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Inverse Agonist and Neutral Antagonist Actions of Antidepressants at Recombinant and Native 5-Hydroxytryptamine$_{2C}$ Receptors: Differential Modulation of Cell Surface Expression and Signal Transduction

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ABSTRACT

Despite the importance of 5-hydroxytryptamine (5-HT)$_{2C}$ (serotonin) receptors in the control of depressive states, actions of antidepressants at these receptors remain poorly characterized. This issue was addressed both in human embryonic kidney (HEK)-293 cells coexpressing unedited human 5-HT$_{2CINI}$ receptors and Gα_q protein and in cultured mouse cortical neurons. Indicative of constitutive activity, the inverse agonist SB206,553 decreased basal inositol phosphate (IP) production in HEK-293 cells. The tetracyclic antidepressants mirtazapine and mianserin likewise suppressed basal IP formation. Conversely, the tricyclics amitriptyline and clomipramine, the *m*-chlorophenylpiperazine derivatives trazodone and nefazodone, and the 5-HT reuptake inhibitors fluoxetine and citalopram were inactive alone, although they blocked 5-HT-induced IP production. Inverse agonist actions of 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole (SB206,553) and mirtazapine were abolished by the neutral antagonist 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-ylcarbamoyl]indoline (SB242,084), which was inactive alone. As assessed by confocal microscopy and enzyme-linked immunosorbent assay, prolonged treatment of HEK-293 cells with SB206,553, mirtazapine, or mianserin, but not the other antidepressants, enhanced cell surface expression of 5-HT$_{2C}$ receptors: 5-HT-induced IP production was also increased, and both these actions were blocked by SB242,084. Cortical neurons were shown by reverse transcription-polymerase chain reaction to predominantly express constitutively active 5-HT$_{2C}$ receptor isoforms. Prolonged pretreatment with SB206,553 or mirtazapine triggered an otherwise absent 5-HT-induced elevation in cytosolic Ca^{2+} concentrations. SB242,084, which was inactive alone, abolished these effects of SB206,553 and mirtazapine. In conclusion, the tetracyclic antidepressants mirtazapine and mianserin, but not other clinically established antidepressants, suppress constitutive activity at recombinant and native 5-HT$_{2C}$ receptors. The clinical significance of inverse agonist versus neutral antagonist properties both during *and* after drug administration will be of interest to elucidate.

5-Hydroxytryptamine$_{2C}$ (serotonin) receptors continue to attract considerable interest in view of their broad physiological role and implication in the actions of psychotropic agents (Giorgetti and Tecott, 2004; Millan, 2005, 2006). It is noteworthy that they show species- and tissue-specific patterns of adenosine-to-inosine mRNA editing, leading to amino acid substitutions within the intracellular loop 2 and the generation of numerous isoforms, ranging from unedited

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); SB206,553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole; SB242,084, 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-ylcarbamoyl]indoline; PLC, phospholipase C; NA, noradrenalin; mCPP, *m*-chlorophenylpiperazine; SSRI, selective serotonin reuptake inhibitor; h, human; HEK, human embryonic kidney; IP, inositol phosphate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ANOVA, analysis of variance; NE, norepinephrine; RTR-PCR, reverse transcription-polymerase chain reaction; SSCP, single-strand conformational polymorphism; PCR, polymerase chain reaction; bp, base pair(s); CHO, Chinese hamster ovary; Ro-60,0175, 2(*S*)-1-(6-chloro-5-fluoro-1*H*-indol-1-yl)-2-propanamine fumarate.

(INI) to the fully edited (VGV) isoforms (Burns et al., 1997; Herrick-Davis et al., 1999; Niswender et al., 1999). The intracellular loop 2 is involved in the interaction of 5-HT$_{2C}$ receptors with G proteins, and specific isoforms of 5-HT$_{2C}$ receptors show contrasting binding and coupling profiles (Herrick-Davis et al., 1999; Niswender et al., 1999). They also show varying degrees of "constitutive activity", ranging from pronounced for the wild-type INIs to intermediate for partially edited isoforms such as VSV to minimal for the fully edited VGV isoforms (Herrick-Davis et al., 1999; Niswender et al., 1999). In certain expression systems, 5-HT$_{2C}$ receptors are constitutively internalized in a G protein-coupled receptor kinase/β-arrestin-dependent manner, and prolonged exposure to SB206,553, an inverse agonist, leads to an increase in their plasma membrane insertion (Marion et al., 2004). Suggesting that constitutive activity at 5-HT$_{2C}$ receptors may be physiologically relevant, SB206,553, but *not* SB242,084 (a "neutral" antagonist), elevates extracellular levels of dopamine in the nucleus accumbens (De Deurwaerdère et al., 2004). Furthermore, inasmuch as dopaminergic pathways control affect (Millan, 2006), excessive signaling at constitutively active 5-HT$_{2C}$ receptors may participate in the induction of depressive states (Millan, 2005). 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 subjects (Gurevich et al., 2002), and mRNA editing is modified by long-term administration of antidepressants to rodents (Englander et al., 2005).

It is noteworthy that the "atypical" antidepressant mianserin behaves as an *inverse* agonist at 5-HT$_{2C}$ receptors coupled to phospholipase C (PLC) and phospholipase A$_2$ (Barker et al., 1994; Devlin et al., 2004). This action of mianserin, together with its blockade of α_2-adrenergic receptors and suppression of noradrenaline (NA) reuptake, reinforces dopaminergic and adrenergic transmission, and it underlies its antidepressant properties in humans (de Boer et al., 1996; Tanda et al., 1996; Millan et al., 2000; Millan, 2006). Mianserin has been largely superseded by the chemically related mirtazapine that lacks actions at NA transporters but that also potently recognizes 5-HT$_{2C}$ (and α_2-adrenergic) receptors (de Boer et al., 1988; Millan et al., 2000), enhances dopaminergic transmission (de Boer et al., 1996; Millan et al., 2000; Devoto et al., 2004), and shows broad therapeutic efficacy in depression (Anttila and Leinonen, 2001; Millan, 2006). It is curious that although mirtazapine blocks actions of 5-HT$_{2C}$ agonists in rodents (de Boer et al., 1988; Millan et al., 2000), it remains unknown whether it behaves as an inverse agonist or as a neutral antagonist. Many tricyclic antidepressants, such as clomipramine and amitriptyline, display comparable affinities for 5-HT$_{2C}$ sites and 5-HT transporters (Sánchez and Hyttel, 1999; Millan, 2006), and they block actions of 5-HT$_{2C}$ agonists in vivo (Jenck et al., 1993). However, despite the contribution of 5-HT$_{2C}$ receptor blockade to the influence of tricyclic antidepressants upon mood (Di Giovanni et al., 1999; Gobert et al., 2000; Di Matteo et al., 2001), their cellular actions at 5-HT$_{2C}$ receptors remain essentially uncharacterized. Likewise, the *m*-chorophenyl-piprazine (mCPP) derivatives and clinically established antidepressants nefazodone and trazodone bind to 5-HT transporters and 5-HT$_{2C}$ receptors with comparable potency (Jenck et al., 1993; Sánchez and Hyttel, 1999; Millan, 2006), but their influence on 5-HT$_{2C}$ receptor coupling and cycling has not been evaluated. Finally, specific serotonin reuptake inhibitors (SSRIs) such as fluoxetine and citalopram indirectly (via 5-HT) *activate* 5-HT$_{2C}$ receptors, underlying their acute anxiogenic properties (Millan, 2005). Furthermore, the progressive functional down-regulation of 5-HT$_{2C}$ receptors is related to the gradual onset of clinical efficacy (Giorgetti and Tecott, 2004; Millan, 2006). Nonetheless, fluoxetine and citalopram attenuate the actions of agonists at native, rat 5-HT$_{2C}$ receptors (Pälvimäki et al., 1996; Ni and Miledi, 1997; Sánchez and Hyttel, 1999), so their actions at constitutively active 5-HT$_{2C}$ receptors would be of interest to determine.

In light of the above-mentioned comments, we evaluated herein the influence of a broad range of antidepressants upon the cell surface expression and coupling to PLC of h5-HT$_{2CINI}$ receptors heterologously expressed in HEK-293 cells. Furthermore, we undertook parallel studies of drug actions at native 5-HT$_{2C}$ receptors in primary cultures of mouse cortical neurons.

Materials and Methods

Plasmids and Drugs. The construct encoding the cMyc-tagged h5-HT$_{2CINI}$ receptor (pRK5/c-Myc-5-HT$_{2CINI}$) has been described previously (Becamel et al., 2001). Using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA), the 5-HT$_{2C}$ receptor was subcloned from the pRK5/c-Myc-5-HT$_{2CINI}$ plasmid into the pCMV-Tag 2B Flag epitope-tagging vector (Stratagene) with the BamHI/SalI restriction sites, yielding the pCMV/Flag-5-HT$_{2CINI}$ construct. The plasmid encoding Gα_q (Gα_q/pRK5) was kindly provided by Dr. J. P. Pin (Centre National de la Recherche Scientifique UMR 5203, Institut de Génomique Fonctionnelle, Montpellier, France). 5-HT (creatinine sulfate), SB206,553, clomipramine, amitriptyline, nor-fluoxetine, and trazodone were purchased from Sigma-Aldrich (St. Louis, MO), and fluoxetine and nefazodone were from Interchim (Montluçon, France). Ro-60,0175, SB242,084, mirtazapine, mianserin, and citalopram were synthesized by Dr. Gilbert Lavielle (Institut de Recherches Servier, Paris, France).

Cell Cultures and Transfection. HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum and antibiotics. They were transfected by electroporation using the V Nucleofactor kit (Amaxa Biosystems, Gaithersburg, MD). Each sample contained 1 μg of either the cMyc-5HT$_{2CINI}$ or the Flag-5-HT$_{2CINI}$ receptor cDNA plus 1 μg of the Gα_q protein cDNA and 10×10^6 cells in 100 μl of V Nucleofactor solution according to the manufacturer's instructions. Experiments were carried out 24 h after transfection. Primary cultures of cortical neurons were prepared as described previously (Weiss et al., 1986). In brief, cells from the cerebral cortex of 16-day-old Swiss mouse embryos were plated in serum-free medium on either 100-mm culture dishes (15×10^6 cells/well) or Lab-Tek II chamber slides (1×10^6 cells/well; Nalge Nunc International, Rochester, NY), coated successively with poly-L-ornithine (mol. wt. = 40,000; 15 μg/ml) and 10% fetal calf serum + 1 μg/ml laminin. The culture medium included a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient supplemented with 33 mM glucose, 2 mM glutamine, 13 mM $NaHCO_3$, 5 mM HEPES buffer, pH 7.4, 5 IU/ml (5 mg/ml) penicillin-streptomycin, and a mixture of salt and hormones containing 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 60 nM putrescine, and 30 nM Na_2SeO_3. Cultures were maintained for 12 days at 37°C in a humidified atmosphere containing 5% CO_2. At this stage, cultures were shown to contain at least 95% of neurons (Weiss et al., 1986).

[^{3}H]Mesulergine Binding. HEK-293 cells were grown in 150-mm culture dishes until confluence, and then they were cotransfected with

cMyc-5HT$_{2CINI}$ receptors and Gα_q. Cells were homogenized using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) for 20 s in 50 mM HEPES buffer, pH 7.4, containing a cocktail of protease inhibitors (Roche Diagnostics, Basel, Switzerland), and homogenates were centrifuged at 20,000*g* for 20 min at 4°C. The pellet (membrane fraction) was resuspended in the binding buffer containing 20 mM HEPES, pH 7.7, 2 mM EDTA, and 0.1% (w/v) ascorbic acid. Membranes (20 μg/assay) were incubated in 96-well OptiPlates (PerkinElmer Life and Analytical Sciences, Boston, MA) with 0.2 to 32 nM [^{3}H]mesulergine (GE Healthcare, Chalfont St. Giles, UK) for 105 min at 22°C in total volume of 300 μl. For the determination of the total number of binding sites, cells were directly homogenized in the binding buffer, and homogenates (20 μg/assay) were incubated with [^{3}H]mesulergine as described above. Assays were terminated by rapid filtration through GF-B filters using a 96-well cell harvester (Brandel Inc., Gaithersburg, MD). Radioactivity was determined by scintillation counting using a Wallac 1450 MicroBeta microplate liquid scintillation counter (Molecular Devices, Sunnyvale, CA). Nonspecific binding was defined with 1 μM mianserin.

Measurement of Inositol Phosphate Production. HEK-293 cells grown in 96-well plates (0.25×10^6 cells/well) and cotransfected with cMyc-5HT$_{2CINI}$ receptors and Gα_q (unless otherwise indicated) were labeled overnight with 0.5 μCi/well *myo*-[^{3}H]inositol (10–20 Ci/mmol; GE Healthcare). Cells were washed twice in Locke's solution containing 150 mM NaCl, 20 mM HEPES, 4.2 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 33 mM glucose, incubated in Locke's solution supplemented with 10 mM LiCl for 10 min, and then exposed to drugs for 30 min. IP generation was terminated by the addition of 0.1 M formic acid. Supernatants were recovered and total [^{3}H]IPs purified in 96-well plates by ion exchange chromatography using a DOWEX AGI·X8 resin (Bio-Rad Laboratories, Hemel Hempstead, UK). The [^{3}H]IPs were then eluted with a solution of 10 M ammonium formate and 0.1 M formic acid. Radioactivity was determined by scintillation counting. Results are expressed as the amount of [^{3}H]IP produced in comparison with radioactivity present in the 10% Triton X-100 and 0.1 M NaOH-solubilized membrane fraction. Data are means ± S.E.M. of values obtained in at least three independent experiments, which were performed in triplicate.

Analysis of 5-HT$_{2C}$ Receptor Cell Surface Expression by Confocal Microscopy and ELISA. HEK-293 cells grown on glass coverslips were cotransfected with Flag-tagged 5-HT$_{2CINI}$ receptors and Gα_q. Four hours after transfection, cells were exposed for 18 h to antagonists, inverse agonists, or both. They were then washed in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature. After three washes with 0.1 M glycine, cells were permeabilized with 0.1% (w/v) Triton X-100 for 5 min. They were then incubated with PBS containing 10% BSA for 30 min at 37°C and overnight at 4°C with a rabbit anti-FLAG antibody (1:1000; Sigma-Aldrich), in PBS supplemented with 3% BSA. Cells were washed three times with PBS + 10% BSA, and then they were incubated for 1 h at room temperature with a cyanine 3-labeled anti-rabbit antibody (1:2000 dilution in PBS + 3% BSA; Invitrogen, Paisley, UK). After three washes, coverslips were mounted on glass slides in Mowiol 4.88 (Calbiochem). Confocal laser scanning microscopy was performed using a 1024 Bio-Rad confocal system. A series of optical sections were collected with a step of 0.40 μm and scanned at 1024 × 1024 pixel resolution. Quantification of receptor cell surface expression was performed by ELISA under nonpermeabilized conditions. After the antagonist/inverse agonist incubation period, cells grown in 96-well plates were fixed with 4% paraformaldehyde for 20 min at room temperature. After two washes, cells were incubated in PBS containing 1% fetal calf serum for 30 min, and they were incubated with a horseradish peroxidase-conjugated anti-FLAG monoclonal antibody (1:5000; Sigma-Aldrich) for 30 min. After five washes, the chromogenic substrate (SuperSignal ELISA Femto; Pierce Biotechnology, Rockford, IL) was added and immunoreactivity detected at 492 nm with a Wallac Victor2 luminescence counter (PerkinElmer Life and Analytical Sciences). Control experiments were performed by omitting the primary antibody or by using cells transfected with empty vectors. Values were also normalized with respect to the total amount of protein. For each data point, four determinations were averaged, and results were analyzed using ANOVA followed by Student-Newman-Keuls test.

Immunoblotting. Proteins, resolved on 12.5% gels, were transferred electrophoretically onto nitrocellulose membranes (Hybond-C; GE Healthcare). Membranes were incubated in blocking buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% Tween 20, and 5% skimmed dried milk) for 1 h at room temperature and overnight with either rabbit anti-Gα_q (C-19) or anti-Gα_{13} (A-20) polyclonal antibodies (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or a mouse anti-Myc monoclonal antibody (clone 9E10, 1:1000; Sigma-Aldrich) in blocking buffer. They were then washed three times with blocking buffer and incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:3000 in blocking buffer; GE Healthcare) for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence kit (enhanced chemiluminescence detection reagent; GE Healthcare).

Calcium Imaging. Cortical neurons, grown in Lab-Tek II chamber slides and pretreated or not for 18 h with 5-HT$_{2C}$ receptor inverse agonists/antagonists, were loaded with Fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, CA) at a final concentration of 12.5 μM for 30 min at 37°C in Locke's solution. The inverse agonists/antagonists were also included into the loading medium. Cells were then rinsed three times in Locke's solution and incubated for an additional 30-min period in dye-free Locke's solution in the absence of drugs. Lab-Teks were then placed on the stage of an IX70 microscope (Olympus, Tokyo, Japan) and continuously superfused with Locke's solution. Imaging of intracellular calcium changes in individual cells was accomplished by ratiometric imaging of Fura-2 fluorescence at 340- and 380-nm excitation using the MetaFluor Imaging system (Molecular Devices). Fluorescence was excited by illumination via a 20× water immersion objective with rapid light wavelength switching provided by a DG4 filter wheel (Sutter Instrument Company, Novato, CA) and detected by a charge-coupled device camera under the control of the MetaFluor software. Before agonist stimulation, images were obtained for 30 s to establish a stable baseline Ca^{2+} measurement. Ca^{2+} responses obtained in representative fields of cells (50–80 cells/field) from at least three experiments performed on different sets of cultured neurons are illustrated.

Determination of 5-HT$_{2C}$ Receptor RNA-Edited Transcripts. Total mRNAs were extracted from cortical neurons grown on 100-mm culture dishes using the RNA Easy kit (QIAGEN GmbH, Hilden, Germany). The relative proportions of mRNAs corresponding to nonedited and differentially edited 5-HT$_{2C}$ receptors were determined after RT-PCR amplification and analysis of 5-carboxyfluorescein and Vic fluorescent final strands after separation by capillary electrophoresis according to their respective single-strand conformational polymorphism (SSCP) (Poyau et al., 2007). Reverse transcription of 5-HT$_{2C}$ receptor mRNAs was carried out with 15 units of reverse transcriptase (ThermoScript; Invitrogen) starting from 0.5 μg of total RNA and using 10 nmol of a gene-specific primer (5′-TTCGTCCCTCAGTCCAATCAC-3′) that hybridizes with exon VI. A 250-bp cDNA fragment was then amplified by PCR with 0.4 unit of Platinum *Taq*DNA polymerase (Invitrogen) in a final volume of 20 μl using a forward primer (5′-TGTCCCTAGCCATTGCTGATATGC-3′) and a reverse primer (5′-GCAATCTTCATGATGGCCTTAGTC-3′), both at a 0.2 μM final concentration. After an initial denaturation step at 95°C for 3 min, PCR was concluded after 35 cycles (15 s at 95°C, 30 s at 60°C, and 20 s at 72°C), and a final elongation step of 2 min at 72°C. The 250-bp fragments and the cloned cDNAs corresponding to the 32 mouse standard isoforms (Poyau et al., 2007) were reamplified by nested PCR for capillary electrophoresis-SSCP analysis. This second PCR step was performed with fluorescent 5-carboxyfluorescein- and Vic-labeled primers (0.3 μM; Applied Biosystems, Foster City, CA) and 0.25 unit of Platinum *Pfx*DNA polymerase (Invitrogen) in a final volume of 20 μl. The resulting fragments were

obtained after an initial denaturation step of 5 min at 94°C, 35 cycles of amplification (15 s at 94°C, 30 s at 55°C, and 20 s at 68°C), and a final elongation step of 2 min at 68°C. Sample and standard fluorescent PCR products (138 bp) were then analyzed according to their SSCP, and the relative proportion of each 5-HT$_{2C}$ receptor mRNA isoform was quantified as described previously (Poyau et al., 2007).

Results

Activity of Nonedited 5-HT$_{2CINI}$ Receptors Expressed in HEK-293 Cells. No significant increase in basal IP production was detected in HEK-293 cells transiently transfected with the nonedited isoform of human 5-HT$_{2C}$ receptors (h5-HT$_{2CINI}$) alone (Fig. 1A), probably because of an insufficient reservoir of Gα_q protein (Lefkowitz et al., 1993). Accordingly, to enhance basal constitutive activity, cells were cotransfected with exogenous Gα_q protein. This yielded a 3.6-fold elevation in its levels as assessed by immunoblotting (13.8 ± 0.1 pmol/mg protein compared with 3.8 ± 0.1 pmol/mg protein in cells transfected with empty vectors) together with an 8-fold increase in basal IP accumulation (Fig. 1A). Overexpression of Gα_q alone did not significantly elevate basal IP production (Fig. 1A). Saturation analyses with [^{3}H]mesulergine demonstrated that the density of 5-HT$_{2C}$ receptors (B_{max}) was 0.31 ± 0.07 pmol/mg total protein, with a K_d value of 0.98 ± 0.1 nM in cotransfected cultures (Fig. 1B).

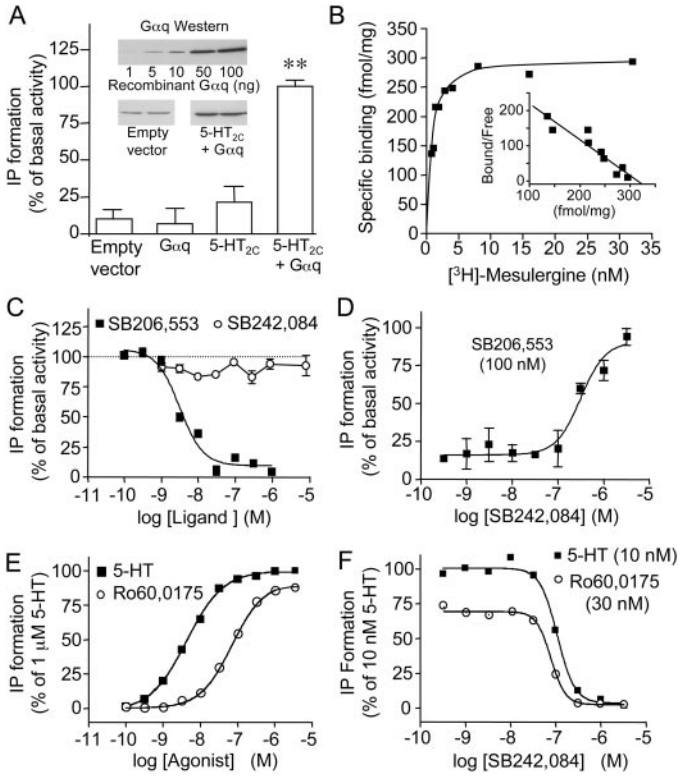

Fig. 1. Constitutive activity of nonedited 5-HT$_{2CINI}$ receptors expressed in HEK-293 cells cotransfected with Gα_q; inverse agonist suppression of inositol phosphate formation. A, HEK-293 cells were transfected with either empty vectors, or with vectors encoding the Myc-tagged 5-HT$_{2CINI}$ receptor or with Gα_q alone, or cotransfected with both vectors. Cells were labeled with *myo*-[^{3}H]inositol, and basal IP formation (30 min) was measured in the presence of 10 mM LiCl. Data are expressed as the percentage of basal IP formation observed in cotransfected cells (defined as 100%). **, $P < 0.01$ versus cells transfected with empty vectors (ANOVA followed by Dunnett's test). Inset, Gα_q subunit expression was determined in HEK-293 cells transfected with empty vectors and in cotransfected cells by immunoblotting and densitometry. The calibration curve was constructed using purified, recombinant Gα_q. B, 5-HT$_{2CINI}$ receptor density in cotransfected cultures (expressed in femtomoles per milligram of protein) was determined by [^{3}H]mesulergine saturation binding (0.2–32 nM). Nonspecific binding was defined by use of 10 μM mianserin. Inset, Scatchard plot of saturation binding data. C to F, cotransfected HEK-293 cells were challenged with drugs for 30 min in the presence of LiCl. In experiments using SB242,084 as an antagonist, cells were exposed to SB242,084 for 10 min before the 30-min incubation period together with the agonist or inverse agonist. All curves were fitted using Prism (version 4.0) software (GraphPad Software Inc., San Diego, CA). Data are means ± S.E.M.s of values obtained in representative experiments performed in quadruplicate. The experiments were repeated three times with similar results.

Basal IP production in cells coexpressing 5-HT$_{2CINI}$ receptors and Gα_q was concentration-dependently reduced by the prototypical 5-HT$_{2C}$ receptor inverse agonist SB206,553 [pEC$_{50}$ = 8.54 ± 0.15; mean of values obtained in three (n) independent experiments, each performed in quadruplicate on different cultures; Fig. 1C]. By contrast, the neutral antagonist SB242,084 did not alter basal IP accumulation (Fig. 1C), and it concentration-dependently inhibited the inverse agonist effects of SB206,553 (Fig. 1D). Both serotonin and the 5-HT$_{2C}$ receptor agonist Ro-60,0175, concentration-dependently increased IP accumulation, with pEC$_{50}$ values of 8.57 ± 0.07 and 7.34 ± 0.14 (n = 3), respectively (Fig. 1E). The induction of IP accumulation by 5-HT and Ro-60,0175 was abolished by SB242,084, with pK_B values of 7.55 ± 0.05 and 7.3 ± 0.09 (n = 3), respectively (Fig. 1F).

Actions of Antidepressants at Constitutively Active 5-HT$_{2CINI}$ Receptors Expressed in HEK-293 Cells. The tetracyclic antidepressant mirtazapine concentration-dependently diminished the constitutive activity of 5-HT$_{2CINI}$ receptors as revealed by a concentration-dependent decrease in the production of IPs (pEC$_{50}$ = 7.02 ± 0.17; n = 3; Fig. 2A). The chemically related antidepressant mianserin similarly decreased basal IP production (pEC$_{50}$ = 7.74 ± 0.20; n = 3), but with a lower maximal effect compared with mirtazapine (58.4 ± 1.6 versus 90.7 ± 2.6%; $P < 0.0001$ in Student's two-tailed t test; Fig. 2C). In line with its neutral antagonist properties, SB242,084 abolished the inverse agonist effects of both mirtazapine and mianserin (Fig. 2, B and D). In contrast to these agents, the tricyclic antidepressants clomipramine (Fig. 2E) and amitriptyline (data not shown), the mCPP derivatives nefazodone (Fig. 2G) and trazodone (data not shown), and the SSRIs fluoxetine (Fig. 2I) and citalopram (data not shown) did not significantly alter IP accumulation. Nonetheless, they all blocked the increased IP production elicited by 5-HT (Fig. 2, F, H, and J; Table 1). Likewise, norfluoxetine, the major active metabolite of fluoxetine (Millan, 2006), inhibited IP production induced by 5-HT with a potency (pEC$_{50}$ = 5.90 ± 0.05; n = 3) similar to that of fluoxetine (Table 1), without affecting basal IP formation, indicating neutral antagonist activity.

Influence of Prolonged Treatment with SB206,553 on the Cell Surface Localization of 5-HT$_{2CINI}$ Receptors Expressed in HEK-293 Cells. Untreated HEK-293 cells coexpressing a Flag-tagged version of the h5-HT$_{2CINI}$ receptor and Gα_q exhibited marked intracellular immunostaining mainly associated with membrane- and vesicular-type structures (Fig. 3A). Incubation of these cells with 1 μM 5-HT for 60 min did not change the apparent cellular distribution of the receptor (Fig. 3A). Nonetheless, ELISA revealed that

5-HT treatment significantly decreased the number of cell surface 5-HT$_{2CINI}$ receptors compared with untreated cells (Fig. 3B). In contrast, prolonged pretreatment of cells with 1 μM SB206,553 for 18 h induced a marked redistribution of 5-HT$_{2CINI}$ receptors to the cell surface (Fig. 3, A and B). Accordingly, 5-HT$_{2C}$ receptors were mostly detected at the plasma membrane (Fig. 3A). Furthermore, this effect of SB206,553 was exerted concentration-dependently (pEC$_{50}$ = 7.0 $\pm$ 0.26; n = 3), with a maximal response observed at a concentration of 1 μM (Fig. 3C), and it was prevented by the neutral antagonist SB242,084 at 1 μM, which did not itself modify the density of receptors at the cell surface (Fig. 3, A and B). As shown in Fig. 3D, the plasma membrane insertion of 5-HT$_{2CINI}$ receptors elicited by SB206,553 increased progressively and time-dependently during an 18-h period of exposure. The reversibility of the effect of SB206,553 was demonstrated by washing the cells to remove the drug: this led to a progressive and time-dependent decrease in the density of 5HT$_{2CINI}$ receptors at the cell surface (Fig. 3E).

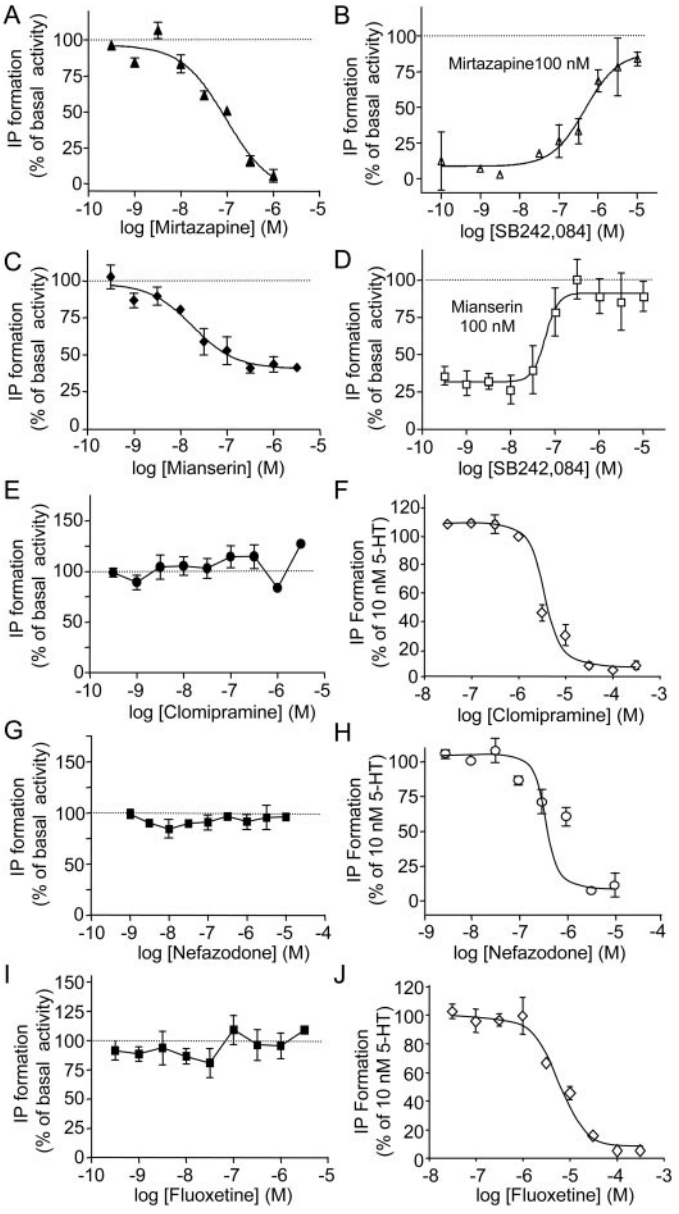

Fig. 2. Contrasting actions of antidepressants at constitutively active 5-HT$_{2CINI}$ receptors expressed in HEK-293 cells as determined by their influence upon inositol phosphate formation. HEK-293 cells cotransfected with Myc-tagged 5-HT$_{2CINI}$ receptors and Gα_q protein were labeled with *myo*-[^{3}H]inositol and incubated with drugs for 30 min in the presence of 10 mM LiCl. In experiments using SB242,084 as an antagonist of mirtazapine and mianserin, cells were exposed to SB242,084 for 10 min before the 30-min incubation period together with the inverse agonist. A, C, E, G, and I show the effects of antidepressants upon constitutive activity. B and D illustrate inhibition by the neutral antagonist SB242,084 of the inverse agonist activities of mirtazapine and mianserin. F, H, and J illustrate the inhibition by neutral antagonist antidepressants of 10 nM 5-HT-induced IP production. Data are means $\pm$ S.E.M.s of values obtained in representative experiments performed in quadruplicate. The experiments were repeated three times with similar results.

Effect of Prolonged Treatment with Antidepressants on the Cell Surface Localization of 5-HT$_{2CINI}$ Receptors Expressed in HEK-293 Cells. Consistent with its inverse agonist properties for suppression of basal IP production, long-term exposure to mirtazapine concentration-dependently increased the plasma membrane density of 5-HT$_{2CINI}$ receptors by a similar magnitude as that seen with SB206,553 (Fig. 3, A and C). The effect of mirtazapine was blocked by 1 μM SB242,084 (Fig. 3, A and B). In a similar manner, prolonged exposure of the cells to 1 μM mianserin for 18 h increased the cell surface expression of 5-HT$_{2CINI}$ receptors, an effect prevented by SB242,084 (Fig. 3, A and B). In line with their neutral antagonist properties (absence of effect on basal IP accumulation), pretreatment of cells with clomipramine, nefazodone, or fluoxetine did *not* significantly alter the intracellular distribution of the receptor (Fig. 3, A and B).

Effect of Prolonged Treatment with SB206,553 and Mirtazapine on the Responsiveness of 5-HT$_{2CINI}$ Receptors Expressed in HEK-293 Cells. Prolonged treatment (18 h) of HEK-293 cells cotransfected with h5-HT$_{2CINI}$ receptors and Gα_q with either 1 μM SB206,553 or 1 μM mirtazapine, followed by extensive washout, resulted in a marked enhancement of serotonin-induced IP production (maximal effect) relative to vehicle (Fig. 4A). The potency of serotonin was not significantly altered by these treatments [pEC$_{50}$ = 8.55 $\pm$ 0.17, 8.67 $\pm$ 0.17, and 8.63 $\pm$ 0.11 (n = 3), in cells pretreated with vehicle, SB206,553, and mirtazapine, respectively; $P > 0.05$]. Conversely, pretreatment of cells with 1 μM SB242,084 did not modify the efficacy of 5-HT-induced IP production (Fig. 4B), although it resulted in an apparent decrease in the potency of 5-HT, probably as a result of incomplete drug washout. When SB206,553 and mirtazapine were preincubated in the presence of SB242,084, their ability to enhance the maximal response to 5-HT was abolished (Fig. 4B). SB206,553 and mirtazapine treatment did not alter the expression of Gα_q or Gα_{13} proteins (Fig. 4C), suggesting that the enhanced responsiveness of 5-HT$_{2C}$ receptors was not due to an "up-regulation" of these G proteins. However, prolonged SB206,553 or mirtazapine treatment was followed by a decrease in the overall level of 5-HT$_{2C}$ receptors, as assessed by immunoblotting (Fig. 4C) and total [^{3}H]mesulergine binding (Fig. 4D).

Influence of Prolonged Exposure to Antidepressants upon Responsiveness of Native 5-HT$_{2C}$ Receptors in Cortical Neurons. Complete quantitative SSCP analysis revealed a complex editing profile of 5-HT$_{2C}$ receptor-specific mRNAs in primary cultures of mouse cortical neurons. Twenty-five differentially edited 5-HT$_{2C}$ receptor mRNAs, corresponding to 17 potentially expressed receptor isoforms were

identified. Nine isoforms were found to be processed by 15 different mRNAs, representing individually more than 1% of the total 5-HT$_{2C}$ receptor mRNAs and globally 93.7 ± 0.36% of total 5-HT$_{2C}$ mRNAs (Fig. 5). Among these nine protein isoforms, five (INI, VNV, VSI, VSV, and INV) are known to display constitutive activity (Herrick-Davis et al., 1999). Their mRNAs represented 65.85 ± 0.96% of the total amount of mRNA encoding 5-HT$_{2C}$ receptors. In contrast, the mRNAs encoding the fully edited isoform (VGV), which is devoid of constitutive activity, represented 1.94 ± 0.17% of the total

TABLE 1

Pharmacological properties of diverse classes of antidepressant at constitutively active 5-HT$_{2CINI}$ receptors

The pK_i values were determined by competition binding experiments using [^{3}H]mesulergine as a radioligand. Isotherms were analyzed by nonlinear regression. The potencies of inverse agonists and neutral antagonists were evaluated upon basal (pEC$_{50}$) and 5-HT-induced IP formation (pK_b), respectively. All data are the mean ± S.E.M.s of three separate experiments each performed in triplicate.

Drug	Class	pK_i	pK_b	pEC$_{50}$	Action (PLC)
Mirtazapine	Tetracyclic	8.22 ± 0.09		7.02 ± 0.17	Inverse agonist
Mianserin	Tetracyclic	8.92 ± 0.04		7.74 ± 0.20	Inverse agonist
Clomipramine	Tricyclic	7.53 ± 0.06	5.90 ± 0.03		Neutral antagonist
Amitriptyline	Tricyclic	8.01 ± 0.13	7.06 ± 0.05		Neutral antagonist
Nefazodone	mCPP derivative	7.37 ± 0.03	6.60 ± 0.04		Neutral antagonist
Trazodone	mCPP derivative	6.81 ± 0.05	5.91 ± 0.09		Neutral antagonist
Fluoxetine	SSRI	7.14 ± 0.07	5.70 ± 0.09		Neutral antagonist
Norfluoxetine	Fluoxetine metabolite	7.01 ± 0.07	5.90 ± 0.05		Neutral antagonist
Citalopram	SSRI	6.46 ± 0.22	5.83 ± 0.07		Neutral antagonist

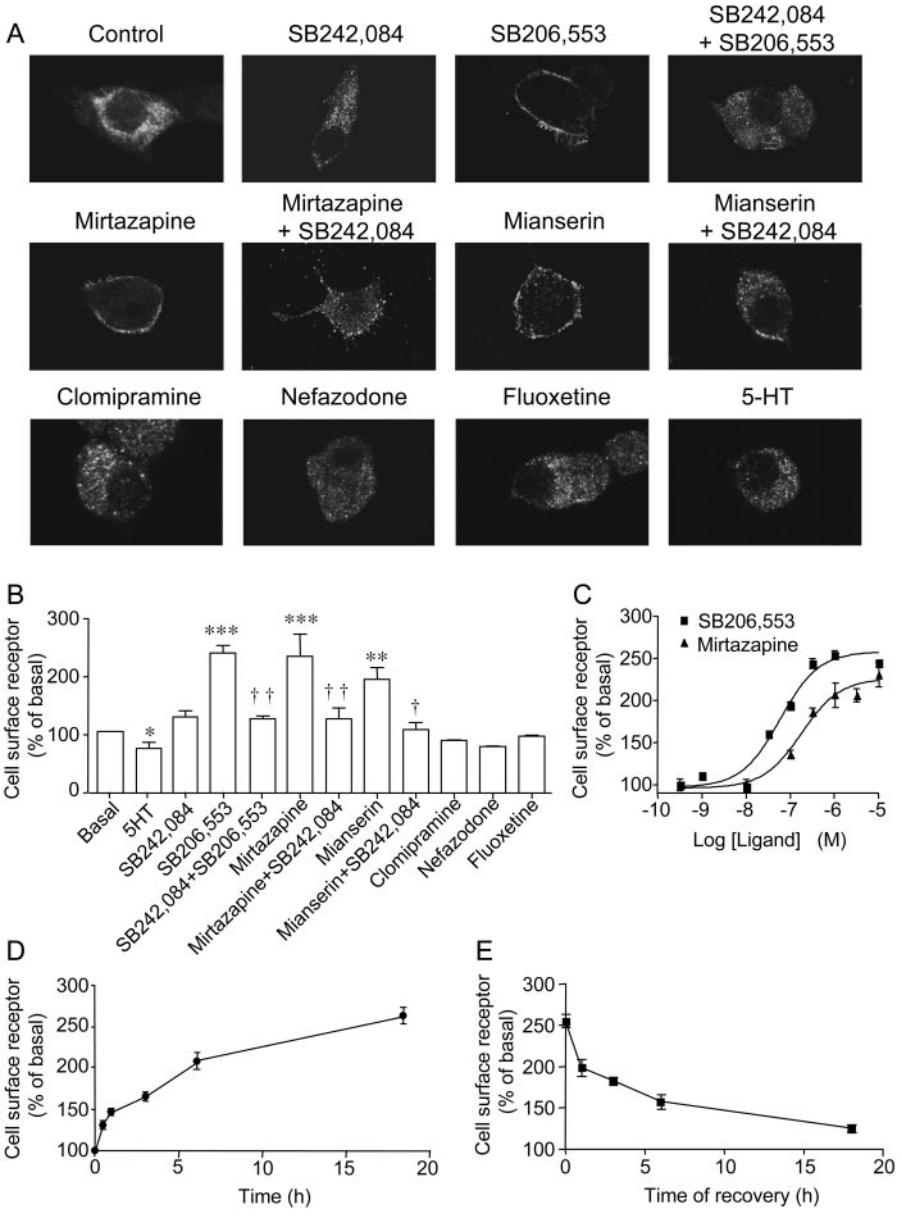

Fig. 3. Influence of prolonged exposure to antidepressants upon the cellular localization of 5-HT$_{2CINI}$ receptors expressed in HEK-293 cells. A, HEK-293 cells transiently transfected with Flag-tagged 5-HT$_{2CINI}$ receptors and Gα_q protein were exposed for 18 h to drugs at the following concentrations: SB206,553, SB242,084, mirtazapine, and mianserin at 1 μM; and clomipramine, trazodone, and fluoxetine at 10 μM. In an additional experiment, cells were exposed for 60 min to 1 μM serotonin. Cellular distribution of 5-HT$_{2CINI}$ receptors was visualized by immunocytochemistry in combination with fluorescence confocal microscopy. Representative images from three independent experiments are shown. Scale bar, 10 μm. B, cells were exposed to the treatments indicated in A. C, cells were exposed for 18 h to incremental concentrations of SB206,553 or mirtazapine. D, cells were treated with 1 μM SB206,553 for the indicated times. E, cells were treated with 1 μM SB206,553 for 18 h, washed extensively, and then incubated in the absence of drugs for the indicated times. In B to E, cell surface expression of 5-HT$_{2CINI}$ receptors was determined by ELISA. Data are means ± S.E.M.s of values obtained in representative experiments performed in quadruplicate. The experiments were repeated four times with similar results. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus untreated cells. †, $P < 0.05$ and ††, $P < 0.01$ versus the corresponding treatment performed in the absence of SB242,084.

5-HT_{2C} receptor mRNA. Thus, the majority of the total pool of 5-HT_{2C} receptor isoforms potentially expressed in cultured cortical neurons were (probably) constitutively active.

Although application of serotonin (up to 1 μM) did *not* induce a detectable increase in cytosolic Ca^{2+} concentration in cultured cortical neurons (Fig. 6A), after pretreatment for 18 h with 100 nM SB206,553, application of serotonin elicited a clear elevation in cytosolic Ca^{2+} concentration in a subpopulation of cultured neurons (Fig. 6, B and J). Furthermore, the percentage of cells responding to 5-HT gradually increased with the time of SB206,553 exposure (Fig. 6J, inset). Serotonin-elicited Ca^{2+} responses were already detectable after a 30-min exposure to SB206,553; and after 18 h of pretreatment, 14.2 ± 1.2% (mean ± S.E.M. of values obtained in three independent experiments performed with different cultures) of SB206,553-treated neurons exhibited increases in cytosolic levels of Ca^{2+} upon serotonin challenge (Fig. 6J). Application of 1 μM SB242,084 blocked the elevation by 5-HT of Ca^{2+} levels after pretreatment with SB206,553, indicating the involvement of 5-HT_{2C} receptors (Fig. 6C). Furthermore, when neurons were pretreated with SB206,553 in the presence of SB242,084, no induction of Ca^{2+} responses to 5-HT was detected (Fig. 6D). By analogy to SB206,553, serotonin induced transient Ca^{2+} elevations in neurons pretreated with 100 nM mirtazapine for 18 h (Fig. 6E). The ability of mirtazapine to generate 5-HT functional responses was prevented by the coapplication of SB242,084 during pretreatment (data not shown). Furthermore, acute application of 1 μM SB242,084 together with 5-HT inhibited 5-HT-evoked Ca^{2+} responses in neurons pretreated with mirtazapine (Fig. 6F). Collectively, these results indicate that prolonged treatment with inverse agonists unmasks functional, 5-HT_{2C} receptor-mediated Ca^{2+} responses in cortical neurons. In contrast, and consistent with their neutral antagonist properties, pretreating neurons for 18 h with either clomipramine, nefazodone, or fluoxetine (10 μM each) did not reveal functional Ca^{2+} responses to 5-HT, and it blocked their induction by SB206,553 (Fig. 6J). Moreover, acute application of either clomipramine, nefazodone, or fluoxetine prevented 5-HT-evoked Ca^{2+} responses in neurons pretreated with SB206,553 (Fig. 6, G–I). The enhanced responsiveness of 5-HT_{2C} receptors followed prolonged exposure to inverse agonists was not related to an up-regulation of G proteins, because prolonged (18-h) treatment with SB206,553 or mirtazapine did not alter the expression of $G\alpha_q$ or $G\alpha_{13}$ in neurons (Fig. 6K).

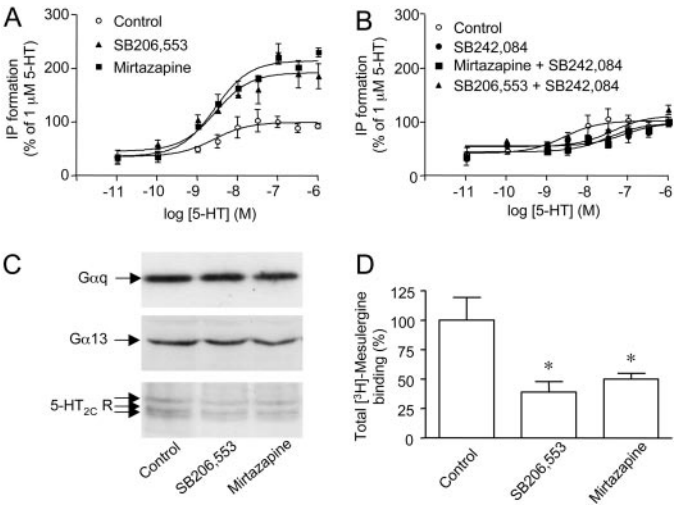

Fig. 4. Influence of prolonged exposure of HEK-293 cells coexpressing 5-HT_{2CINI} receptors and $G\alpha_q$ to SB206,553 and mirtazapine upon 5-HT-induced IP production. A and B, HEK-293 cells transiently transfected with Myc-tagged 5-HT_{2CINI} receptors and $G\alpha_q$ protein, labeled with *myo*-[^{3}H]inositol, were exposed for 18 h to either vehicle (control), 1 μM SB206,553, or 1 μM mirtazapine in the absence or presence of 1 μM SB242,084. Cells were then subjected to three 10-min washes in Locke's solution, incubated for an additional 10-min period in Locke's solution in the presence of 10 mM LiCl, and the absence of drugs, and then they were exposed to incremental concentrations of serotonin for 30 min in the presence of LiCl. Data are means ± S.E.M.s of values obtained in representative experiments performed in quadruplicate. The experiments were repeated three times with similar results. C, $G\alpha_q$, $G\alpha_{13}$, and Myc-5-HT_{2CINI} receptor expression in cells treated or not with either SB206,553 or mirtazapine for 18 h was analyzed by immunoblotting. The data are representative of three independent experiments performed on different cultures. D, total 5-HT_{2C} receptor density was determined under the same conditions by [^{3}H]mesulergine binding as described in the legend to Fig. 1. Data are expressed as a percentage of values obtained in untreated cells (690 ± 26 fmol/mg protein), and they are means ± S.E.M. of values obtained in three independent experiments performed in quadruplicate. $P < 0.05$ versus untreated cells (ANOVA followed by Dunnett's test).

Discussion

Constitutive Activity of h5-HT_{2CINI} Receptors in HEK-293 Cells. The density of 5-HT_{2CINI} receptors in transfected HEK-293 cells was similar to that measured in the choroid plexus, a structure rich in 5-HT_{2C} receptors and possessing principally constitutively active isoforms (Burns et al., 1997; Herrick-Davis et al., 1999; Marazziti et al., 1999). In line with the lack of constitutive activity of 5-HT_{2C} receptors expressed at a similar density in CHO cells (Berg et

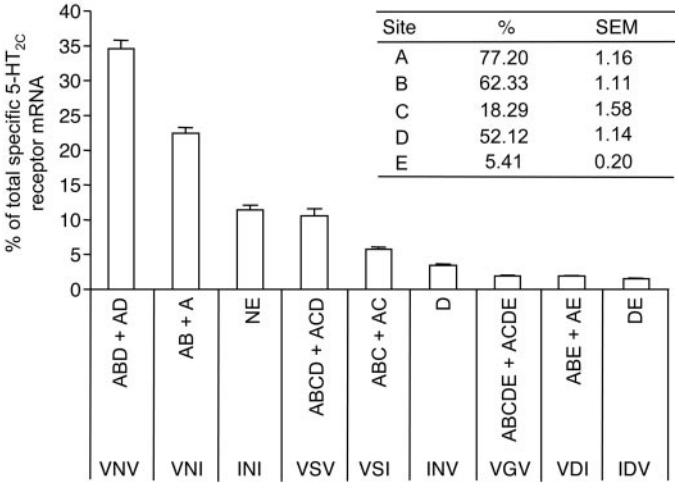

Fig. 5. Expression of differentially edited 5-HT_{2C} receptor isoforms in primary cultures of mice cortical neurons. The relative amounts of mRNA encoding individual, differentially edited isoforms of the 5-HT_{2C} receptor were determined by quantitative RT-PCR followed by SSCP separation via capillary electrophoresis. Pooling specific mRNAs coding for the same 5-HT_{2C} receptor protein isoforms revealed nine sets of mRNA (depicted on the figure) each representing more than 1% of total 5-HT_{2C} receptor mRNA. Data, expressed as a percentage of total specific receptor mRNA, are means ± S.E.M.s of results obtained in four independent experiments performed on different sets of cultured neurons. The proportion of edition for each individual site is indicated in the inset. The values for individual mRNA isoforms are as follows: AB, 30.6 ± 1.1; AD, 3.9 ± 0.3; AB, 14.9 ± 0.9; A, 7.5 ± 0.19; nonedited (NE), 11.4 ± 0.79; ABCD, 9.9 ± 1.08; ACD, 0.7 ± 0.08; ABC, 5.14 ± 0.19; AC, 0.6 ± 0.2; D, 3.5 ± 0.24; ACDE, 1.02 ± 0.1; ABCDE, 0.92 ± 0.12; AE, 1.1 ± 0.29; ABE, 0.82 ± 0.07; and DE, 1.5 ± 0.07.

al., 1999), no increase in basal IP formation was seen in HEK-293 cells transfected with the receptor alone. However, cotransfection of Gα_q to enhance "spontaneous" coupling at 5-HT$_{2CINI}$ receptors led to an augmentation of basal PLC activity: this reflected an increase in constitutive activity at 5-HT$_{2CINI}$ receptors, because it was concentration-dependently abolished by the inverse agonist SB206,553. This observation extends similar findings of "negative" intrinsic activity of SB206,553 at 5-HT$_{2CINI}$ receptors coupled to IP and arachidonic acid generation in CHO cells (Berg et al., 1999; De Deurwaerdère et al., 2004). It is noteworthy that the suppression of basal IP production by SB206,553 was blocked by SB242,084, supporting neutral antagonist properties of this agent at 5-HT$_{2C}$ receptors signaling via PLC (Kennett et al., 1997; De Deurwaerdère et al., 2004; Schlag et al., 2004; Millan, 2005). However, note that SB242,084 possesses modest inverse agonist properties at 5-HT$_{2C}$ receptors signaling via phospholipase A$_2$ (De Deurwaerdère et al., 2004), suggesting that it may be actually a protean agonist.

Inverse-Agonist and Neutral Antagonist Properties of Antidepressants. By analogy to SB206,553, mianserin likewise suppressed basal IP production, underpinning reports of inverse agonist properties at 5-HT$_{2C}$ receptors in a variety of other procedures (Berg et al., 1999; Rauser et al., 2001; Schlag et al., 2004). However, this is the first demonstration that the chemically related "tetracyclic" antidepressant mirtazapine similarly behaves as an inverse agonist at 5-HT$_{2C}$ receptors. Inverse agonist properties of mirtazapine might participate in its marked elevation in extracellular dopamine and NA levels in rodents (Millan et al., 2000). In contrast to mianserin and mirtazapine, the tricyclics amitriptyline and clomipramine and the mCPP derivatives nefazodone and trazodone all failed to modify basal IP production, despite blocking its elevation by 5-HT at concentrations similar to their affinities for 5-HT$_{2C}$ sites (Pälvimäki et al., 1996; Sànchez and Hyttel, 1999; Millan, 2006), indicative of neutral antagonist properties at h5-HT$_{2CINI}$ receptors. Extending observations at rat 5-HT$_{2C}$ receptors (Pälvimäki et al.,

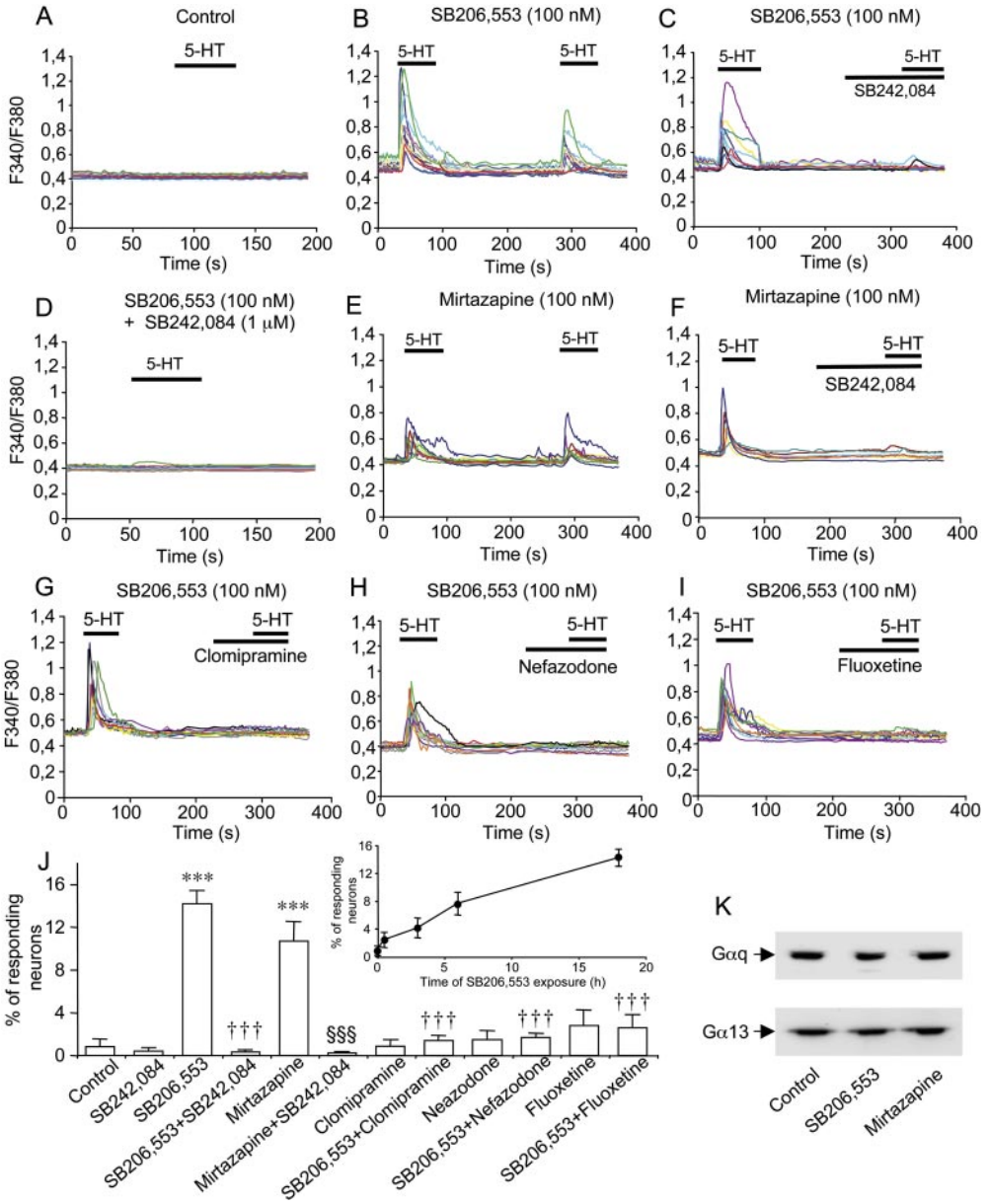

Fig. 6. Influence of prolonged treatment with 5-HT$_{2C}$ receptor inverse agonists and/or neutral antagonists upon serotonin-evoked Ca^{2+} response in mice cortical neurons. A to I, representative recordings of variations in cytosolic Ca^{2+} levels in response to a 1 μM 5-HT challenge for 60 s in cortical neurons pretreated or not (control) with either 100 nM SB206,553 or 100 nM mirtazapine in the absence or presence of 1 μM SB242,084 for 18 h. To confirm that Ca^{2+} responses were indeed mediated by 5-HT$_{2C}$ receptors, a second 5-HT challenge was performed in the absence or presence of 1 μM SB242,084 after a 3-min washout period. This washout period permitted complete repletion of intracellular Ca^{2+} stores (data not shown). In G to I, the second 5-HT challenge was performed in the presence of either clomipramine, nefazodone or fluoxetine (each at 10 μM) to assess their antagonist activity at native 5-HT$_{2C}$ receptors. J, percentage of neurons in which a 5-HT challenge induced an increase in cytosolic Ca^{2+} concentration after 18-h treatment with the indicated compound. Inset, neurons were pretreated with 100 nM SB206,553 for the indicated times before the 5-HT challenge. Data are means ± S.E.M. of values obtained in three independent experiments performed on different sets of cultured neurons (four fields containing each 50–80 neurons counted per experiment). ***, $P < 0.001$ versus untreated neurons; †††, $P < 0.001$ versus cells treated with SB206,553 alone; and §§§, $P < 0.001$ versus cells treated with mirtazapine alone (ANOVA followed by Student-Newman-Keuls test. K, Gα_q and Gα_{13} protein expression in neurons pretreated with either SB206,553 or mirtazapine for 18 h was analyzed by immunoblotting. The data are representative of three independent experiments performed on different sets of cultured neurons.

1996; Ni and Miledi, 1997), fluoxetine inhibited the generation of IP by 5-HT with modest potency, although it failed to influence basal levels of IP, suggesting neutral antagonist properties. Likewise, weak neutral antagonist actions of a further antidepressant, citalopram, were observed, consistent with its low affinity at 5-HT_{2C} receptors (Pälvimäki et al., 1996; Sánchez and Hyttel, 1999; Millan, 2006).

Increased Cell Surface Expression of 5-HT_{2CINI} Receptors after Exposure to Inverse Agonists. A predominantly intracellular compartmentalization has been observed for several classes of constitutively active G protein-coupled receptors (Morris et al., 2004; Leterrier et al., 2006). By analogy, confocal microscopy revealed intracellular localization of 5-HT_{2CINI} receptors in HEK-293 cells, probably reflecting their constitutive phosphorylation and endocytosis by G protein-coupled receptor kinase 2 and β-arrestin2 (Marion et al., 2004). Although application of inverse agonists did not modify the cellular distribution of edited 5-HT_{2CVSV} receptors in HEK-293 cells (Schlag et al., 2004), exposure to a single concentration of 1 μM SB206,553 led to a marked relocation of 5-HT_{2CINI} receptors to the plasma membrane (Marion et al., 2004). The present studies extend these findings in demonstrating by confocal microscopy and quantitative ELISA approaches that SB206,553 concentration-dependently increases cell surface expression of 5-HT_{2CINI} receptors. Moreover, in line with their suppression of basal IP accumulation, mianserin and mirtazapine similarly increased the density of 5-HT_{2CINI} receptors at the plasma membrane, an effect abolished by SB242,084. Conversely, and in line with their neutral antagonist properties, tricyclic antidepressants, *m*-CPP derivatives, and SSRIs all *failed* to alter the subcellular localization of 5-HT_{2CINI} receptors. Although the findings of Marion et al. (2004) were restricted to short (30- and 60-min) preincubation times, herein the actions of SB206,553 were shown to progressively develop over 0.5 to 18 h of preincubation. It is possible that interference with the 5-HT_{2C} receptor/β-arrestin2 complex, thereby interrupting the continuous endocytosis of 5-HT_{2CINI} receptors from the cell surface, participates in the increase in 5-HT_{2C} receptors cell surface density upon inverse agonist treatment.

Enhanced Responsiveness of 5-HT_{2CINI} Receptors to 5-HT after Prolonged Exposure to Inverse Agonists. Increases in 5-HT_{2CINI} receptor cell surface density was associated with enhanced signaling, as revealed by the increase in the maximal effect of 5-HT for stimulation of IP production in cells pretreated with SB206,553 or mirtazapine. These results corroborate data showing that prolonged (24-h) exposure of CHO cells stably expressing 5-HT_{2C} receptors to SB206,553 or mianserin enhanced 5-HT-elicited IP (but not arachidonic acid) production (Berg et al., 1999; Devlin et al., 2004). However, in distinction to previous data obtained on CHO cells (Berg et al., 1999), the mechanism contributing to the enhanced receptor responsiveness after prolonged exposure of HEK-293 cells to SB206,553 or mirtazapine did not involve augmented expression of $G\alpha_q$ (or $G\alpha_{13}$) protein levels. Furthermore, despite the increase in plasma membrane insertion of 5-HT_{2C} receptors after prolonged (18-h) exposure to inverse agonists, these treatments decreased their *total* levels in the cells: that it, including those located in *cytosolic* and plasma membrane compartments. It is clear that additional study of molecular mechanisms underlying the influence of long-term exposure to inverse agonists upon cellular cycling of 5-HT_{2C} receptors would be of interest, including measures of levels and turnover of mRNA encoding 5-HT_{2C} receptors. Nonetheless, this observation is reminiscent of studies of cultured rat choroid plexus 5-HT_{2C} receptors where their density was reduced by sustained treatment with mianserin or SB206,553 (Barker et al., 1994). It also mirrors the progressive decrease in functional transmission at 5-HT_{2C} receptors seen upon chronic administration of mianserin or clozapine (an inverse agonist at 5-HT_{2C} receptors) in vivo (Van Oekelen et al., 2003; Millan, 2006).

Inverse Agonist Actions at Cortical Neurons Bearing 5-HT_{2C} Receptors. Mimicking the above-mentioned observations, a time-dependent induction of 5-HT-evoked Ca^{2+} response was detected in primary cultures of mouse cortical neurons after prolonged treatment with SB206,553 and mirtazapine. These 5-HT-mediated Ca^{2+} signals were abolished by SB242,084, underscoring the involvement of 5-HT_{2C} receptors. Moreover, preincubation of neurons with SB242,084 prevented the inducing action of SB206,553. As in HEK-293 cells, and in contrast to CHO cells (Berg et al., 1999), enhancement of 5-HT_{2C} receptor signaling in mouse cortical neurons did not reflect an increase in G protein expression. Owing to the very low density of 5-HT_{2C} receptors in these neurons, it was not technically possible to demonstrate altered cell surface expression of constitutively internalized 5-HT_{2C} receptors. Nonetheless, in line with the notion of intracellular localization, RT-PCR analysis revealed that the majority of 5-HT_{2C} receptor mRNAs present in cortical neurons encoded constitutively active receptors. It is noteworthy that cortical 5-HT_{2C} receptors interact via their C termini with a set of postsynaptic density 95/disc-large/zona occludens proteins that affect their membrane stability (Bécamel et al., 2002; Gavarini et al., 2006). It is possible, then, that β-arrestin and other postsynaptic proteins regulate the constitutive and ligand-induced internalization and membrane insertion of 5-HT_{2C} receptors (Marion et al., 2004; Gavarini et al., 2006). Although molecular substrates underlying these observations in *neuronal* cultures bearing *native* 5-HT_{2C} receptors remain to be directly elucidated, these findings offer a "bridge" to studies of constitutively active 5-HT_{2C} receptors in vivo (De Deurwaerdère et al., 2004).

Functional Significance of Inverse Agonist Properties at 5-HT_{2C} Receptors. Inverse agonists may be therapeutically more effective than neutral antagonists in blocking "hyperactive" 5-HT_{2C} receptors triggering depressed mood, such as populations inhibiting corticolimbic dopaminergic pathways (Millan, 2005, 2006; Esposito, 2006). However, they may exert more marked side effects than neutral antagonists, including obesity (Giorgetti and Tecott, 2004). Moreover, abrupt cessation of inverse agonist treatment may be associated with an overactivity of "unmasked," constitutively active 5-HT_{2C} receptors, leading to a recrudescence of depressed affect. Likewise, discontinuation of treatment with inverse agonists possessing short elimination times should be performed gradually. Although mianserin and mirtazapine do *not* seem to present problems of withdrawal, their half-lives are long, and this issue has not been systematically addressed (Millan, 2006). Moreover, it is difficult to decipher the significance of inverse agonist versus neutral antagonist properties of currently used antidepressants in view of their other actions at, for example, 5-HT_{2A} receptors, α_2-adreno-

ceptors, and monoamine transporters (Millan, 2005). For a clearer assessment of the significance of constitutive activity at 5-HT$_{2C}$ receptors, there is a need for comparative studies of selective inverse agonists versus neutral antagonists in rodents (including mice genetically lacking 5-HT$_{2C}$ receptors) and, ultimately, in humans.

In conclusion, this study provides novel and compelling support for constitutive activity at both recombinant, and, importantly, native cerebral 5-HT$_{2C}$ receptors. Although most clinically used antidepressants behaved as neutral antagonists, mirtazapine and mianserin mimicked the inverse agonist actions of SB206,553, and it would be interesting to extend their characterization to other intracellular signals, such as phospholipase A$_2$. The functional and clinical significance of contrasting inverse agonist versus neutral antagonist properties of antidepressants at 5-HT$_{2C}$ receptors is an important issue justifying careful investigation.

Acknowledgments

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References

Anttila SA and Leinonen EV (2001) A review of the pharmacological and clinical profile of mirtazapine. *CNS Drug Rev* **7:**249–264.

Barker EL, Westphal RS, Schmidt D, and Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. *J Biol Chem* **269:**11687–11690.

Bécamel C, Alonso G, Galeotti N, Demey E, Jouin P, Ullmer C, Dumuis A, Bockaert J, and Marin P (2002) Synaptic multiprotein complexes associated with 5-HT(2C) receptors: a proteomic approach. *EMBO J* **21:**2332–2342.

Becamel C, Figge A, Poliak S, Dumuis A, Peles E, Bockaert J, Lubbert H, and Ullmer C (2001) Interaction of serotonin 5-hydroxytryptamine type 2C receptors with PDZ10 of the multi-PDZ domain protein MUPP1. *J Biol Chem* **276:**12974–12982.

Berg KA, Stout BD, Cropper JD, Maayani S, and Clarke WP (1999) Novel actions of inverse agonists on 5-HT2C receptor systems. *Mol Pharmacol* **55:**863–872.

Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, and Emeson RB (1997) Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* **387:**303–308.

de Boer TH, Maura G, Raiteri M, de Vos CJ, Wieringa J, and Pinder RM (1988) Neurochemical and autonomic pharmacological profiles of the 6-aza-analogue of mianserin, Org 3770 and its enantiomers. *Neuropharmacology* **27:**399–408.

de Boer TH, Nefkens F, van Helvoirt A, and van Delft AM (1996) Differences in modulation of noradrenergic and serotonergic transmission by the alpha-2 adrenoceptor antagonists, mirtazapine, mianserin and idazoxan. *J Pharmacol Exp Ther* **277:**852–860.

De Deurwaerdère P, Navailles S, Berg KA, Clarke WP, and Spampinato U (2004) Constitutive activity of the serotonin2C receptor inhibits in vivo dopamine release in the rat striatum and nucleus accumbens. *J Neurosci* **24:**3235–3241.

Devlin MG, Smith NJ, Ryan OM, Guida E, Sexton PM, and Christopoulos A (2004) Regulation of serotonin 5-HT2C receptors by chronic ligand exposure. *Eur J Pharmacol* **498:**59–69.

Devoto P, Flore G, Pira L, Longu G, and Gessa GL (2004) Mirtazapine-induced corelease of dopamine and noradrenaline from noradrenergic neurons in the medial prefrontal and occipital cortex. *Eur J Pharmacol* **487:**105–111.

Di Giovanni G, De Deurwaerdére P, Di Mascio M, Di Matteo V, Esposito E, and Spampinato U (1999) Selective blockade of serotonin-2C/2B receptors enhances mesolimbic and mesostriatal dopaminergic function: a combined in vivo electrophysiological and microdialysis study. *Neuroscience* **91:**587–597.

Di Matteo V, De Blasi A, Di Giulio C, and Esposito E (2001) Role of 5-HT(2C) receptors in the control of central dopamine function. *Trends Pharmacol Sci* **22:**229–232.

Englander MT, Dulawa SC, Bhansali P, and Schmauss C (2005) How stress and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. *J Neurosci* **25:**648–651.

Esposito E (2006) Serotonin-dopamine interaction as a focus of novel antidepressant drugs. *Curr Drug Targets* **7:**177–185.

Gavarini S, Bécamel C, Altier C, Lory P, Poncet J, Wijnholds J, Bockaert J, and Marin P (2006) Opposite effects of PSD-95 and MPP3 PDZ proteins on serotonin 5-hydroxytryptamine2C receptor desensitization and membrane stability. *Mol Biol Cell* **17:**4619–4631.

Giorgetti M and Tecott LH (2004) Contributions of 5-HT(2C) receptors to multiple actions of central serotonin systems. *Eur J Pharmacol* **488:**1–9.

Gobert A, Rivet JM, Lejeune F, Newman-Tancredi A, Adhumeau-Auclair A, Nicolas JP, Cistarelli L, Melon C, and Millan MJ (2000) Serotonin(2C) receptors tonically suppress the activity of mesocortical dopaminergic and adrenergic, but not serotonergic, pathways: a combined dialysis and electrophysiological analysis in the rat. *Synapse* **36:**205–221.

Gurevich I, Englander MT, Adlersberg M, Siegal NB, and Schmauss C (2002) Modulation of serotonin 2C receptor editing by sustained changes in serotonergic neurotransmission. *J Neurosci* **22:**10529–10532.

Herrick-Davis K, Grinde E, and Niswender CM (1999) Serotonin 5-HT2C receptor RNA editing alters receptor basal activity: implications for serotonergic signal transduction. *J Neurochem* **73:**1711–1717.

Jenck F, Moreau JL, Mutel V, Martin JR, and Haefely WE (1993) Evidence for a role of 5-HT1C receptors in the antiserotonergic properties of some antidepressant drugs. *Eur J Pharmacol* **231:**223–229.

Kennett GA, Wood MD, Bright F, Trail B, Riley G, Holland V, Avenell KY, Stean T, Upton N, Bromidge S, et al. (1997) SB 242084, a selective and brain penetrant 5-HT2C receptor antagonist. *Neuropharmacology* **36:**609–620.

Lefkowitz RJ, Cotecchia S, Samama P, and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.

Leterrier C, Laine J, Darmon M, Boudin H, Rossier J, and Lenkei Z (2006) Constitutive activation drives compartment-selective endocytosis and axonal targeting of type 1 cannabinoid receptors. *J Neurosci* **26:**3141–3153.

Marazziti D, Rossi A, Giannaccini G, Zavaglia KM, Dell'Osso L, Lucacchini A, and Cassano GB (1999) Distribution and characterization of [3H]mesulergine binding in human brain postmortem. *Eur Neuropsychopharmacol* **10:**21–26.

Marion S, Weiner DM, and Caron MG (2004) RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2c receptor isoforms. *J Biol Chem* **279:**2945–2954.

Millan MJ (2005) Serotonin 5-HT2C receptors as a target for the treatment of depressive and anxious states: focus on novel therapeutic strategies. *Therapie* **60:**441–460.

Millan MJ (2006) Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacol Ther* **110:**135–370.

Millan MJ, Gobert A, Rivet JM, Adhumeau-Auclair A, Cussac D, Newman-Tancredi A, Dekeyne A, Nicolas JP, and Lejeune F (2000) Mirtazapine enhances frontocortical dopaminergic and corticolimbic adrenergic, but not serotonergic, transmission by blockade of alpha2-adrenergic and serotonin2C receptors: a comparison with citalopram. *Eur J Neurosci* **12:**1079–1095.

Morris DP, Price RR, Smith MP, Lei B, and Schwinn DA (2004) Cellular trafficking of human alpha1a-adrenergic receptors is continuous and primarily agonist-independent. *Mol Pharmacol* **66:**843–854.

Ni YG and Miledi R (1997) Blockage of 5HT2C serotonin receptors by fluoxetine (Prozac). *Proc Natl Acad Sci U S A* **94:**2036–2040.

Niswender CM, Copeland SC, Herrick-Davis K, Emeson RB, and Sanders-Bush E (1999) RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. *J Biol Chem* **274:**9472–9478.

Pälvimäki EP, Roth BL, Majasuo H, Laakso A, Kuoppamaki M, Syvalahti E, and Hietala J (1996) Interactions of selective serotonin reuptake inhibitors with the serotonin 5-HT2c receptor. *Psychopharmacology* **126:**234–240.

Poyau A, Vincent L, Berthomme H, Paul C, Nicolas B, Pujol JF, and Madjar JJ (2007) Identification and relative quantification of adenosine to inosine editing in serotonin 2c receptor mRNA by CE. *Electrophoresis* **28:**2843–2852.

Rauser L, Savage JE, Meltzer HY, and Roth BL (2001) Inverse agonist actions of typical and atypical antipsychotic drugs at the human 5-hydroxytryptamine(2C) receptor. *J Pharmacol Exp Ther* **299:**83–89.

Sánchez C and Hyttel J (1999) Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. *Cell Mol Neurobiol* **19:**467–489.

Schlag BD, Lou Z, Fennell M, and Dunlop J (2004) Ligand dependency of 5-hydroxytryptamine 2C receptor internalization. *J Pharmacol Exp Ther* **310:**865–870.

Tanda G, Bassareo V, and Di Chiara G (1996) Mianserin markedly and selectively increases extracellular dopamine in the prefrontal cortex as compared to the nucleus accumbens of the rat. *Psychopharmacology* **123:**127–130.

Van Oekelen D, Luyten WH, and Leysen JE (2003) 5-HT2A and 5-HT2C receptors and their atypical regulation properties. *Life Sci* **72:**2429–2449.

Weiss S, Pin JP, Sebben M, Kemp DE, Sladeczek F, Gabrion J, and Bockaert J (1986) Synaptogenesis of cultured striatal neurons in serum-free medium: a morphological and biochemical study. *Proc Natl Acad Sci U S A* **83:**2238–2242.

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