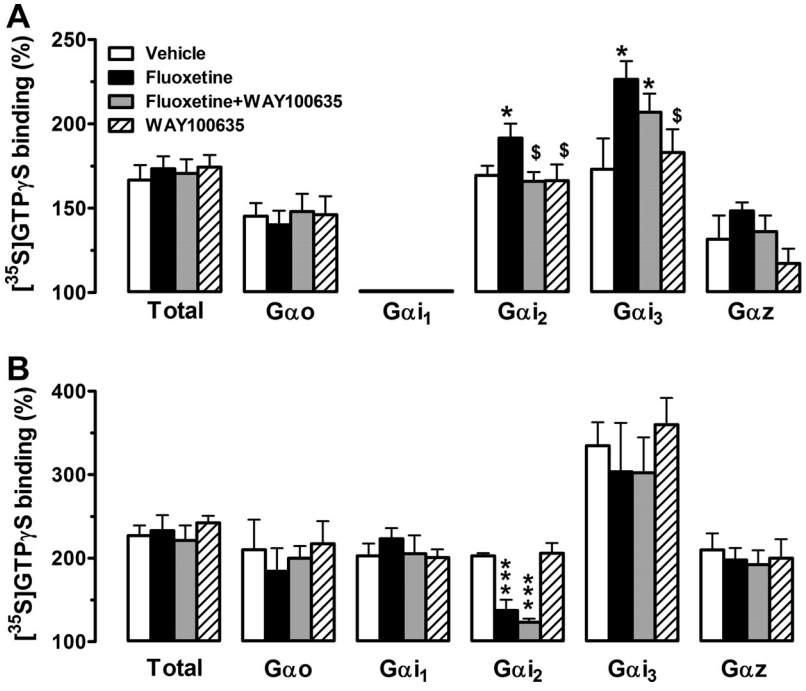

Fig. 2. CB_1 receptor-mediated activation of $G\alpha$ subunits in the rat brain is modulated by long-term fluoxetine: stimulation of $G\alpha_0$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$ protein subunits by the cannabinoid agonist WIN55,212-2 (10 μM) in the PFC (A) and cerebellum (B) of rats continuously treated with vehicle, fluoxetine, WAY100635, or the combination of both compounds. Activation of G protein subunits was determined with anti-$G\alpha_0$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$ 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 Gα 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; ***, $p < 0.001$).

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, CB_1 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 CB_1 receptor coupling to $G\alpha_i$ 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-HT_{1A} 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 CB_1 Receptors in the Antidepressant Efficacy of Long-Term Fluoxetine Treatment in the OBX Depression Model. To test whether the observed modulation of CB_1 receptor coupling to specific $G\alpha_i$ protein sub-

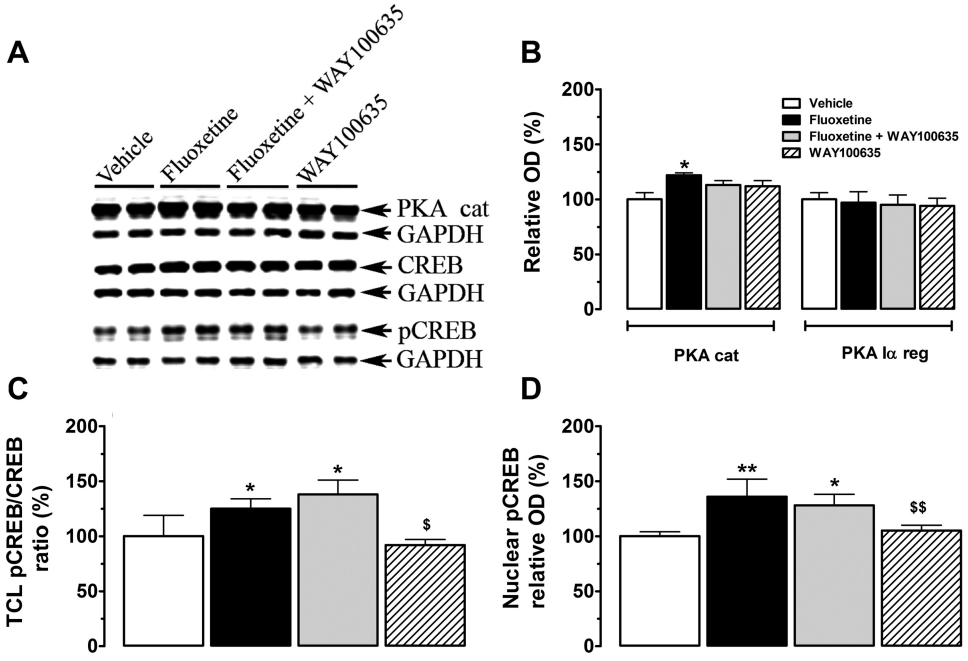

Fig. 3. Long-term fluoxetine modulates the expression of PKA catalytic subunit, CREB, and pCREB in the rat PFC. A, representative Western blot analyses of PKA catalytic subunit (PKA cat), CREB, and pCREB levels in TCLs from vehicle, fluoxetine, fluoxetine + WAY100635, or WAY100635 alone-treated rats. 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. *, $p < 0.05$; **, $p < 0.0$ versus vehicle; $, $p < 0.05$; $$, $p < 0.01$ versus fluoxetine (Newman-Keuls post-ANOVA).

units/AC in the PFC by long-term fluoxetine treatment could participate in the therapeutic efficacy of this SSRI, we studied the behavioral consequences of long-term CB_1 receptor activation in the OBX depression model. Two weeks after sham ($n = 8$) or OBX ($n = 32$) surgery and before the initiation of the long-term treatments, OBX-induced hyperactivity was confirmed on the open field test (data not shown). The next day, sham animals were treated with vehicle, and OBX animals were treated with either vehicle, fluoxetine (10 mg/kg/day), the CB_1 receptor agonist Δ^9-THC (10 mg/kg/day), or the combination of both compounds for another 14 days, and the behavioral effects of the treatments were evaluated in the open field (Table 6). In agreement with our previous observations (Rodríguez-Gaztelumendi et al., 2009), long-term fluoxetine treatment induced a complete reversion of the increased number of ambulations in OBX rats relative to the vehicle-treated OBX group (Table 6; #, $p < 0.05$). In contrast, we detected no difference in total ambulation scores between Δ^9-THC-treated and vehicle-treated OBX rats (Table 6). Finally, long-term treatment with Δ^9-THC did not modulate the ability of fluoxetine to reduce hyperactivity in OBX rats (Table 6). Thus, long-term fluoxetine treatment rectified the locomotor hyperactivity in the OBX rats, whereas long-term Δ^9-THC was without effect and did not affect the behavioral effects of fluoxetine in this depression model.

Discussion

The present results constitute the first evidence that long-term treatment with an antidepressant compound enhances CB_1 receptor coupling to specific $G\alpha_i$ subunits and to AC 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 CB_1 receptor density. Some of them suggest an enhancement of CB_1 receptor expression in brain areas with a well established role in depression (Hill et al., 2006a,b; Hill et al., 2007). Increased CB_1 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 CB_1 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 CB_1 receptor coupling to $G\alpha_i$ proteins/AC strengthen the idea that long-term administration of chemical antidepressants enhance CB_1 receptor activity in this brain area. When interpreting these findings, it must also be considered that reduced CB_1 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 CB_1 receptor transduction cascade.

Stimulation of CB_1 receptors by WIN 55212-2 resulted in the activation of at least five different $G\alpha_{i/o}$ proteins 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 CB_1 receptor coupling to the activation of $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits, and not to $G\alpha_o$ subunits, in the PFC of fluoxetine-treated rats. The absence of fluoxetine effect on CB_1 receptor-stimulated [^{35}S]GTPγS binding strengthens this idea, because these assays mainly detect the activation of $G\alpha_o$ subunits (Jiang et al., 2001), which might be related to the fact that $G\alpha_o$ are in significant excess over $G\alpha_i$ in the brain (Sternweis and Robishaw, 1984; Spicher et al., 1992). It is noteworthy that both CB_1 receptor stimulation of $G\alpha_{i2}$ 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 CB_1 receptor coupling to $G\alpha_i$ proteins may contribute to the observed changes in the level of CB_1 receptor-dependent modulation of AC activity. The fact that opposite modulation of CB_1 receptor coupling to $G\alpha_i$ proteins/AC by long-term flu-

TABLE 4

Effect of long-term treatment with fluoxetine and/or WAY100635 on CREB, pCREB, ERK1/2, and pERK1/2 expression in total cell lysate fractions from rat prefrontal cortex.

Values are means ± S.E.M. corresponding to densitometric analysis of the different proteins in total cell lysates of prefrontal cortex 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.

	Vehicle	Fluoxetine	Fluoxetine + WAY100635	WAY100635
CREB	100 ± 10	135 ± 11*	125 ± 19	109 ± 12
pCREB	100 ± 10	141 ± 7***	151 ± 8***	109 ± 6#
ERK1	100 ± 8	110 ± 8	103 ± 5	93 ± 5
ERK2	100 ± 3	107 ± 4	105 ± 5	107 ± 5
pERK1	100 ± 28	117 ± 25	97 ± 32	125 ± 25
pERK2	100 ± 16	108 ± 13	98 ± 13	112 ± 12

* $p < 0.05$ versus vehicle.
*** $p < 0.001$ versus vehicle.
# $P < 0.05$ versus fluoxetine (Newman-Keuls post-ANOVA).

TABLE 5

Long-term treatment with fluoxetine and/or WAY100635 does not modulate PKA_c, PKAr1α, CREB, pCREB, ERK1/2, and pERK1/2 expression in total cell lysate fractions of rat cerebellum

Values are means ± S.E.M. corresponding to densitometric analysis of the different proteins in total cell lysate fractions of cerebellum from rats treated with fluoxetine, fluoxetine + WAY100635, or WAY100635 alone, expressed as percentage of the same proteins in vehicle-treated animals. Two independent experiments were performed in brain samples from five animals for each condition.

	Vehicle	Fluoxetine	Fluoxetine + WAY100635	WAY100635
PKA_c	100 ± 7	114 ± 13	92 ± 11	99 ± 7
PKA_r1α	100 ± 6	91 ± 9	96 ± 7	98 ± 5
CREB	100 ± 11	100 ± 20	97 ± 25	118 ± 18
pCREB	100 ± 5	102 ± 12	104 ± 10	113 ± 10
ERK1	100 ± 16	105 ± 7	119 ± 7	115 ± 11
ERK2	100 ± 12	90 ± 2	86 ± 5	94 ± 4
pERK1	100 ± 4	93 ± 8	102 ± 3	102 ± 6
pERK2	100 ± 4	93 ± 10	115 ± 12	114 ± 10

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 $G\alpha_{i2}$ proteins and to AC in the PFC. It is noteworthy that WAY100635 did not prevent desensitization of CB_1 receptor signaling at the $G\alpha_{i2}$ 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 $G\alpha_{i2}$ proteins underlies the observed changes at the AC level. In addition, these findings strongly suggest that the enhanced CB_1 signaling at the $G\alpha_{i2}$ 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 $G_{i/o}$ 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.

TABLE 6

Behavioral effects induced by long-term treatment with fluoxetine and/or Δ^9-THC in OBX animals assessed in the open-field test

Data represent mean ± S.E.M.

	Ambulation Scores
Vehicle-sham	70.3 ± 7.5
Vehicle-OBX	152.3 ± 15.3***
Fluoxetine-treated OBX	99.1 ± 9.5#
Fluoxetine + THC-treated OBX	101.7 ± 12.2#
THC-treated OBX	151.3 ± 18.2***

$F = 7.8$, $p < 0.001$ (ANOVA).
*** $p < 0.001$ versus vehicle-sham rats.
# $p < 0.05$ versus vehicle-OBX rats (Newman-Keuls post-ANOVA).

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 ($G\alpha_s$) to AC (Donati and Rasenick, 2003) in the presence of persistent activation of various $G_{i/o}$-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 $G\alpha_{i2}$ 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-

dressing the antidepressant effects of CB_1 cannabinoid agonists and antagonists in humans are lacking, the high incidence of depression and anxiety in clinical trials with the CB_1 receptor antagonist rimonabant for 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 CB_1 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 CB_1 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 Δ^9-THC injection before the initiation of long-term treatment with fluoxetine significantly decreased hyperactivity in OBX rats, indicating that direct activation of CB_1 receptors elicits antidepressant-like effects in this depression model (Rodríguez-Gaztelumendi et al., 2009). In contrast, here we report that long-term Δ^9-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 Δ^9-THC is expected to induce adaptive changes in the CB_1 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 CB_1 receptors in the rat brain in a region-specific manner. Up-regulated CB_1 receptor signaling at the $G\alpha_i$ 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 $5\text{-}HT_{1A}$ receptors, because it is prevented by coadministration of WAY100635 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.

Acknowledgments

The technical assistance of Beatriz Romero and Rebeca Madureira is kindly acknowledged.

References

Aso E, Renoir T, Mengod G, Ledent C, Hamon M, Maldonado R, Lanfumey L, and Valverde O (2009) Lack of CB1 receptor activity impairs serotonergic negative feedback. *J Neurochem* **109:**935–944.

Bambico FR, Katz N, Debonnel G, and Gobbi G (2007) Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex. *J Neurosci* **27:**11700–11711.

Bortolato M, Mangieri RA, Fu J, Kim JH, Arguello O, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2007) Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biol Psychiatry* **62:**1103–1110.

Blier P and de Montigny C (1994) Current advances and trends in the treatment of depression. *Trends Pharmacol Sci* **15:**220–226.

Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Vogt LJ, and Sim-Selley LJ (1999) Chronic Δ^9-tetrahydrocannabinol treatment produces a time-dependent loss of cannabinoid receptors and cannabinoid receptor-activated G proteins in rat brain. *J Neurochem* **73:**2447–2459.

Castro E, Díaz A, Rodriguez-Gaztelumendi A, Del Olmo E, and Pazos A (2008) WAY100635 prevents the changes induced by fluoxetine upon the 5-HT1A receptor functionality. *Neuropharmacology* **55:**1391–1396.

Donati RJ and Rasenick MM (2003) G protein signaling and the molecular basis of antidepressant action. *Life Sci* **73:**1–17.

Elena Castro M, Diaz A, del Olmo E, and Pazos A (2003) Chronic fluoxetine induces opposite changes in G protein coupling at pre and postsynaptic 5-HT1A receptors in rat brain. *Neuropharmacology* **44:**93–101.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, Tontini A, Tarzia G, Mor M, Trezza V, Goldberg SR, Cuomo V, and Piomelli D (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci U S A* **102:**18620–18625.

Griebel G, Stemmelin J, and Scatton B (2005) Effects of the cannabinoid CB1 receptor antagonist rimonabant in models of emotional reactivity in rodents. *Biol Psychiatry* **57:**261–267.

Hill MN and Gorzalka BB (2005) Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test. *Eur Neuropsychopharmacol* **15:**593–599.

Hill MN, Ho WS, Sinopoli KJ, Viau V, Hillard CJ, and Gorzalka BB (2006a) Involvement of the endocannabinoid system in the ability of long-term tricyclic antidepressant treatment to suppress stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Neuropsychopharmacology* **31:**2591–2599.

Hill MN, Barr AM, Ho WS, Carrier EJ, Gorzalka BB, and Hillard CJ (2007) Electroconvulsive shock treatment differentially modulates cortical and subcortical endocannabinoid activity. *J Neurochem* **103:**47–56.

Hill MN, Carrier EJ, McLaughlin RJ, Morrish AC, Meier SE, Hillard CJ, and Gorzalka BB (2008a) Regional alterations in the endocannabinoid system in an animal model of depression: effects of concurrent antidepressant treatment. *J Neurochem* **106:**2322–2336.

Hill MN, Ho WS, Hillard CJ, and Gorzalka BB (2008b) Differential effects of the antidepressants tranylcypromine and fluoxetine on limbic cannabinoid receptor binding and endocannabinoid contents. *J Neural Transm* **115:**1673–1679.

Hill MN, Sun JC, Tse MT, and Gorzalka BB (2006b) Altered responsiveness of serotonin receptor subtypes following long-term cannabinoid treatment. *Int J Neuropsychopharmacol* **9:**277–286.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, and Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54:**161–202.

Hungund BL, Vinod KY, Kassir SA, Basavarajappa BS, Yalamanchili R, Cooper TB, Mann JJ, and Arango V (2004) Upregulation of CB1 receptors and agonist-stimulated [35S]GTPγS binding in the prefrontal cortex of depressed suicide victims. *Mol Psychiatry* **9:**184–190.

Jiang M, Spicher K, Boulay G, Wang Y, and Birnbaumer L (2001) Most central nervous system D2 dopamine receptors are coupled to their effectors by Go. *Proc Natl Acad Sci U S A* **98:**3577–3582.

Malberg JE and Blendy JA (2005) Antidepressant action: to the nucleus and beyond. *Trends Pharmacol Sci* **26:**631–638.

Marco EM, Pérez-Alvarez L, Borcel E, Rubio M, Guaza C, Ambrosio E, File SE, and Viveros MP (2004) Involvement of 5-HT1A receptors in behavioural effects of the cannabinoid receptor agonist CP 55,940 in male rats. *Behav Pharmacol* **15:**21–27.

Martin M, Ledent C, Parmentier M, Maldonado R, and Valverde O (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. *Psychopharmacology* (*Berl*) **159:**379–387.

Mato S, Aso E, Castro E, Martín M, Valverde O, Maldonado R, and Pazos A (2007) CB1 knockout mice display impaired functionality of 5-HT1A and 5-HT2A/C receptors. *J Neurochem* **103:**2111–2120.

McLaughlin RJ, Hill MN, Morrish AC, and Gorzalka BB (2007) Local enhancement of cannabinoid CB1 receptor signalling in the dorsal hippocampus elicits an antidepressant-like effect. *Behav Pharmacol* **18:**431–438.

Mitchell PB and Morris MJ (2007) Depression and anxiety with rimonabant. *Lancet* **370:**1671–1672.

Morrish AC, Hill MN, Riebe CJ, and Gorzalka BB (2009) Protracted cannabinoid administration elicits antidepressant behavioral responses in rats: role of gender and noradrenergic transmission. *Physiol Behav* **98:**118–124.

Pazos A and Palacios JM (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res* **346:**205–230.

Pompeiano M, Palacios JM, and Mengod G (1992) Distribution and cellular localization of mRNA coding for $5\text{-}HT_{1A}$ receptor in the rat brain: correlation with receptor binding. *J Neurosci* **12:**440–453.

Prather PL, Martin NA, Breivogel CS, and Childers SR (2000) Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein alpha-subunits with different potencies. *Mol Pharmacol* **57:**1000–1010.

Rodríguez-Gaztelumendi A, Rojo ML, Pazos A, and Díaz A (2009) Altered CB receptor-signaling in prefrontal cortex from an animal model of depression is reversed by chronic fluoxetine. *J Neurochem* **108:**1423–1433.

Rubino T, Forlani G, Viganò D, Zippel R, and Parolaro D (2004) Modulation of extracellular signal-regulated kinases cascade by chronic delta 9-tetrahydrocannabinol treatment. *Mol Cell Neurosci* **25:**355–362.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, and Hen R (2003) Requirement of hip-

pocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301:**805–809.
Scatchard G (1949) The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* **51:**660–672.
Spicher K, Nuernberg B, Jäger B, Rosenthal W, and Schultz G (1992) Heterogeneity of three electrophoretically distinct Go alpha-subunits in mammalian brain. *FEBS Lett* **307:**215–218.
Steiner MA, Marsicano G, Nestler EJ, Holsboer F, Lutz B, and Wotjak CT (2008) Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. *Psychoneuroendocrinology* **33:**54–67.
Sternweis PC and Robishaw JD (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J Biol Chem* **259:**13806–13813.
Urigüen L, Pérez-Rial S, Ledent C, Palomo T, and Manzanares J (2004) Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. *Neuropharmacology* **46:**966–973.
Valdizán EM, Castro E, and Pazos A (2009) Agonist-dependent modulation of G-protein coupling and transduction of 5-HT1A receptors in rat dorsal raphe nucleus. *Int J Neuropsychopharmacol* doi: 10.1017/S1461145709990940.
Vinod KY and Hungund BL (2006) Role of the endocannabinoid system in depression and suicide. *Trends Pharmacol Sci* **27:**539–545.
Witkin JM, Tzavara ET, Davis RJ, Li X, and Nomikos GG (2005) A therapeutic role for cannabinoid CB1 receptor antagonists in major depressive disorders. *Trends Pharmacol Sci* **26:**609–617.

Address correspondence to: Dr. Angel Pazos, Department of Physiology and Pharmacology, University of Cantabria, Avda. Cardenal Herrera Oria s/n, 39011 Santander, Cantabria, Spain. E-mail: pazosa@unican.es