Constitutive Activity of Serotonin\textsubscript{2C} Receptors at G Protein-Independent Signaling: Modulation by RNA Editing and Antidepressants\textsuperscript{S}

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ABSTRACT

Serotonin (5-HT)\textsubscript{2C} receptor is a G\textsubscript{q}-coupled receptor exhibiting a high degree of constitutive activity toward phospholipase C effector pathway, a process regulated by receptor mRNA editing. In addition to G protein-dependent signaling, 5-HT\textsubscript{2C} receptors also activate the extracellular signal-regulated kinase (ERK) 1/2 pathway independently of receptor coupling to G proteins. Constitutive activity at ERK signaling has not yet been explored. Transient expression of unedited 5-HT\textsubscript{2C-INI} receptors in human embryonic kidney (HEK) 293 cells resulted in a marked increase in ERK1/2 phosphorylation compared with nontransfected cells. No increase in ERK1/2 phosphorylation was measured in cells expressing fully edited (5-HT\textsubscript{2C-VGV}) receptors. Basal ERK1/2 phosphorylation in 5-HT\textsubscript{2C-INI} receptor-expressing cells was abolished by 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-\textit{f}]indole (SB206,553), a 5-HT\textsubscript{2C} inverse agonist toward phospholipase C. This effect was prevented by the neutral antagonist 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yl)oxy]pyridin-3-ylcarbamoyl]indoline (SB242,084), which alone did not alter basal activity. Similar observations were made in vivo in mouse choroid plexus, a structure rich in constitutively active 5-HT\textsubscript{2C} receptors. Reminiscent of agonist-induced ERK1/2 phosphorylation, basal activity in HEK 293 cells was unaffected by cellular depletion of G\textsubscript{q/11} and G\textsubscript{13} proteins but strongly reduced in cells expressing a dominant-negative β-arrestin or depleted of β-arrestin by RNA interference and in cells expressing a dominant-negative calmodulin or a 5-HT\textsubscript{2C-INI} receptor mutant not capable of interacting with calmodulin. The tetracyclic antidepressants mirtazapine and mianserin likewise reduced basal ERK activation. On the other hand, the m-chlorophenylpiperazine derivative trazodone and the selective serotonin reuptake inhibitor fluoxetine were inactive alone but blocked 5-HT-induced ERK1/2 phosphorylation. Together, these data provide the first evidence of constitutive activity of a G protein-coupled receptor toward G-independent, β-arrestin-dependent, receptor signaling.

Introduction

Among the G protein-coupled receptors (GPCRs) activated by serotonin (5-hydroxytryptamine, 5-HT), 5-HT\textsubscript{2C} receptors still raise particular attention in view of their implication in many physiological functions and behaviors and a spectrum of psychiatric disorders, including anxiodepressive states, schizophrenia, and obesity. Accordingly, they represent an important site of action for numerous psychoactive compounds such as antidepressants, anxiolytics, antipsychotics, and food intake inhibitors (Giogetti and Tecott, 2004; Millan, 2005; Di Giovanni et al., 2006).

The 5-HT\textsubscript{2C} receptor is the only GPCR whose mRNA undergoes adenosine-to-inosine editing, leading to amino acid substitutions within the intracellular loop 2 and the generation of a great number of isoforms (14 in human), ranging from the unedited (INI) to the fully edited (VGV) one and exhibiting different regional distributions (Burns et al., 2003; Di Giovanni et al., 2006). The 5-HT\textsubscript{2C} receptor is the only GPCR whose mRNA undergoes adenosine-to-inosine editing, leading to amino acid substitutions within the intracellular loop 2 and the generation of a great number of isoforms (14 in human), ranging from the unedited (INI) to the fully edited (VGV) one and exhibiting different regional distributions (Burns et al., 2003; Di Giovanni et al., 2006).
1997). RNA editing decreases binding affinity of agonists and alters some coupling, ligand-functional selectivity, and signaling characteristics of the receptor (Herrick-Davis et al., 1999; Niswender et al., 1999; Berg et al., 2008a; Werry et al., 2008a). Differentially edited receptors exhibit varying degrees of constitutive activity at G protein-dependent signaling, ranging from the highest for the nonedited 5-HT2C-INI receptor to intermediate for partially edited isoforms and negligible for the fully edited 5-HT2C-VOV receptor (Herrick-Davis et al., 1999; Niswender et al., 1999). RNA editing also affects the recruitment of β-arrestin by 5-HT2C receptor variants and their subcellular distribution. The ability of variants to spontaneously associate with β-arrestin is strongly correlated with their degree of constitutive activity. 5-HT2C-INI receptors bind to β-arrestin in an agonist-independent manner, a process resulting in constitutive receptor internalization and their predominant localization in intracellular compartments (Marion et al., 2004). Moreover, constitutive interaction with β-arrestin was reversed by inverse agonist treatments, which promote receptor redistribution to the plasma membrane. In contrast, the fully edited 5-HT2C-VOV isoform, which displays the lowest degree of constitutive activity, does not spontaneously associate with β-arrestin, is mainly localized at the cell surface under basal conditions and only undergoes agonist-dependent endocytosis (Marion et al., 2004).

Microdialysis studies revealed a prominent role of constitutive activity in the tonic inhibition by 5-HT2C receptors of mesocorticolimbic dopaminergic neurons and dopamine release in the nucleus accumbens, providing the first demonstration of 5-HT2C receptor constitutive activity in vivo (De Deurwaerdere et al., 2004; Navailles et al., 2006; Aloyo et al., 2009). Excessive signaling at constitutively active 5-HT2C receptors inhibiting dopaminergic pathways, which exert a positive influence upon mood, might be involved in the induction of depressive states (Millan, 2005, 2006; Berg et al., 2008b; Aloyo et al., 2009). Underlining the possible relevance of constitutively active 5-HT2C receptors and mRNA editing to affective disorders, the relative proportion of 5-HT2C receptor isoforms is altered in the cortex of patients with depression (Niswender et al., 2001; Gurevich et al., 2002; Iwamoto and Kato, 2003), and mRNA editing is modified by the long-term administration of antidepressants in rodents (Englander et al., 2005; Iwamoto et al., 2005).

5-HT2C receptors are prototypically coupled to phospholipase (PL) C via Goq. In addition, they activate PLA2, possibly via Gtalphaq that also recruits a RhoA/PLD pathway (McGrew et al., 2002). 5-HT2C receptors are also capable of stimulating the extracellular signal-regulated kinase (ERK) 1/2 pathway in various cell backgrounds, including transfected Chinese hamster ovary K1 and human embryonic kidney (HEK) 293 cells as well as choroid plexus epithelial cells, which express native receptors (Werry et al., 2005, 2008b; Labasque et al., 2008). It is noteworthy that 5-HT2C receptor-operated ERK1/2 signaling in HEK 293 cells was entirely independent of PLC and of G proteins known to be coupled to the receptor (Labasque et al., 2008). Rather, ERK1/2 activation required physical association of both calmodulin (probably a dimer) and β-arrestin with the receptor. Constitutive activity of 5-HT2C receptor at G protein-independent signaling remains largely unexplored. Here, we have addressed this issue in HEK 293 cells transiently expressing 5-HT2C receptor and in vivo, in mouse choroid plexus cells, which express the highest receptor densities. We show that 5-HT2C receptors constitutively activate ERK1/2 signaling independently of receptor coupling to their cognate G proteins. Reminiscent of the agonist-evoked response, constitutive activation of ERK pathway was dependent of recruitment of both β-arrestin and CaM by the receptor. Experiments were also carried out to examine the effect of mRNA editing and the effects of various antidepressant subclasses on constitutive activity of 5-HT2C receptors at ERK signaling.

Materials and Methods

Chemicals, Plasmid Vectors, and Antibodies. 5-HT (creatinine sulfate), 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-|j]indole (SB206,553), clomipramine, amitriptyline, fluoxetine, mirtazapine, trazodone, and α-[aminol[4-aminophenyl]-thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile (SL327) were purchased from Sigma-Aldrich (I’lle d’Abeau Chesnes, France). 6-Chloro-5-methyl-1-[6-(2-methylpyridin-3-yl)pyridin-3-ylcarbamoyl]indoline (SB242,084), mirtazapine, and mianserin were synthesized by Dr. Gilbert Lavielle (Institut de Recherches Servier, Paris, France). 7-[2-Amino-1-oxo-3-thio-propyl]-8-cyclohexylmethyl-2-phenyl-5,6,7,8-tetrahydro-imidazo-[1,2-a]pyrazine dimer, hydrochloride (BIM-46,187) was kindly provided by Dr. J. P. Pin (Institut de Génomique Fonctionnelle, Montpellier, France). For in vivo experiments, drugs were injected intraperitoneally in a volume of 10 ml/kg to male Swiss mice (~30 g, purchased from Janvier, Le Genest Saint Isle, France). SL327 was dissolved in 25% (v/v) DMSO and diluted twice in NaCl 0.9% (final concentration of DMSO, 12.5%). SB242,084 was dissolved in DMSO and then diluted eight times in a solution of Tween 80 [5% (v/v)] and NaCl 0.9%. Control mice were injected in parallel with appropriate vehicles.

The plasmids encoding cMyc-tagged human (h)5-HT2C-INI receptor (pRK5/cMyc-5-HT2C-INI) and cMyc-(h)5-HT2C-VOV receptor (pRK5/cMyc-5-HT2C-VOV) have been described elsewhere (Gavirini et al., 2006). The plasmids encoding YFP-tagged β-arrestin 2 (pDNA-farr 2-YFP) and the dominant-negative mutant of β-arrestin 2 (pCDNA-DN-β-arrestin 2) were generously provided by Dr. M. Bovier (University of Montreal, Montreal, QC, Canada) and the plasmid encoding the Ca2+-insensitive mutant of CaM (CaM-2,3,4-pJPA7r/CaM-DEF1234A) by Dr. J. P. Adelman (Oregon Health and Science University, Portland, OR).

Rabbit polyclonal anti-ERK1/2 and anti-phospho-ERK1/2 (Thr202/Tyr204) antibodies were from Cell Signaling Technology (Oxford, France). The rabbit polyclonal anti-Galphaq, and the rabbit polyclonal anti-Galphaq, antibodies were from Santa Cruz Technology (Delaware, CA), the monoclonal anti-actin antibody was from Interchim (Montluçon, France), the rabbit polyclonal anti-GFP antibody was from Invitrogen (Carlsbad, CA), the mouse monoclonal anti-GFP antibody was from Roche Applied Science (Meylan, France), and the monoclonal anti-Myc antibody (clone 4G10) was from Sigma-Aldrich. The rabbit polyclonal anti-β-arrestin AICT antibody was a gift from Dr. R. J. Lefkowitz (Duke University Medical Center, Durham, NC). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from GE Healthcare (Orsay, France), and the Alexa Fluor 488-conjugated goat-anti rabbit antibody was from Invitrogen.

Cell Cultures and Transfections. HEK 293 cells, grown in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% dialyzed, heat-inactivated fetal calf serum and antibiotics, were transfected at 60 to 70% confluence either by electroporation for microinjection or using Lipofectamine 2000 (Invitrogen) for experiments using siRNA, as described previously (Chanrion et al., 2008). Cells were transfected at 60 to 70% confluence either by electroporation for microinjection or using Lipofectamine 2000 (Invitrogen) for experiments using siRNA, as described previously (Chanrion et al., 2008). The DNA ratio used for cotransfection was 1:2 (receptor cDNAs versus cDNAs encoding dominant-negative forms of β-arrestin 2 or
CaM). Immunofluorescence experiments indicated that under these conditions, more than 95% of cells expressing recombinant 5-HT2C receptors also expressed the cotransfected protein (data not shown).

**Small Interfering RNA Transfection.** HEK 293 cells were seeded in six-well dishes (100,000 cells/well) 48 h before their co-transfection with the pRc/CMV-5-HT2C plasmid and siRNAs (Eugentec, Seraing, Belgium) targeted against either β-arrestin 1 (positions 439–459) (5′-AAAGCCCUCCGGCGGCGAGAAGG-3′), β-arrestin 2 (positions 201–221) (5′-AAGGCACCUGAGGUCUGUGUG-3′), Gαq11 (positions 931–951) (5′-AAAGATGCTTCGGACCCCTGACC-3′), Gαq12 (positions 96–114) (5′-GGGATCTGCAATTGTCCG-3′), and with control siRNA (5′-AAGUGGACCUGUAGGAUGGCG-3′) using Lipofectamine 2000, as described previously (Labasque et al., 2008). All of these siRNAs have been validated in previous studies (Birukova et al., 2004; Barnes et al., 2005; Kara et al., 2006; Labasque et al., 2008). Immunoblotting assays were performed 3 days after siRNA transfection.

**Immunoprecipitation.** Transfected HEK 293 cells were lysed in radioimmunoprecipitation assay buffer ( Sigma-Aldrich) supplemented by replacing the incubation medium with 100 mM LiCl for 10 min and then exposed to 4°C. Samples were incubated with 40 μl of protein A-Sepharose beads (GE Healthcare) for 1 h at 4°C, and immune complexes were collected by centrifugation, washed five times with radioimmunoprecipitation assay buffer, eluted with SDS sample buffer, and analyzed by immunoblotting.

**Immunoblotting.** Proteins resolved by SDS-polyacrylamide gel electrophoresis were electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk diluted in PBS containing 0.2% Tween 20 and incubated successively with the primary antibodies (anti-pERK1/2, 1:1000; anti-ERK1/2, 1:1000; anti-Gαq, 1:1000; anti-Gα12, 1:1000; AICT, 1:500; anti-Myc, 1:500; anti-GFP, 1:500; and anti-actin 1:2000) overnight at 4°C and with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence method (ECL Plus detection reagent; GE Healthcare). Immunoreactive bands were quantified by densitometry using the ImageJ software (http://rswebw.nih.gov/ij/). Statistical analyses were performed with ANOVA, followed by Student Newman-Keuls test. A p value of <0.05 was considered statistically significant.

**Analysis of Inositol Phosphate Formation and ERK1/2 Phosphorylation in Freshly Removed Choroid Plexus.** Male Sprague-Dawley rats (~200 g; Janvier) were anesthetized with pentobarbital (100 mg/kg i.p.; Ceva Santé Animale, Libourne, France) and decapitated. Choroid plexuses located in lateral ventricles and in choroid plexus of the third ventricle were rapidly dissected and labeled for 4 h with 4 μCi/ml [3H]inositol (10–20 Ci/mmol; GE Healthcare) in 0.5 ml of Krebs bicarbonate buffer containing 124 mM NaCl, 26.3 mM NaHCO3, 1.25 mM K3HPO4, 3.5 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, and 10 mM glucose equilibrated previously with 95% O2/5% CO2 in 12-well plates (one choroid plexus per well). After two washes in Krebs bicarbonate buffer, they were incubated in the same buffer supplemented with 10 mM LiCl for 10 min and then exposed to treatments for 30 min. Inositol phosphate (IP) generation was terminated by replacing the incubation medium with 100 μl of formic acid (0.1 M). [3H]IPs were purified in 96-well plates by ion-exchange chromatography using a DOWEX AG1-X8 resin (Bio-Rad Laboratories, Hercules, CA) and eluted with a solution of 10 M ammonium formate/0.1 M formic acid. Radioactivity was determined by scintillation counting. Results were expressed as the amount of [3H]IPs produced compared with radioactivity present in the 10% Triton X-100/0.1 M NaOH-solubilized membrane fraction (phosphatidyl inositol-containing fraction). For analysis of ERK1/2 phosphorylation, freshly removed choroid plexuses were incubated for 1 h in Krebs bicarbonate buffer in 12-well plates and then exposed to drugs for 10 min. They were then lysed in 70 μl of lysis buffer containing Tris-HCl (10 mM, pH 7.4) and 1% SDS. Protein concentration was determined using the bicinchoninic acid procedure and ERK1/2 phosphorylation was analyzed by sequential immunoblotting with the anti-pERK1/2 and anti-ERK1/2 antibodies.

**Analysis of ERK1/2 Phosphorylation In Vivo.** Swiss mice were rapidly anesthetized with pentobarbital (100 mg/kg i.p.) and perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 1 mM Na+ vanadate. Brains were fixed overnight in the same solution and stored at 4°C. Sections (50 μm thick) were cut with a Vibratome (Leica, Nussloch, Germany) and stored at 4°C in PBS containing NaN3 (0.05%) until they were processed for immunofluorescence. Free-floating sections were rinsed in PBS and incubated for 48 h at 4°C with the anti-phospho-ERK1/2 (1:400) in PBS containing 20% goat serum and 0.1% Triton X-100. After three washes in PBS (10 min at room temperature), they were incubated with Alexa Fluor 488-conjugated goat-anti-rabbit antibody (1:2000) for 3 h at room temperature, washed three times with PBS, and mounted on glass slides in Mowiol 4.88 (purchased from Merck Biosciences, Nottingham, UK). Immunofluorescence staining was observed with a Zeiss Axiophot2 microscope (Carl Zeiss GmbH, Jena, Germany) equipped with epifluorescence (475 ± 40 and 530 ± 50 nm for excitation and emission, respectively). Images were acquired using Metamorph software (Molecular Devices, Sunnyvale, CA) driving a CoolSNAP charge-coupled device camera (Photometrics, Tucson, AZ), and fluorescence quantification was performed with the ImageJ software.

**Results**

**Constitutive Activation of ERK1/2 Signaling in HEK 293 Cells Transiently Expressing 5-HT2C-INI Receptor.** Transient expression of 5-HT2C-INI receptors in HEK 293 cells resulted in a decrease in basal ERK1/2 phosphorylation compared with that measured in non-transfected cells (Fig. 1A). Basal phosphorylation level, which represented ~20% of that elicited by a maximally effective concentration of 5-HT (1 μM), was inhibited in a concentration-dependent manner by SB206,553 (pEC50 = 8.51 ± 0.35, mean of values obtained in three independent experiments), a prototypical inverse agonist of 5-HT2C receptor at Gαq-dependent signaling (Fig. 1, A and D). In contrast and in line with its neutral antagonist properties at PLC, SB242,084 also totally inhibited the 5-HT-elicited ERK1/2 phosphorylation (Fig. 1A). No increase in basal ERK1/2 phosphorylation was measured in cells expressing the fully edited 5-HT2C-VGV receptor (Fig. 1B), but 5-HT (1 μM) activated ERK to an extent similar to that measured in 5-HT2C-INI receptor-expressing cells (Fig. 1C). As observed in cells expressing 5-HT2C-INI receptor, application of SB242,084 completely abolished the 5-HT-evoked response (Fig. 1B).

**Constitutive Activation of ERK Signaling by 5-HT2C-INI Receptor Is Independent of Its Coupling to Heterotrimeric G Proteins.** 5-HT2C-INI receptors are known to primarily couple to Gαq protein in both recombinant systems and native tissues and to exhibit a high degree of constitutive activity at Gαq-operated signaling. Transfection of HEK 293 cells with siRNA directed against Gαq, but not with control siRNA, efficiently reduced the level of endogenous Gαq (as assessed 24 h after transfection; Fig. 2A) and inhibited Gαq-dependent signaling (Labasque et al., 2008). However, transfecting Gαs siRNA did not alter basal ERK1/2 phosphorylation (Fig. 2B), reminiscent of its lack of effect upon 5-HT-elicited ERK1/2 phosphorylation (Labasque et al., 2008).
5-HT$_{2C}$ receptors can also activate Go$_{13}$, a process involved in receptor-mediated activation of PLD and rearrangement of the actin cytoskeleton (McGrew et al., 2002). Transfection of HEK 293 cells expressing 5-HT$_{2C,INIR}$ receptor with siRNA directed against human Go$_{13}$ almost completely abolished Go$_{13}$ expression compared with control siRNA-transfected cells (Fig. 2A). As observed previously for 5-HT-elicited ERK1/2 phosphorylation (Labasque et al., 2008), Go$_{13}$ knockdown did not attenuate basal ERK1/2 phosphorylation (Fig. 2B).

Constitutive Activation of ERK Signaling by 5-HT$_{2C,INIR}$ Receptor Is Dependent on β-Arrestins and Calmodulin. In line with the spontaneous association of 5-HT$_{2C,INIR}$ Receptor with β-arrestins (Marion et al., 2004, 2006) and the implication of β-arrestins in 5-HT-induced elevation of ERK1/2 phosphorylation (Labasque et al., 2008), transfecting HEK 293 cells expressing 5-HT$_{2C,INIR}$ receptor with either β-arrestin 1 or β-arrestin 2 siRNA, which strongly decreased expression of the corresponding β-arrestin (Fig. 2A), also significantly reduced constitutive ERK1/2 phosphorylation (Fig. 2B). As observed with the spontaneous association of 5-HT$_{2C,INIR}$ receptor with β-arrestins in HEK 293 cells transfected with control (CTL), Go$_{13}$, β-arrestin 1, or β-arrestin 2 siRNA, which strongly decreased expression of the corresponding β-arrestin (Fig. 2A), also significantly reduced constitutive ERK1/2 phosphorylation (Fig. 2B). Corroborating this result, basal ERK1/2 phosphorylation was similarly decreased by the expression of a dominant-negative mutant of β-arrestin known to affect clathrin-
We demonstrated previously that expression of either a dominant-negative CaM mutant (CaM1,2,3,4) or a 5-HT2C receptor mutant unable to bind to CaM (5-HT2C-2CR376/377A) strongly decreased recruitment of β-arrestin 2 to the receptor upon agonist treatment in HEK 293 cells (Labasque et al., 2008). Coexpression of CaM1,2,3,4 with 5-HT2C receptor likewise decreased spontaneous association of β-arrestin 2 with the receptor (Fig. 2D). Moreover, 5-HT2C-376/377A receptor bound more weakly to β-arrestin 2 in the absence of agonist than did wild-type receptors (Fig. 2D). Accordingly, basal ERK1/2 phosphorylation, which was dependent on β-arrestins, was also strongly decreased in HEK 293 cells coexpressing CaM1,2,3,4 and wild-type 5-HT2C-INI receptor or in cells expressing 5-HT2C-376/377A receptor compared with that measured in cells expressing wild-type 5-HT2C-INI receptor alone (CTL, Fig. 2E). Together, these results indicate that constitutive activation of the ERK pathway by the 5-HT2C-INI receptor, which is independent of G proteins known to be coupled to the receptor, requires association of both CaM and β-arrestin with the unliganded receptor.

Effects of Antidepressants on Constitutive Activity of 5-HT2C-INI Receptor at ERK Signaling. Several lines of evidence support potential antidepressant effect of 5-HT2C antagonists. Moreover, clinically proven antidepressants from different subclasses, such as tricyclics, tetracyclics, m-chlorophenylpiperazine derivatives and selective serotonin reuptake inhibitors (SSRIs), which display relatively high affinities for 5-HT2C receptors (Chanrion et al., 2008), behave as neutral antagonists or inverse agonists toward the Gq-arrestin with the unliganded receptor. The tetracyclic antidepressants mianserin and mirtazapine concentration-dependently decreased basal ERK1/2 phosphorylation (pEC50 = 10.3 ± 0.65 and 9.35 ± 0.46, n = 3, respectively; Fig. 3, A and C) and 5-HT-elicted ERK1/2 phosphorylation (Fig. 3B) in HEK 293 cells. The inverse agonist effects of both compounds were abolished by the neutral antagonist SB242,084. By contrast, the tricyclic clomipramine (10 μM) and amitriptyline (30 μM) increased ERK1/2 phosphorylation, whereas the m-CPP derivative trazodone (10 μM) and the SSRI fluoxetine (10 μM) did not significantly alter basal ERK phosphorylation. Nonetheless, both trazodone and fluoxetine blocked the increase in ERK1/2 phosphorylation elicited by 5-HT, indicating neutral antagonist activity (Fig. 3B). We were surprised to find that the ability of clomipramine and amitriptyline to increase ERK1/2 phosphorylation was not suppressed by SB242,084 (1 μM; Supplemental Fig. S1A), suggesting that this stimulatory effect was not mediated by 5-HT2C receptors. Consistent with this hypothesis, amitriptyline and clomipramine still enhanced ERK1/2 phosphorylation in nontransfected HEK 293 cells (Supplemental Fig. S1B). These 5-HT2C receptor-independent effects were only detected at relatively high concentrations.

Constitutive Activation of ERK by Native 5-HT2C Receptors Expressed in Choroid Plexus. Together, the above findings demonstrate constitutive activity of recombinant, nonedited 5-HT2C-INI receptors at G protein-independent, β-arrestin-dependent, ERK signaling. We next examined whether native 5-HT2C receptors expressed in mice brain would also constitutively activate the ERK pathway by immunofluorescence analysis of phosphorylated ERK1/2. No immunoreactivity was detected in all brain areas of mice injected with vehicle, except for choroid plexus epithelial cells, which are known to express the highest density of 5-HT2C receptors, principally constitutively active forms (Burns et al., 1997; Marazziti et al., 1999). Consistent with specific labeling of phosphorylated ERK1/2, immunostaining of choroid plexus cells was indistinguishable from background in mice injected with SL327 (50 mg/kg i.p.), a brain-penetrating “specific” inhibitor of mitogen-activated protein kinase/ERK kinase, the enzyme that selectively activates ERK (Fig. 4). Systemic administration of SB206,553 (5 mg/kg i.p.), which behaved as an inverse agonist in transfected HEK 293 cells, likewise abolished immunofluorescent staining of choroid plexus cells (Fig. 4). In contrast, a strong immunostaining was detected in choroid plexus of mice in-
jected with the neutral antagonist SB242,084 (10 mg/kg i.p.) and of mice injected with both SB242,084 and SB206,553 (administered 30 min after SB242,084; Fig. 4). Activation of ERK1/2 signaling by choroid plexus 5-HT2C receptors was further confirmed by Western blotting (Fig. 5A). Exposure of freshly removed choroid plexus to 5-HT (1 μM) or SB206,553 (1 μM) increased or inhibited ERK1/2 phosphorylation, respectively (Fig. 5A). Neither basal nor 5-HT-elicited ERK1/2 phosphorylation was affected by treatment of choroid plexus with BIM-46,187 (10 μM), an inhibitor of GPCR signaling mediated by all heterotrimeric G protein families (Ayoub et al., 2009). In contrast, BIM-46,187 inhibited both constitutive and 5-HT-elicited IP production (Fig. 5B), consistent with previous observations made in transfected cells (Ayoub et al., 2009). Moreover, exposure to SB206,553 (1 μM), which inhibited basal IP formation, did not further reduce the level of IPs in BIM-46,187-treated choroid plexuses. Together, these results suggest that engagement of ERK1/2 signaling by choroid plexus 5-HT2C receptors is G protein-independent and that 5-HT2C receptors constitutively activate this pathway in vivo.

**Discussion**

It is now well established that activated GPCRs can transduce signals independently of coupling to heterotrimeric G proteins. In some cases, G protein-independent signaling is intimately related to the ability of receptor to bind to β-arrestin upon agonist stimulation. We demonstrated previously that engagement of ERK1/2 signaling by 5-HT2C receptor, which is independent of the principal G proteins known to couple to the receptor (Ga9 and Gα13, respectively), is strongly dependent on the recruitment of β-arrestin 1 and 2 by the stimulated receptor (Labasque et al., 2008), even though additional mechanisms such as transactivation of tyrosine kinase receptors can also contribute to receptor-operated ERK signaling, depending on the cell background.

![Fig. 4. 5-HT2C receptor constitutively activates ERK1/2 signaling in mice choroid plexus.](image)

Fluorescence intensity (arbitrary units) in at least 100 cells originating from five sections per mouse was determined and subtracted from background using the ImageJ software. Data are means ± S.E.M. of values obtained in n = 4 mice per treatment, *p < 0.05 versus basalo.

![Fig. 5. G protein-independent activation of ERK1/2 signaling by 5-HT2C receptor in choroid plexus.](image)

A. freshly removed choroid plexuses were exposed or not to BIM-46,187 (10 μM) for 1 h and then to either 5-HT (1 μM) or SB206,553 (1 μM) for 10 min. ERK1/2 phosphorylation was analyzed by sequential immunoblotting with the anti-pERK1/2 and anti-ERK1/2 antibodies. Representative immunoblots of three independent experiments performed with choroid plexus located in lateral ventricles are illustrated. Similar results were obtained in choroid plexus located in the third ventricle. Data are means ± S.E.M. of values obtained in three independent experiments. *p < 0.05 versus basalo.

B. choroid plexuses labeled with [myo-3H]inositol, were exposed to either 5-HT (1 μM) or SB206,553 (1 μM) for 30 min in absence or presence of BIM-46,187 (10 μM; added 1 h before the 5-HT or SB206,553 exposure). Data are means ± S.E.M. of values obtained in three independent experiments performed in duplicate in choroid plexus located in lateral ventricles. Similar results were obtained in choroid plexus located in the third ventricle. *p < 0.01 versus basalo; $p < 0.05 versus corresponding control condition.
the receptor isofrom, and the agonist used to stimulate the receptor (Werry et al., 2005; Werry et al., 2008a).

Here, we demonstrated that 5-HT$_{2C}$ receptors also constitutively activated this pathway: expression of 5-HT$_{2C-INI}$ receptors in HEK 293 cells induced by itself a clear elevation of basal ERK1/2, which represented ~20% of the level measured after stimulation of cells with a maximally effective concentration of 5-HT and was concentration-dependently abolished by the prototypic 5-HT$_{2C}$ receptor inverse agonist SB206,553. Moreover, suppression of basal ERK1/2 phosphorylation by SB206,553 was blocked by SB242,084, which alone did not affect basal ERK1/2 phosphorylation, supporting neutral antagonist properties of this compound at receptor-operated ERK1/2 signaling. Rminiscient of agonist-elicited ERK activation (Labasque et al., 2008), basal ERK1/2 phosphorylation was not affected by cellular depletion of Go$_{i}$ and Go$_{13}$. Moreover and differing from constitutive activity at G protein-dependent signaling in the same expression system (Chanrion et al., 2008), the increase in basal ERK1/2 phosphorylation in 5-HT$_{2C-INI}$ receptor-expressing cells did not require concomitant overexpression of the corresponding G proteins. These findings, which revealed constitutive activation of G protein-independent signaling by 5-HT$_{2C-INI}$ receptors, provide the first demonstration of constitutivity of a GPCR toward a G protein-independent signaling pathway.

By analogy to agonist-induced ERK1/2 activation, basal activity was dependent on both β-arrestins 1 and 2 and thus reflected spontaneous association of 5-HT$_{2C-INI}$ receptors with β-arrestins, a phenomenon responsible for constitutive receptor internalization and its intracellular distribution (Marion et al., 2004). Despite the higher affinity of 5-HT$_{2C-INI}$ receptor for β-arrestin 2 than β-arrestin 1 (Marion et al., 2004), basal ERK1/2 phosphorylation was greatly reduced by the cellular depletion of either β-arrestin 1 or β-arrestin 2. These results suggest that overexpressed 5-HT$_{2C}$ receptors can spontaneously form complexes with both β-arrestin 1 and 2, which thereby positively contribute to basal ERK1/2 phosphorylation, and are consistent with previous findings indicating that both β-arrestin isoforms are required for G protein-independent ERK signaling mediated by various GPCRs such as β$_{2}$-adrenergic receptors and parathyroid hormone type 1 receptors (Gesty-Palmer et al., 2006; Shenoy et al., 2006). Further supporting implication of β-arrestins, basal ERK1/2 phosphorylation was strongly diminished in cells expressing a β-arrestin mutant not capable of interacting with clathrin. Moreover, the distinct effects of the majority antidepressants tested in the present study on ERK phosphorylation were correlated to their contrasting actions on the subcellular distribution of 5-HT$_{2C-INI}$ receptors, which reflect their effect on spontaneous association of receptors with β-arrestins. By analogy to SB206,553, mianserin and mirtazapine induced a relocation of 5-HT$_{2C-INI}$ receptors to the plasma membrane (Chanrion et al., 2008), an effect that probably results from their inverse agonist effect on β-arrestin recruitment, and inhibited constitutive ERK activation. In contrast, in line with their absence of effect on the subcellular distribution of 5-HT$_{2C-INI}$ receptors (Chanrion et al., 2008), the m-chlorophenylpiperazine derivative trazodone and the SSRI fluoxetine behaved as neutral antagonists. Only stimulatory effects of tricyclics on the ERK pathway were not correlated to their action on receptor trafficking. Nonetheless, these effects were clearly receptor-independent and might contribute to the induction of neurogenesis in hippocampus in response to long-term treatment with tricyclics, an effect possibly involved in their antidepressant action (Peng et al., 2008; Boldrini et al., 2009).

Agonist-independent association of 5-HT$_{2C}$ receptor with β-arrestins is also profoundly affected by receptor mRNA editing (Marion et al., 2004). Editing affects the second intracellular loop region that surrounds a proline residue critical for β-arrestin recruitment, and in contrast to the nonedited INI isofrom, fully edited 5-HT$_{2C-INI}$ receptors are not capable of spontaneously associating with β-arrestins (Marion et al., 2004, 2006). Consistent with these observations, expression of 5-HT$_{2C-INI}$ receptor in HEK 293 cells did not result in a detectable increase in basal ERK1/2 phosphorylation, whereas comparable levels of phosphorylated ERK1/2 were detected in cells expressing nonedited and fully edited receptors upon agonist exposure. These observations indicate that mRNA editing similarly affects constitutive activity at G protein-dependent and G protein-independent (β-arrestin-dependent) 5-HT$_{2C}$ receptor signaling.

In addition to β-arrestins, CaM has also been involved in the activation of ERK pathway upon the stimulation of various GPCRs, including μ-opioid, 5-HT$_{1A}$, and 5-HT$_{2C}$ receptors (Della Rocca et al., 1999; Belcheva et al., 2001; Labasque et al., 2008). CaM associated with the juxtamembrane region of 5-HT$_{2C}$ receptor C terminus was found to promote and/or stabilize the association of β-arrestins to the receptor and to act in concert with β-arrestins to activate the ERK pathway upon receptor activation (Labasque et al., 2008). Here, we showed that association of β-arrestins with 5-HT$_{2C}$ receptor in the absence of agonist was also dependent on CaM bound to the receptor C-terminal domain. Indeed, the expression of a dominant-negative CaM mutant strongly decreased the spontaneous association of β-arrestin 2 to 5-HT$_{2C-INI}$ receptor, and 5-HT$_{2C-INI}$ receptor mutant not able to interact with CaM bound more weakly to β-arrestin 2 than wild-type receptor. Consistent with the critical role of β-arrestins in constitutive activation of ERK pathway, basal ERK phosphorylation was likewise inhibited in cells expressing either the CaM mutant or 5-HT$_{2C-INI}$ receptor not capable of associating with CaM. Although the interaction of CaM with 5-HT$_{2C}$ receptor is a dynamic process induced by agonist treatment (Labasque et al., 2008), the present findings suggest that a fraction of unliganded receptors is associated with CaM, which then permits β-arrestin recruitment.

In the HEK 293 cell expression system used, 5-HT$_{2C-INI}$ receptors were expressed at a density equivalent to that measured in the 5-HT$_{2C}$ receptor-rich choroid plexus (Labasque et al., 2008), suggesting that their coupling and signal transduction properties might reflect those of native receptors. Analysis of immunoreactivity against phosphorylated ERK1/2 throughout mice brain revealed specific immunofluorescence signals only in choroid plexus epithelial cells. Mimicking their inverse agonist and neutral antagonists activities on ERK signaling in HEK 293 cells, systemic administration of SB206,553 suppressed basal ERK1/2 phosphorylation, whereas SB242,084, which was devoid of intrinsic activity, blocked the action of SB206,553. These findings, together with the expression of high proportions of nonedited (or partially edited) 5-HT$_{2C}$
5-HT2C Receptor Constitutive Activity at 0 G Signaling


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