Inhibition of Serotonin 5-Hydroxytryptamine2C Receptor Function through Heterodimerization

RECEPTOR DIMERS BIND TWO MOLECULES OF LIGAND AND ONE G-PROTEIN*

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Although dimerization appears to be a common property of G-protein-coupled receptors (GPCRs), it remains unclear whether a GPCR dimer binds one or two molecules of ligand and whether ligand binding results in activation of one or two G-proteins when measured using functional assays in intact living cells. Previously, we demonstrated that serotonin 5-hydroxytryptamine2C (5-HT2C) receptors form homodimers (Herrick-Davis, K., Grinde, E., and Mazurkiewicz, J. (2004) Biochemistry 43, 13963–13971). In the present study, an inactive 5-HT2C receptor was created and coexpressed with wild-type 5-HT2C receptors to determine whether we demonstrated that serotonin 5-hydroxytryptamine2C (5-HT2C) ligand binding results in activation of one or two G-proteins when GPCR dimer binds one or two molecules of ligand and whether G-protein-coupled receptors (GPCRs), it remains unclear whether a wide variety of therapeutic agents. Recent studies investigating GPCR families of signaling proteins in the human genome and are targets for a functional significance of dimerization is essential for 5-HT receptor function.

In this study, radioligand binding, inositol phosphate (IP) signaling, confocal imaging of receptor endocytosis, and fluorescence resonance energy transfer (FRET) were evaluated in HEK293 cells coexpressing wild-type and inactive mutant S138R 5-HT2C receptors to determine the effect of dimerization on receptor function and to determine the ligand/dimer/G-protein stoichiometry in living cells. Mutagenesis of Ser138 to Arg (S138R) produced a 5-HT2C receptor incapable of binding ligand or stimulating inositol phosphate (IP) signaling. Confocal fluorescence imaging revealed plasma membrane expression of yellow fluorescent protein-tagged S138R receptors. Expression of wild-type 5-HT2C receptors in an S138R-expressing stable cell line had no effect on ligand binding to wild-type 5-HT2C receptors, but inhibited basal and 5-HT-stimulated IP signaling as well as constitutive and 5-HT-stimulated endocytosis of wild-type 5-HT2C receptors. M1 muscarinic receptor activation of IP production was normal in the S138R-expressing cells. Heterodimerization of S138R with wild-type 5-HT2C receptors was visualized in living cells using confocal fluorescence resonance energy transfer (FRET). FRET was dependent on the donor/acceptor ratio and independent of the receptor expression level. Therefore, inactive 5-HT2C receptors inhibit wild-type 5-HT2C receptor function by forming nonfunctional heterodimers expressed on the plasma membrane. These results are consistent with a model in which one GPCR dimer binds two molecules of ligand and one G-protein and indicate that dimerization is essential for 5-HT receptor function.

G-protein-coupled receptors (GPCRs) represent one of the largest subfamilies of GPCRs. There are 14 different 5-HT receptor subtypes widely distributed throughout the peripheral and central nervous systems, representing therapeutic targets for drugs used to treat anxiety, depression, schizophrenia, obesity, irritable bowel syndrome, migraine, emesis, and other disorders (reviewed in Refs. 8 and 9). If dimerization plays a role in regulating the function of 5-HT receptors, it could have important implications for the development of novel serotonergic therapeutic agents. Immunoprecipitation and Western blot experiments suggest that 5-HT1 receptors may form homodimers and heterodimers (10, 11). Previously, we used biochemical and biophysical techniques to demonstrate that 5-HT2C receptors are present as constitutive homodimers on the plasma membrane of living human embryonic kidney 293 (HEK293) cells (12). However, the functional significance of 5-HT receptor dimerization (if any) remains unknown. Also, it remains unclear whether a GPCR dimer binds one or two molecules of ligand and whether ligand binding results in receptor dimer activation of one or two G-proteins when measured using functional assays in an intact cell system.

In this study, radioligand binding, inositol phosphate (IP) signaling, confocal imaging of receptor endocytosis, and fluorescence resonance energy transfer (FRET) were evaluated in HEK293 cells coexpressing wild-type and inactive mutant S138R 5-HT2C receptors to determine the effect of dimerization on receptor function and to determine the ligand/dimer/G-protein stoichiometry. The results of this study show that S138R receptors form heterodimers with wild-type 5-HT2C receptors in living cells and have an inhibitory effect on 5-HT2C receptor function. Previous studies have reported a dominant-negative effect of splice variant, truncated, or mutant receptors on wild-type receptor function through heterodimerization, resulting in trapping of wild-type receptors in the endoplasmic reticulum (13–16), or through sequestration of G-proteins (17). In contrast, our study describes a dominant-
negative effect of an inactive mutant GPCR on wild-type receptor function through heteromerization, resulting in the expression of nonfunctional receptor complexes on the plasma membrane. Although several studies have reported attenuation of receptor function following coexpression of different GPCR subtypes (18–20), the ligand/dimer/G-protein stoichiometry was not evaluated. Other studies using isolated membranes (21) and purified receptors (22) suggest that each GPCR dimer interacts with a single G-protein. We tested this model in intact living cells following coexpression of wild-type and inactive 5-HT2C receptors. Our results suggest that each GPCR dimer binds two molecules of ligand, resulting in the activation of one G-protein, and indicate that dimerization is essential for 5-HT receptor function.

**MATERIALS AND METHODS**

**Cell Culture**—HEK293 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; CellGro) with 10% fetal bovine serum (CellGro) at 37 °C and 5% CO2. HEK293 cells were plated at 5 × 10⁵ cells/100-mm dish 24 h prior to transfection. Cells were transfected with the indicated plasmid DNAs using Lipofectamine reagent (Invitrogen) for 5 h at 37 °C in serum-free medium and then returned to DMEM with serum. All experiments were performed 36–42 h post-transfection, and cells were cultured in serum-free medium for the final 16 h prior to the experiment.

cDNAs—The human 5-HT2C-VSV receptor cDNA (from Dr. Elaine Sanders-Bush) was used as the starting template for creating receptor fusion proteins and for site-directed mutagenesis to create the mutant S138R receptor. The S138R mutation in the 5-HT2C receptor was created by PCR following the Stratagene protocol using complementary 5-HT2C/YFP, and 5-HT2C/HA fusion proteins as described previously (12). S138R and wild-type 5-HT2C receptor cDNAs were ligated into the pECFP-N1 and/or pEYFP-N1 vector (Clontech), and the M1 muscarinic receptor was ligated into pcDNA3 (Clontech). All constructs were confirmed by DNA sequencing (Center for Functional Genomics, Albany, NY).

**S138R 5-HT2C/YFP-expressing Stable Cell Line**—A stable cell line expressing S138R 5-HT2C/YFP was created by transfecting 2 × 10⁶ HEK293 cells with 10 μg of S138R 5-HT2C/YFP plasmid DNA by calcium phosphate precipitation. Two days post-transfection, cells were cultured in DMEM containing 400 μg/ml G418 for 2 weeks. Colonies expressing YFP-tagged S138R receptors on the plasma membrane were identified by confocal fluorescence microscopy.

**Radioligand Binding**—HEK293 cells (4 × 10⁶ cells/100-mm dish) were transfected with 1 μg of the indicated plasmid DNAs using 20 μl of Lipofectamine. For control experiments, cells were transfected independently with either 5-HT2C/YFP or 5-HT2C/HA and mixed together after transfection. Cross-linking of receptors was performed by incubating intact cells in 5 mM bis(sulfosuccinimidyl) suberate (a cell membrane-impermeable cross-linker; Pierce) for 30 min at 37 °C and 5% CO2. Cells were lysed, and membranes were solubilized in 10 mM CHAPS as described previously (12). Solubilized membrane proteins were immunoprecipitated overnight with 10 μl of anti-HA (Santa Cruz Biotechnology, Inc.) antibody-agarose (Santa Cruz Biotechnology, Inc.) according to the manufacturer’s protocol.

**IP Assay**—For IP assays, HEK293 or S138R-expressing stable cells were seeded at 2 × 10⁵ cells/well in 24-well plates (Biocoat, Inc.) and transfected 3 h later with 5-HT2C or M1 muscarinic receptor plasmid DNA (25 ng/well) and Lipofectamine (1 μl/well). For IP assays performed in parallel with the radioligand binding assays, 4 × 10⁶ cells/100-mm dish were transfected with 0.2 μg of 5-HT2C receptor plasmid DNA; half the dish was used for the radioligand binding assay, and the other half was replated at 2 × 10⁵ cells/well in 24-well plates for the IP assay. Twenty-four hours post-transfection, 0.5 μCi of [3H]myoinositol in isositol-free, serum-free DMEM was added to each well for an additional 16 h. Cells were washed with phosphate-buffered saline and incubated in serum-free DMEM with lithium chloride in the absence or presence of agonist for 35 min. Total [3H]IP production was measured by anion exchange chromatography as described previously (24). Data were analyzed using GraphPad Prism software.

**Confocal Microscopy**—HEK293 cells (4 × 10⁶ cells/100-mm dish) were transfected with 0.2 μg of wild-type or S138R 5-HT2C/YFP plasmid DNA using 20 μl of Lipofectamine. Twenty-four hours post-transfection, cells were plated overnight in serum-free medium on polylysine-coated glass coverslips. The cells were viewed live in phosphate-buffered saline on a Noran Oz confocal microscope system using a Nikon Diaphot 200 inverted microscope with a ×X60, 1.4-numerical aperture oil immersion objective. The 488-nm laser line from a krypton/argon laser was used to excite YFP, and fluorescence was imaged with a 488-nm dichroic mirror and a 500-nm long-pass filter. Time-lapse images were collected every 15 s over a 10-min period. After 10 min, 10 μM 5-HT was added to the cells, and images were collected every 15 s for 10 min. HEK293 cells coexpressing wild-type 5-HT2C/CFP and S138R 5-HT2C/YFP were viewed using a Zeiss LSM 510 META confocal imaging system with linear unmixing of CFP and YFP emission spectra following excitation at 458 nm using the same conditions and settings as described for the FRET experiments.

**Immunoprecipitation and Western Blotting**—HEK293 cells (4 × 10⁶ cells/100-mm dish) were transfected with 1 μg of the indicated plasmid DNAs using 20 μl of Lipofectamine. For control experiments, cells were transfected independently with either 5-HT2C/YFP or 5-HT2C/HA and mixed together after transfection. Cross-linking of receptors was performed by incubating intact cells in 5 mM bis(sulfosuccinimidy) suberate (a cell membrane-impermeable cross-linker; Pierce) for 30 min at 37 °C and 5% CO2. Cells were lysed, and membranes were solubilized in 10 mM CHAPS as described previously (12). Solubilized membrane proteins were immunoprecipitated overnight with 10 μl of anti-HA (Y-11) antibody-agarose (Santa Cruz Biotechnology, Inc.) as described previously (12). Pellets were resuspended in 50 μl of 1× nonreducing Laemmli sample buffer and heated at 70 °C for 10 min, and 10-μl samples were run on a 10% Tris-HCl Ready Gel (Bio-Rad). Blots were probed with horseradish peroxidase-conjugated anti-green fluorescent protein (B-2) antibody (Santa Cruz Biotechnology, Inc.) diluted 1:3000 and visualized by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer’s protocol.

**FRET**—HEK293 cells (4 × 10⁶ cells/100-mm dish) were cotransfected with S138R 5-HT2C/CFP (donor) and wild-type 5-HT2C/YFP (acceptor) or with wild-type 5-HT2C/CFP (donor) and wild-type 5-HT2C/YFP (acceptor) at a donor/acceptor ratio of 1:1 (0.4 μg + 0.4 μg of plasmid DNA) or 1:2 (0.3 μg + 0.6 μg of plasmid DNA) using 20 μl of Lipofectamine. Twenty-four hours post-transfection, cells were plated overnight in serum-free medium on polylysine-coated glass coverslips.
prior to the FRET assay. The cells were viewed live in phosphate-buffered saline at room temperature using a Zeiss LSM 510 META confocal imaging system with a 30-milliwatt argon laser and a ×63, 1.4-numerical aperture oil immersion objective at ×2 zoom. CFP and YFP emission spectra were collected (from cells expressing 5-HT2C/CFP or 5-HT2C/YFP alone) following excitation at 458 nm and were used as reference spectra for linear unmixing of CFP and YFP emission spectra in cotransfected cells using the Zeiss META detector and Zeiss AIM software as described previously (12).

FRET was measured by acceptor photobleaching (25). Confocal microscopy was used to visualize a 2-μm optical slice through the middle of a living HEK293 cell expressing both 5-HT2C/CFP and 5-HT2C/YFP. A small region of plasma membrane (typically 3–4 μm in length) was selected for photobleaching. Pre-bleach CFP and YFP images were collected simultaneously following excitation at 458 nm (38% laser intensity, detector gain = 740). The selected region of plasma membrane was irradiated with the 514-nm laser line (100% intensity, 60 iterations, using a 458/514-nm dual dichroic mirror) for 10 s to photobleach YFP, typically resulting in a 95% decrease in YFP fluorescence. Post-bleach CFP and YFP images were collected simultaneously (at 458 nm) within 1 s following photobleaching. In an independent experiment, YFP fluorescence recovery after photobleaching was measured and found to be negligible 1 s following photobleaching. The short photobleach time and the automated rapid collection of pre- and post-bleach images make this technique suitable for use in living cells. FRET was measured as an increase in CFP fluorescence intensity (donor dequenching) following YFP (acceptor) photobleaching. FRET efficiency was calculated as 100 × [(CFP pre-bleach − CFP post-bleach)/CFP pre-bleach] using the FRET macro in the Zeiss AIM software package, taking into account CFP and YFP background noise in each channel. FRET efficiencies were calculated in cells expressing both CFP and YFP, in cells expressing CFP alone, and in non-bleached regions of the cell membrane as a control.

RESULTS

Mutation of Ser138 to Arg Produces an Inactive Form of the 5-HT2C Receptor—Site-directed mutagenesis was used to create an inactive form of the 5-HT2C receptor to test the hypothesis that 5-HT2C receptors function as dimeric or oligomeric complexes. Computer modeling and mutagenesis studies have provided evidence that Ser159 in transmembrane domain (TMD) III plays an important role in 5-HT binding to the 5-HT2A receptor (26). Therefore, we mutated the analogous serine (amino acid 138) in the 5-HT2C receptor to arginine (S138R) in an attempt to create an inactive receptor. [3H]Mesulergine binding and IP production assays were used to evaluate receptor expression and function in HEK293 cells. Specific [3H]mesulergine binding was detected in cells transfected with plasmid containing wild-type 5-HT2C receptor cDNA, but not in cells transfected with plasmid containing S138R 5-HT2C receptor cDNA (Fig. 1). Similarly, 5-HT stimulated [3H]IP production in HEK293 cells transfected with plasmid containing wild-type 5-HT2C receptor cDNA, but not in cells transfected with plasmid containing S138R 5-HT2C receptor cDNA (Fig. 1). Although wild-type 5-HT2C receptors had moderate levels of basal activity in the IP assay, S138R receptors were devoid of basal activity. These results demonstrate that mutation of Ser138 to Arg eliminates the ability of the 5-HT2C receptor to bind ligand and to stimulate phospholipase C.

Plasma Membrane Localization of S138R and Wild-type 5-HT2C Receptors—Fluorescently tagged 5-HT2C receptors were created to allow direct visualization of S138R and wild-type 5-HT2C receptors in living cells by confocal microscopy. A previous study has shown that a fluorescent tag (YFP) expressed as a fusion protein on the C terminus of the 5-HT2C receptor does not alter 5-HT binding affinity or potency (12). S138R or wild-type 5-HT2C/YFP fusion protein was expressed in HEK293 cells, and live time-lapse images were collected (Fig. 2, A–D).

To assess receptor trafficking activity subsequent to delivery to the plasma membrane, receptor movement was monitored in the absence and presence of 5-HT. Time-lapse images were captured every 15 s over a 10-min period. In cells expressing wild-type 5-HT2C receptors, significant trafficking was observed between the plasma membrane and intracellular compartments in the form of bidirectional vesicular movement. Selected frames from a representative time-lapse movie are illustrated in Fig. 2A. Within the first minute following the addition of 5-HT, vesicular trafficking increased, with a net increase in the retrograde movement of receptor-containing vesicles as evidenced by an increase in the fluorescence intensity of the intracellular compartment. This was followed by an overall cellular shape change that was clearly evident 3 min after the addition of 5-HT (Fig. 2B). The increase in intracellular fluorescence intensity suggests the induction of down-regulation of ligand-bound receptor in the form of endocytosis. HEK293 cells expressing S138R 5-HT2C/YFP showed little or no observable receptor trafficking in the absence of 5-HT (Fig. 2C). Overall movement of the cell was observed, as would be expected from a living cell over a 10-min period. However, there was no significant receptor endocytosis subsequent to the addition of 5-HT (Fig. 2D). When cells were cotransfected with wild-type 5-HT2C/CFP and S138R 5-HT2C/YFP, both wild-type and mutant receptors were expressed on the plasma membrane (Fig. 2E). The addition of 50 μM 5-HT produced a small increase in intracellular wild-type 5-HT2C/CFP fluorescence (Fig. 2F), in contrast to the large redistribution of wild-type 5-HT2C receptors observed when expressed in the absence of S138R receptors (Fig. 2B). There was no change in the distribution of S138R 5-HT2C/YFP fluorescence following the addition of 5-HT to the cotransfected cells (Fig. 2F). These results suggest that the ability of wild-type 5-HT2C receptors to respond to 5-HT stimulation is impaired when coexpressed with inactive S138R 5-HT2C receptors.

S138R 5-HT2C Receptors Inhibit the Function of Wild-type 5-HT2C Receptors—Wild-type 5-HT2C receptors were expressed in HEK293 cells and in a stable cell line expressing S138R 5-HT2C receptors to determine whether wild-type 5-HT2C receptor function is altered when expressed in the presence of inactive S138R receptors. 5-HT dose-response curves for stimulation of IP production in HEK293 cells and in the S138R-expressing stable cell line transfected with equal amounts of wild-type 5-HT2C receptor plasmid DNA are shown in Fig. 3A. Although the 5-HT EC50 values for stimulation of IP production by wild-type 5-HT2C receptors expressed in HEK cells (1.3 ± 0.2 nM) and in
S138R-expressing cells (2.3 ± 0.4 nm) were similar, 5-HT-stimulated IP production was drastically reduced in the S138R-expressing cells. Radioligand binding studies were performed to measure wild-type 5-HT_{2C} receptor expression following transfection into the S138R-expressing stable cell line using the same saturation experiments as described for the IP assay. [3H]Mesulergine saturation analyses yielded B_{max} values ranging from 4.1 to 5.0 pmol/mg of protein and K_{D} values ranging from 0.8 to 1.0 nM in three independent transfection experiments.

Additional radioligand binding studies were performed to compare wild-type 5-HT_{2C} receptor expression levels in HEK293 cells and in the S138R-expressing stable cell line. Four saturation experiments were performed in parallel: two with 0.2 μg and two with 0.5 μg of wild-type 5-HT_{2C} receptor plasmid DNA transfected into HEK293 cells or into the S138R-expressing stable cell line (Fig. 3B). B_{max} values for 0.2 μg of wild-type 5-HT_{2C} receptor plasmid DNA transfected into HEK293 and S138R-expressing stable cells were 4.9 and 4.1 pmol/mg of protein, respectively. B_{max} values for 0.5 μg of native 5-HT_{2C} receptor plasmid DNA transfected into HEK293 and S138R-expressing stable cells were 7.9 and 9.5 pmol/mg of protein, respectively. [3H]Mesulergine K_{D} values were similar in all four saturation experiments and ranged from 0.7 to 1.0 nM.

Because S138R receptors do not bind radioligand, the plasma membrane expression level of S138R receptors in the S138R 5-HT_{2C}/YFP-expressing stable cell line was determined by fluorescence microscopy. Plasma membrane YFP fluorescence intensity in the S138R-expressing stable cell line (1573 ± 52, n = 64) was similar to that in the wild-type 5-HT_{2C}/YFP-expressing stable cell line (1665 ± 50, n = 71) with a 70–80% decrease in 5-HT-stimulated [3H]IP production for native receptors expressed in the S138R-expressing stable cell line (Figs. 3A and 4A).

When expressed in HEK293 cells, wild-type 5-HT_{2C} receptors displayed moderate levels of basal and 5-HT-stimulated IP production (Fig. 4A). However, when wild-type 5-HT_{2C} receptors were expressed in the S138R-expressing stable cell line, basal IP production was eliminated, and the maximal 5-HT-stimulated IP response was dramatically reduced. In contrast, both basal and carbachol-stimulated IP production were similar in HEK293 and S138R-expressing stable cells following transfection with M1 muscarinic receptor plasmid (Fig. 4B). These results indicate that the reduced wild-type 5-HT_{2C} receptor signaling observed in the S138R-expressing stable cell line is due to decreased native receptor expression, abnormal function of the G_{q} proteins endogenously expressed in the S138R-expressing stable cell line, or sequestration of G_{q} proteins through binding to inactive S138R receptors.
Co-immunoprecipitation of S138R and Wild-type 5-HT2C Receptors—The results of the coexpression studies raised the possibility that there is an interaction between S138R and wild-type 5-HT2C receptors when they are coexpressed in the same cell. Co-immunoprecipitation and Western blot studies were performed to determine whether S138R 5-HT2C receptors associate with wild-type 5-HT2C receptors. HEK293 cells were transfected with 5-HT2C/HA and/or 5-HT2C/YFP plasmid DNA as indicated in Fig. 5. Previously, we demonstrated that 5-HT2C receptor homodimers are detergent-sensitive (12). Therefore, in these experiments, intact cells were pretreated with the bis(sulfosuccinimidyl) suberate cross-linker prior to membrane solubilization and immunoprecipitation with anti-HA antibody. When the HA-immunoprecipitated proteins were separated by PAGE, YFP immunoreactive bands were not identified in samples prepared from cells individually expressing either 5-HT2C/HA or 5-HT2C/YFP, even when the transfected cells were mixed together prior to solubilization and immunoprecipitation. However, YFP immunoreactive bands at the approximate predicted size of 5-HT2C dimers were the predominant species identified in samples prepared from cells cotransfected with wild-type 5-HT2C/HA and/or 5-HT2C/YFP or with wild-type 5-HT2C/HA and S138R 5-HT2C/YFP plasmid DNAs.

FRET between S138R and Wild-type 5-HT2C Receptors—FRET experiments were performed to determine whether S138R 5-HT2C receptors form heterodimers with wild-type 5-HT2C receptors on the plasma membrane of living cells. FRET operates on the principle that, if two fluorescent proteins (donor and acceptor) with overlapping emission and excitation spectra are within 100 Å of each other and if their dipoles are oriented appropriately, energy emitted by the donor (in its excited state) will be transferred to the acceptor (27), resulting in acceptor excitation and quenching of donor fluorescence. When the acceptor is removed by photobleaching, the donor is dequenched, and an increase in donor fluorescence is observed (25). In this study, S138R 5-HT2C/CFP was used as the donor, and wild-type 5-HT2C/YFP was used as the acceptor. A pre-bleach image of a living HEK293 cell coexpressing S138R 5-HT2C/CFP and wild-type 5-HT2C/YFP was captured (Fig. 6, A, C, and E). A region of plasma membrane (indicated by white boxes) was photobleached at 514 nm for 10 s. CFP and YFP post-bleach images were collected simultaneously following excitation at 458 nm (B, D, and F). Plasma membrane CFP fluorescence increased from 1000 to 1400 arbitrary fluorescence intensity units following YFP photobleaching (B and F), producing a FRET efficiency of 28.6%.

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**FIGURE 4.** [3H]IP production was measured in the absence (Basal) and presence of agonist (1 μM) in 2 × 10⁵ HEK293 cells and in the S138R-expressing stable cell line following transfection with 25 ng of wild-type 5-HT2C receptor (A) or M1 muscarinic receptor (B) plasmid DNA. Data represent the means ± S.E. of five to nine transfections.* p < 0.01 versus HEK293 cells.

**FIGURE 5.** Co-immunoprecipitation of wild-type and S138R 5-HT2C receptors. HEK293 cells were transfected with wild-type 5-HT2C/HA (2C/HA) or 5-HT2C/YFP (2C/YFP) cDNA or with S138R 5-HT2C/YFP cDNA (S138R/YFP) as indicated. Cells were pretreated with the bis(sulfosuccinimidyl) suberate cross-linker prior to membrane solubilization in CHAPS. All samples were immunoprecipitated (Immppt) with anti-HA antibody, and the blot was probed with horseradish peroxidase-conjugated anti-green fluorescent protein (GFP) antibody.

**FIGURE 6.** Confocal microscopy was used to visualize a 2-μm-thick optical section through the middle of a living HEK293 cell coexpressing S138R 5-HT2C/CFP (S138R/CFP; donor) and wild-type 5-HT2C/YFP (2C/YFP; acceptor). CFP fluorescence is shown in green, and YFP in red. CFP and YFP pre-bleach images were collected simultaneously following excitation at 458 nm (A, C, and E). A region of plasma membrane (indicated by white boxes) was photobleached at 514 nm for 10 s. CFP and YFP post-bleach images were collected simultaneously following excitation at 458 nm (B, D, and F). Plasma membrane CFP fluorescence increased from 1000 to 1400 arbitrary fluorescence intensity units following YFP photobleaching (B and F), producing a FRET efficiency of 28.6%.
TABLE ONE

| DNA transfected                  | % FRET | uD/A ratio | CFP fluorescence | YFP fluorescence |
|----------------------------------|--------|------------|------------------|------------------|
| S138R 5-HT2c/CFP + WT 5-HT2c/YFP | 30.5 ± 1.2 | 0.87 ± 0.05 | 1392 ± 70         | 1702 ± 79         |
| WT 5-HT2c/CFP + 5-HT2c/YFP      | 28.4 ± 1.0 | 0.90 ± 0.04 | 1499 ± 69         | 1785 ± 83         |

FRET in HEK293 cells expressing S138R and wild-type 5-HT2c receptors

FRET was measured (following acceptor photobleaching) on the plasma membrane of live cells cotransfected with S138R 5-HT2c/CFP (donor) and wild-type (WT) 5-HT2c/YFP (acceptor) or with wild-type 5-HT2c/CFP and 5-HT2c/YFP. Data represent the means ± S.E. from 65 cells.

and wild-type 5-HT2c/YFP or wild-type 5-HT2c/CFP and wild-type 5-HT2c/YFP. The mean FRET efficiencies were similar for both groups of cells (TABLE ONE). The plasma membrane fluorescence of wild-type 5-HT2c/YFP was similar regardless of whether it was coexpressed with wild-type or S138R 5-HT2c/CFP, indicating that coexpression with S138R does not influence the amount of wild-type 5-HT2c receptor expressed on the plasma membrane (TABLE ONE). Fig. 7 (A and B) shows the relationship between FRET efficiency and the donor/acceptor ratio (unquenched post-bleach donor fluorescence divided by pre-bleach acceptor fluorescence (uD/A)). FRET efficiency was dependent on the uD/A ratio, but was independent of the receptor expression level as measured by acceptor fluorescence (Fig. 7C).

Because the FRET experiments were performed in living cells, FRET was measured in cells expressing wild-type 5-HT2c/CFP alone to determine whether receptor migration within the plasma membrane during the 10-s photobleaching period could give rise to a false-positive FRET signal. 5-HT2c/CFP pre- and post-bleach plasma membrane fluorescence intensities were measured in 14 living cells, and the mean FRET efficiency was calculated in the same manner as described for cells coexpressing CFP- and YFP-tagged receptors. For cells expressing only 5-HT2c/CFP, the mean FRET efficiency was 0.14 ± 0.22%, indicating that receptor migration within the plasma membrane during the short photobleach period does not give rise to a false-positive FRET signal.

**DISCUSSION**

This study was performed to determine whether dimerization plays a functional role in regulating the activity of 5-HT receptors and to determine the ligand/dimer/G-protein stoichiometry. We addressed these issues by examining the function of wild-type 5-HT2c receptors when coexpressed with an inactive form of the receptor. Mutation of Ser138 to Arg eliminated [3H]mesulergine binding and IP production. Time-lapse confocal fluorescence imaging of HEK293 cells expressing S138R receptors fused to YFP revealed a predominantly plasma membrane localization. In contrast to wild-type 5-HT2c receptors, the S138R receptors displayed minimal constitutive receptor cycling, and 5-HT did not promote internalization of the S138R receptors. When 5-HT was added to cells coexpressing S138R and wild-type 5-HT2c receptors, intracellular trafficking of wild-type receptors was diminished, and S138R receptors remained on the plasma membrane. These results suggest that, in the presence of S138R receptors, wild-type 5-HT2c receptors lose the ability to respond to 5-HT stimulation and appear to be trapped on the plasma membrane.

IP production was measured in cells coexpressing S138R and wild-type 5-HT2c receptors to test the hypothesis that wild-type 5-HT2c receptor function is compromised in the presence of S138R receptors. When wild-type 5-HT2c receptors were expressed in the S138R-expressing stable cell line, both basal and 5-HT-stimulated IP production were greatly reduced, with little change in 5-HT potency. Coexpression of S138R and wild-type 5-HT2c receptors did not decrease wild-type receptor expression on the plasma membrane, as demonstrated by confocal fluorescence microscopy and radioligand binding, indicating that the decrease in IP production observed for wild-type receptors following expression in the S138R-expressing cell line is not due to a decrease in the amount of wild-type receptor expressed on the plasma membrane. In addition, M1 muscarinic receptors exhibited normal IP signaling in the S138R-expressing cell line. These results indicate that Goq proteins in the S138R-expressing cell line function normally and that the mutant S138R receptors do not sequester Goq proteins, in contrast to studies with mutant α1-adrenergic receptors that exhibit a dominant-negative effect on native receptor function through G-protein sequestration (17).

FRET (measured by acceptor photobleaching) demonstrated coex-
Ligand/Dimer/G-protein Stoichiometry

expression of both S138R and wild-type 5-HT$_{2C}$ receptors in close proximity on the plasma membrane of living cells. Laser scanning confocal microscopy allowed the photobleaching to be confined to a small region of the plasma membrane, thereby minimizing the time required for photobleaching and making the technique suitable for living cells. A positive FRET signal resulting from random proximity (overexpression) of donor and acceptor has been reported to be dependent on the amount of acceptor expressed on the plasma membrane, whereas FRET produced by clustered proteins (such as dimers or oligomers) is predicted to be independent of acceptor expression levels and dependent on the uD/A ratio (28, 29). FRET efficiencies measured in cells coexpressing S138R and wild-type 5-HT$_{2C}$ receptors were independent of the acceptor expression level and dependent on the uD/A ratio, as predicted for receptors in a clustered distribution. These results indicate that S138R receptors form heterodimers with wild-type 5-HT$_{2C}$ receptors when they are coexpressed in the same cell.

The results of the immunoprecipitation and FRET studies suggest that S138R receptors decrease the function of wild-type 5-HT$_{2C}$ receptors by forming inactive heterodimers that are expressed on the plasma membrane. As a result, the effective concentration of active wild-type receptor homodimers is reduced, resulting in decreased IP signaling. Therefore, heterodimerization between inactive and active GPCRs can have a dominant-negative effect on receptor function when the heterodimers are expressed on the plasma membrane. This is in contrast to previous studies demonstrating a dominant-negative effect of inactive splice variants or mutant receptors on wild-type receptor function through heterodimerization, resulting in trapping of the wild-type receptors in the endoplasmic reticulum (13–16). Although our study examined S138R and wild-type 5-HT$_{2C}$ receptor interactions only on the plasma membrane, it is possible that the heterodimers form in the endoplasmic reticulum and are transported to the plasma membrane, as described previously for other GPCRs (16, 30).

GPCR activation has been reported to involve the coordinated movements of TMDs III and VI (17, 31–33). The S138R mutation is located in TMD III of the 5-HT$_{2C}$ receptor. As a result, this mutation may directly interfere with the ability of TMD III to adopt the proper conformation required for an active state of the receptor. This is supported by the observation that the S138R mutation eliminates receptor basal activity. This mutation does not appear to alter the ability of 5-HT$_{2C}$ receptors to dimerize, as similar FRET efficiencies were observed for homodimerization of wild-type receptors and heterodimerization between S138R and wild-type receptors. Presently, the site(s) of interaction between 5-HT receptor dimers/oligomers remains unknown. Previous studies using cysteine cross-linking and mutagenesis have provided evidence for the involvement of TMD IV in the formation of D$_2$-dopamine receptor homodimers (34, 35), and atomic force microscopy has provided evidence for rhodopsin receptor homodimer interfaces at TMDs IV and V (21, 36). Studies of the $\alpha_{1A}$-adrenergic receptor suggest that the homodimerization interface for this receptor may involve TMDs I and IV (37, 38). FRET experiments performed using chimeric $\alpha_{1A}/\beta_2$-adrenergic receptors suggest that TMD III does not play a role in $\alpha_{1A}$-adrenergic receptor homodimerization (37). These findings are consistent with the results of our study, in which the S138R mutation in TMD III did not appear to alter 5-HT$_{2C}$ receptor dimerization.

In our experiments, the S138R receptor was incapable of adopting the appropriate active conformation necessary to generate basal or agonist-stimulated IP production. Our observation that coexpression of S138R and wild-type 5-HT$_{2C}$ receptors eliminated basal activity and greatly diminished agonist-stimulated IP production is consistent with a model in which a single G-protein interacts with one GPCR dimer (Fig. 8). In this model, the S138R receptor in the wild-type/S138R 5-HT$_{2C}$ receptor heterodimer is not capable of forming the complementary G-protein interaction required for heterodimer activation of a single G-protein. Thus, the heterodimer remains silent with respect to G-protein activation and IP signaling.

If two G-proteins bound to each dimer (in other words, one G-protein/receptor monomer), then equal wild-type 5-HT$_{2C}$ receptor expression levels in the HEK293 and S138R-expressing stable cells would be predicted to result in equal IP production. However, IP signaling was 70–80% lower when wild-type 5-HT$_{2C}$ receptors were expressed in the S138R-expressing stable cell line. Expression of wild-type 5-HT$_{2C}$ receptors in the S138R-expressing stable cell line resulted in a plasma membrane receptor expression ratio of 1:2 for wild-type/S138R 5-HT$_{2C}$ receptors, predicting that ~80% of the wild-type 5-HT$_{2C}$ receptors would be engaged as heterodimers with S138R receptors. This corresponds to the 70–80% decrease in IP signaling observed when wild-type 5-HT$_{2C}$ receptors were expressed in the S138R-expressing stable cell line, consistent with the wild-type/S138R 5-HT$_{2C}$ receptor heterodimers being inactive with respect to G-protein coupling and signaling. If the wild-type/S138R 5-HT$_{2C}$ receptor heterodimers exhibited impaired G-protein coupling or activation, then the 5-HT dose-response curve for IP production would be shifted to the right, with 5-HT displaying lower potency upon expression of wild-type 5-HT$_{2C}$ receptors in the S138R-expressing stable cell line. However, 5-HT potency for IP stimulation was the same when wild-type 5-HT$_{2C}$ receptors were expressed in HEK293 cells or in the S138R-expressing stable cell line. These results indicate that the heterodimers are inactive with respect to G-protein coupling and signaling and are consistent with the model in Fig. 8, showing a receptor dimer/G-protein stoichiometry of 1:1.

This model is consistent with studies based on the crystalline structure of rhodopsin and atomic force microscopy studies suggesting that rhodopsin receptor dimerization may be necessary to form a G-protein-binding site that is large enough to accommodate a single heterotrimeric G-protein (21, 36). In addition, studies using chemical cross-linking and purified leukotriene B$_4$ receptors indicate that one G-protein associates with each leukotriene B$_4$ receptor homodimer (22). These results are supported by functional studies showing that D$_1$/D$_2$-dopamine receptor heterodimers can form a novel signaling complex capable of stimulating a different G protein than D$_1$- or D$_2$-dopamine receptor homodimers (39).

Given that the S138R receptor is incapable of binding ligand, if wild-type/S138R 5-HT$_{2C}$ receptor heterodimers formed a single binding
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In conclusion, Ser138 plays a critical role in ligand binding and the ability of the 5-HT$_2C$ receptor to adopt the active conformation required for G-protein activation. Plasma membrane expression of an inactive mutant receptor with wild-type receptors can have a dominant-negative effect on the function of the wild-type receptors, indicating that dimerization is essential for 5-HT receptor function. These results are consistent with a model in which one GPCR dimer binds two molecules of ligand, resulting in the binding and activation of one G-protein. Studies with the inactive S138R receptor suggest that dimerization may be independent of the active state of the 5-HT$_2C$ receptor. Additional studies comparing dimerization of 5-HT$_2C$ receptors with high basal activity and no basal activity (INI and VGV isoforms, respectively) are in progress to determine the relationship between dimer formation and the active state of the 5-HT$_2C$ receptor.

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