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Serotonin 5-HT_{2C} receptor homodimerization is not regulated by agonist or inverse agonist treatment

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Abstract

Serotonin 5-HT_{2C} receptors represent targets for therapeutics aimed at treating anxiety, depression, schizophrenia, and obesity. Previously, we demonstrated that 5-HT_{2C} receptors function as homodimers. Herein, we investigated the effect of agonist and inverse agonist treatment on the homodimer status of two naturally occurring 5-HT_{2C} receptor isoforms, one without basal activity (VGV) and one with constitutive activity (INI) with respect to G_{αq} signaling. Cyan- and yellow-fluorescent proteins were used to monitor VGV and INI homodimer formation by western blot, and in living cells using bioluminescence and fluorescence resonance energy transfer (BRET and FRET). Western blots of solubilized membrane proteins revealed equal proportions of homodimeric receptor species from HEK293 cells transfected with either the VGV or INI isoform in the absence and presence of 5-HT. BRET ratios measured in HEK293 cells transfected with the VGV or INI isoform were the same and were not modulated by 5-HT. Similarly, FRET efficiencies were the same regardless of whether measured in cells expressing the VGV or INI isoform in the absence or presence of 5-HT or clozapine. The results indicate that serotonin 5-HT_{2C} receptors form homodimers regardless of whether they are in an inactive or active conformation and are not regulated by drug treatment.

Index Words

Serotonin 5-HT_{2C} receptor; homodimers; fluorescence resonance energy transfer

1. Introduction

G-protein-coupled receptors are expressed on the plasma membrane of all cells and play vital roles in cell communication and survival. They are one of the largest families of signaling proteins and are targets for approximately 50% of all currently marketed pharmaceuticals. Therefore, significant emphasis has been placed on understanding molecular mechanisms that regulate G-protein-coupled receptor function. Over the last decade, a large body of evidence has been accumulating which suggests that G-protein-coupled receptors function as dimeric or oligomeric complexes (Angers et al., 2000; George et al., 2002; Guo et al., 2003; Milligan 2004; Goudet et al., 2005; Fotiadis et al., 2006). Biochemical and biophysical techniques have been used to identify homo- and hetero-dimers in cell lysates and intact, living cells. For some receptors, dimer formation has been shown to be critical for normal trafficking and expression

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of functional receptors on the plasma membrane (Jones et al., 1998; Margeta-Mitrovic et al., 2000; Uberti et al., 2003). Recent studies suggest that the dimer may represent the basic metabotropic signaling unit (Baneres et al., 2003; Liang et al., 2003; Kniazeff et al., 2004; Herrick-Davis et al., 2005). In addition, heterodimerization has been reported to alter the pharmacology of individual receptors within the heterodimer (Devi, 2001; Waldhoer et al., 2005) and to alter G-protein coupling specificity (Lee et al., 2004). However, it remains unclear as to how ligand binding and the transition to an active conformation of the receptor influences or regulates the dimeric/oligomeric complex. For example, ligand binding has been reported to increase, decrease or have no effect on receptor dimerization. Somatostatin and gonadotropin-releasing hormone receptors have been reported to be expressed as monomers on the plasma membrane and to undergo ligand-induced dimerization (Rocheville et al., 2000; Cornea et al., 2001), while many other receptors have been reported to be constitutively dimerized on the plasma membrane in the absence of ligand (McVey et al., 2001; Babcock et al., 2003; Dinger et al., 2003; Guo et al., 2003; Terrillon et al., 2003; Berthouze et al., 2005; Goin and Nathanson, 2006). Agonist binding has been reported to have no effect or to promote dissociation of delta opioid receptor homomers (Cvejic et al., 1997; McVey et al., 2001), and thyrotropin homomers (Latif et al., 2002). It is possible that the conflicting findings arise from differences in the methods used, differences in the interpretation of the experimental results, or they may represent true differences in the operational characteristics of individual G-protein-coupled receptors.

The serotonin 5-HT_{2C} receptor is widely distributed throughout the brain (Mengod et al., 1990) and couples to G α_q (Chang et al., 2000), arachidonic acid metabolism (Berg et al., 1996) and phospholipase D (McGrew et al., 2002). Many different classes of psychoactive agents interact with the 5-HT_{2C} receptor including hallucinogens, antipsychotics, antidepressants, anxiolytics and anorectic agents, and as such, the 5-HT_{2C} receptor has been identified as a potential target for drugs used to treat anxiety, depression, schizophrenia, and obesity (Herrick-Davis et al., 2000; Jones and Blackburn, 2002; Hoyer et al., 2002; Li et al., 2005; Miller, 2005). Therefore, the present study was performed to investigate the effect of drug treatment on the homodimer status of the 5-HT_{2C} receptor. To address this issue, we compared the homodimer status of two naturally occurring isoforms of the 5-HT_{2C} receptor, one that has no basal activity and the other which is constitutively active with respect to G α_q signaling, in the presence and absence of agonist and inverse agonist treatment. RNA editing occurs in the second intracellular loop of the 5-HT_{2C} receptor, changing amino acids 156, 158 and 160 from INI to VGV in the fully edited isoform (Burns et al., 1997) and results in the expression of 14 different 5-HT_{2C} receptor isoforms in the human brain (Niswender et al., 1999; Fitzgerald et al., 1999) with varying levels of constitutive activity (Herrick-Davis et al., 1999). The unedited INI isoform has high basal activity, while the fully edited VGV isoform has very low basal activity. Herein, a combination of biochemical and biophysical techniques were employed to assess the homodimer status of the VGV and INI isoforms of the 5-HT_{2C} receptor in the absence and presence of agonist and inverse agonist treatment. CFP and YFP incorporated into the C-terminal tail of the receptor were used to monitor VGV and INI homodimer formation in plasma membrane fractions by western blot, and in intact living cells using bioluminescence and fluorescence resonance energy transfer techniques (BRET and FRET) in the absence and presence of 5-HT and clozapine.

2. Materials and Methods

2.1. Cell culture

HEK293 cells from the American Type Culture Collection (ATCC) were cultured in DMEM (Cellgro) with 10% fetal bovine serum at 37°C, 5% CO₂. Transfections were performed using lipofectamine reagent (Invitrogen) according to the manufacture's protocol. All experiments

were performed 36 to 48 h post-transfection, with cells cultured in serum-free medium for the final 12–16 h prior to the experiment.

2.2. Receptor fusion proteins

Studies were performed using the VGV or INI isoform of the human 5-HT_{2C} receptor as indicated. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were attached to the C-terminal end of the 5-HT_{2C} receptor or the M₄-muscarinic receptor by ligation into pECFP-N1 and pEYFP-N1 vectors (Clontech), as previously described (Herrick-Davis et al., 2004). For the BRET studies, Renilla luciferase was attached to the C-terminal end of the 5-HT_{2C} receptor by ligation into pRLuc (Promega) to create the 5-HT_{2C}/RLuc fusion protein, as previously described (Herrick-Davis et al., 2004). Beta-arrestin2 (a generous gift from Dr. Robert Lefkowitz) was ligated into the pECFP-N1 vector to create the beta-arrestin2/CFP fusion protein. All constructs were confirmed by DNA sequencing (Center for Functional Genomics, Albany, NY). YFP attached to the C-terminus of the receptor has no effect on ligand binding or inositol phosphate production (Herrick-Davis et al., 2004).

2.3. 5-HT_{2C} receptor expression level

[³H]-Mesulergine binding was performed to monitor receptor expression levels following transient transfection of HEK293 cells as described below for the IP, BRET and FRET assays. Twenty-four hours post transfection, cells were incubated in serum-free DMEM over night prior to the assay. Membranes were prepared and [³H]-mesulergine binding was performed as previously described (Herrick-Davis et al., 1999). Protein was measured by BCA (Pierce). Data were analyzed using GraphPad Prism software. The mean 5-HT_{2C} receptor expression level from 12 independent transfections performed in parallel with the IP, BRET and FRET experiments was 6.6 ± 0.4 pmol/mg protein. At a 40% transfection efficiency, this is slightly higher than endogenous 5-HT_{2C} receptor expression levels of 10 pmol/mg protein reported for the choroid plexus (Sanders-Bush and Breeding, 1990).

2.4. Inositol tri-phosphate (IP₃) production

HEK 293 cells (4 × 10⁶ cells/100mm dish) were co-transfected with 5-HT_{2C}/CFP (0.2 µg) and 5-HT_{2C}/YFP (0.4 µg) using lipofectamine reagent (Invitrogen). Twenty-four h post transfection, cells were plated at 2 × 10⁵ cells/well in 24 well plates (Biocoat; Becton Dickinson). The cells were incubated in inositol-free, serum-free DMEM with [³H]-myoinositol (0.5 µCi/well) over night prior to the assay. Wells were washed with phosphate-buffered saline and incubated in serum-free DMEM (containing 10mM lithium chloride) in the absence or presence of drug for 35 min. Total [³H]-IP₃ production was measured by anion exchange chromatography, as previously described (Herrick-Davis et al., 1999). Data were analyzed using GraphPad Prism software.

2.5. Sucrose density gradient and Western blot

HEK293 stable cell lines expressing the VGV or INI isoform of 5-HT_{2C}/YFP at 20 pmol/mg protein were used for the Western blot experiments. 2 × 10⁷ cells were resuspended in 2 mls of cold lysis buffer [50 mM Tris (pH 7.6), 1 mM EDTA, 10 mM Iodoacetamide (Sigma) and protease inhibitor cocktail 1:100 (Sigma)], sonicated 40 s on ice, and centrifuged five min at 1,000xg to pellet nuclei and unlysed cells. The supernatant was collected and 2 M sucrose was added to achieve a final concentration of 0.2 M. The sample was applied to the top of a discontinuous step gradient (0.5M – 2.0M sucrose). Samples were centrifuged in a Beckman SW28 rotor at 100,000xg, for 16 h at 4°C. Following centrifugation, 5 ml was removed from the top of the gradient and discarded, and 3 ml fractions were collected (from top to bottom of the gradient). Lysis buffer (8 mls) was added to each fraction and the samples were centrifuged at 100,000xg for two h at 4°C. Each pellet was resuspended in 50 µl of lysis buffer and combined

with 50 μ l of non-reducing Laemmli sample buffer. Samples were heated at 70°C for 15 min and run on a 10% Tris-HCl BioRad Ready Gel (5 μ g protein/lane) at 95 V for 70 min. Gel proteins were transferred to nitrocellulose (Hybond ECL, Amersham), probed with GFP(B-2)-HRP antibody (1:3000 dilution, Santa Cruz) and were visualized by enhanced chemiluminescence (Amersham). Fractions rich in plasma membrane were detected with antibodies that recognize the α subunit of the Na⁺/K⁺ - ATPase pump (1:250 dilution, Sigma).

2.6. Bioluminescence Resonance Energy Transfer (BRET)

HEK 293 cells (4×10^6 cells/100mm dish), co-transfected with 1 μ g each of the VGV or INI isoform of 5-HT_{2C}/Rluc and 5-HT_{2C}/YFP, were cultured in serum-free media overnight prior to assay. At the start of the assay, cells were incubated at 37°C/5%CO₂ in the absence or presence of 1 μ M 5-HT for 15 min. Cells were lifted in 1ml of PBS/EDTA and added to a cuvette containing 5 μ M coelenterazine f (Molecular Probes). Emission spectra were collected using a Perkin Elmer LS-50B luminescence spectrophotometer, as previously described (Herrick-Davis K et al., 2004). Three consecutive emission spectra (400nm–600nm) were collected at one min intervals immediately following the addition of coelenterazine f. BRET ratios were calculated from the emission spectra using the following equation: [(emission at 510–590nm) – (emission at 440–500nm) $\times$ cf]/(emission at 440–500nm) where cf = (emission at 510–590nm) – (emission at 440–500nm) for 5-HT_{2C}/Rluc+pcDNA3 vector control, as previously described (Angers et al., 2000).

2.7. Fluorescence Resonance Energy Transfer (FRET)

HEK 293 cells (4×10^6 cells/100mm dish) were co-transfected with 5-HT_{2C}/CFP (0.2 μ g or 0.4 μ g) and 5-HT_{2C}/YFP (0.4 μ g) using lipofectamine reagent (Invitrogen). Twenty-four h post-transfection, cells were plated in serum-free media on poly-lysine coated glass cover slips overnight prior to the FRET assay. Transfected cells were imaged live (in HEPES-buffered Minimal Essential Medium without phenol) using a Zeiss LSM 510Meta confocal imaging system with a 30mW argon laser and a 63 $\times$ 1.4 NA oil immersion objective. CFP and YFP emission spectra were collected (from cells expressing 5-HT_{2C}/CFP or 5-HT_{2C}/YFP alone) following excitation at 458nm and were used as reference spectra for on-line fingerprinting and linear unmixing of CFP and YFP emission spectra in co-transfected cells using the Zeiss META detector and Zeiss AIM software, as previously described (Herrick-Davis et al., 2004). FRET was measured by acceptor photo-bleaching (Bastiaens et al., 1996), with the following modifications. Confocal microscopy was used to visualize a 2 μ m optical slice through the middle of live HEK293 cells co-expressing 5-HT_{2C}/CFP (donor) and 5-HT_{2C}/YFP (acceptor). Pre-bleach CFP and YFP images were collected simultaneously following excitation at 458nm (38% laser intensity; detector gain = 740). A selected region of plasma membrane was irradiated with the 514nm laser line (100% intensity, 60 iterations, using a 458nm/514nm dual dichroic mirror) for 5–10 s to photo-bleach YFP. Post-bleach CFP and YFP images were collected simultaneously (at 458nm) immediately following photo-bleaching. FRET was measured as an increase in CFP fluorescence intensity following YFP photo-bleaching. FRET efficiency was calculated as $100 \times [(CFP \text{ post-bleach} - CFP \text{ pre-bleach})/CFP \text{ post-bleach}]$ using the FRET Macro in the Zeiss Aim software package, taking into account CFP and YFP background noise in each channel. For live cells expressing 5-HT_{2C}/CFP alone, the mean FRET efficiency was $0.3 \pm 1.8\%$, indicating that receptor migration within the region of interest during the short photobleach period does not give rise to a false positive FRET signal.

2.8. Beta-arrestin2 recruitment

HEK293 cells co-expressing beta-arrestin2/CFP and VGV 5-HT_{2C}/YFP were cultured in serum-free medium for 24 h and imaged following excitation at 458nm and linear unmixing

of the CFP and YFP emission spectra, as described for the FRET experiments. An image was captured then 1 μ M 5-HT was added to the cells and images were captured for 15 min.

3. Results

3.1. Constitutive Activity

RNA editing of the 5-HT_{2C} receptor has been shown to alter constitutive activation of G α_q and subsequent inositol tri-phosphate (IP₃) production (Herrick-Davis et al., 1999). In the present study, IP₃ production was measured in HEK293 cells expressing the VGV or INI isoform of the 5-HT_{2C} receptor with CFP or YFP attached to the C-terminus of the receptor. HEK293 cells were transfected with the VGV or INI isoform of the human 5-HT_{2C}/YFP receptor and [³H]-IP₃ production was measured. The VGV isoform displayed a robust response to treatment with 5-HT and minimal basal activity (Fig. 1). The basal activity for the VGV isoform represented only 4% of the maximal response to 5-HT. In contrast, the INI isoform displayed significant basal activity, representing 72% of the maximal response to 5-HT. Clozapine displayed inverse agonist activity and inhibited 83% of INI basal activity (Fig. 1).

3.2. Sucrose gradient and western blot

Sucrose density gradient centrifugation was used to isolate membranes from HEK293 cells expressing the VGV or INI isoform of the 5-HT_{2C}/YFP receptor, grown in serum-free culture medium. Transfected cells were lysed by sonication and loaded on a 0.5M – 2.0M sucrose gradient. Following centrifugation, the top six fractions containing plasma membranes (0.5M – 1.2M sucrose) were run on a denaturing acrylamide gel (Fig 2). GFP-immunoreactive bands the predicted size of 5-HT_{2C}/YFP monomers were visible at 90kD, and bands the predicted size of homodimers were also present. Na⁺K⁺/ATPase, a plasma membrane protein, was detected in fractions 2–5 of the INI samples. Densitometry analyses of the bands were performed to compare homodimerization of the VGV and INI 5-HT_{2C} receptor isoforms. The mean monomer:dimer ratio (expressed as % homodimer) of fractions 2–4 was calculated from four different sucrose gradient experiments. Densitometry analyses (mean $\pm$ standard deviation, n=4) revealed no difference in homodimerization for VGV (42.0 $\pm$ 8.1%) or INI (38.8 $\pm$ 6.1%). 5-HT did not alter homodimerization of VGV (39.5 $\pm$ 3.8%) or INI (40.4 $\pm$ 2.7%) receptor isoforms.

3.3. Bioluminescence resonance energy transfer (BRET)

BRET is a proximity-based assay that can be used in living cells to determine if two proteins are close enough to associate with one another. When a bioluminescent donor protein and a fluorescent acceptor protein with overlapping emission and excitation spectra are within 1–10nm of each other and their dipoles aligned, resonance energy will be transferred from the donor to the acceptor. The transfer of energy excites the acceptor, resulting in acceptor emission, and BRET is measured as a ratio of acceptor and donor emissions (Xu et al., 1999). 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 an M₄-muscarinic/YFP fusion protein. HEK293 cells were co-transfected with Rluc- and YFP-tagged receptors to compare BRET ratios for the VGV and INI isoforms of the 5-HT_{2C} receptor in living cells. Minimal BRET was observed in cells co-expressing the INI isoform tagged with Rluc (INI/Rluc) and the YFP vector, and a weak BRET signal was observed with M₄-muscarinic/YFP (Fig. 3A). However, co-expression of INI/Rluc with INI/YFP or VGV/Rluc with VGV/YFP resulted in a robust BRET signal. The co-transfection experiments were performed using three different ratios of Rluc to YFP plasmid DNAs as indicated in Fig. 3A. There was no difference between VGV and INI isoforms, regardless of the ratio of donor to acceptor transfected. In subsequent experiments, BRET was

measured in HEK293 cells co-transfected with VGV/Rluc and VGV/YFP in the absence and presence of 5-HT (Fig. 3B). Pre-treatment of the cells with 1 μ M 5-HT for 15 min did not change the BRET ratio. Similarly, there was no change in BRET ratio measured in cells co-expressing INI/Rluc and INI/YFP in the absence or presence of 5-HT (Fig. 3B).

3.4. Fluorescence resonance energy transfer (FRET)

A confocal microscopy-based FRET method was used to compare homodimerization of the VGV and INI 5-HT_{2C} receptor isoforms in the absence and presence of 5-HT and clozapine. FRET operates on the same principle as BRET, with the exception that FRET uses a fluorescent donor excited by a laser. In the present study, acceptor photo-bleaching (Bastiaens et al., 1996) was used to measure FRET between CFP- and YFP-tagged VGV or INI 5-HT_{2C} receptors. This method involves the selective irradiation of YFP fluorescence using the 514nm laser line. If 5-HT_{2C}/CFP (donor) and 5-HT_{2C}/YFP (acceptor) are within 10nm of each other and their dipoles are aligned, resonance energy will be transferred from CFP to YFP. YFP photo-bleaching will result in enhanced CFP fluorescence, due to the dequenching of CFP following the removal of YFP. HEK293 cells co-expressing the VGV isoform of 5-HT_{2C}/CFP and 5-HT_{2C}/YFP were imaged live using a Zeiss LSM-510 Meta laser scanning confocal microscope (Fig. 4). CFP and YFP fluorescence were imaged simultaneously following excitation at 458nm and subsequent linear unmixing of emission spectra using the Zeiss Meta detector. A region of plasma membrane (marked by the white rectangle in Fig. 4) was selectively irradiated for 10 s using the 514nm laser line and a post-bleach image was captured at 458nm. An increase in CFP fluorescence was observed following YFP photo-bleaching (arrow in Fig. 4), corresponding to a FRET efficiency of 40%. Live-cell acceptor photo-bleaching FRET experiments, as shown in Fig. 4, were performed on cells co-expressing the VGV (n=80) or INI (n=60) isoform of 5-HT_{2C}/CFP and 5-HT_{2C}/YFP. The specificity of the FRET signal was verified by demonstrating the dependence of FRET efficiency on the ratio of CFP to YFP expressed in a given cell (uD/A ratio) and the independence of FRET efficiency on YFP fluorescence (receptor expression level). FRET efficiency measured on the plasma membrane was plotted versus the uD/A ratio (post-bleach CFP fluorescence/pre-bleach YFP fluorescence). Non-linear regression analyses produced correlation coefficients of 0.88 and 0.90 for VGV and INI isoforms, respectively, indicating that FRET efficiency is dependent on the uD/A ratio (Fig. 5A,B). When FRET efficiency was plotted vs YFP fluorescence (by uD/A ratio) linear regression analyses revealed no correlation between the amount of FRET observed and the amount of YFP-tagged receptor expressed on the plasma membrane (Fig. 5C,D).

In order to make a meaningful comparison, the FRET data presented in Fig. 5A and B were divided into six separate groups based on their uD/A ratios (table 1). The mean FRET efficiencies for each group were different, again demonstrating the dependence of FRET efficiency on the uD/A ratio. However, the mean FRET efficiencies for VGV and INI were not significantly different when compared within a given uD/A range (table 1).

3.5. The effect of 5-HT and clozapine on CFP/YFP ratio and FRET

Confocal fluorescence imaging was performed on HEK293 cells co-expressing CFP- and YFP-tagged VGV or INI 5-HT_{2C} receptors in the absence and presence of 5-HT or clozapine. For these experiments, the CFP/YFP ratio was used as an indicator of FRET. The CFP/YFP ratio was measured on the plasma membrane following linear unmixing of the CFP and YFP emission spectra. Live-cell confocal imaging of CFP and YFP fluorescence was performed at 37°C using a heated stage. 5-HT was added and imaging was continued at one minute intervals for 15 min (Fig. 6A). There was no change in CFP/YFP ratios prior to or following the addition of 1 μ M 5-HT. Experiments performed over a 20 min period following the addition of 5-HT revealed no change in the CFP/YFP ratio (data not shown). Similar experiments were

performed in HEK293 cells co-expressing CFP- and YFP-tagged INI 5-HT_{2C} receptors. The CFP/YFP ratio was measured prior to and at one minute intervals following the addition of 1 μ M clozapine (Fig. 6B). Treatment with clozapine did not alter the CFP/YFP ratio monitored over a 15 minute period. [³H]-clozapine (2.5nM) association kinetics monitored in HEK293 cells expressing YFP-tagged INI 5-HT_{2C} receptors revealed a $t_{1/2}$ of 3.4 and 3.8 min in two independent experiments (data not shown), indicating that the time course chosen for the CFP/YFP ratio experiments was adequate to observe changes related to clozapine occupancy of 5-HT_{2C} receptors.

3.6. Beta-arrestin recruitment

To confirm that 5-HT was in fact binding to and activating the VGV 5-HT_{2C} receptor during the time course used for the CFP/YFP ratio experiments, a control experiment was performed to determine the time-course for 5-HT-stimulated beta-arrestin recruitment to the plasma membrane. HEK293 cells co-expressing beta-arrestin/CFP and VGV 5-HT_{2C}/YFP were imaged live (as described above) in the absence and presence of 5-HT. Prior to the addition of 5-HT (time 0), beta-arrestin/CFP was purely cytosolic and 5-HT_{2C}/YFP was present on the plasma membrane, with no overlap of CFP (shown as green) and YFP (shown as red) in the merged image (Fig. 7). 5-HT was added to the cell and images were captured over a 15 min period. Treatment with 5-HT caused a redistribution of beta-arrestin/CFP fluorescence (CFP fluorescence decreased within the cytosolic compartment and increased around the plasma membrane) within two min following 5-HT treatment. In the merged image, the plasma membrane changed from red to yellow indicating the presence of both CFP and YFP fluorescence at the plasma membrane. Vesicular trafficking of the 5-HT_{2C} receptor was readily visible by five min post 5-HT application. These results demonstrate the successful binding of 5-HT and activation of the 5-HT_{2C} receptor within the time frame used for the CFP/YFP ratio experiments shown in Fig. 6.

4. Discussion

Previous studies have largely confirmed that dimer/oligomer formation is a relatively common phenomenon among G-protein-coupled receptors. For class C GABA_B receptors it is clear that dimerization is an obligatory step in the expression of a functional receptor complex on the plasma membrane (Jones et al., 1998; Margeta-Mitrovic et al., 2000; Uberti et al., 2003). However, for many class A G-protein-coupled receptors the relationship between receptor activation and homodimer formation remains unclear. RNA editing produces multiple 5-HT_{2C} receptor isoforms in different conformations, ranging from inactive in the absence of 5-HT (VGV) to constitutive activation (INI) of G α_q (Herrick-Davis et al., 1999; Niswender et al., 1999). We used this system to monitor the effect of drug treatment on 5-HT_{2C} receptor dimerization by comparing the dimer/oligomer status of the VGV and INI isoforms following treatment with agonist and inverse agonist.

Western blots of solubilized membrane proteins from HEK293 cells expressing 5-HT_{2C} receptors revealed the presence of both monomeric and dimeric species, with equal proportions of dimeric species for VGV and INI in the absence and presence of 5-HT. Previously, we have shown that 5-HT_{2C} receptor homodimers are detergent sensitive, and that increasing detergent stringency results in a higher proportion of monomers solubilized from plasma membrane (Herrick-Davis et al., 2004). Thus the presence of monomeric species on the western blot in this study is likely the result of detergent solubilization and does not necessarily indicate the existence of monomeric receptors in intact plasma membrane.

Resonance energy transfer techniques (BRET and FRET) were used to compare homodimer formation of VGV and INI isoforms of the 5-HT_{2C} receptor in living cells. BRET ratios were the same regardless of whether measured in cells expressing VGV or INI 5-HT_{2C} receptors.

Consistent with these results, FRET was observed on the plasma membrane of living cells expressing either the VGV or INI isoform of the 5-HT_{2C} receptor. The specificity of the FRET signal was verified by demonstrating the dependence of FRET efficiency on the ratio of CFP to YFP expressed in a given cell (uD/A ratio) and the independence of FRET efficiency on YFP fluorescence (receptor expression level). These results indicate that the observed FRET signal results from receptors in a clustered distribution and not from random proximity due to receptor over-expression (Kenworthy and Edidin 1998). FRET efficiencies were the same for VGV and INI expressing cells across all uD/A ratios examined. Following the addition of 5-HT to cells expressing the VGV isoform of the 5-HT_{2C} receptor, there was no change in the BRET ratio and no change in the CFP/YFP ratio. Likewise, clozapine had no effect on the CFP/YFP ratio measured in live HEK293 cells expressing the INI isoform of the 5-HT_{2C} receptor, indicating no change in the relative proximity of Rluc and YFP in the BRET assay or CFP and YFP fluorophores in the FRET assay following agonist or inverse agonist treatment. While these results indicate that 5-HT_{2C} receptor homodimer status is insensitive to drug treatment, it must be considered that ligand binding may produce subtle conformational changes within the individual protomers of the homodimer without changing the homodimeric status and without changing the relative proximity between the fluorescent tags located on the C-terminus of the receptor.

Previous studies have produced conflicting results concerning the effect of ligand binding on the homodimer formation. Studies using somatostatin and gonadotropin-releasing hormone receptors reported that these receptors are expressed as monomers and undergo ligand-induced dimerization on the plasma membrane (Rocheville et al., 2000; Cornea et al., 2001). Thyrotropin releasing hormone, cholecystokinin, and beta₂-adrenergic receptors have been reported to form constitutive homomers on the plasma membrane which are sensitive to agonist treatment (Angers et al., 2000; Cheng et al., 2001; Kroeger et al., 2001). The thyrotropin and beta₂-adrenergic homomers displayed an increase in BRET signal following the addition of agonist, while agonist application resulted in a decrease in BRET signal for cholecystokinin homomers. In contrast, agonist binding has been reported to promote homodimer dissociation of delta opioid receptors (Cvejic et al., 1997), thyrotropin receptors (Latif et al., 2002) and somatostatin receptors (Grant et al., 2004). These observations may reflect differences in the operational characteristics of individual G-protein-coupled receptors or differences in the methods used and interpretation of the experimental results. It should be noted that the majority of the studies performed to date have used fluorescent antibodies or tags on the N- or C-terminus of the receptor. However, the ligand binding domain and dimer interface for class A G-protein-coupled receptors are predicted to involve transmembrane domain regions of the receptor (Guo et al., 2003; Milligan 2004; Fotiadis et al., 2006), thus raising the possibility that changes in receptor conformation following ligand binding may or may not be mirrored in the N- or C-terminus of the receptor. This could in part be responsible for some of the reported discrepancies in the literature concerning the effect of ligands on receptor homodimer formation measured using BRET or FRET.

In the present study, we were unable to find any difference in homodimer status between the VGV and INI isoforms of the 5-HT_{2C} receptor in the presence of agonist or inverse agonist using western blot, BRET and FRET assays. Mutagenesis and cysteine cross-linking experiments involving the D₂-dopamine receptor suggest that conformational changes occur at the dimer interface in transmembrane domain four during receptor activation and inactivation (Guo et al., 2005). In addition, agonist binding to the leukotriene B₄ receptor has been reported to induce conformational changes in the unliganded protomer of the dimer (Mesnier et al., 2004), indicating that conformational changes resulting from receptor activation are translated across the dimer interface. These results are consistent with reports of trans-activation of G-proteins following ligand binding to one protomer of the dimer (Goudet et al., 2005; Hlavackova et al., 2005). Rhodopsin receptor homodimers have been reported to be organized

in higher order structures and the oligomeric organization of rhodopsin appears to be related to receptor activation, with rhodopsin being the most active in preparations containing the highest proportion of oligomers (Jastrzebska et al., 2006). Whether activation of monoamine receptors leads to a reorganization of homodimers into higher order oligomeric complexes remains to be determined.

The results of the present study demonstrate that serotonin 5-HT_{2C} receptors are expressed on the plasma membrane as homodimers regardless of whether they are in an inactive or active conformation and do not dissociate upon agonist or inverse agonist binding. These results are consistent with a model in which class A G-protein-coupled receptor homodimer formation occurs prior to receptor expression on the plasma membrane and remains unaltered following ligand binding. It remains to be determined whether bivalent ligands will have improved clinical efficacy over the current therapeutic agents designed to bind to a single protomer within a given receptor dimer/oligomer complex.

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Abbreviations

BRET

bioluminescence resonance energy transfer

FRET

fluorescence resonance energy transfer

Figure 1.

Inositol tri-phosphate (^3H -IP₃) production in HEK293 cells expressing the VGV or INI isoform of 5-HT_{2C}/YFP. ^3H -IP₃ production was measured in the absence (basal) and presence of 5-HT (1 μM) or clozapine (1 μM). Background levels of ^3H -IP₃ production were measured in cells transfected with vector only (typically 2,500 dpm) and were subtracted out from ^3H -IP₃ produced in cells transfected with the VGV or INI isoform. Data represent the mean $\pm$ sem of three independent experiments each performed in triplicate. * $p < 0.01$ vs Basal.

Figure 2.

Western blot of membrane proteins prepared from HEK293 cells expressing the VGV or INI isoform of the 5-HT_{2C}/YFP receptor. Membrane proteins were separated on a 0.5M–2.0M sucrose gradient. The first six fractions collected from the top of the gradient (0.5M–1.2M) were run on a 10% polyacrylamide gel (lanes 1–6), and probed with antibodies to GFP or Na⁺K⁺/ATPase.

Figure 3.

A) Bioluminescence resonance energy transfer (BRET) was measured in HEK293 cells following co-transfection with Rluc- and YFP-tagged receptors in a 1:1, 1:2, or 1:3 ratio as indicated. BRET ratios were calculated as described in materials and methods. Data represent the mean $\pm$ sem from three to five independent experiments. B) BRET measured in HEK293 cells co-transfected with VGV Rluc- and YFP-tagged receptors (in a 1:2 ratio) or INI Rluc- and YFP-tagged receptors (in a 1:3 ratio) in the absence (open bars) and presence (closed bars) of 1 μ M 5-HT. Data represent the mean $\pm$ sem from three independent experiments.

Figure 4.

Acceptor photobleaching FRET. Fluorescence confocal microscopy was used to visualize a 2 μm thick optical cross-section of live HEK293 cells co-expressing the VGV isoform of 5-HT_{2C}/CFP (donor) and 5-HT_{2C}/YFP (acceptor) on the plasma membrane. CFP (green) and YFP (red) images were captured simultaneously following excitation at 458 nm and linear unmixing of their emission spectra (pre-bleach). A region of plasma membrane (marked by the white rectangle) was photobleached at 514 nm for 10 s. Post-bleach images were captured simultaneously following excitation at 458 nm. FRET is visualized as an increase in CFP fluorescence following YFP photobleaching (marked by arrow).

Figure 5.

FRET was measured on the plasma membrane of live HEK293 cells expressing the VG or INI isoform of CFP- and YFP-tagged 5-HT_{2C} receptors. A,B) FRET efficiency plotted versus uD/A ratio (post-bleach CFP fluorescence/pre-bleach YFP fluorescence) for VG (n=80) or INI (n=60). Non-linear regression analyses (one-phase exponential decay) were performed using GraphPad Prism software. C,D) FRET efficiency plotted versus acceptor (YFP) fluorescence by uD/A ratio for the same cells as shown in A and B above. Linear regression analyses performed using GraphPad Prism software yielded slopes that did not significantly differ from zero for all uD/A ratios examined.

Figure 6.

A) The CFP/YFP ratio (CFP fluorescence/YFP fluorescence) was measured on the plasma membrane of live HEK293 cells co-expressing CFP- and YFP-tagged VGV 5-HT_{2C} receptors at one min intervals. Two images were captured (time = -1 and 0 min), then 1 μ M 5-HT was added and the same cells were imaged for an additional 15 min. Data represent the mean $\pm$ sem from four cells. Linear regression analyses (performed using GraphPad Prism) slope not significantly different from zero. B) CFP/YFP ratios were measured on the plasma membrane of live HEK293 cells co-expressing CFP- and YFP-tagged INI 5-HT_{2C} receptors at 1 min intervals prior to and following the addition of 1 μ M clozapine. Data represent the mean $\pm$ sem from four cells. Linear regression analyses (performed using GraphPad Prism) slope not significantly different from zero.

Figure 7. HEK293 cells co-expressing beta-arrestin2/CFP and VGV 5-HT_{2C}/YFP were imaged live following excitation at 458nm and subsequent linear unmixing of CFP (shown as green) and YFP (shown as red) emission spectra, in the absence and presence of 1 μ M 5-HT for the indicated times (T). The red scale bar represents 10 μ m. Multiple cells from four independent 5-HT time course experiments are shown.

Table 1FRET efficiency by uD/A ratio for INI and VGV isoforms of the 5-HT_{2C} receptor.

uD/A Ratio	%FRET Efficiency	
	INI	VGV
0.20 – 0.30	49.9 +/- 1.1 (10)	49.3 +/- 1.5 (8)
0.31 – 0.39	40.3 +/- 2.0 (5)	38.6 +/- 3.8 (5)
0.40 – 0.59	33.8 +/- 1.4 (14)	29.9 +/- 0.9 (23)
0.60 – 0.80	23.9 +/- 1.4 (12)	22.0 +/- 0.8 (17)
0.81 – 0.99	17.6 +/- 2.9 (5)	17.3 +/- 2.3 (5)
1.00 – 2.00	12.8 +/- 0.7(14)	11.7 +/- 0.7 (22)

FRET was measured on the plasma membrane of live HEK293 cells expressing the INI (n=60) or VGV (n=80) isoforms of 5-HT_{2C}/CFP (donor) and 5-HT_{2C}/YFP (acceptor). FRET efficiencies were divided into six groups based on the ratio of donor to acceptor (uD/A) expressed on the plasma membrane in a given cell. Data represent the mean +/- sem for the number of cells indicated in parentheses.