Dimerization is a common property of G-protein-coupled receptors (GPCR). While the formation of GPCR dimers/oligomers has been reported to play important roles in regulating receptor expression, ligand binding, and second messenger activation, less is known about how and where GPCR dimerization occurs. The present study was performed to identify the precise cellular compartment in which class A GPCR dimer/oligomer biogenesis occurs. We addressed this issue using confocal microscopy and fluorescence resonance energy transfer (FRET) to monitor GPCR proximity within discrete intracellular compartments of intact living cells. Time-lapse confocal imaging was used to follow CFP- and YFP-tagged serotonin 5-HT$_{2C}$ receptors during biosynthesis in the endoplasmic reticulum (ER), trafficking through the Golgi apparatus and subsequent expression on the plasma membrane. Real-time monitoring of FRET between CFP- and YFP-tagged 5-HT$_{2C}$ receptors was performed by acceptor photobleaching within discrete regions of the ER, Golgi, and plasma membrane. The FRET signal was dependent on the ratio of CFP- to YFP-tagged 5-HT$_{2C}$ receptors expressed in each region and was independent of receptor expression level, as predicted for proteins in a non-random, clustered distribution. FRET efficiencies measured in the ER, Golgi and plasma membrane were similar. These experiments provide direct evidence for homodimerization/oligomerization of class A GPCR in the ER and Golgi of intact living cells, and suggest that dimer/oligomer formation is a naturally occurring step in 5-HT$_{2C}$ receptor maturation and processing.
expression with β2-adrenergic receptors has been reported to be necessary for expression of α1D-adrenergic receptors on the plasma membrane (24). Additional evidence supporting intracellular dimerization of class A GPCR is provided by studies showing that non-trafficking, mutant receptors decrease the plasma membrane expression of their wild-type counterparts (25-29).

Dimerization has been proposed as a general mechanism necessary for proper trafficking of class A GPCR to the plasma membrane (8, 9, 24). Until recently, experimental evidence in favor of this model has been limited to studies using immunoprecipitation of solubilized receptors prepared from whole cell lysates and confocal microscopy showing co-localization of fluorescent-tagged GPCR in endomembranes of fixed cells. More recently, this issue has been addressed using bioluminescence resonance energy transfer (BRET) following sucrose density gradient centrifugation. Positive BRET signals were detected in both plasma membrane and endomembrane-enriched fractions prepared from HEK293 cells expressing vasopressin or β2-adrenergic receptors (14, 30). While these results suggest that GPCR dimerization may occur within intracellular compartments, direct experimental evidence demonstrating the formation of class A GPCR homodimers in the ER and Golgi of intact living cells is still lacking.

The present study was performed to test the hypothesis that class A GPCR form dimers/oligomers in the ER and Golgi of intact living cells. Previously, we have shown that 5-HT2C receptors are present as constitutive homodimers on the plasma membrane (15). Herein, we used a combination of biochemical and biophysical techniques to determine if 5-HT2C receptors form homodimers in the ER prior to trafficking to the Golgi and plasma membrane. Immunoprecipitation with differentially tagged 5-HT2C receptors revealed the presence of 5-HT2C receptor bands from ER and plasma membrane, and confocal fluorescence imaging was used to quantify fluorescence resonance energy transfer (FRET) in the ER and Golgi of living HEK293 cells expressing fluorescent-tagged 5-HT2C receptors. The confocal microscopy-based FRET approach allows specific intracellular compartments to be examined, without compromising cellular integrity, and real-time monitoring of GPCR dimerization in the ER and Golgi apparatus. These experiments are the first to use time-lapse confocal imaging of transfected HEK293 cells to provide real-time visualization of fluorescent-tagged 5-HT2C receptors during biosynthesis in the ER, trafficking through the Golgi, and subsequent expression on the plasma membrane at physiologically relevant receptor expression levels. Our results demonstrate that class A GPCR can form homodimeric/oligomeric complexes in the ER and Golgi of intact living cells, indicating that dimerization/oligomerization may be a naturally occurring step in the maturation and processing of 5-HT2C receptors.

Materials and Methods

Cell culture and transfection: HEK293 cells from the American Type Culture Collection (ATCC) were cultured in DMEM (Cellgro) with 10% fetal bovine serum at 37°C, 5% CO2. HEK293 cells were plated at 5 x 10^5 cells per well in six-well plates containing poly-lysine treated glass coverslips. Transfections were performed using lipofectamine reagent (Invitrogen) according to the manufacture’s protocol. Cells were transfected for five hours and cultured post-transfection in serum-free DMEM. All experiments were performed in serum-free culture media 12 to 36 hours post-transfection.

Fusion proteins: All studies were performed using the INI isoform of the human 5-HT2C receptor. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were attached to the C-terminal end of the 5-HT2C or CCR5-chemokine receptor by ligation into pECFP-N1 or pEYFP-N1 vectors (Clontech) to create 5-HT2C/CFP, 5-HT2C/YFP, and CCR5/YFP as previously described (15). The 5-HT2C/HA fusion protein was created by PCR to add the 10 amino acid HA sequence to the C-terminal end of the receptor, as previously described (15). All constructs were confirmed by DNA sequencing (Center for Functional Genomics, Albany, NY). YFP and HA attached to the C-terminus of the receptor had no effect on ligand binding or inositol phosphate production (15).

Confocal Microscopy: HEK293 cells (5 x 10^5 cells/well) were transfected with 40ng of the indicated plasmid DNAs and imaged live (at room temperature in HEPES-buffered Minimal Essential
Medium without phenol, Cellgro) using a Zeiss LSM 510Meta confocal imaging system with a 30mW argon laser and a 63x 1.4 NA oil immersion objective at 2x zoom. The pEYFP-ER plasmid (Clontech) encoding a YFP fusion protein containing the ER targeting sequence of calreticulin with a KDEL retention sequence and the pEYFP-Golgi plasmid (Clontech) encoding a YFP fusion protein that specifically targets the trans-medial region of the Golgi apparatus were used as markers to visualize ER and Golgi, respectively, following excitation at 514nm. Real-time imaging of CFP- and YFP-tagged 5-HT_{2C} receptors was performed 12 to 24 hours post-transfection to visualize 5-HT_{2C} receptors following biosynthesis in the ER, trafficking through the Golgi, and subsequent expression on the plasma membrane. Cells co-expressing 5-HT_{2C}/CFP and 5-HT_{2C}/YFP were imaged following excitation at 458nm. CFP and YFP fluorescence were separated using on-line finger printing and linear unmixing with the Zeiss Meta detector and Zeiss Aim Software, as previously described (15).

Fluorescence Resonance Energy Transfer (FRET): HEK293 cells, plated at 5 x 10^5 cells per well in six-well plates containing poly-lysine treated glass coverslips, were co-transfected with 5-HT_{2C}/CFP and 5-HT_{2C}/YFP plasmid DNA in the following ratios: 1:1 (40ng + 40ng); and 1:2 (20ng + 40ng) using lipofectamine (Invitrogen). For control experiments, cells were transfected with 5-HT_{2C}/CFP and 5-HT_{2C}/YFP plasmids in the following ratios: 1:1 (40ng + 40ng) or 1:2 (20ng + 40ng) using lipofectamine (Invitrogen). For control experiments, cells were co-expressing 5-HT_{2C}/CFP and 5-HT_{2C}/YFP, in cells expressing 5-HT_{2C}/CFP alone, and in cells co-expressing 5-HT_{2C}/CFP with ER/YFP or CCR5/YFP. For live cells expressing only 5-HT_{2C}/CFP the mean FRET efficiency was 1.3 +/- 0.7%, indicating that receptor migration within the region of interest during the 5-10 second photobleach period does not give rise to a false positive FRET signal.

Radioligand binding assay: HEK293 cells were plated and transfected as described above for the FRET experiments. Membranes were prepared from transfected cells and 3H-mesulergine binding was performed as previously described (32). Protein was measured by BCA (Pierce). Data were analyzed using GraphPad Prism software.

Immunoprecipitation and PNGase digest: HEK293 cells (4 x 10^6 cells/100mm dish) were pretreated with or without 1uM Brefeldin A (Sigma) for over-night prior to transfection with 1ug of 5-HT_{2C}/YFP and 1ug of 5-HT_{2C}/HA plasmid DNA using 20ul of lipofectamine reagent (InVitrogen). As a control, separate dishes of cells were transfected independently with either 5-HT_{2C}/YFP or 5-HT_{2C}/HA and the cells were mixed together after transfection. Transfected cells were washed with PBS and scraped in 2mls of PBS and centrifuged at 1500g for 5 minutes. Cells were resuspended in 0.5ml of lysis buffer containing 50mM TrisHCl pH 7.6, 1mM EDTA, 10ul of
protease inhibitor cocktail (Sigma), sonicated for 30 seconds on ice, and centrifuged at 1500g for 5 minutes. The supernatant was centrifuged at 21,000g for 30 minutes at 4ºC. The membrane pellet was resuspended in 0.2ml of solubilization buffer (50mM TrisHCl pH 7.6, 1mM EDTA, 150mM NaCl, 10mM iodoacetamide, 5ul protease inhibitor cocktail, 10mM CHAPS). The membrane proteins were solubilized for 60 minutes on ice and centrifuged at 21,000g for 30 minutes at 4ºC to pellet insoluble material. Solubilized membrane proteins were immunoprecipitated overnight with 10ul of HA(Y-11)-agarose (Santa Cruz) at 4ºC. Samples were centrifuged at 4,000g for 5 minutes, the pellet was washed twice with PBS containing protease inhibitor cocktail, resuspended in 50ul of denaturing Laemmli sample buffer. For the PNGase digest, solubilized membrane protein was diluted in 50mM NaPO₄ (pH 7.5) and incubated with 2ul of PNGase (NewEngland Biolabs) for three hours at 37ºC. Samples were run on a Tris-HCl ready gel (BioRad) at 100V for 70 minutes under denaturing conditions. The gel was transferred to nitrocellulose (BioRad), probed with GFP(B-2)-HRP antibody (Santa Cruz) diluted 1:3,000 and visualized by enhanced chemiluminescence (Amersham).

**Results**

**Confocal fluorescence imaging of ER and Golgi membranes in living cells:** HEK293 cells were transfected with plasmid DNA containing a mutant YFP protein engineered with an ER targeting and retention sequence (ER-YFP) or with a YFP variant that specifically targets the trans-medial region of the Golgi apparatus (Golgi-YFP). Live-cell confocal fluorescence imaging was performed to visualize ER and Golgi membranes in 2um thick optical slices through the middle of HEK293 cells expressing the ER-YFP or Golgi-YFP marker. In HEK293 cells, ER membranes appear as a diffuse reticular network spreading outward from the nucleus throughout the cytosol towards the plasma membrane (figure 1A). In contrast, Golgi membranes are visualized as dense areas of intense fluorescence confined to a discrete perinuclear region of the cytosol (figure 1B).

**Real-time confocal fluorescence imaging of 5-HT₂C receptors trafficking from the ER to the Golgi and plasma membrane:** Fluorescent proteins were used as markers to visualize 5-HT₂C receptors following biosynthesis in the ER and subsequent trafficking to the Golgi and plasma membrane. HEK293 cells co-transfected with 5-HT₂C/CFP and the ER/YFP marker showed co-localization of CFP and YFP fluorescence in the ER 12 hours post-transfection (figure 1 C-E). Cells co-transfected with 5-HT₂C/CFP and the Golgi/YFP marker showed co-localization of CFP and YFP fluorescence in the Golgi 16 hours post-transfection (figure 1 F-H). Twenty hours post-transfection, 5-HT₂C/CFP fluorescence was on the plasma membrane and the Golgi/YFP marker remained in the Golgi (figure 1 I-K). Based on these results, a time-course was performed in cells co-transfected with 5-HT₂C/CFP and 5-HT₂C/YFP. The cells were imaged live using confocal fluorescence imaging at various time points post-transfection. Ten hours after the addition of transfection reagents, 5-HT₂C/CFP and 5-HT₂C/YFP receptor fluorescence began to emerge. Twelve to 16 hours post-transfection, confocal fluorescence imaging revealed many cells with a diffuse reticular pattern of fluorescence (figure 2A), identical to HEK293 cells expressing the ER-YFP marker (figure 1A), demonstrating 5-HT₂C receptor expression in the ER. Confocal fluorescence imaging 16 to 20 hours post-transfection revealed many cells with a more clustered, dense pattern of labeling with a distinct perinuclear distribution (figure 2B), identical to HEK293 cells expressing the Golgi-YFP marker (figure 1B). By 20 hours post-transfection, many cells were expressing 5-HT₂C receptors on the plasma membrane (figure 2C). At this time, 5-HT₂C receptor expression appears to have reached a steady-state level with the majority of the receptor expressed on the plasma membrane and little intracellular fluorescence remaining. This phenomenon has been observed in every one of our transient transfections and in all of our stable cell lines. Whether this is due to mRNA stability or some other form of feedback regulation by the receptors once they reach the plasma membrane is unknown. While the time-course for receptor expression is likely to vary for different receptors in different cell lines and different culture conditions, our results indicate that it is possible to monitor receptor trafficking through discrete intracellular compartments at early time points post-transfection.
5-HT<sub>2C</sub> receptor homodimer/oligomer formation in the ER of living cells: A confocal microscopy-based FRET method was used to monitor 5-HT<sub>2C</sub> homodimers in the ER of living cells 12 to 16 hours post-transfection. HEK293 cells co-transfected with 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP were visualized live by confocal fluorescence imaging using a Zeiss LSM-510 Meta laser scanning confocal microscope. CFP and YFP fluorescence were imaged simultaneously following excitation at 458nm and subsequent linear unmixing of emission spectra using the Zeiss Meta detector (figure 3A,B). CFP- and YFP-tagged 5-HT<sub>2C</sub> receptors were co-localized in the ER of a living HEK293 cell (figure 3C). Acceptor photobleaching was used to measure FRET between 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP in the ER. This method involves the selective irradiation of YFP fluorescence using the 514nm laser line. If 5-HT<sub>2C</sub>/CFP (donor) and 5-HT<sub>2C</sub>/YFP (acceptor) are within 10nm of each other and the fluorophore dipoles are aligned, resonance energy can be transferred from CFP to YFP. If FRET occurs, then photobleaching of YFP fluorescence will result in enhanced CFP fluorescence, due to the dequenching of CFP following the removal of YFP. To perform acceptor photobleaching, a pre-bleach image was captured using the 458nm laser line (figure 3A-C). A region of the ER (marked by the white rectangle in figure 3E) was selectively irradiated using the 514nm laser line. A 458nm/514nm dual dichroic mirror was used to allow rapid, automated image acquisition immediately after YFP photobleaching (figure 3D-F). An increase in CFP fluorescence was observed following YFP photobleaching (indicated by the arrow in figure 3D) and the FRET efficiency was 28.5%. Acceptor photobleaching experiments, as shown in figure 3, were performed on 50 living HEK293 cells co-expressing 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP. All FRET measurements were made 12 to 16 hours after transfection, when 5-HT<sub>2C</sub> receptor expression in the ER was visible. FRET efficiency was plotted versus the uD/A ratio (post-bleach CFP fluorescence / pre-bleach YFP fluorescence). The amount of FRET observed within a given cell was dependent on the uD/A ratio (figure 4A). When FRET efficiency was plotted vs acceptor fluorescence by uD/A ratio, there was no correlation between the amount of FRET observed and 5-HT<sub>2C</sub> receptor expression level in the ER (figure 4B).

FRET efficiency was also measured in cells co-expressing 5-HT<sub>2C</sub>/CFP with the ER-YFP marker or the CCR5-chemokine receptor (CCR5/YFP) as controls (table 1). FRET efficiencies measured for cells co-expressing 5-HT<sub>2C</sub>/CFP with the ER-YFP marker or CCR5/YFP were negligible. CCR5/YFP fluorescence levels (1517±/−82; n=10) and 5-HT<sub>2C</sub>/YFP fluorescence levels (1794+/−96; n=50) were not statistically different (p=0.1), indicating similar receptor expression levels.

Real-time visualization of 5-HT<sub>2C</sub> receptor homodimers/oligomers trafficking through the Golgi apparatus of living cells: Confocal imaging and acceptor photobleaching were used to visualize and quantify FRET between CFP- and YFP-tagged 5-HT<sub>2C</sub> receptors in the Golgi apparatus of living cells. HEK293 cells were co-transfected with 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP and imaged 16 to 20 hours following transfection. Differentially tagged 5-HT<sub>2C</sub> receptors were clearly visible and co-localized within the Golgi apparatus (figure 5A-C). A region of the Golgi (marked by the white rectangle in figure 5E) was selectively irradiated using the 514nm laser line and a post-bleach image was captured (figure 5D-E). An increase in CFP fluorescence was observed following YFP photobleaching (indicated by the arrow in figure 5D) and the FRET efficiency was 40.6%. Acceptor photobleaching experiments, as shown in figure 5, were performed on 50 living HEK293 cells co-expressing 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP. All FRET measurements were made 16 to 20 hours after transfection, when 5-HT<sub>2C</sub> receptor expression in the Golgi was visible. FRET efficiency was plotted versus the uD/A ratio. The amount of FRET observed within a given cell was dependent on the uD/A ratio (figure 6A). FRET efficiency plotted vs acceptor fluorescence by uD/A ratio revealed no correlation between FRET efficiency and 5-HT<sub>2C</sub> receptor expression (figure 6B).

Comparison of 5-HT<sub>2C</sub> receptor FRET efficiencies measured in ER, Golgi and plasma membrane: As shown in figure 3, the FRET efficiency for 5-HT<sub>2C</sub> receptors expressed in the ER was 28.5%, while in figure 5 the reported FRET efficiency for 5-HT<sub>2C</sub> receptors expressed in the Golgi was 40.6%.
Comparing the FRET efficiencies from these two experiments would lead to the erroneous conclusion that FRET efficiency increases as 5-HT\textsubscript{2C} receptors traffic from the ER to the Golgi. However, as shown in figures 4A and 6A FRET efficiency is dependent on the ratio of donor to acceptor expressed in a given cell. Therefore, a meaningful comparison of FRET efficiencies can only be made between cells with similar uD/A ratios. When the FRET data presented in figures 4A and 6A were divided into five separate groups, based on their uD/A ratios, the mean FRET efficiencies for each group were different (table 2), again demonstrating the dependence of FRET efficiency on the uD/A ratio. However, the mean FRET efficiencies for ER and Golgi were not significantly different when compared within a given uD/A range, except for the 1.3 to 2.0 range where FRET efficiencies were less than 10%. FRET was measured on the plasma membrane of HEK293 cells co-expressing 5-HT\textsubscript{2C}/CFP and 5-HT\textsubscript{2C}/YFP, as described above for ER and Golgi (table 3). The overall mean uD/A ratios and FRET efficiencies were similar for CFP- and YFP-tagged 5-HT\textsubscript{2C} receptors expressed in the ER, Golgi, and on the plasma membrane (table 3).

**5-HT\textsubscript{2C} receptor expression level:** Radioligand binding studies were performed in parallel with FRET experiments to monitor 5-HT\textsubscript{2C} receptor expression levels in transfected cells used in the FRET assay. \textsuperscript{3}H-Mesulergine binding yielded 5-HT\textsubscript{2C} receptor expression levels of 6.9+/-.0.6 pm/mg protein from three independent transfection experiments performed in parallel with transfections for the FRET experiments. The 5-HT\textsubscript{2C} receptor expression levels in the transfected cells used for the FRET experiments are similar to endogenous 5-HT\textsubscript{2C} receptor expression (10pm/mg) in choroid plexus epithelial cells (33).

**Immunoprecipitation and PNGase digest:** Immunoprecipitation of differentially tagged 5-HT\textsubscript{2C} receptors was performed to confirm the FRET results by demonstrating a physical association between 5-HT\textsubscript{2C} receptors. Solubilized membrane proteins from HEK293 cells separately transfected or co-transfected with 5-HT\textsubscript{2C}/HA and 5-HT\textsubscript{2C}/YFP were immunoprecipitated with HA-agarose. Following immunoprecipitation, the samples were denatured, separated on a 10% polyacrylamide gel, and immunoblotted with GFP-HRP antibody (figure 7A). Denaturation of the immunoprecipitate resulted in the appearance of GFP immunoreactive bands the predicted size of mature (fully glycosylated) 5-HT\textsubscript{2C}/YFP (90kD) and immature 5-HT\textsubscript{2C}/YFP (70kD). A PNGase digest was performed to confirm the identity of the 90kD and 70kD bands as mature and immature forms of the 5-HT\textsubscript{2C} receptor (figure 7B). The sample was digested with PNGase and run on a 7.5% acrylamide gel for better separation of the different glycosylation states of the 5-HT\textsubscript{2C} receptor. Digest with PNGase resulted in a single GFP immunoreactive band the predicted size of unglycosylated 5-HT\textsubscript{2C}/YFP. When the cells were pretreated with Brefeldin A (to prevent receptor trafficking to the plasma membrane) and then immunoprecipitated, a GFP immunoreactive band the predicted size of unglycosylated/immature 5-HT\textsubscript{2C}/YFP (70kD) appeared only in samples from cells co-transfected with 5-HT\textsubscript{2C}/HA and 5-HT\textsubscript{2C}/YFP (figure 7C).

**Discussion**

While GPCR are generally conceptualized as forming dimeric/oligomeric complexes, very little is understood about the molecular interactions between dimers/oligomers, the cellular compartment in which dimerization occurs, and how receptor dimerization regulates GPCR function. The present study was performed to identify the cellular compartment in which class A GPCR dimerization occurs. Our approach to understanding the biogenesis of GPCR dimers is unique in that we used a confocal microscopy-based FRET technique that allows the determination of protein:protein proximity within specific intracellular compartments, such as ER and Golgi, of living cells. Time-lapse confocal imaging of transfected HEK293 cells provided real-time visualization of fluorescent-tagged 5-HT\textsubscript{2C} receptors during biosynthesis in the ER, trafficking through the Golgi, and subsequent expression on the plasma membrane.

FRET combined with confocal microscopy was used to determine the proximity of CFP- and YFP-tagged 5-HT\textsubscript{2C} receptors within the ER and Golgi, and on the plasma membrane of living cells. Resonance energy can be transferred from CFP to YFP if they are within 10nm of each other and
their dipoles are appropriately aligned. Energy transfer from CFP (donor) to YFP (acceptor) results in the quenching of CFP fluorescence, an increase in YFP fluorescence, and can be measured as an increase in CFP fluorescence following selective irradiation of the YFP fluorophore. This method of measuring FRET is called acceptor photobleaching (31). Laser scanning confocal microscopy allows the photobleaching to be confined to very discrete intracellular regions. This minimizes the time required for irradiation of the acceptor fluorophore and makes the technique suitable for live cell imaging. A specific FRET signal resulting from proteins in a clustered distribution, such as dimers/oligomers, has been shown to be dependent on the ratio of donor to acceptor expressed in the cell, while FRET resulting from over-expression of randomly distributed proteins in close proximity to one another can be distinguished by a dependence on acceptor fluorescence or total protein expression level (34, 35). In the present study, real-time FRET efficiencies measured for 5-HT2C/CFP and 5-HT2C/YFP fusion proteins in the ER and Golgi apparatus of living HEK293 cells were dependent on the ratio of donor to acceptor (uD/A) expressed within a given cell and independent of receptor expression level (34, 35). When FRET efficiencies were divided into five groups based on their uD/A ratios, the mean FRET efficiencies increased as uD/A decreased and were similar for ER and Golgi. In addition, the overall mean uD/A ratios and FRET efficiencies were similar for ER, Golgi, and plasma membrane. The similar mean FRET efficiencies determined for 5-HT2C/CFP and 5-HT2C/YFP fusion proteins in the ER, Golgi, and plasma membrane suggest that 5-HT2C receptor homodimers/oligomers form in the ER and then traffic through the secretory pathway to the plasma membrane. The FRET results were confirmed by immunoprecipitation of both immature and mature glycosylated forms of the 5-HT2C receptor.

Previous studies demonstrating the presence of constitutive dimers/oligomers on the plasma membrane (5, 12-18), studies using mutant receptors that retain their wild-type counterparts within intracellular compartments (25-29), and BRET studies using sub-cellular membrane fractions (14, 30) suggest that GPCR dimers/oligomers may form prior to trafficking to the plasma membrane. The present study provides direct visualization of class A GPCR dimer/oligomer biogenesis occurring within discrete intracellular compartments (ER and Golgi) of living cells, and subsequent trafficking through the secretory pathway to the plasma membrane. This may be a naturally occurring step in GPCR biosynthesis, it may be required for trafficking out of the ER, and/or it may be essential for forming a functional signaling unit. At the present time the functional significance of this phenomenon remains unknown.

The obligatory dimerization of GABAB receptors critical for expression of a functional signaling unit, the intracellular retention of wild-type GPCR by their mutant counterparts, and the evidence provided by the current live-cell study indicate that dimerization may be a prerequisite for normal receptor trafficking and expression on the plasma membrane. Dimer/oligomer formation following receptor biosynthesis may be necessary for passing ER quality control check-points that determine functionality. Proteins can be retained in the ER if they are not folded properly or assembled with other proteins critical for the formation of a functional complex. In the case of GABAB receptors, dimerization is essential for masking an ER retention motif located in the c-terminal region of the GABAB2R1 receptor, allowing export of a functional receptor complex from the ER (22). It is also possible that dimerization in the ER may be a prerequisite for trafficking to the plasma membrane as dimers may represent the basic metabotropic signaling unit.

Studies involving the rhodopsin receptor support the hypothesis that the dimer may represent the basic signaling unit. Atomic force microscopy has been used to visualize rhodopsin receptors in native mammalian membranes as rows of dimeric complexes (6, 7). Also, the distance between the α- and γ-subunits of a single heterotrimeric G-protein, which are the reported regions of contact with GPCR, is predicted to be too large to accommodate a single rhodopsin receptor (36, 37). Studies using chemical cross-linking and purified leukotriene B4 receptors (LTB4) have demonstrated that an LTB4 homodimer forms a pentameric complex with a single heterotrimeric G-protein (38). We have recently reported that the
predicted ligand:dimer:G-protein stoichiometry is 2:1:1 for 5-HT₂c receptors expressed in HEK293 cells (39). In addition, the D1-D2 dopamine receptor heterodimer has been reported to form a novel signaling complex in which ligand binding to both protomers results in Ga₃ activation, but blockade of either protomer alone is sufficient to block signaling (40). The results of these experiments are consistent with a model in which class A GPCR dimers interact with a single G-protein and suggest that the dimer may represent the basic signaling unit. Elegant studies involving trafficking and non-trafficking wild-type and mutant class C metabotropic glutamate receptors have demonstrated that ligand binding to one protomer of the dimer can result in G-protein activation (41). Recent studies involving the D₂ dopaminergic receptor have established that conformational changes occur at the dimer interface during receptor activation and inactivation (42), indicating that conformational changes at the dimer interface may provide a means for trans-activation of G-proteins and may explain how heterodimerization between two different GPCR can produce a unique pharmacological profile.

In conclusion, our results indicate that class A GPCR dimer/oligomer biogenesis occurs at an early time point during receptor biosynthesis and processing in the ER and Golgi. Live-cell confocal microscopy combined with FRET provided real-time visualization of dimer/oligomer formation and trafficking through the secretory pathway to the plasma membrane, suggesting that homodimerization/oligomerization is a naturally occurring step in 5-HT₂c receptor maturation and processing. Future studies aimed at identifying the molecular interface between protomers of 5-HT₂c receptor homodimers, and subsequent disruption of dimer formation, will provide a model system which can be used for determining the importance of homodimerization in GPCR trafficking and signaling.
References:

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Figure Legends

Figure 1: Live-cell confocal fluorescence imaging of ER and Golgi apparatus. The red scale bar represents 10µm. A) HEK293 cells 24 hrs post-transfection with a YFP variant containing ER targeting and retention sequences (ER/YFP). B) HEK293 cells 24 hrs post-transfection with a YFP variant containing a Golgi targeting sequence (Golgi/YFP) indicated by white arrows. C-E) HEK293 cells co-transfected with 5-HT<sub>2C</sub>/CFP (green) and the ER/YFP marker (red). Twelve hours post-transfection, live cells were imaged following excitation at 458nm and linear unmixing of CFP and YFP emission spectra. F-H) HEK293 cells co-transfected with 5-HT<sub>2C</sub>/CFP (green) and the Golgi/YFP marker (red). Cells were imaged live as in C-E above, 16 hours post-transfection. I-K) HEK293 cells co-transfected with 5-HT<sub>2C</sub>/CFP (green) and the Golgi/YFP marker (red). Cells were imaged live as in C-E above, 20 hours post-transfection.

Figure 2: Confocal fluorescence imaging of living HEK293 cells co-transfected with 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP. A) 5-HT<sub>2C</sub> receptors expressed in the ER. B) 5-HT<sub>2C</sub> receptors expressed in the Golgi. C) 5-HT<sub>2C</sub> receptors on the plasma membrane. The red scale bar represents 10um.

Figure 3: Acceptor photobleaching FRET in the ER. Fluorescence confocal microscopy was used to visualize a 2 µm thick optical cross-section of a living HEK293 cell co-expressing 5-HT<sub>2C</sub>/CFP (donor) and 5-HT<sub>2C</sub>/YFP (acceptor) in the ER. CFP (green) and YFP (red) images were captured simultaneously following excitation at 458 nm and linear unmixing of their emission spectra (A, B, C). A region of ER (marked by the white rectangle) was photobleached at 514 nm for five seconds. Post-bleach images were captured simultaneously following excitation at 458 nm (D, E, F). FRET is visualized as an increase in CFP fluorescence following YFP photobleaching (marked by arrow in D). Red scale bar represents 10um.

Figure 4: FRET was measured in the ER of living HEK293 cells expressing fluorescent-tagged 5-HT<sub>2C</sub> receptors. A) FRET efficiency was plotted versus uD/A ratio (post-bleach CFP fluorescence / pre-bleach YFP fluorescence) for cells co-expressing 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP (n=50). Non-linear regression analysis (one-phase exponential decay) R<sup>2</sup>=0.92 was determined using GraphPad Prism software. B) FRET efficiency plotted versus acceptor fluorescence by uD/A ratio for the same 50 cells as shown in figure 4A above. Linear regression analysis (GraphPad Prism) revealed slopes not different from zero.

Figure 5: Acceptor photobleaching FRET in the Golgi. Fluorescence confocal microscopy was used to visualize a 2 µm thick optical cross-section of a living HEK293 cell co-expressing 5-HT<sub>2C</sub>/CFP (donor) and 5-HT<sub>2C</sub>/YFP (acceptor) in the Golgi. CFP (green) and YFP (red) images were captured simultaneously following excitation at 458 nm and linear unmixing of their emission spectra (A, B, C). A region of Golgi (marked by the white rectangle) was photobleached at 514 nm for five seconds. Post-bleach images were captured simultaneously following excitation at 458 nm (D, E, F). FRET is visualized as an increase in CFP fluorescence following YFP photobleaching (marked by arrow in D). Red scale bar represents 10um.

Figure 6: FRET was measured in the Golgi of living HEK293 cells expressing fluorescent-tagged 5-HT<sub>2C</sub> receptors. A) FRET efficiency was plotted versus uD/A ratio (post-bleach CFP fluorescence / pre-bleach YFP fluorescence) for cells co-expressing 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP (n=50). Non-linear regression analysis (one-phase exponential decay) R<sup>2</sup>=0.92 was determined using GraphPad Prism software. B) FRET efficiency plotted versus acceptor fluorescence by uD/A ratio for the same 50 cells as shown in figure 6A above. Linear regression analysis (GraphPad Prism) revealed slopes not different from zero.

Figure 7: Immunoprecipitation of differentially tagged 5-HT<sub>2C</sub> receptors. A) Solubilized membrane proteins were immunoprecipitated with HA(Y-11)-agarose, denatured, run on a 10% polyacrylamide gel, and immunoblotted with GFP-HRP antibody. Lane 1: HEK293 cells co-transfected with 5-HT<sub>2C</sub>/HA and 5-HT<sub>2C</sub>/YFP. Lane 2: HEK293 cells separately transfected with 5-HT<sub>2C</sub>/HA or 5-HT<sub>2C</sub>/YFP and cells
mixed post-transfection prior to solubilization. B) Solubilized membrane proteins in the absence (Lane 1) and presence (Lane 2) of PNGase run on a 7.5% polyacrylamide gel for separation of multiple glycosylation states of the 5-HT\textsubscript{2C} receptor. C) Cells were pretreated with Brefeldin A and immunoprecipitated as described in section A above. Lane 1: HEK293 cells co-transfected with 5-HT\textsubscript{2C}/HA and 5-HT\textsubscript{2C}/YFP. Lane 2: HEK293 cells separately transfected with 5-HT\textsubscript{2C}/HA or 5-HT\textsubscript{2C}/YFP and cells mixed post-transfection.
Table 1: FRET controls.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>%FRET</th>
<th>uD/A</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;/CFP</td>
<td>-1.3 ± 0.7</td>
<td>N.A.</td>
<td>10</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;/CFP + ER/YFP marker</td>
<td>2.3 ± 1.5</td>
<td>1.09 ± 0.13</td>
<td>10</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;/CFP + CCR5/YFP</td>
<td>3.3 ± 1.1</td>
<td>0.83 ± 0.04</td>
<td>10</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2C&lt;/sub&gt;/CFP + 5-HT&lt;sub&gt;2C&lt;/sub&gt;/YFP</td>
<td>21.4 ± 1.4</td>
<td>0.81 ± 0.06</td>
<td>50</td>
</tr>
</tbody>
</table>

FRET was measured in the ER of living HEK293 cells following transfection with the indicated plasmid DNAs. Data represent the mean ± sem as indicated (n). N.A. = not applicable.

Table 2: FRET efficiency by uD/A ratio for 5-HT<sub>2C</sub> receptors expressed in the ER and Golgi.

<table>
<thead>
<tr>
<th>uD/A ratio</th>
<th>%FRET ER</th>
<th>%FRET Golgi</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30 – 0.45</td>
<td>34.4 ± 1.2 (11)</td>
<td>36.0 ± 1.0 (10)</td>
</tr>
<tr>
<td>0.46 – 0.59</td>
<td>28.9 ± 1.0 (9)</td>
<td>26.5 ± 1.1 (11)</td>
</tr>
<tr>
<td>0.60 – 0.85</td>
<td>20.3 ± 0.9 (12)</td>
<td>17.5 ± 1.3 (7)</td>
</tr>
<tr>
<td>0.86 – 1.29</td>
<td>13.3 ± 0.5 (11)</td>
<td>12.1 ± 0.6 (15)</td>
</tr>
<tr>
<td>1.30 – 2.00</td>
<td>6.3 ± 0.7 (7)</td>
<td>8.6 ± 0.8* (7)</td>
</tr>
</tbody>
</table>

FRET efficiencies measured in the ER and Golgi of living HEK293 cells co-expressing 5-HT<sub>2C</sub>/CFP (donor) and 5-HT<sub>2C</sub>/YFP (acceptor) were divided into five groups based on the ratio of donor to acceptor (uD/A). Data represent the mean ± sem for the number of cells indicated (n).

*p<0.05 versus ER, Student’s t-test.

Table 3: Comparison of mean FRET efficiencies for 5-HT<sub>2C</sub> receptors expressed in the ER, Golgi and on the plasma membrane.

<table>
<thead>
<tr>
<th>Region</th>
<th>uD/A</th>
<th>%FRET</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>0.81 ± 0.06</td>
<td>21.4 ± 1.4</td>
<td>50</td>
</tr>
<tr>
<td>Golgi</td>
<td>0.81 ± 0.06</td>
<td>20.3 ± 1.5</td>
<td>50</td>
</tr>
<tr>
<td>Plasma Membrane</td>
<td>0.80 ± 0.05</td>
<td>21.5 ± 1.5</td>
<td>24</td>
</tr>
</tbody>
</table>

FRET between 5-HT<sub>2C</sub>/CFP and 5-HT<sub>2C</sub>/YFP was measured in co-transfected HEK293 cells. The donor/acceptor ratio (uD/A) and FRET efficiency (%FRET) were measured in selected regions of the ER, Golgi apparatus, and plasma membrane of living cells. Data represent the mean ± sem for the number of cells indicated (n).
Figure 1

![Image of Figure 1 showing ER/YFP and Golgi/YFP markers with time post-transfection.]  

12hr 16hr 20hr

Figure 2

![Image of Figure 2 showing 2C/CFP and 2C/YFP with time post-transfection.]  

12 hrs 16 hrs 20 hrs
Figure 3

Figure 4
Figure 5

Figure 6
Figure 7
Serotonin 5-HT2c receptor homodimer biogenesis in the endoplasmic reticulum: Real-time visualization with confocal fluorescence resonance energy transfer
Katharine Herrick-Davis, Barbara A. Weaver, Ellinor Grinde and Joseph E. Mazurkiewicz

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