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Constitutive Activity of Serotonin$_{2C}$ Receptors at G Protein-Independent Signaling: Modulation by RNA Editing and Antidepressants

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Running title: 5-HT_{2C} receptor constitutive activity at 0 G signaling

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Abbreviations: CaM, calmodulin; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine; IP; inositol phosphate; mCPP, m-chlorophenylpiperazine; PLA2, phospholipase A2 ; PLC, phospholipase C; SSRI, selective serotonin reuptake inhibitor

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Abstract

Serotonin (5-HT)$_{2C}$ receptor is a Gq-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$_{2C}$ 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$_{2C-INI}$ receptors in HEK-293 cells resulted in a marked increase in ERK1,2 phosphorylation, compared with non-transfected cells. No increase in ERK1,2 phosphorylation was measured in cells expressing fully edited (5-HT$_{2C-VGV}$) receptors. Basal ERK1,2 phosphorylation in 5-HT$_{2C-INI}$ receptor-expressing cells was abolished by SB206,553, a 5-HT$_{2C}$ inverse agonist toward phospholipase C. This effect was prevented by the neutral antagonist 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$_{2C}$ receptors. Reminiscent to agonist-induced ERK1,2 phosphorylation, basal activity in HEK-293 cells was unaffected by cellular depletion of G$\alpha_{q/11}$ and Gα_{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$_{2C-INI}$ receptor mutant not capable of interacting with calmodulin. The tetracyclic antidepressants mirtazapine and mianserin likewise reduced basal ERK activation. Conversely, the *m*-chlorophenylpiperazine derivative trazodone and the SSRI fluoxetine were inactive alone but blocked 5-HT-induced ERK1,2 phosphorylation. Collectively, these data provide the first evidence of constitutive activity of a G protein-coupled receptor toward G-independent, β-arrestin-dependent, receptor signaling.

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Introduction

Among the G protein-coupled receptors (GPCRs) activated by serotonin (5-hydroxytryptamine, 5-HT), 5-HT_{2C} receptors still raise particular attention in view of their implication in many physiological functions and behaviors as well as a spectrum of psychiatric disorders, including anxio-depressive 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 (Di Giovanni et al., 2006; Giorgetti and Tecott, 2004; Millan, 2005).

The 5-HT_{2C} receptor is the only GPCR, whose mRNA undergoes adenosine-to-inosine editing, leading to amino acid substitutions within the intracellular loop 2 (i2) 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., 1997). RNA editing decreases binding affinity of agonists and alters some coupling, ligand functional selectivity and signaling characteristics of the receptor (Berg et al., 2008a; Herrick-Davis et al., 1999; Niswender et al., 1999; Werry et al., 2008a). Differentially edited receptors exhibit varying degrees of constitutive activity at G protein-dependent signaling, ranging from the highest for the non-edited $5\text{-HT}_{2C\text{-INI}}$ receptor to intermediate for partially-edited isoforms and negligible for the fully-edited $5\text{-HT}_{2C\text{-VGV}}$ receptor (Herrick-Davis et al., 1999; Niswender et al., 1999). RNA editing also affects recruitment of β-arrestin by 5-HT_{2C} 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. $5\text{-HT}_{2C\text{-INI}}$ 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 $5\text{-HT}_{2C\text{-VGV}}$ isoform, which displays the lowest degree of constitutive activity, does not spontaneously associate with β-arrestin, is mainly localized at the cell

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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-HT_{2C} receptors of mesocorticolimbic dopaminergic neurons and dopamine release in the nucleus accumbens, providing the first demonstration of 5-HT_{2C} receptor constitutive activity *in vivo* (Aloyo et al., 2009; De Deurwaerdere et al., 2004; Navailles et al., 2006). Excessive signaling at constitutively active 5-HT_{2C} receptors inhibiting dopaminergic pathways, which exert a positive influence upon mood, might be involved in the induction of depressive states (Aloyo et al., 2009; Berg et al., 2008b; Millan, 2005; Millan, 2006). Underlining the possible relevance of constitutively-active 5-HT_{2C} receptors and mRNA editing to affective disorders, the relative proportion of 5-HT_{2C} receptor isoforms is altered in the cortex of depressed patients (Gurevich et al., 2002; Iwamoto and Kato, 2003; Niswender et al., 2001) and mRNA editing is modified by long-term administration of antidepressants in rodents (Englander et al., 2005; Iwamoto et al., 2005).

5-HT_{2C} receptors are prototypically coupled to phospholipase (PL)C via $G\alpha_q$. In addition, they activate PLA2, possibly *via* $G\alpha_{13}$ that also recruits a RhoA/PLD pathway (McGrew et al., 2002). 5-HT_{2C} receptors are also capable of stimulating the extracellular signal-regulated kinase (ERK)1,2 pathway in various cell backgrounds, including transfected Chinese Hamster Ovary (CHO-K1) and human embryonic kidney (HEK)-293 cells as well as choroid plexus epithelial cells, which express native receptors (Labasque et al., 2008; Werry et al., 2005; Werry et al., 2008b). Importantly, 5-HT_{2C} 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-HT_{2C} receptor at G protein-independent signaling remains largely unexplored. Here, we have addressed this issue in HEK-293 cells transiently expressing 5-HT_{2C} receptor and *in vivo*, in mouse choroid plexus cells, which express the highest receptor densities. We show that 5-HT_{2C} receptors constitutively activate ERK1,2 signaling independently of receptor coupling to

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their cognate G proteins. Reminiscent to 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 impact of mRNA editing and the effects of various antidepressant subclasses on constitutive activity of 5-HT_{2C} receptors at ERK signaling.

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Materials and Methods

Chemicals, plasmid vectors and antibodies. 5-HT (creatinine sulfate), SB206,553, clomipramine, amitriptyline, fluoxetine, mirtazapine, trazodone and SL327 were purchased from Sigma-Aldrich (l'Isle d'Abeau Chesnes, France). SB242,084, mirtazapine and mianserin were synthesized by Dr. Gilbert Lavielle (Institut de Recherches Servier, Paris, France). BIM-46,187 (7-[2-amino-1-oxo-3-thio-propyl]-8-cyclohexylmethyl-2-phenyl-5,6,7,8-tetrahydro-imidazo-[1,2a]-pyrazine dimer, hydrochloride) was kindly provided by Dr J.P. Pin (IGF, Montpellier). For *in vivo* experiments, drugs were injected intraperitonally 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% DMSO (vol/vol) and diluted twice in NaCl 0.9% (final concentration of DMSO, 12.5%). SB242,084 was dissolved in DMSO and then diluted 8 times in a solution of Tween 80 (5 % vol/vol) and NaCl 0.9%. Control mice were injected in parallel with appropriate vehicles.

The plasmids encoding cMyc-tagged human (h)5-$HT_{2C\text{-}INI}$ receptor (pRK5/cMyc-5-$HT_{2C\text{-}INI}$) and cMyc-(h)5-$HT_{2C\text{-}VGV}$ receptor (pRK5/cMyc-5-$HT_{2C\text{-}VGV}$) have been described elsewhere (Gavarini et al., 2006). The plasmids encoding YFP-tagged β-arrestin 2 (pcDNA-βarr 2-YFP) and the dominant-negative mutant of β-arrestin 2 (pcDNA-DN-β-arr 2-(319-418), generated in the laboratory of Dr. J.L. Benovic) were generously provided by Dr. M. Bouvier (University of Montreal, Canada) and the plasmid encoding the Ca^{2+}-insensitive mutant of CaM ($CaM_{1,2,3,4}$, pJPA7/rCaM-DEF1234A) by Dr. J.P. Adelman (Oregon Health and Science University, Portland, USA).

Rabbit polyclonal anti-ERK1,2 and anti-phospho-ERK1,2 (Thr202/Tyr204) antibodies were from Cell Signaling Technology (Ozyme, France). The rabbit polyclonal anti-$G\alpha_q$ and the rabbit polyclonal anti-$G\alpha_{13}$ antibodies were from Santa Cruz Technology (Delaware, CA), the monoclonal anti-actin antibody from Interchim (Montluçon, France), the rabbit polyclonal anti-GFP antibody from Invitrogen (Fisher Scientific, France), the mouse monoclonal anti-GFP antibody from Roche Applied Science (Meylan, France) and the monoclonal anti-Myc

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antibody (clone 4G10) from Sigma. The rabbit polyclonal anti-β-arrestins A1CT 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 from Invitrogen.

Cell cultures and transfections. HEK-293 cells, grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% dialyzed, heat-inactivated fetal calf serum and antibiotics, were transfected at 60-70% confluence either by electroporation for immunoblotting, or using Lipofectamine™ 2000 (Invitrogen) for experiments using siRNA, as previously described (Chanrion et al., 2008). The DNA ratio used for co-transfection was 1:2 (receptor cDNAs *vs.* 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-HT_{2C} receptors also expressed the co-transfected protein (not illustrated).

Small interfering RNA transfection. HEK-293 cells were seeded in 6-well dishes (100,000 cells/well) 48 hr before their co-transfection with the pRK5/cMyc-5-HT_{2C} plasmid and siRNAs (Eurogentec) targeted against either β-arrestin 1 (positions 439-459) (5'-AAAGCCUUCUGCGCGGAGAAU-3') or β-arrestin 2 (positions 201-221) (5'-AAGGACCGCAAAGUGUUUGUG-3') or $G\alpha_{q/11}$ (positions 931-951) (5'-AAGATGTTCGTGGACCTGAAC-3') or $G\alpha_{13}$ (positions 96-114) (5'-GGAGATCGACAAATGCCTG-3'), or with control siRNA (5'-AAGUGGACCCUGUAGAUGGCG-3'), using Lipofectamine™ 2000, as previously described (Labasque *et al.*, 2008). All these siRNAs have been validated in previous studies (Barnes et al., 2005; Birukova et al., 2004; Kara et al., 2006; Labasque et al., 2008). Immunoblotting assays were performed three days after siRNA transfection.

Immunoprecipitation. Transfected HEK-293 cells were lysed in RIPA buffer (Sigma Aldrich) supplemented with $CaCl_2$ (1 mM). Cell lysates were centrifuged at 14,000 × *g* for

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25 min and solubilized proteins were incubated with the rabbit polyclonal anti-GFP antibody (4 µg) overnight at 4 °C. Samples were incubated with 40 µl of protein A-Sepharose beads (GE Healthcare) for 1 hr at 4°C and immune complexes were collected by centrifugation, washed five times with RIPA buffer, eluted with SDS sample buffer and analyzed by immunoblotting.

Immunoblotting. Proteins resolved by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk diluted in PBS containing 0.2% Tween 20 and incubated successively with the primary antibodies (anti-pERK1,2, 1: 1,000; anti-ERK1,2, 1: 1,000; anti-$G\alpha_q$, 1: 1,000; anti-$G\alpha_{13}$, 1: 1,000; A1CT, 1:5,000; anti-Myc, 1: 500; anti-GFP, 1: 500; anti-actin 1: 2,000) overnight at 4°C and with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1 hr 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 (National Institutes of Health, USA). Statistical analyses were performed with ANOVA, followed by Student Newman Keul's 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, purchased from Janvier, Le Genest Saint Isle, France) were anaesthetized with pentobarbital (100 mg/kg i.p., Ceva Santé Animale, Libourne, France) and decapitated. Choroid plexuses located in lateral ventricles and in the third ventricle were rapidly dissected, and labeled for 4 hrs with 4 µCi/ml [^{3}H]-*myo*-inositol (10-20 Ci/mmol, GE Healthcare) in 0.5 ml Krebs bicarbonate buffer containing (in mM) NaCl, 124; $NaHCO_3$, 26.3; K_2HPO_4, 1.25; KCl, 3.5; $MgSO_4$, 1.2; $CaCl_2$, 1.2 and glucose, 10, previously equilibrated with 95% O_2/5% CO_2, 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 formic acid (0.1 M). [^{3}H]-IPs were purified in 96-well plates by ion exchange

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chromatography using a DOWEX AGI-X8 resin (Bio-Rad) 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 [^{3}H]-IPs produced in comparison to radioactivity present in the 10% Triton X-100/0.1 M NaOH-solubilized membrane fraction (phosphatidyl inositol (PI)-containing fraction). For analysis of ERK1,2 phosphorylation, freshly removed choroid plexuses were incubated for 1 hr in Krebs bicarbonate buffer in 12-well plates and then exposed to drugs for 10 min. They were then lyzed in 70 µl 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 anaesthetized 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 NaF and 1 mM Na^+-vanadate. Brains were post-fixed overnight in the same solution and stored at 4°C. 50-µm thick sections were cut with a vibratome (Leica, Nussloch, Germany) and stored at 4°C in PBS containing NaN_3 (0.05 %) until they were processed for immunofluorescence. Free-floating sections were rinsed in PBS and incubated for 48 hr 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 (1:2,000) for 3 hr at room temperature, washed thrice with PBS were mounted on glass slides in Mowiol 4. 88. Immunofluorescence staining was observed with a Zeiss Axiophot2 microscope 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 CCD camera (Photometrics) and fluorescence quantification was performed with the ImageJ software.

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Results

Constitutive activation of ERK1,2 signaling in HEK-293 cells transiently expressing 5-HT_{2C-INI} receptor. Transient expression of 5-HT_{2C-INI} receptors in HEK-293 cells resulted in an increase 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 (pEC_{50} = 8.51 ± 0.35, mean of values obtained in three independent experiments), a prototypical inverse agonist of 5-HT_{2C} receptor at Gq-dependent signaling (Fig. 1A and D). In contrast, and in line with its neutral antagonist properties at PLC, SB242,084 (1 µM) did not alter basal ERK1,2 phosphorylation but prevented the inverse agonist effect of SB206,553 (Fig. 1A). 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-HT_{2C-VGV} receptor (Fig. 1B), but 5-HT (1 µM) activated ERK to an extend similar to that measured in 5-HT_{2C-INI} receptor-expressing cells (Fig. 1C). As observed in cells expressing 5-HT_{2C-INI} receptor, application of SB242,084 completely abolished the 5-HT-evoked response (Fig. 1B).

Constitutive activation of ERK signaling by 5-HT_{2C-INI} receptor is independent of its coupling to heterotrimeric G proteins. 5-HT_{2C-INI} receptors are known to primarily couple to $G\alpha_q$ protein in both recombinant systems and native tissues and to exhibit a high degree of constitutive activity at Gq-operated signaling. Transfection of HEK-293 cells with siRNA directed against $G\alpha_q$, but not with control siRNA, efficiently reduced the level of endogenous $G\alpha_q$ (as assessed 24 hrs after transfection, Fig. 2A) and inhibited $G\alpha_q$-dependent signaling (Labasque et al., 2008). However, transfecting $G\alpha_q$ siRNA did not alter basal ERK1,2 phosphorylation (Fig. 2B), reminiscent to its lack of effect upon 5-HT-elicited ERK1,2 phosphorylation (Labasque et al., 2008). 5-HT_{2C} receptors can also activate $G\alpha_{13}$, 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-INI} receptor with

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siRNA directed against human $G\alpha_{13}$ almost completely abolished $G\alpha_{13}$ expression compared with control siRNA-transfected cells (Fig. 2A). As previously observed for 5-HT-elicited ERK1,2 phosphorylation (Labasque et al., 2008), $G\alpha_{13}$ knockdown did not attenuate basal ERK1,2 phosphorylation (Fig. 2B).

Constitutive activation of ERK signaling by 5-$HT_{2C\text{-}INI}$ receptor is dependent on β-arrestins and calmodulin. In line with the spontaneous association of 5-$HT_{2C\text{-}INI}$ receptor with β-arrestins (Marion et al., 2006; Marion et al., 2004) 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-$HT_{2C\text{-}INI}$ 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. 2C). Corroborating this result, basal ERK1,2 phosphorylation was similarly decreased by expression of a dominant-negative mutant of β-arrestin known to affect clathrin-dependent GPCR internalization (319-418, DN β-arr, Fig. 2C). Residual ERK1,2 phosphorylation measured in β-arrestin siRNA- or DN β-arr-transfected cells was further decreased by application of SB206,553 (0.1 μM, Fig. 2C).

We previously demonstrated that expression of either a dominant negative CaM mutant ($CaM_{1,2,3,4}$) or a 5-HT_{2C} receptor mutant unable to bind to CaM (5-$HT_{2CR376/377A}$) strongly decreased recruitment of β-arrestin 2 to the receptor upon agonist treatment in HEK-293 cells (Labasque et al., 2008). Co-expression of $CaM_{1,2,3,4}$ with 5-HT_{2C} receptor likewise decreased spontaneous association of β-arrestin2 with the receptor (Fig. 2D). Moreover, 5-$HT_{2CR376/377A}$ receptor bound more weakly to β-arrestin2 in 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 co-expressing $CaM_{1,2,3,4}$ and wild type 5-$HT_{2C\text{-}INI}$ receptor or in cells expressing 5-$HT_{2CR376/377A}$ receptor, compared with that measured in cells expressing wild type 5-$HT_{2C\text{-}INI}$ receptor alone (CTL, Fig. 2E). Collectively, these results indicate that constitutive activation of the ERK pathway by the 5-

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HT_{2C-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-HT_{2C-INI} receptor at ERK signaling. Several lines of evidence support potential antidepressant effect of 5-HT_{2C} antagonists. Moreover, clinically proven antidepressants from different subclasses, such as tricyclics, tetracyclics, m-chlorophenylpiperazine (mCPP) derivatives and specific serotonin reuptake inhibitors (SSRIs), which display relatively high affinities for 5-HT_{2C} receptors (Chanrion et al., 2008), behave as neutral antagonists or inverse agonists toward the Gq-PLC pathway. The tetracyclic antidepressants mianserin and mirtazapine concentration-dependently decreased basal ERK1,2 phosphorylation (pEC_{50} = 10.3 ± 0.65 and 9.35 ± 0.46, n = 3, respectively, Figs. 3A and C), as well as 5-HT-elicited 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 tricyclics 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). Surprisingly, the ability of clomipramine and amitriptyline to increase ERK1,2 phosphorylation was not suppressed by SB242,084 (1 μM, Fig. S1A), suggesting that this stimulatory effect was not mediated by 5-HT_{2C} receptors. Consistent with this hypothesis, amitriptyline and clomipramine still enhanced ERK1,2 phosphorylation in non-transfected HEK-293 cells (Fig. S1B). These 5-HT_{2C} receptor-independent effects were only detected at relatively high concentrations.

Constitutive activation of ERK by native 5-HT_{2C} receptors expressed in choroid plexus. Collectively, the above findings demonstrate constitutive activity of recombinant, non-edited 5-HT_{2C-INI} receptors at G protein-independent, β-arrestin-dependent, ERK signaling. We next examined whether native 5-HT_{2C} receptors expressed in mice brain would also constitutively activate the ERK pathway by immunofluorescence analysis of phosphorylated ERK1,2. No

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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-HT_{2C} 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 undistinguishable from background in mice injected with SL327 (50 mg/kg, i.p.), a brain-penetrating "specific" inhibitor of MAP-kinase/ERK kinase (MEK), 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 injected with the neutral antagonist SB242,084 (10 mg/kg, i.p.) and of mice injected with both SB242,084 and SB205,553 (administrated 30 min after SB242,084, Fig. 4). Activation of ERK1,2 signaling by choroid plexus 5-HT_{2C} 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 were 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. Collectively, these results suggested that engagement of ERK1,2 signaling by choroid plexus 5-HT_{2C} receptors is G protein-independent and that 5-HT_{2C} receptors constitutively activate this pathway *in vivo*.

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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 previously demonstrated that engagement of ERK1,2 signaling by 5-HT_{2C} receptor, which is independent of the principal G proteins known to couple to the receptor ($G\alpha_q$ and $G\alpha_{13}$, 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, the receptor isoform 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-$HT_{2C\text{-}INI}$ 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. Reminiscent to agonist-elicited ERK activation (Labasque et al., 2008), basal ERK1,2 phosphorylation was not affected by cellular depletion of $G\alpha_q$ and $G\alpha_{13}$. 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-$HT_{2C\text{-}INI}$ 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-$HT_{2C\text{-}INI}$ receptors, provide the first demonstration of constitutive activity of a GPCR toward a G protein-independent signaling pathway.

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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). In spite of 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 impact 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 mCPP derivative trazodone and the SSRI fluoxetine behaved as neutral antagonists. Only stimulatory effects of tricyclics on ERK pathway were not correlated to their action on receptor trafficking. Nonetheless, these effects were clearly receptor-independent and might contribute to induction of neurogenesis in hippocampus in response to chronic treatment with tricyclics, an effect possibly involved in their antidepressant action (Boldrini et al., 2009; Peng et al., 2008).

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

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intracellular loop region that surrounds a proline residue critical for β-arrestin recruitment and in contrast to the non-edited INI isoform, fully edited 5-HT_{2C-VGV} receptors are not capable of spontaneously associating with β-arrestins (Marion et al., 2006; Marion et al., 2004). Consistent with these observations, expression of 5-HT_{2C-VGV} 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 non-edited and fully edited receptors upon agonist exposure. These observations indicate that mRNA editing similarly impacts 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 activation of ERK pathway upon stimulation of various GPCRs, including mu-opioid, 5-HT_{1A} and 5-HT_{2C} receptors (Belcheva et al., 2001; Della Rocca et al., 1999; Labasque et al., 2008). CaM associated with the juxtamembrane region of 5-HT_{2C} receptor C-terminus was found to promote and/or stabilize 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 absence of agonist was also dependent on CaM bound to the receptor C-terminal domain. Indeed, expression of a dominant-negative CaM mutant strongly decreased spontaneous association of β-arrestin2 to 5-HT_{2C-INI} receptor and 5-HT_{2C-INI} receptor mutant not able to interact with CaM bound more weakly to β-arrestin2 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 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 to CaM, which then permits β-arrestin recruitment.

In the HEK-293 cell expression system employed, 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 signal 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 expression of high proportions of non-edited (or partially edited) 5-HT_{2C} receptors in choroid plexus, compared with other brain regions (Burns et al., 1997), strongly suggest that basal ERK phosphorylation results from receptor constitutive activity rather than from their stimulation by the relative high concentrations of 5-HT present in cerebrospinal fluid. 5-HT_{2C} receptors expressed in choroid plexus reduce the rate of CSF secretion, *via* inhibition of K^+ channel activity (Speake et al., 2004), and stimulate the production of transferrin by choroid plexus epithelial cells (Esterle and Sanders-Bush, 1992). The role of constitutive activity at 5-HT_{2C} receptors in these processes remains to be established. Reciprocally, 5-HT_{2C} receptor functional status is inhibited by insulin, *via* the recruitment of ERK1,2 pathway, in choroid plexus cells (Hurley et al., 2003). Activation of the ERK by 5-HT_{2C} receptors might thus serve as a negative feedback preventing excessive signaling at constitutively active receptors.

Constitutive activity at 5-HT_{2C} receptors has been clearly established in other brain regions such as ventral tegmental area, nucleus accumbens and prefrontal cortex, where it has physiological relevance on dopaminergic neurotransmission (De Deurwaerdere et al., 2004; Leggio et al., 2009; Navailles et al., 2006). Lack of detection of phospho-ERK1,2 immunoreactivity in neurons from these regions might be due to insufficient sensitivity of the method to detect constitutively activated ERK1,2 in cells expressing moderate 5-HT_{2C} receptor density. Therefore, additional experiments are needed to definitively demonstrate constitutive activity toward the ERK1,2 pathway of 5-HT_{2C} receptors controlling the activity of dopaminergic neurons. The possible contribution of this pathway *vs.* the Gq-PLC effector pathway to tonic inhibition of dopamine release is another important issue requiring further investigation.

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Footnote

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Figure legends

Fig. 1. Non-edited 5-HT_{2C-INI} receptor, but not fully edited 5-HT_{2C-VGV} receptor, constitutively activates ERK1,2 in HEK-293 cells. HEK-293 cells, transfected with empty vector (Mock) or with plasmids encoding either non-edited 5-HT_{2C-INI} receptor (A, C and D), or fully edited 5-HT_{2C-VGV} receptor (B and C), were treated for 10 min with either vehicle (Basal) or 5-HT (1 µM) or SB206,553 (0.1 µM in A and B, at indicated concentrations in D). In experiments using SB242,084, cells were pretreated with SB242,084 (1 µM, 10 min) and then exposed to agonist (5-HT) or inverse agonist (SB206,553) for 10 min. ERK1,2 phosphorylation was analyzed by sequential immunoblotting with the polyclonal antibody against phospho-Thr202/Tyr204 ERK1,2 and the polyclonal antibody recognizing ERK1,2 independently of their phosphorylation state. Representative immunoblots of three independent experiments performed on different sets of cultured cells are illustrated. Immunoreactive signals were quantified by densitometry. Data, expressed in % of basal ERK1,2 phosphorylation in cells expressing 5-HT_{2C-INI} receptor (A, C and D) or of 5-HT-elicited response (B), correspond to the ratio of phosphorylated ERK1,2 (pERK1,2) to total ERK1,2 signals. Each data point represents means ± SEM of values obtained in three experiments. In D, the dose-response curve was fitted using the Prism software. ∗, $p < 0.05$, *vs.* basal. $, $p < 0.05$, *vs.* cells exposed to SB206,553 alone (ANOVA, followed by Student Newman Keul's test).

Fig. 2. Constitutive activation of ERK1,2 signaling by 5-HT_{2C-INI} receptor is independent of G proteins and dependent on both β-arrestins and calmodulin. (A) Endogenous $G\alpha_q$, $G\alpha_{13}$ and β-arrestins 1 and 2 expression in HEK-293 cells transfected with control (CTL), $G\alpha_{q/11}$, $G\alpha_{13}$, β-arrestin 1 or β-arrestin 2 siRNA was analyzed by immunoblotting. The data are representative of three independent experiments performed on different cultures. (B, C and E) HEK-293 cells were transfected with the pRK5/cMyc-5-HT_{2C-INI} plasmid alone (CTL) or co-transfected with pRK5/cMyc-5-HT_{2C-INI} plasmid and either the indicated siRNAs (B, C) or the plasmid encoding a dominant-negative mutant of β-arrestin 2 (DN-β-arr) (C) or the plasmid

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encoding Ca^{2+}-insensitive CaM mutant ($CaM_{1,2,3,4}$) (E), or transfected with the plasmid encoding the 5-$HT_{2C\text{-}INIR376/377A}$ receptor alone (E). Cells were then exposed to sham treatment or SB206,553 (0.1 μM) for 10 min and ERK1,2 phosphorylation was analyzed as indicated in the legend to Fig. 1. Values, normalized to total ERK immunoreactivity, are means ± SEM of results obtained in three independent experiments. They were expressed in % of basal ERK1,2 phosphorylation measured in cells co-transfected with the plasmid encoding 5-$HT_{2C\text{-}INI}$ receptor and the control siRNA (CTL siRNA, B) or in % of basal ERK1,2 phosphorylation measured in cells transfected with the plasmid encoding 5-$HT_{2C\text{-}INI}$ receptor alone (CTL, C and E). *, $p < 0.01$, *vs.* CTL. $, $p < 0.05$, *vs.* corresponding value in untreated cells. (D) Solubilized proteins from HEK-293 cells co-transfected with pcDNA-βarr2-YFP plasmid and the indicated constructs were immunoprecipitated with the polyclonal anti-GFP antibody. Immunoprecipitated proteins were analyzed by Western blotting using monoclonal anti-GFP and anti-Myc antibodies. The multiple bands are due to heterogeneous glycosylation of 5-HT_{2C} receptor, as incubation of solubilized proteins with N-Glycosidase F overnight at 37°C resulted in a shift of the upper bands to the lower apparent molecular weight (data not shown). Inputs represent 5% of the total protein amount used in immunoprecipitations. Data are representative of three independent experiments performed on different cultures.

Fig. 3. Contrasting effects of antidepressants on 5-$HT_{2C\text{-}INI}$ receptor-mediated ERK1,2 activation. (A) HEK-293 cells, transfected with the pRK5/cMyc-5-$HT_{2C\text{-}INI}$ plasmid, were treated for 10 min with either vehicle or mianserin (Mian, 0.1 μM) or mirtazapine (Mirta, 0.1 μM), or clomipramine (Clomi, 10 μM), or amitriptyline (Ami, 30 μM) or trazodone (10 μM) or fluoxetine (10 μM). (B) Cells were subjected to same treatments before the 10 min 5-HT (1 μM) exposure. (C) Cells were exposed for 10 min to increasing concentrations of mianserin or mirtazapine. Dose-response curves were fitted using the Prism software. (D) Cells pretreated or not with SB242,084 (1 μM, 10 min) were then exposed for 10 min to either sham treatment (Basal) or mianserin or mirtazapine (both at 0.1 μM). ERK1,2 phosphorylation was analyzed as indicated in the legend to Fig. 1. Values, normalized to total

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ERK immunoreactivity, are means ± SEM of results obtained in three independent experiments performed on different sets of cultured cells. ∗, $p < 0.01$, *vs.* basal (A and D) or 5-HT-elicited (B) ERK1,2 phosphorylation.

Fig. 4. 5-HT_{2C} receptor constitutively activates ERK1,2 signaling in mice choroid plexus. Immunofluorescent detection and quantification of phosphorylated ERK1,2 in choroid plexus epithelial cells from mice injected with either Vehicle (12.5% DMSO in 0.9% NaCl) or SL327 (50 mg/kg i.p.) or SB206,553 (5 mg/kg i.p.) or SB242,084 (10 mg/kg i.p.) are represented. In mice treated with both SB242,084 and SB206,553, SB242,084 was administrated 30 min before SB206,553. The top left panel represents a section from a mouse injected with vehicle and not incubated with the anti phospho-ERK1,2 antibody. Fluorescence intensity in at least 100 cells originating from 5 sections per mouse was determined and subtracted from background using the ImageJ software. Data are means ± SEM of values obtained in n = 4 mice per treatment. ∗, $p < 0.01$ *vs.* SL327. Statistical significance was determined by ANOVA followed by Dunnett's test. Scale bar: 40 µm.

Fig. 5. G protein-independent activation of ERK1,2 signaling by 5-HT_{2C} receptor in choroid plexus. (A) Freshly removed choroid plexuses were exposed or not to BIM-46,187 (10 µM) for 1 hr 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, normalized to total ERK1,2 immunoreactivity and expressed in % of basal ERK1,2 phosphorylation measured in absence of BIM-46,187, are means ± SEM of values obtained in three independent experiments. ∗, $p < 0.05$, *vs.* basal. (B) Choroid plexuses labeled with [^{3}H]-*myo*-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,

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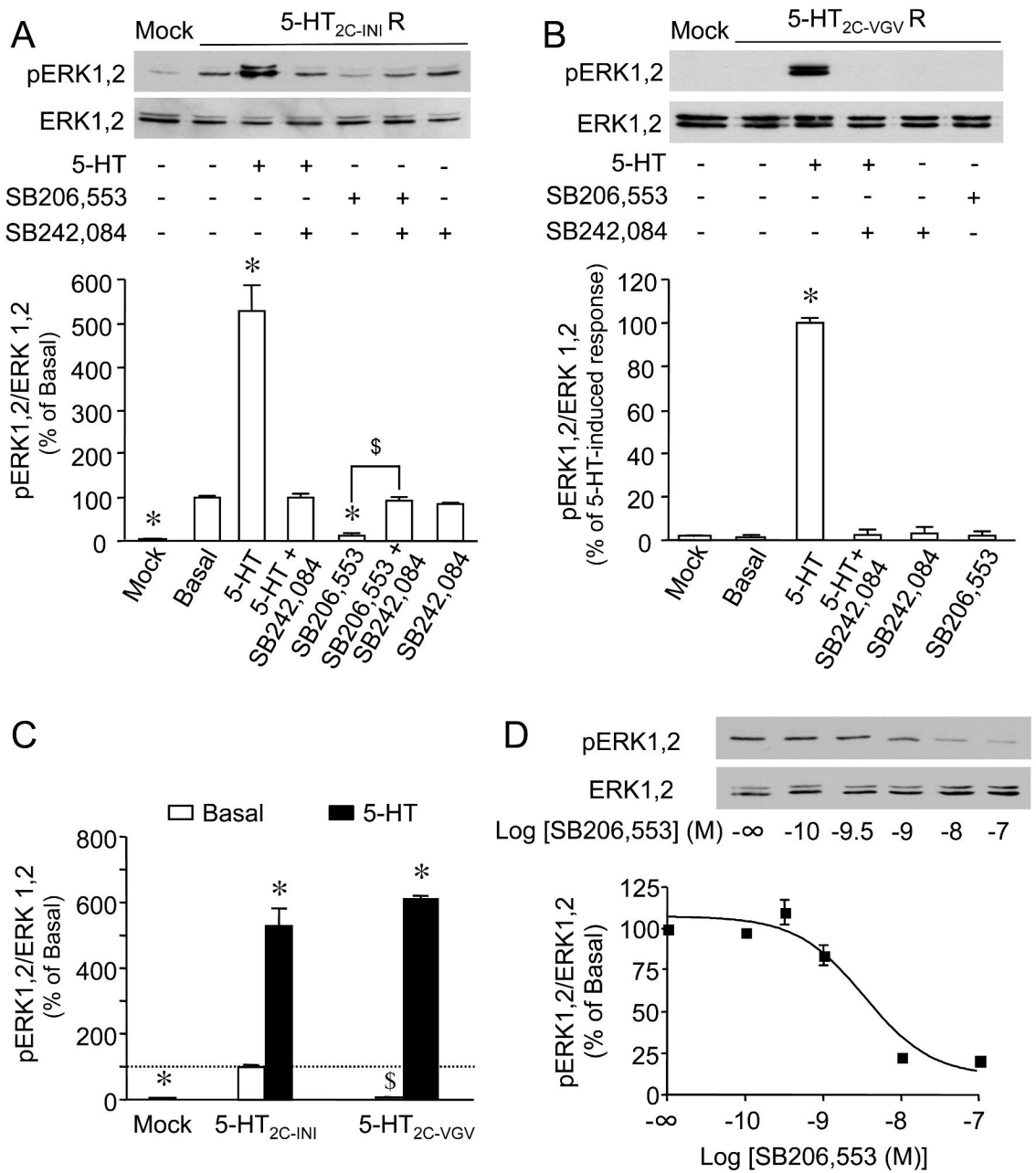
added 1 hr before the 5-HT or SB206,553 exposure). Data are means ± SEM of values obtained in three independent experiments performed in duplicate in choroid plexus located in lateral ventricles. Similar results were obtained in choroid plexus located in the third ventricle. ∗, $p < 0.01$, *vs.* basal. $, $p < 0.05$, *vs.* corresponding control condition.


A
Mock
5-HT2C-INI R
pERK1,2
ERK1,2
5-HT - - + + - - -
SB206,553 - - - - + + -
SB242,084 - - - + - + +
pERK1,2/ERK 1,2 (% of Basal)
600
500
400
300
200
100
0
*
*
$
*
Mock
Basal
5-HT
5-HT + SB242,084
SB206,553
SB206,553 + SB242,084
SB242,084
B
Mock
5-HT2C-VGV R
pERK1,2
ERK1,2
5-HT - - + + - -
SB206,553 - - - - - +
SB242,084 - - - + + -
pERK1,2/ERK 1,2 (% of 5-HT-induced response)
120
100
80
60
40
20
0
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Mock
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5-HT
5-HT+ SB242,084
SB242,084
SB206,553
C
Basal
5-HT
pERK1,2/ERK 1,2 (% of Basal)
800
600
400
200
0
*
*
*
$
Mock
5-HT2C-INI
5-HT2C-VGV
D
pERK1,2
ERK1,2
Log [SB206,553] (M) -∞ -10 -9.5 -9 -8 -7
pERK1,2/ERK1,2 (% of Basal)
125
100
75
50
25
0
-∞
-10
-9
-8
-7
Log [SB206,553 (M)]

Figure 1

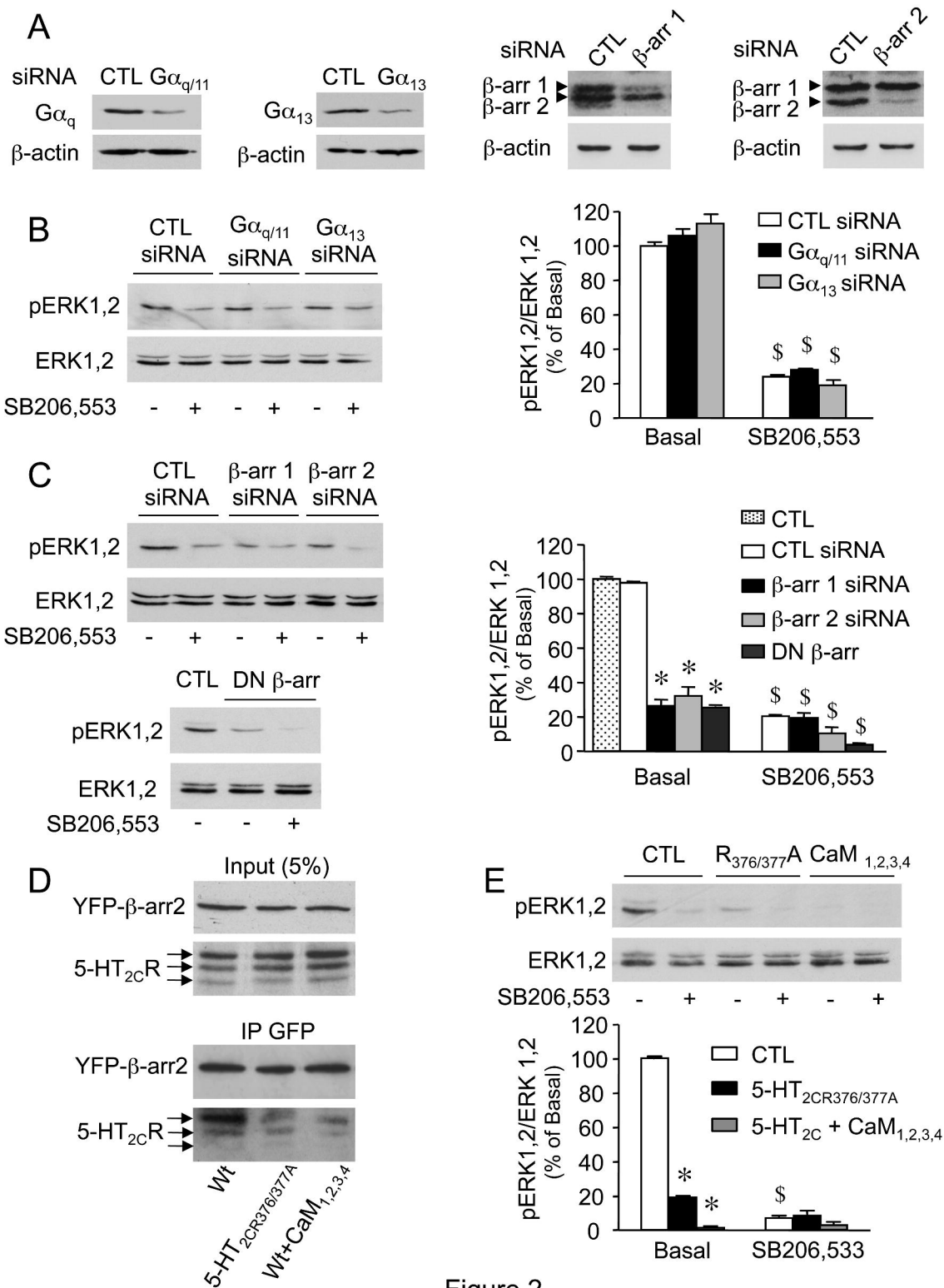

A
siRNA
CTL
Gαq/11
Gαq
β-actin
CTL
Gα13
Gα13
β-actin
siRNA
CTL
β-arr 1
β-arr 1
β-arr 2
β-actin
siRNA
CTL
β-arr 2
β-arr 1
β-arr 2
β-actin
B
CTL siRNA
Gαq/11 siRNA
Gα13 siRNA
pERK1,2
ERK1,2
SB206,553 - + - + - +
pERK1,2/ERK 1,2 (% of Basal)
120
100
80
60
40
20
0
CTL siRNA
Gαq/11 siRNA
Gα13 siRNA
$ $ $
Basal
SB206,553
C
CTL siRNA
β-arr 1 siRNA
β-arr 2 siRNA
pERK1,2
ERK1,2
SB206,553 - + - + - +
CTL
DN β-arr
pERK1,2
ERK1,2
SB206,553 - - +
pERK1,2/ERK 1,2 (% of Basal)
120
100
80
60
40
20
0
CTL
CTL siRNA
β-arr 1 siRNA
β-arr 2 siRNA
DN β-arr
* * *
$ $ $ $
Basal
SB206,553
D
Input (5%)
YFP-β-arr2
5-HT2CR
IP GFP
YFP-β-arr2
5-HT2CR
Wt
5-HT2CR376/377A
Wt+CaM1,2,3,4
E
CTL
R376/377A
CaM 1,2,3,4
pERK1,2
ERK1,2
SB206,553 - + - + - +
pERK1,2/ERK 1,2 (% of Basal)
120
100
80
60
40
20
0
CTL
5-HT2CR376/377A
5-HT2C + CaM1,2,3,4
* * $
Basal
SB206,533

Figure 2

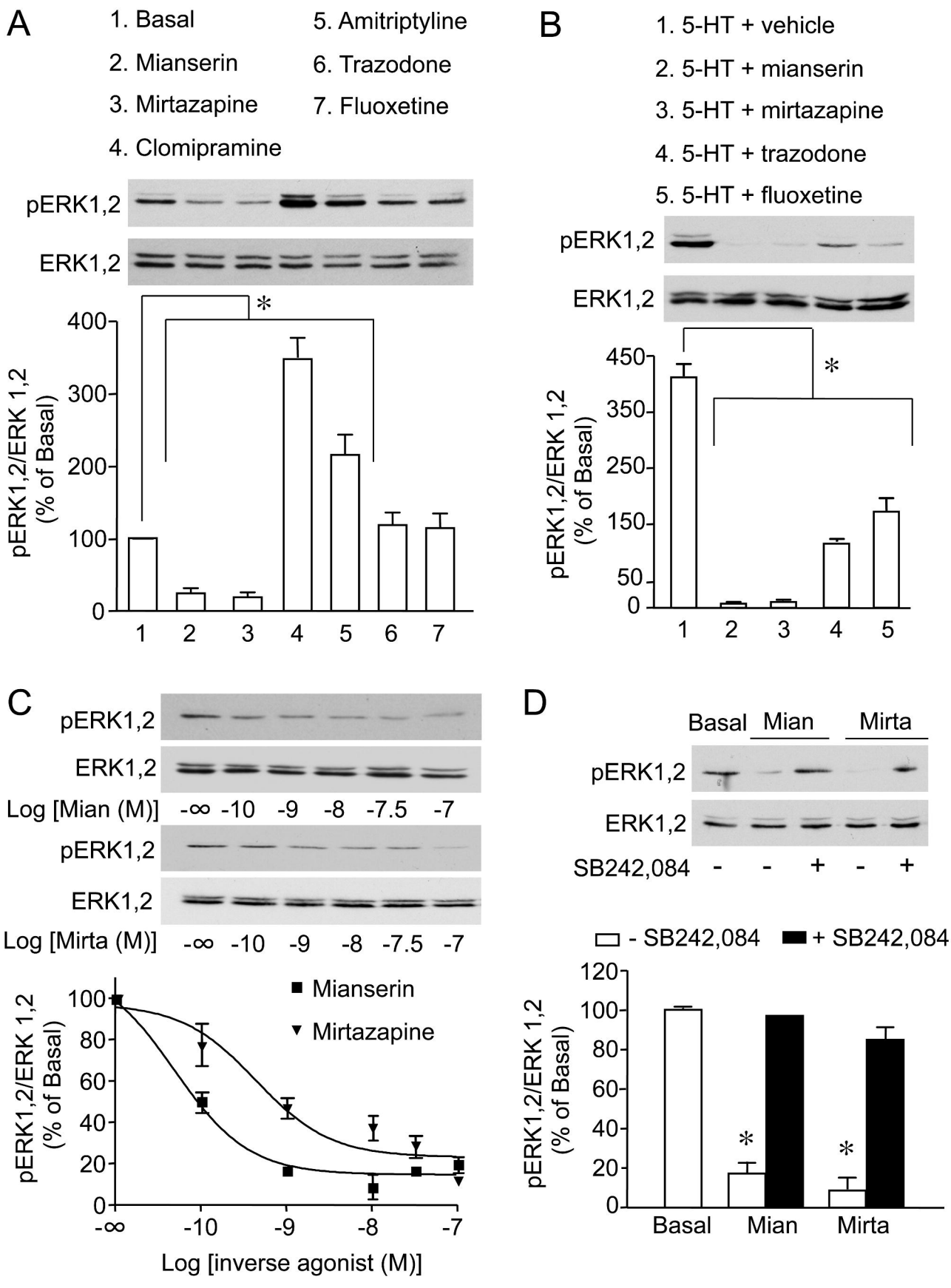
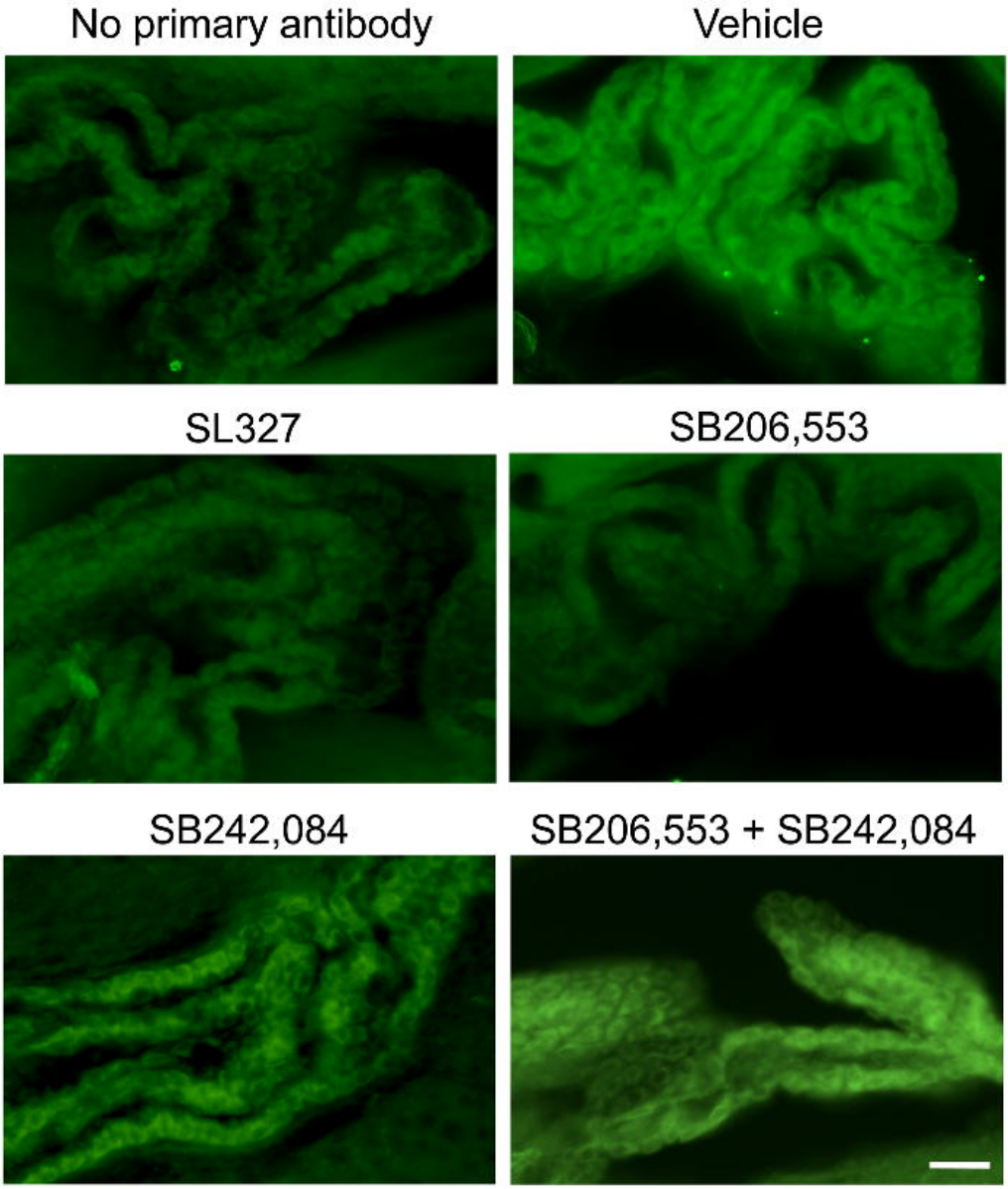

A
1. Basal
2. Mianserin
3. Mirtazapine
4. Clomipramine
5. Amitriptyline
6. Trazodone
7. Fluoxetine
pERK1,2
ERK1,2
pERK1,2/ERK 1,2 (% of Basal)
B
1. 5-HT + vehicle
2. 5-HT + mianserin
3. 5-HT + mirtazapine
4. 5-HT + trazodone
5. 5-HT + fluoxetine
C
Log [Mian (M)] -∞ -10 -9 -8 -7.5 -7
Log [Mirta (M)] -∞ -10 -9 -8 -7.5 -7
Mianserin
Mirtazapine
Log [inverse agonist (M)]
D
Basal Mian Mirta
SB242,084 - - + - +
- SB242,084
+ SB242,084
Basal
Mian
Mirta

Figure 3


No primary antibody
Vehicle
SL327
SB206,553
SB242,084
SB206,553 + SB242,084

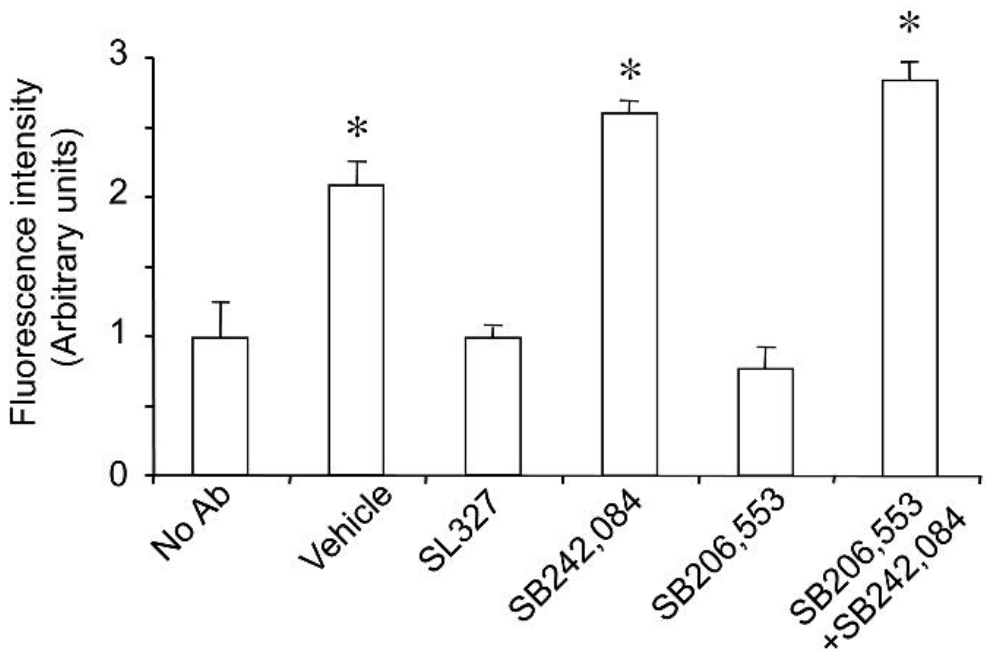

Fluorescence intensity
(Arbitrary units)
0
1
2
3
*
*
*
No Ab
Vehicle
SL327
SB242,084
SB206,553
SB206,553
+SB242,084

Figure 4

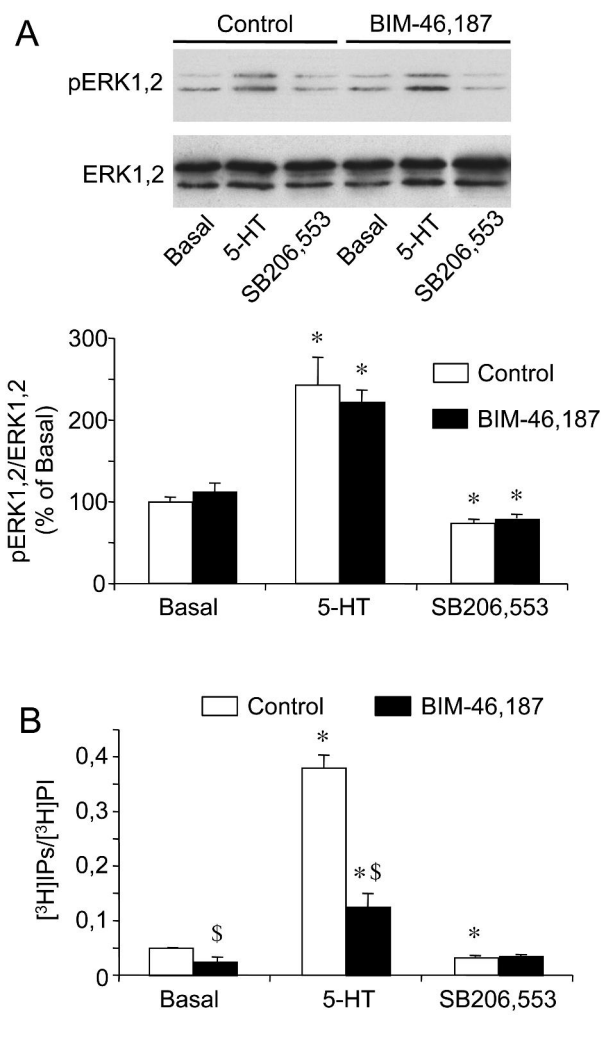

A
Control
BIM-46,187
pERK1,2
ERK1,2
Basal
5-HT
SB206,553
Basal
5-HT
SB206,553
pERK1,2/ERK1,2
(% of Basal)
300
200
100
0
Control
BIM-46,187
Basal
5-HT
SB206,553
B
Control
BIM-46,187
[3H]IPs/[3H]PI
0,4
0,3
0,2
0,1
0
Basal
5-HT
SB206,553

Figure 5