Biochemical and Biophysical Characterization of Serotonin 5-HT_{2C} Receptor Homodimers on the Plasma Membrane of Living Cells†

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ABSTRACT: While many studies have provided evidence of homodimerization and heterodimerization of G-protein-coupled receptors (GPCRs), few studies have used fluorescence resonance energy transfer (FRET) combined with confocal microscopy to visualize receptor dimerization on the plasma membrane, and there have been no reports demonstrating the expression of serotonin receptor dimers/oligomers on the plasma membrane of living cells. In the study presented here, biochemical and biophysical techniques were used to determine if 5-HT_{2C} receptors exist as homodimers on the plasma membrane of living cells. Immunoprecipitation followed by Western blotting revealed the presence of immunoreactive bands the predicted size of 5-HT_{2C} receptor monomers and homodimers that were detergent and cross-linker sensitive. Bioluminescence resonance energy transfer (BRET) was assessed in HEK293 cells expressing 5-HT_{2C} receptors labeled with Renilla luciferase and yellow fluorescent protein. BRET levels were not altered by pretreatment with serotonin. Confocal microscopy provided direct visualization of FRET on the plasma membrane of live cells expressing 5-HT_{2C} receptors labeled with cyan (donor) and yellow (acceptor) fluorescent proteins. FRET, assessed by acceptor photobleaching, was dependent on the donor/acceptor ratio and independent of acceptor expression levels, indicating that FRET resulted from receptor clustering and not from overexpression of randomly distributed receptors, providing evidence for GPCR dimers/oligomers in a clustered distribution on the plasma membrane. The results of this study suggest that 5-HT_{2C} receptors exist as constitutive homodimers on the plasma membrane of living cells. In addition, a confocal-based FRET method for monitoring receptor dimerization directly on the plasma membrane of living cells is described.

G-Protein-coupled receptor (GPCR)[1] dimerization is an emerging area of research in which molecular mechanisms involved in receptor activation are investigated. Early studies examining GPCR dimerization using membrane solubilization and Western blotting were criticized because of the potential for nonspecific protein aggregation during membrane preparation and solubilization. In addition, the issue was complicated by the fact that many of the higher-molecular weight bands observed on the Western blots, presumed to be receptor dimers/oligomers, were insensitive to detergents and reducing agents. However, subsequent studies using co-immunoprecipitation (*1*−*5*) and biophysical techniques (*6*−*12*) have provided stronger evidence of the existence of GPCR dimers/oligomers. There is evidence supporting functional roles for GPCR dimerization/oligomerization in the regulation of ligand binding, receptor signaling, and receptor trafficking (*13*−*20*).

Fourteen different serotonin (5-HT) receptors have been identified, and they represent targets for drugs used to treat anxiety, depression, schizophrenia, obesity, and other disorders (reviewed in refs *21* and *22*). If 5-HT receptors function as oligomeric complexes, it could have important implications for future drug design and development. The first evidence suggesting that 5-HT receptors may form homodimers was provided by Western blots of a solubilized membrane protein from Sf9 insect cells expressing 5-HT_{1B} or 5-HT_{1D} receptors (*23*, *24*). In a subsequent study, co-immunoprecipitation of differentially tagged 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors suggested that 5-HT_1 receptors may form heterodimers (*25*). To date, there have been no reports demonstrating the presence of 5-HT receptor dimers/oligomers on the plasma membrane of living cells.

This study was performed to determine if 5-HT_{2C} receptors form homodimers, to determine the physical nature of the protein−protein interaction, and to determine if homodimers are present on the plasma membrane of living cells. A combination of biochemical (immunoprecipitation and Western blot) and biophysical (bioluminescence and fluorescence resonance energy transfer, BRET and FRET, respectively) techniques were used to address this issue. While each technique has its own limitations, the use of all three techniques provides the best available combination for evaluating GPCR dimerization/oligomerization. Immuno-

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[1] Abbreviations: 5-HT, serotonin; GPCR, G-protein-coupled receptor; FRET, fluorescence resonance energy transfer; BRET, bioluminescence energy transfer.

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precipitation provides evidence for protein−protein interactions; BRET has an advantage in that it can be performed in intact living cells and does not require the use of an external excitation source such as a laser, and FRET combined with confocal microscopy can be used to determine the exact cellular location of the FRET signal.

Several studies have used FRET as an indicator of GPCR dimerization (*7*−*9*, *26*−*30*). However, few studies have combined FRET with confocal microscopy to demonstrate a plasma membrane localization of the FRET signal as evidence of GPCR dimerization on the plasma membrane of live cells. In addition, these studies have not demonstrated that the observed FRET efficiency is dependent on the donor/acceptor ratio and independent of the acceptor expression level, two conditions that should be satisfied before one could conclude that the FRET signal originates from receptor clustering (dimers/oligomers) and not from randomly distributed receptors densely packed in the proximity of one another as a result of receptor overexpression. This study examines the relationship between FRET efficiency, donor/acceptor ratio, and acceptor expression, providing evidence for GPCR dimers/oligomers in a clustered distribution on the plasma membrane. A confocal-based FRET method that can be used to monitor GPCR dimerization on the plasma membrane of living cells is described. This method provides evidence of 5-HT_{2C} receptor homodimerization and can be used for direct visualization of FRET occurring between GPCR dimers/oligomers on the plasma membrane of living cells.

MATERIALS AND METHODS

5-HT_{2C} Receptor Fusion Proteins. YFP, CFP, and Rluc (Renilla luciferase) fusion proteins were created using cDNAs for the human 5-HT_{2C-INI} receptor (from E. Sanders-Bush), the β_2-adrenergic receptor (from R. Lefkowitz), and the M_4-muscarinic receptor (PCR amplified from genomic DNA). For the YFP and CFP constructs, cDNAs were PCR amplified with a 5′ primer containing an EcoRI site followed by the first 20 nucleotides of the open reading frame, and a 3′ primer complementary to the last 25 nucleotides of the open reading frame with a BamHI site in place of the stop codon. Following TA cloning (Invitrogen) and miniprep (Qiagen), plasmid DNA was digested with EcoRI and BamHI and ligated into pEYFP or pECFP (Clonetech) upstream and in frame with the fluorescent protein so that the fluorescent protein would be expressed on the C-terminus of the receptor. For Rluc fusion proteins, cDNAs were PCR amplified with a 5′ primer containing an NheI site followed by a kozak sequence and the first 19 nucleotides of the open reading frame, and a 3′ primer complementary to the last 11 nucleotides of the open reading frame with an NheI site in place of the stop codon followed by 11 bases of 3′ UTR. Following TA cloning and miniprep, plasmid DNA was digested with NheI and ligated into pRluc (Promega) in frame and upstream of the Renilla luciferase gene. The 5-HT_{2C}−HA fusion protein was made by PCR using the same 5′ primer that was used for the CFP and YFP fusion proteins along with a 3′ primer to remove the stop codon of the 5-HT_{2C} receptor and add the 10-amino acid sequence for the HA tag to the C-terminal end of the receptor. DNA sequencing was performed to verify the accuracy of all fusion constructs created for this study.

Cell Culture. HEK293 cells from ATCC were cultured in DMEM (Cellgro) with 10% fetal bovine serum (Cellgro) at 37 °C in 5% CO_2. A 5-HT_{2C} stable cell line was created by transfecting 10^6 HEK293 cells with 5 μg of 5-HT_{2C}−YFP plasmid DNA (linearized with ALWN1) by calcium phosphate precipitation. Transfected colonies were identified by fluorescence microscopy and characterized by radioligand binding and inositol phosphate production. For radioligand binding, immunoprecipitation, BRET, and FRET experiments, HEK293 cells were plated at a density of 2×10^6 cells per 100 mm dish 24 h prior to transfection. Cells were transfected using lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. All experiments were performed 36−42 h post-transfection, and cells were cultured in serum-free medium for the final 18 h prior to the experiment.

Radioligand Binding. Membranes were prepared from transfected HEK293 cells, and 1 nM [^{3}H]mesulergine was used to label 5-HT_{2C} receptors as previously described (*31*). Competition experiments were performed by incubating membranes (10 μg of protein), 1 nM [^{3}H]mesulergine, and 5-HT (1 nM to 10 μM) in 0.5 mL of buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, 0.5 mM EDTA, and 0.1% ascorbate) for 30 min at 37 °C followed by filtration through glass fiber filters (Schleicher & Schuell) on a brandel cell harvester and liquid scintillation counting in Ecoscint cocktail (National Diagnostics). Data were analyzed using graphpad prism software.

Inositol Phosphate Assay. For inositol phosphate (IP) assays, cells were seeded at a density of 2×10^5 cells/well in 24-well plates (Biocoat) and transfected 3 h later with 50 ng of plasmid DNA and 1 μL of lipofectamine per well. Twenty-four hours post-transfection, 0.5 μCi of [^{3}H]myo-inositol in inositol-free, serum-free DMEM was added to each well. Total [^{3}H]IP production was measured by anion exchange chromatography as previously described (*31*). Data were analyzed using graphpad prism software.

Membrane Preparation and Western Blot. A confluent 100 mm dish of the HEK293 stable cell line expressing the human 5-HT_{2C-INI}−YFP receptor was washed with PBS, and cells were lifted with a cell scraper in 2 mL of PBS and centrifuged at 1500*g* for 5 min. For studies using the BS^3 cross-linker (Pierce), intact cells were incubated in a 5 mM BS^3/PBS solution for 30 min at 37 °C in 5% CO_2 prior to lifting and centrifugation. Cells were resuspended in 0.5 mL of lysis buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 10 μL of protease inhibitor cocktail (Sigma)], sonicated for 30 s on ice, and centrifuged at 1500*g* for 5 min. The supernatant was centrifuged at 21000*g* for 30 min at 4 °C. The membrane pellet was resuspended in 0.2 mL of solubilization buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 150 mM NaCl, 10 mM iodoacetamide, and 5 μL of protease inhibitor cocktail] containing one of the following detergents: 10 mM CHAPS, 1% Triton X-100, RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), or 1% SDS. Cells were solubilized for 60 min on ice and centrifuged at 21000*g* for 30 min at 4 °C. The supernatant (10 μL, approximately 5 μg of protein determined by BCA, Pierce) was combined with 10 μL of 2× nonreducing Laemmli sample buffer (*32*), heated at 70 °C for 10 min, and run on a 10% Tris-HCl ready gel (Bio-Rad) at 100 V for 70 min. Gels were transferred to nitrocellulose (Nitrobind,

Osmonics), probed with GFP(B-2)-HRP antibody (Santa Cruz) diluted 1/3000 or HA(Y-11)-HRP (Santa Cruz) diluted 1/4000, and visualized by enhanced chemiluminescence (Amersham).

Immunoprecipitation. HEK293 cells (4×10^6 cells/100 mm dish) were transfected with 1 μg of 5-HT$_{2C-INI}$−YFP and/or 1 μg of HA−5-HT$_{2C-INI}$ plasmid DNA using 20 μL of lipofectamine. For control experiments, cells were transfected independently with either 5-HT$_{2C-INI}$−YFP or HA−5-HT$_{2C-INI}$ fusion protein and mixed after transfection. Cross-linking of receptors was performed by incubating intact cells in a 5 mM BS^3/PBS solution for 30 min at 37 °C in 5% CO_2 prior to lifting and centrifugation. Cells were lysed, and membranes were solubilized in 10 mM CHAPS as described above. Solubilized membrane proteins were immunoprecipitated overnight with 10 μL of HA(Y-11)-agarose (Santa Cruz) on a rocker platform at 4 °C. Samples were centrifuged at 4000*g* for 5 min; the pellet was washed twice with PBS containing protease inhibitor cocktail, resuspended in 50 μL of 1× nonreducing Laemmli sample buffer, and heated at 70 °C for 10 min, and 10 μL was analyzed by Western blot as described above.

Bioluminescence Resonance Energy Transfer (BRET). HEK293 cells (4×10^6 cells/100 mm dish) were cotransfected with 1 μg of the donor Rluc fusion protein (5-HT$_{2C}$−Rluc or β_2−Rluc) and 3 μg of the acceptor YFP fusion protein (5-HT$_{2C}$−YFP, β_2−YFP, or M$_4$−YFP) using 20 μL of lipofectamine. Twenty-four hours post-transfection, cells were cultured in serum-free medium overnight prior to the BRET assay. Cells were washed in PBS, lifted in 1 mL of PBS/EDTA, and placed in a cuvette with 5 μM coelenterazine f (Molecular Probes). For experiments involving 5-HT, cells were pretreated with 1 μM 5-HT for 10 min at 37 °C prior to lifting. Emission spectra were collected using a Perkin-Elmer LS-50B luminescence spectrophotometer. Three consecutive emission spectra (400−600 nm) were collected at 1 min intervals immediately following the addition of coelenterazine f. BRET ratios were calculated from the emission spectra using the equation [(emission at 510−590 nm − emission at 440−500 nm) × cf]/(emission at 440−500 nm), where cf = emission at 510−590 nm − emission at 440−500 nm for 5-HT$_{2C}$−Rluc+pcDNA3 control, as previously described (*6*).

Fluorescence Resonance Energy Transfer (FRET). HEK293 cells (4×10^6 cells/100 mm dish) were cotransfected with 0.2 μg of the donor CFP fusion protein (5-HT$_{2C}$−CFP) and 0.4 μg of the acceptor YFP fusion protein (5-HT$_{2C}$−YFP) using 20 μL of lipofectamine. This typically resulted in 5-HT$_{2C}$ receptor expression levels of approximately 5 pmol/mg of protein measured by [^{3}H]mesulergine binding, similar to endogenous receptor expression levels in the choroid plexus (*33*). Twenty-four hours post-transfection, cells were plated in serum-free medium on polylysine-coated glass cover slips overnight prior to the FRET assay. The cells were viewed live in phosphate-buffered saline using a Zeiss LSM-510 META confocal imaging system with a 30 mW argon laser and 63 × 1.4 NA oil immersion objective (2× zoom). All confocal imaging experiments were performed at 20 °C. Cells expressing 5-HT$_{2C}$−CFP or 5-HT$_{2C}$−YFP protein were imaged using the Zeiss META detector with the 458 nm laser line over an emission wavelength range from 462.9 to 602 nm, using bandwidths of 10.7 nm (pinhole of 1.32 Airy units and a *Z* resolution of 2 μm) to generate a λ stack (*34*). These spectra served as CFP and YFP reference emission signatures and were used in a linear unmixing algorithm (Zeiss AIM software) to separate the fluorescence contribution of CFP and YFP (on a pixel by pixel basis) in images of cells coexpressing 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP protein. FRET assessed by acceptor photobleaching (*35*) combined with linear unmixing of CFP and YFP emission spectra using the Zeiss META detector (*34*, *36*) eliminates the need for filters to separate CFP and YFP emission spectra, and eliminates spectral cross talk and bleed through which contribute to false FRET signals. Confocal microscopy was used to visualize a 2 μm optical slice through the middle of a live HEK293 cell expressing both 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP protein. Prebleach CFP and YFP images were collected (at 12 bit resolution over 512 × 512 pixels with a pixel dwell time of 6.4 μs) using the argon laser with a 458 nm/514 nm dual dichroic. A selected region of interest (ROI) on the plasma membrane was irradiated with the 514 nm laser line (100% intensity) for 30 s (60 iterations) to photobleach YFP. Postbleach images were captured immediately following YFP photobleaching. FRET was assessed as an increase in CFP fluorescence intensity (donor dequenching) following YFP (acceptor) photobleaching. FRET efficiency was calculated using the equation 100(CFP postbleach − CFP prebleach)/(CFP postbleach). FRET efficiencies were calculated in cells expressing both CFP and YFP, in cells expressing CFP alone, and in nonbleached regions of the cell membrane as a control.

Table 1: 5-HT Affinity and Potency for 5-HT$_{2C}$ Receptor Fusion Proteins[a]

transfection	K_i (nM)	EC_{50} (nM)
5-HT$_{2C}$	111 ± 23	2.8 ± 0.9
5-HT$_{2C}$−YFP	118 ± 20	2.4 ± 0.5
5-HT$_{2C}$−HA	140 ± 14	3.0 ± 1.0
5-HT$_{2C}$−Rluc	122 ± 14	2.4 ± 0.5

[a] Radioligand binding and IP assays were performed in HEK293 cells transfected with the indicated cDNAs. [^{3}H]Mesulergine (1 nM) was used to label 5-HT$_{2C}$ receptors. Data represent the mean ± the standard deviation from three to five experiments, each performed in triplicate.

RESULTS

5-HT$_{2C}$ receptor fusion proteins were created for use in immunoprecipitation, BRET, and FRET experiments designed to determine if 5-HT$_{2C}$ receptors are present as homodimers or oligomers on the cell membrane of living cells. Each fusion protein was evaluated for functionality following expression in HEK293 cells using radioligand binding and inositol phosphate (IP) production assays. The presence of the YFP, HA, or Rluc tags did not alter the binding affinity of 5-HT for [^{3}H]mesulergine-labeled 5-HT$_{2C}$ receptors and did not alter 5-HT potency for stimulating IP production (Table 1).

For the Western blot studies, an HEK293 stable cell line expressing the 5-HT$_{2C}$−YFP fusion protein was created and tested for functionality as described above (data not shown). The 5-HT$_{2C}$ receptor has an approximate mass of 45−60 kDa depending on the degree of glycosylation (*37*). Therefore, the 5-HT$_{2C}$−YFP fusion protein would be predicted to have an approximate mass of 75−90 kDa. Cell membranes were

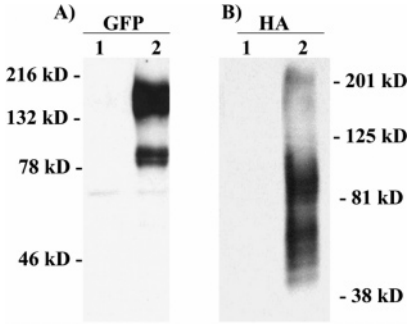

FIGURE 1: Western blots of solubilized 5-HT_{2C} receptors. (A) CHAPS soluble membrane proteins from untransfected HEK293 cells (lane 1) and from a 5-HT_{2C}−YFP HEK293 stable cell line (lane 2) were evaluated via 10% PAGE and probed with the GFP antibody. (B) CHAPS soluble membrane proteins from untransfected HEK293 cells (lane 1) and from cells transfected with 5-HT_{2C}−HA (lane 2) were evaluated via 7.5% PAGE and probed with the HA antibody.

FIGURE 2: Detergent and cross-linker sensitivity of 5-HT_{2C} receptors. Cell membranes from a 5-HT_{2C}−YFP HEK293 stable cell line were solubilized with different detergents as indicated (+), evaluated via 10% PAGE, and probed with the GFP antibody: lanes 1−4, 5-HT_{2C}−YFP monomers (80−90 kDa) and dimers (160−180 kDa); and lane 5, cells pretreated with the BS^3 cross-linker prior to solubilization in CHAPS.

prepared from the 5-HT_{2C}−YFP stable cell line and from untransfected cells. Membrane proteins were solubilized in 10 mM CHAPS and run on a 10% polyacrylamide gel under nonreducing conditions, and the blot was probed with the GFP antibody. Immunoreactive bands the approximate size of 5-HT_{2C}−YFP monomers are visible, along with higher-molecular weight (MW) bands which are the approximate predicted size of 5-HT_{2C}−YFP dimers (Figure 1A). A similar experiment was performed using solubilized membrane proteins from HEK293 cells transfected with the 5-HT_{2C}−HA fusion protein (45−60 kDa). Following separation on a 7.5% gel, HA immunoreactive bands at approximately 50 and 100 kDa, and a very faint band at 200 kDa, were observed in samples prepared from transfected cells but not from untransfected cells (Figure 1B). The immunoreactive bands are the approximate predicted sizes for 5-HT_{2C}−HA receptor monomers, dimers, and tetramers.

Cell membranes were prepared from the 5-HT_{2C}−YFP stable cell line, solubilized using different detergents, and analyzed by Western blotting probed with the GFP antibody (Figure 2). All samples were prepared in the presence of 10 mM iodoacetamide to prevent disulfide interchange during cell membrane preparation. As shown in Figure 2, immunoreactive bands the approximate size of 5-HT_{2C}−YFP monomers are visible, along with higher-MW bands which are the approximate predicted size of 5-HT_{2C}−YFP dimers,

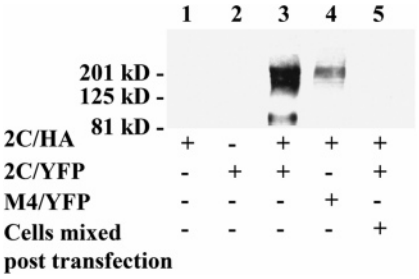

FIGURE 3: Co-immunoprecipitation of 5-HT_{2C} receptors. HEK293 cells were transfected with 5-HT_{2C}−HA and 5-HT_{2C}−YFP or M_4-muscarinic−YFP cDNAs as indicated (+). Cells were pretreated with the BS^3 cross-linker prior to membrane solubilization in CHAPS. All samples were immunoprecipitated with the HA antibody, evaluated via 10% PAGE, and probed with the GFP antibody.

when membrane proteins were solubilized using 10 mM CHAPS. When the detergent stringency was increased, by solubilization in 1% Triton X-100, RIPA, or 1% SDS, the monomeric species was the predominant form identified on the blot. No immunoreactive bands were detected in untransfected cells. All samples shown in Figure 2 were run under nonreducing conditions. The addition of 10 mM DTT or 1% β-mercaptoethanol to samples solubilized with CHAPS did not reduce the higher-MW immunoreactive bands (data not shown). These results indicate that the higher-MW immunoreactive bands are detergent sensitive and do not contain disulfide bonds. When intact cells were pretreated for 30 min with 5 mM BS^3 (a membrane impermeable protein cross-linker), prior to cell lysis and solubilization, the higher-MW immunoreactive species was the predominant form observed on the blot (Figure 2).

Co-immunoprecipitation studies were performed to determine if the higher-MW immunoreactive bands observed in Figure 2 may be the result of a protein−protein interaction between 5-HT_{2C} receptors expressed on the cell membrane. For these experiments, HEK293 cells were transfected with 5-HT_{2C}−HA and/or 5-HT_{2C}−YFP protein. The transfected cells were pretreated with 5 mM BS^3 prior to cell membrane preparation and solubilization in 10 mM CHAPS, as described above. CHAPS soluble membrane proteins were immunoprecipitated with the HA antibody conjugated to agarose, run under nonreducing conditions on 10% PAGE, and probed with GFP antibody (Figure 3). No immunoreactive bands were observed in samples prepared from cells transfected with 5-HT_{2C}−HA or 5-HT_{2C}−YFP. However, immunoreactive bands the approximate predicted size of 5-HT_{2C}−HA:5-HT_{2C}−YFP dimers were visible in samples prepared from cells cotransfected with 5-HT_{2C}−HA and 5-HT_{2C}−YFP. A faint immunoreactive band was observed in samples prepared from cells cotransfected with 5-HT_{2C}−HA and M_4-muscarinic−YFP, possibly indicating a weak interaction between 5-HT_{2C} receptors and M_4-muscarinic receptors. More importantly, no immunoreactive bands were observed when cells were transfected with 5-HT_{2C}−HA or 5-HT_{2C}−YFP and then mixed prior to solubilization and immunoprecipitation (Figure 3).

Since the immunoprecipitation and Western blot studies suggest that 5-HT_{2C} receptors may form homodimers when expressed in a recombinant cell system, bioluminescence resonance energy transfer (BRET) experiments were performed to determine if similar results could be obtained in

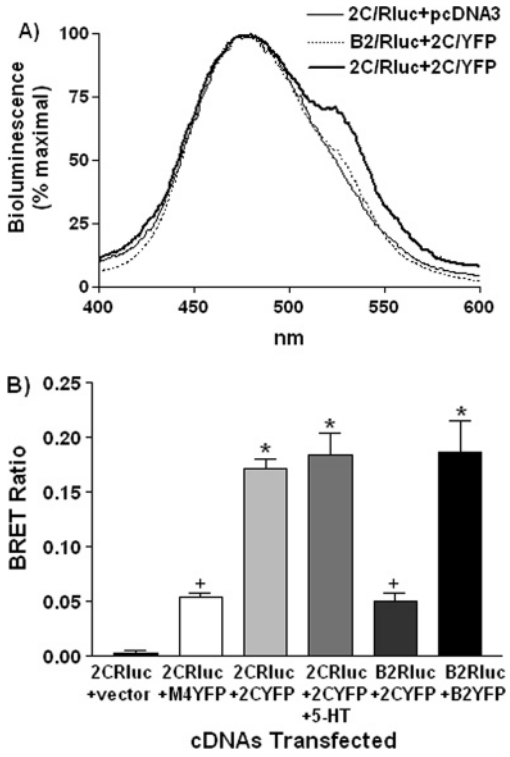

FIGURE 4: 5-HT$_{2C}$ receptor BRET. HEK293 cells were transfected with 5-HT$_{2C}$ (2C), β_2-adrenergic (B2), or M$_4$-muscarinic (M4) receptors tagged with Renilla luciferase (Rluc) and/or YFP as indicated. Coelenterazine f (5 μM) was added to transfected cells, and emission spectra were collected using a luminescence spectrophotometer. (A) When BRET occurs, the acceptor (YFP) emission spectrum (λ_{max} = 525 nm) appears in addition to the donor (Rluc) emission spectrum (λ_{max} = 475 nm). Data represent the mean of three independent experiments. (B) BRET ratios were calculated for HEK293 cells expressing the indicated cDNAs. The designation +5-HT indicates cells were pretreated with 1 μM 5-HT for 10 min. Data represent the mean ± the standard error of the mean of four to six experiments. (+) $p < 0.01$ vs 2C−Rluc and pcDNA3. (*) $p < 0.01$ vs 2C−Rluc with M4−YFP and B2−Rluc with 2C−YFP.

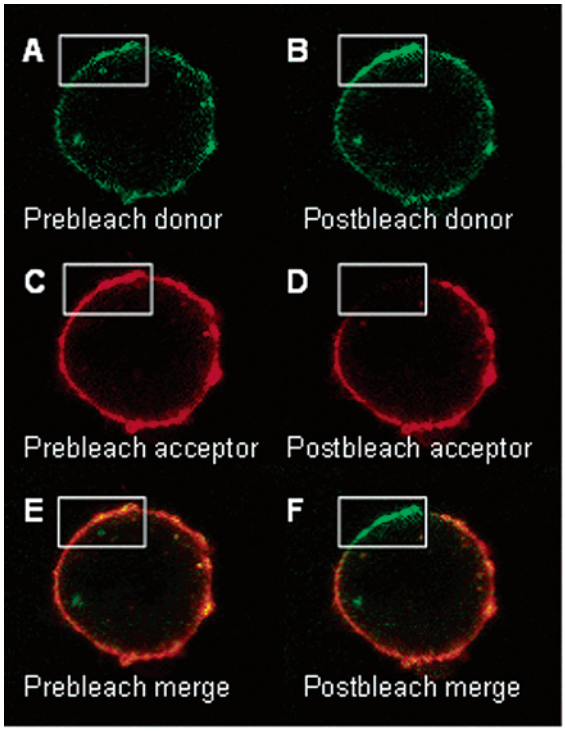

FIGURE 5: 5-HT$_{2C}$ receptor FRET. Confocal microscopy was used to visualize a 2 μm optical slice through the middle of a live HEK293 cell coexpressing 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP. CFP and YFP were excited with an argon laser using a 458 nm/514 nm dual dichroic. Linear unmixing of CFP and YFP emission spectra was performed using the Zeiss LSM-510 META detector. CFP fluorescence (donor) appears green and YFP fluorescence (acceptor) red. A region of plasma membrane was selected (white rectangle), and prebleach images were collected (A, C, and E). YFP fluorescence was photobleached by scanning with a 514 nm laser for 30 s, and postbleach images were collected (B, D, and F). An increase in plasma membrane donor fluorescence following acceptor photobleaching is shown in panel B.

intact living cells. If two proteins, a bioluminescent donor and a fluorescent acceptor, with overlapping emission and excitation spectra are within 1−10 nm of each other with their dipoles appropriately oriented, light emitted from the donor (in its excited state) will be transferred to the acceptor, resulting in acceptor excitation and the appearance of the acceptor emission spectra (*38*). For our BRET experiments, Renilla luciferase (Rluc) expressed as a fusion protein on the C-terminus of the 5-HT$_{2C}$ receptor was used as the donor and YFP expressed as a fusion protein on the C-terminus of the 5-HT$_{2C}$ receptor served as the acceptor. In addition, control experiments were performed using Rluc and YFP fusion proteins made with the M$_4$-muscarinic and β_2-adrenergic receptors. HEK293 cells were transfected with the donor/acceptor pairs as indicated in Figure 4. In the presence of Rluc and oxygen, coelenterazine f is converted to coelenteramide, producing a bioluminescence emission spectra with a λ_{max} of 475 nm. When HEK293 cells were cotransfected with 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP and mixed with coelenterazine f, the characteristic emission spectra of YFP (λ_{max} = 525 nm) were observed, indicating that BRET had occurred between 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP (Figure 4A). BRET ratios were calculated for cells cotransfected with different combinations of Rluc and YFP donor/acceptor pairs as indicated in Figure 4B. Coexpression of 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP produced a significant increase in the BRET ratio over cells transfected with 5-HT$_{2C}$−Rluc and pcDNA$_3$ vector, or 5-HT$_{2C}$−Rluc and M$_4$-muscarinic−YFP. Pretreatment with 1 μM 5-HT did not produce a significant change in the BRET ratio. BRET ratios obtained from cells cotransfected with β_2-adrenergic−Rluc and 5-HT$_{2C}$−YFP were one-third of the BRET ratios obtained from cells cotransfected with 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP (Figure 4B). BRET ratios obtained from cells cotransfected with β_2-adrenergic−Rluc and β_2-adrenergic−YFP were not significantly different from BRET ratios obtained for cells cotransfected with 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP.

Fluorescence resonance energy transfer (FRET) experiments were performed to determine if 5-HT$_{2C}$ receptor homodimers could be visualized on the plasma membrane of living cells. FRET operates on the same principle as BRET, with the exception that FRET uses a fluorescent donor excited by an external laser. In this study, 5-HT$_{2C}$−CFP was used as the donor and 5-HT$_{2C}$−YFP was used as the acceptor. FRET was assessed by acceptor photobleaching (*35*). HEK293 cells were transfected with donor and acceptor plasmids, in a 1/2 ratio, and viewed live in phosphate-buffered saline. Prebleach CFP and YFP images were collected (Figure 5A,C,E). For better color contrast and visualization, the CFP fluorescence (donor) is shown as green

Table 2: 5-HT_{2C} Receptor FRET Efficiency[a]

transfection	% FRET	D/A	*n*
5-HT_{2C}−CFP and 5-HT_{2C}−YFP	18.6 ± 1.4	0.80 ± 0.04	30
5-HT_{2C}−CFP and M4−YFP	5.1 ± 1.4	0.88 ± 0.06	10
5-HT_{2C}−CFP	0.3 ± 1.8	NA[b]	10

[a] FRET was assessed by acceptor photobleaching in live HEK293 cells transfected with the indicated cDNAs. The FRET efficiency (% FRET) and donor/acceptor ratio (D/A) were measured in selected regions of the plasma membrane. Data represent the mean ± the standard error of the mean for the number of cells indicated (*n*). M4 is M_4-muscarinic. [b] Not applicable.

and YFP (acceptor) as red in Figure 5. A selected region of plasma membrane was photobleached with the 514 nm laser line for 30 s, and a postbleach image was collected (Figure 5B,D,F). Acceptor photobleaching resulted in a 34% increase in donor fluorescence (Figure 5B) and an 85% decrease in acceptor fluorescence (Figure 5D).

Acceptor photobleaching experiments were performed on selected regions of plasma membrane, as described above, in 30 live cells coexpressing 5-HT_{2C}−CFP and 5-HT_{2C}−YFP proteins, 10 cells coexpressing 5-HT_{2C}−CFP and M_4-muscarinic−YFP proteins, and 10 cells expressing 5-HT_{2C}−CFP protein alone. The FRET efficiency was calculated as the donor fluorescence postbleach minus donor fluorescence prebleach, divided by the donor fluorescence postbleach. The average FRET efficiency was 18.6% for cells transfected with 5-HT_{2C}−CFP and 5-HT_{2C}−YFP, compared to 5.1% for cells transfected with 5-HT_{2C}−CFP and M_4−YFP (Table 2). In our experiments, we used laser scanning confocal microscopy which allows the photobleaching to be confined to a small region of plasma membrane, thereby minimizing the time required for photobleaching and making the technique acceptable for use in living cells. Previous FRET studies have used cells fixed in paraformaldehyde because a potential caveat of using live cells is that during the time required for photobleaching receptors may be moving within the plasma membrane, resulting in the movement of the donor and acceptor into or out of the volume of cell being imaged.

Fluorescence recovery following photobleaching for 30 s was measured by imaging 5-HT_{2C}−YFP fluorescence every 10 s over a 10 min period. Fluorescence recovery began within 1 min, indicating that there is protein diffusion within the plasma membrane. However, the rate of protein diffusion is much slower than that recently reported for membrane raft-associated proteins (*39*). The difference in rates of protein diffusion may be attributed to differences in the extent of photobleaching and ambient temperature. Our study used a much more aggressive photobleaching regime (for assessing FRET), and our studies were performed at 23 °C, instead of at 37 °C, the temperature used by Kenworthy and colleagues.

To determine if receptor movement within the plasma membrane could contribute to the FRET observed in our experiments, donor fluorescence was measured in 10 cells transfected with 5-HT_{2C}−CFP alone, prior to and after photobleaching for 30 s. The average FRET efficiency was 0.3%, with a range from −3.6 to +2.9%. Similar results were obtained when FRET was assessed in nonbleached regions of the cell membrane. These results indicate that receptor movement into and out of the region of the membrane being imaged (during the photobleaching period) could alter the FRET efficiency measured in a single cell by as much as ±3−4%, but would not contribute significantly to the average FRET efficiency measured in our experiments.

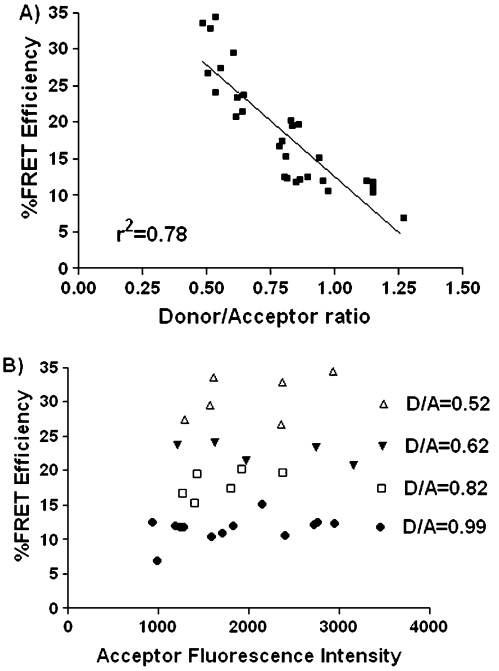

FIGURE 6: Relationship between FRET efficiency, donor/acceptor ratio, and acceptor fluorescence. FRET efficiencies were measured (by acceptor photobleaching) on the plasma membrane of 30 live HEK293 cells coexpressing 5-HT_{2C}−CFP and 5-HT_{2C}−YFP. Cells were transfected with a fixed 1/2 ratio of donor/acceptor plasmid DNA. (A) FRET efficiencies plotted as a function of the donor/acceptor ratio measured as the donor fluorescence divided by the acceptor fluorescence. (B) FRET efficiencies plotted as a function of acceptor fluorescence for cells with the indicated donor/acceptor ratios (D/A).

Figure 6A shows the relationship between FRET efficiency and the donor/acceptor ratio (plasma membrane donor fluorescence divided by acceptor fluorescence) measured in 30 cells coexpressing 5-HT_{2C}−CFP and 5-HT_{2C}−YFP. There was a good correlation between FRET efficiency and the donor/acceptor ratio (r^2 = 0.78). FRET efficiency was independent of the total amount of 5-HT_{2C}−YFP (acceptor fluorescence) expressed on the cell membrane (Figure 6B). When the 30 cells shown in Figure 6A were divided into four separate groups on the basis of their donor/acceptor ratios, the average FRET efficiency increased from 11.6 to 29.8% as the donor/acceptor ratio decreased from 1.0 to 0.5 (Table 3). However, when the cells were divided into four groups on the basis of acceptor fluorescence alone, the average FRET efficiency was the same for all four groups even though the acceptor fluorescence intensity increased 3-fold from 1000 to 3000 (Table 3).

DISCUSSION

This study was performed to determine if 5-HT_{2C} receptors exist as monomers or homodimers/oligomers on the plasma membrane of living cells. Western blots of solubilized membrane proteins from a stable cell line expressing 5-HT_{2C}−YFP receptors and from cells transiently expressing 5-HT_{2C}−HA receptors revealed the presence of immunoreactive bands the approximate predicted size of 5-HT_{2C} receptor monomers, dimers, and oligomers. Studies using

Table 3: Relationship between FRET Efficiency, Donor/Acceptor Ratio, and Acceptor Expression Level[a]

D/A	% FRET	*n*	acceptor	% FRET	*n*
0.99 ± 0.04	11.6 ± 0.5	13	950−1500	15.8 ± 2.0	10
0.82 ± 0.01[b]	18.2 ± 0.8[b]	6	1501−2000	19.5 ± 2.5	10
0.62 ± 0.01[c]	23.8 ± 1.6[c]	5	2001−2500	20.4 ± 3.0	5
0.52 ± 0.01[d]	29.8 ± 1.7[d]	6	2501−3200	20.7 ± 2.9	5

[a] FRET efficiencies measured in 30 HEK293 cells expressing 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP (data from Figure 6) were divided into four groups on the basis of their donor/acceptor ratios (D/A) and acceptor fluorescence intensities (acceptor). Data represent the mean ± the standard error of the mean for the indicated number of cells per group (*n*). [b] $p < 0.01$ vs D/A = 0.99. [c] $p < 0.01$ vs D/A = 0.82. [d] $p < 0.01$ vs D/A = 0.62.

different detergents, cross-linkers, and reducing agents were performed to determine the chemical nature of the presumed 5-HT$_{2C}$ receptor homodimers. These studies demonstrate that the higher-MW bands observed on the Western blot are detergent sensitive and reducing agent insensitive. Experiments using a plasma membrane impermeable cross-linker (BS^3) show that the proteins that make up the higher-MW bands are close enough to one another to allow cross-linking, and that the presumed 5-HT$_{2C}$ receptor homodimers are the predominant species present on the plasma membrane.

It is possible that the higher-MW bands observed on the Western blots may represent 5-HT$_{2C}$ receptor homodimers or may result from the copurification of accessory proteins with the 5-HT$_{2C}$ receptor or both. Immunoprecipitation studies using differentially tagged 5-HT$_{2C}$ receptors revealed the presence of immunoreactive bands the predicted size of 5-HT$_{2C}$ receptor homodimers only in samples prepared from cotransfected cells and not in samples prepared from singly transfected cells mixed post-transfection. The Western blot results suggest that 5-HT$_{2C}$ receptors form homodimers and that the observed results are not likely to be an artifact of membrane preparation and solubilization. The immunoprecipitation of differentially tagged receptors from cell membranes of cotransfected cells is evidence that favors receptor homodimers, but it does not eliminate the possibility that the receptors are held together by accessory proteins.

Previous studies have suggested that family 1 GPCRs may form homodimers through hydrophobic interactions involving their transmembrane domains (TMDs). Studies with the β_2-adrenergic receptor have suggested that TMD VI may represent part of the receptor dimer interface, since TMD VI peptides reduced the frequency of appearance of receptor dimers on Western blots and inhibited agonist-mediated receptor activation of adenylate cyclase (*1*). Studies using mutant D_2-dopamine receptors (*40*) and cysteine cross-linking (*41*) have provided evidence for the involvement of TMD IV in the formation of D_2-dopamine receptor homodimers. Atomic force microscopy has provided evidence for rhodopsin receptor dimer interfaces at TMD IV and V (*42*). In the study presented here, the higher-MW bands observed on the Western blots were detergent sensitive, consistent with the hypothesis that hydrophobic interactions play a role in the formation of 5-HT$_{2C}$ receptor homodimers. It remains to be determined if TMDs are involved in 5-HT$_{2C}$ receptor homodimer formation, as described for other members of GPCR family 1.

Biophysical techniques, BRET and FRET, were used to determine if the 5-HT$_{2C}$ receptor homodimers suggested by Western blots can be detected in intact living cells. BRET and FRET experiments can be performed using living cells coexpressing bioluminescent or fluorescent donor and acceptor proteins fused to the protein of interest. A positive BRET or FRET signal results when light emitted by the donor, in its excited state, is able to excite the acceptor. This can occur only if the donor and acceptor have overlapping emission and excitation spectra and are within 1−10 nm of each other and their dipoles are appropriately oriented (*43*). In the study presented here, coexpression of the 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP receptor fusion proteins resulted in a positive BRET signal, indicating that they are close to one another when coexpressed in living cells. The specificity of this effect is demonstrated by a decrease in the BRET ratio observed in cells coexpressing β_2−Rluc and 5-HT$_{2C}$−YFP or 5-HT$_{2C}$−Rluc and M_4−YFP. Pretreatment with 5-HT did not alter the BRET ratio observed in cells coexpressing 5-HT$_{2C}$−Rluc and 5-HT$_{2C}$−YFP, suggesting that agonist treatment does not alter 5-HT$_{2C}$ receptor homodimerization. The results of our study indicate that 5-HT$_{2C}$ receptors are present as constitutive homodimers/oligomers on the cell membrane prior to agonist treatment. From these experiments, it is not possible to determine if 5-HT$_{2C}$ receptor homodimers are formed once the receptors reach the plasma membrane or if they form in the ER and are transported to the cell membrane as homodimers, as has been described for several other GPCRs (*44*−*47*).

FRET and confocal microscopy were performed to determine if 5-HT$_{2C}$ receptor homodimers/oligomers could be visualized on the plasma membrane of living cells coexpressing 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP. FRET can occur when a fluorescent donor is excited with an external laser and the light emitted by the donor is transferred to the acceptor, resulting in excitation of the acceptor and a quenching of donor fluorescence. When the acceptor is removed by photobleaching, the donor becomes dequenched and an increase in donor fluorescence is observed (*35*). Using the method of acceptor photobleaching, we were able to directly visualize FRET between 5-HT$_{2C}$−CFP and 5-HT$_{2C}$−YFP expressed at physiological levels on the plasma membrane of live HEK293 cells.

Two different patterns of receptor distribution on the plasma membrane can give rise to a positive FRET signal. A positive FRET signal can result from specific protein−protein interactions, such as dimer/oligomer formation (receptor clustering), or from protein overexpression resulting in high levels of donor and acceptor sufficiently close to produce FRET because they are tightly packed in a small region of the membrane (random proximity effect). The random proximity and clustering models have been tested experimentally in FRET studies using the membrane-anchored protein 5′-nucleotidase (*48*) and the IgA receptor (*49*). These studies have suggested that FRET resulting from the random proximity of the donor and acceptor is dependent on the amount of acceptor expressed on the plasma membrane. However, FRET resulting from clustered proteins, such as dimers/oligomers, should be independent of acceptor expression levels and dependent on the ratio of the donor to acceptor expressed on the cell membrane. In our FRET studies with 5-HT$_{2C}$ receptors, FRET efficiency was independent of acceptor fluorescence and dependent on the donor/acceptor ratio. These results indicate that the FRET signal

produced by 5-HT_{2C}−CFP and 5-HT_{2C}−YFP is independent of receptor expression level and results from the proximity of receptor proteins in a clustered distribution on the plasma membrane, providing evidence for 5-HT_{2C} receptor homodimerization. While many studies have used FRET to examine GPCR dimerization, this is the first study to examine the relationship between FRET efficiency, donor/acceptor ratio, and acceptor expression on the plasma membrane, providing evidence for GPCR dimers/oligomers in a clustered distribution on the plasma membrane of living cells.

The results of this study suggest that for GPCR dimers/oligomers, FRET efficiency is dependent on the donor/acceptor ratio. Therefore, the amount of FRET produced in a single cell will vary depending on the ratio of donor to acceptor expressed in that cell (Table 3). This is an important consideration when interpreting the results of BRET and FRET experiments designed to determine the effect of ligand on GPCR dimer/oligomer formation. The data shown in Figure 6A are from transfections performed with a fixed 1/2 ratio of donor to acceptor plasmid DNA (donor/acceptor = 0.5). However, when 30 cells were chosen at random for analysis, not surprisingly the donor/acceptor ratio ranged from 0.5 to 1.2. As a result, the FRET efficiencies ranged from 34 to 7%. If FRET efficiencies from cells treated with the agonist are compared with FRET efficiencies from untreated cells, and the donor/acceptor ratios for the treated and untreated cells are not the same, it would not be possible to determine if differences in FRET efficiency result from drug treatment or result from differences in the donor/acceptor ratios. Previous studies have reported that the extent of GPCR dimerization increases, decreases, or does not change upon ligand binding (*6*−*11*, *23*−*30*, *45*, *50*). Experimental variables such as differences in donor/acceptor ratios between treatment groups, as well as differences in receptor expression levels, could contribute to the wide range of effects reported for agonist treatment on receptor dimerization measured by BRET and FRET. This study highlights the importance of measuring the donor/acceptor ratio in the transfected cells (even when a fixed ratio of donor to acceptor cDNA is used for transfection) and using cells with similar donor/acceptor ratios when BRET or FRET efficiencies are to be compared.

In summary, immunoprecipitation, BRET and FRET experiments provide evidence for 5-HT_{2C} receptor homodimerization. The dimers/oligomers are detergent sensitive and appear to be the predominant form of the receptor expressed on the plasma membrane. Confocal microscopy provided direct visualization of FRET on the plasma membrane of living cells expressing fluorescently tagged 5-HT_{2C} receptors. These studies provide evidence for constitutive 5-HT_{2C} receptor homodimerization on the plasma membrane of living cells. Additional studies are required to determine if dimerization plays a functional role in 5-HT_{2C} receptor ligand binding, signal transduction, and trafficking.

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