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Oligomerization of G-protein-coupled Receptors Shown by Selective Co-immunoprecipitation*

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Recent studies have shown that G-protein-coupled receptors (GPCRs) can assemble as high molecular weight homo- and hetero-oligomeric complexes. This can result in altered receptor-ligand binding, signaling, or intracellular trafficking. We have co-transfected HEK-293 cells with differentially epitope-tagged GPCRs from different subfamilies and determined whether oligomeric complexes were formed by co-immunoprecipitation and immunoblot analysis. This gave the surprising result that the $5HT_{1A}$ receptor was capable of forming hetero-oligomers with all GPCRs tested including the $5HT_{1B}$, $5HT_{1D}$, EDG_1, EDG_3, GPR_{26}, and $GABA_{B2}$ receptors. The testing of other GPCR combinations showed similar results with hetero-oligomer formation occurring for the $5HT_{1D}$ with the $5HT_{1B}$ and EDG_1 receptor. Control studies showed that these complexes were present in co-transfected cells before the time of lysis and that the hetero-oligomers were comprised of GPCRs at discrete stoichiometries. These findings suggest that GPCRs have a natural tendency to form oligomers when co-transfected into cells. Future studies should therefore investigate the presence and physiological role of GPCR hetero-oligomers in cells in which they are endogenously expressed.

Recent studies have shown that G-protein-coupled receptors (GPCRs)[1] may form dimers or higher order oligomers (1–7). This has led to some re-evaluation of the mechanisms thought to be involved in GPCR function. Co-expression studies with chimeric α_{2C}-adrenergic/M3 muscarinic receptor constructs have shown that intermolecular interactions can occur between different GPCR subtypes (1, 2). More direct studies have shown that the $GABA_BR1$ and $GABA_BR2$ receptors are not functional as separate units and can only form a functional receptor complex with the correct pharmacological properties and plasma membrane expression when they are co-expressed (8–12). The finding that co-transfection of the κ and δ opioid receptors results in the formation of heterodimers with ligand binding and functional properties that are distinct from singly transfected receptors suggests that heterodimerization may be involved in modulation of GPCR function (13). Also, Western blot studies have shown that the $5HT_{1D}$ and $5HT_{1B}$ receptors form homodimers when expressed alone although they preferentially form heterodimers when co-expressed (14). In the present study, we have directly examined whether $5HT_1$ receptors are capable of forming oligomeric complexes with a variety of GPCRs by specific immunoprecipitation of each receptor followed by identification of the co-precipitated proteins using immunoblot analysis. The main objective was to determine the specificity of the interactions across different GPCR subfamilies to gain further insight into the mechanism of GPCR hetero-oligomerization.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and other DNA-modifying enzymes were from New England Biolabs (Beverly, MA) or Amersham Biosciences. Dulbecco's modified Eagle's medium and fetal calf serum were from Invitrogen. All other biochemicals were obtained from Sigma unless specified otherwise.

Construction of Epitope-tagged GPCRs and Transient Transfections—The c-Myc epitope (EQKLISEEDL) was inserted at the amino termini of $5HT_{1A}$ and $5HT_{1D}$ receptors by PCR mutagenesis using a Stratagene Robocycler with Hot-Top assembly (Amsterdam, Holland) as described by Nelson and Long (15). The FLAG epitope (DYKDDDDK) was inserted by a similar procedure into the amino termini of $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, EDG_1, and EDG_2 receptors. FLAG-tagged EDG_3, GPR_{26}, and $GABA_{B2}$ receptors were a gift from Dr. Kevin Lynch (University of Virginia, Charlottesville, VA). PCR products were cloned into the eucaryotic expression vector $pCDNA3.1^+$ (Invitrogen) using standard techniques (16). Inserts were sequenced with an ABI Prism dye terminator cycle sequencing kit and analyzed on an Applied Biosystems 373A stretch DNA sequencer. Transient transfection of HEK-293 cells with the epitope-tagged constructs was performed by calcium phosphate-mediated gene transfer as described previously (17).

Immunoprecipitation—Forty-eight hours after transfection cells were washed three times in phosphate-buffered saline, harvested by scraping, and centrifuged for 5 min at 500 × *g*. The pelleted cells were homogenized at 4 °C by drawing up and down through a 20-gauge syringe needle in 1 ml of cell lysis buffer (Sigma). The homogenates were centrifuged for 20 min at 14,000 × *g* at 4 °C, and the supernatants were combined with 12.5 μl (packed gel) of either anti-c-Myc or anti-FLAG M2 affinity agarose (Sigma) and mixed overnight at 4 °C. The immunoadsorbents were recovered by centrifugation for 5 min at 700 × *g* and washed three times by resuspension and centrifugation (5 min at 700 × *g*) in cell lysis buffer and two times in 50 mM Tris (pH 7.5) containing 0.1% (w/v) SDS and 150 mM NaCl. The samples were eluted into 60 μl of SDS loading buffer (Sigma).

Immunoblot Analysis—Eluted samples were heated for 3 min at 100 °C and subjected to SDS-PAGE on slab gels (180 × 160 × 1.5 mm) polymerized from 10% (w/v) acrylamide and 0.1% *N*,*N*′-methylenebisacrylamide in Tris/glycine/SDS buffer (Novex, San Diego, CA) using the discontinuous buffer system of Laemmli (18). The electrophoresed proteins were subjected to semi-dry electrophoretic transfer onto polyvinylidene difluoride membranes as described previously (19). The membranes were incubated with either mouse c-Myc antibodies (1:1000) or mouse FLAG antibodies (1:1000) and then with peroxidase-conjugated sheep anti-mouse serum (1:1000) for detection of immunoreactive bands

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[1] The abbreviation used is: GPCR, G-protein-coupled receptor.

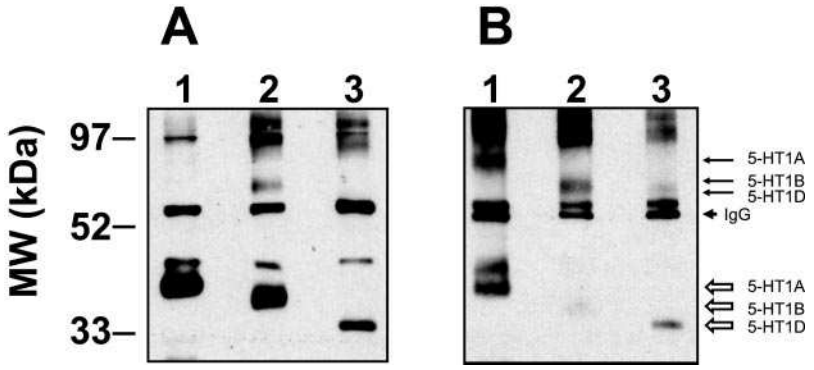

FIG. 1. **Immunoblot analysis of transfected HEK-293 cells showing homo-oligomerization of $5HT_{1A}$, $5HT_{1B}$, and $5HT_{1D}$ receptors.** *A*, HEK-293 cells (5×10^7) expressing the FLAG-tagged $5HT_{1A}$ (*lane 1*), $5HT_{1B}$ (*lane 2*), or $5HT_{1D}$ receptor (*lane 3*) were immunoprecipitated and subjected to immunoblot analysis using FLAG antibodies as described under "Experimental Procedures." *B*, the same immunoprecipitated samples were subjected to immunoblot analysis under non-reducing (minus dithiothreitol) conditions. The migration of monomers is indicated by *open arrows* (approximate molecular sizes are: $5HT_{1A}$, 46 kDa; $5HT_{1B}$, 44 kDa; $5HT_{1D}$, 42 kDa), the putative dimers are indicated by *solid arrows*, and immunoglobulin heavy chains are indicated by an *arrowhead*. The molecular size markers are shown in kDa. The immunoblots shown are representative of at least three independent experiments.

by enhanced chemiluminescence (Amersham Biosciences). Molecular size calibration was achieved with the MultiMark standards (Novex).

RESULTS

Homo-oligomerization of $5HT_{1A}$, $5HT_{1B}$, and $5HT_{1D}$ Receptors—HEK-293 cells transfected with either FLAG-tagged $5HT_{1A}$, $5HT_{1B}$, or $5HT_{1D}$ receptor constructs were immunoprecipitated with anti-FLAG-agarose, and immunoblot analysis of the precipitates revealed the presence of 46, 44, and 42-kDa FLAG-immunoreactive bands, respectively (Fig. 1*A*). Higher molecular weight immunoreactive forms were also present, which might represent oligomeric forms of the $5HT_1$ receptors. An immunoreactive band that migrated at a size predicted for a FLAG-tagged $5HT_{1A}$ receptor dimer (92 kDa) showed a strong increase in immunostaining with the reciprocal decrease of the 46-kDa monomer band when the disulfide bond-reducing agent dithiothreitol was omitted from the sample loading buffer (Fig. 1*B*). An immunoreactive band that migrated at a size predicted for a FLAG-tagged $5HT_{1B}$ receptor dimer (88 kDa) showed increased immunostaining with the loss of the 44-kDa monomer band when the samples were electrophoresed in the absence of dithiothreitol (Fig. 1*B*). Similarly, a possible FLAG-tagged $5HT_{1D}$ receptor dimer (82 kDa) showed an increase in immunostaining on non-reducing gels with the reciprocal disappearance of the 41-kDa monomer band (Fig. 1*B*). Similar results have been shown previously for the $5HT_{1B}$ and $5HT_{1D}$ receptors, which led to the suggestion that they form homodimers (14). The mechanism of $5HT_{1B}$ receptor homodimerization appeared to differ from that of the $5HT_{1A}$ and $5HT_{1D}$ receptors because a high proportion $5HT_{1B}$ receptor homodimer was observed even when gels were run under disulfide bond-reducing conditions (Fig. 1*A*).

Hetero-oligomerization of $5HT_{1A}$ and $5HT_{1D}$ Receptors with Other GPCRs— To directly test for hetero-oligomerization of different GPCR combinations we used sequential co-immunoprecipitation and immunoblot analyses of cells co-transfected with differentially epitope-tagged receptors. This showed that the c-Myc-tagged $5HT_{1A}$ receptor co-precipitated with FLAG-tagged versions of all receptors tested including the $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, EDG_1, EDG_3, GPR_{26}, and $GABA_{B2}$ receptors (Fig. 2, *A* and *B*). The same finding was observed for the c-Myc-tagged $5HT_{1D}$ receptor, which was co-precipitated with FLAG-tagged versions of the $5HT_{1B}$ and EDG_1 receptors (Fig. 2, *A* and *B*). Previous studies have also shown that the $5HT_{1B}$ and $5HT_{1D}$ receptors form heterodimers (14). The finding that the FLAG- and c-Myc-tagged versions of the $5HT_{1A}$ receptor were co-precipitated provides direct evidence that this GPCR forms a homo-oligomer. This was also found for the $5HT_{1D}$ receptor using the c-Myc- and FLAG-tagged versions of this GPCR (data not shown). Co-precipitation of high molecular weight FLAG-immunoreactive $5HT_{1B}$, $5HT_{1D}$, EDG_1, and EDG_3 species with the c-Myc-$5HT_{1A}$ receptor (Fig. 2*A*) suggests that these GPCRs might be present as homodimers and trimers in higher order hetero-oligomeric complexes comprised of at least one c-Myc-$5HT_{1A}$ receptor and two or more of the FLAG-tagged constructs.

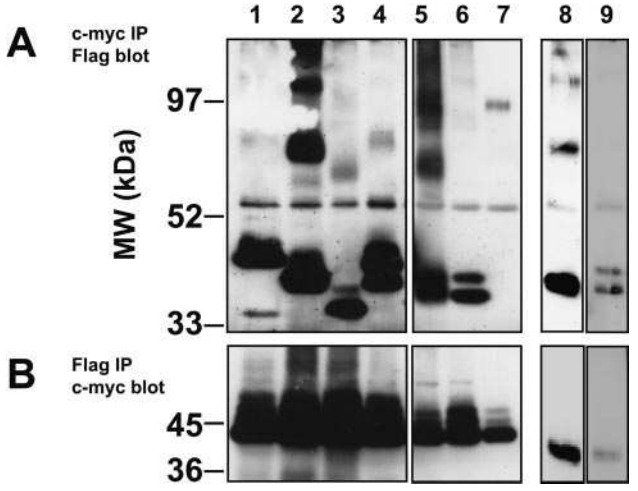

FIG. 2. **Sequential immunoprecipitation/immunoblot analysis of transfected HEK-293 cells showing oligomerization of $5HT_{1A}$ and $5HT_{1D}$ receptors with other GPCRs.** HEK-293 cells (5×10^7) either co-expressing the c-Myc-tagged $5HT_{1A}$ receptor with FLAG-tagged versions of the $5HT_{1A}$ (*lane 1*), $5HT_{1B}$ (*lane 2*), $5HT_{1D}$ (*lane 3*), EDG_1 (*lane 4*), EDG_3 (*lane 5*), GPR_{26} (*lane 6*), and $GABA_{B2}$ (*lane 7*) receptors or co-expressing the c-Myc-tagged $5HT_{1D}$ receptor with FLAG-tagged versions of the $5HT_{1B}$ (*lane 8*) and EDG_1 (*lane 9*) receptors were immunoprecipitated using either anti-c-Myc (*A*) or anti-FLAG-agarose (*B*), and the precipitates were subjected to immunoblot analysis using FLAG (*A*) or c-Myc (*B*) antibodies as described under "Experimental Procedures." The molecular size markers are shown in kDa (approximate molecular sizes are: $5HT_{1A}$, 46 kDa; $5HT_{1B}$, 44 kDa; $5HT_{1D}$, 42 kDa; EDG_1, 43 kDa; EDG_3, 42 kDa; GPR_{26}, 38 kDa; $GABA_{B2}$, 106 kDa). The immunoblots shown are representative of four independent experiments. *IP*, immunoprecipitate.

Sequential Immunoprecipitation/Immunoblot Analysis of Mixed Singly Transfected HEK-293 Cells—To rule out the possibility that the observed hetero-oligomerizations were caused by nonspecific aggregation of receptors, extracts from cells separately expressing the c-Myc-tagged $5HT_{1A}$ and FLAG-tagged $5HT_{1B}$ receptors were mixed, immunoprecipitated with anti-c-

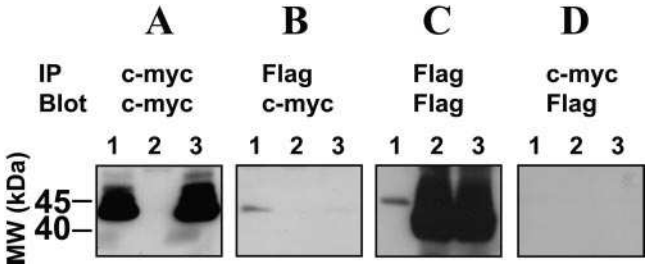

FIG. 3. **Sequential immunoprecipitation/immunoblot analysis of mixed singly transfected HEK-293 cells.** HEK-293 cells (5×10^7) expressing either the c-Myc-tagged $5HT_{1A}$ receptor (*lane 1*), the FLAG-tagged $5HT_{1B}$ receptor (*lane 2*), or a mixture of both membranes (*lane 3*) were immunoprecipitated (*IP*) with either anti-c-Myc agarose (*A* and *D*) or anti-FLAG agarose (*B* and *C*), and the precipitates were subjected to immunoblot (*Blot*) analysis with either c-Myc (*A* and *B*) or FLAG (*C* and *D*) antibodies as described under "Experimental Procedures." The immunoblots shown are representative of at least three independent experiments.

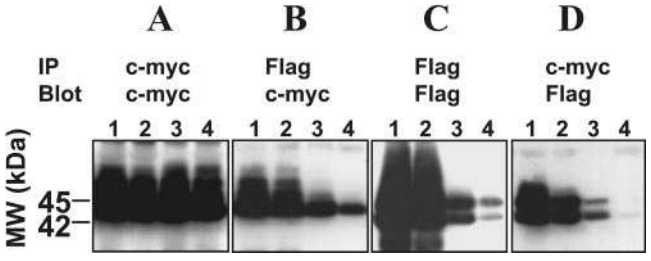

FIG. 4. **Sequential immunoprecipitation/immunoblot analysis of HEK-293 cells transfected with varying amounts of $5HT_{1A}$ and EDG_1 receptors.** HEK-293 cells (5×10^7) were co-transfected with 25 μg of the c-Myc-tagged $5HT_{1A}$ receptor and either 25 (*lane 1*), 5 (*lane 2*), 1 (*lane 3*), or 0.2 (*lane 4*) μg of the FLAG-tagged EDG_1 receptor followed by immunoprecipitation (*IP*) using either anti-c-Myc agarose (*A* and *D*) or anti-FLAG agarose (*B* and *C*), and the precipitates were subjected to immunoblot (*Blot*) analysis using c-Myc (*A* and *B*) or FLAG (*C* and *D*) antibodies as described under "Experimental Procedures." The molecular sizes of the c-Myc-$5HT_{1A}$ (45 kDa) and FLAG-EDG_1 (42 kDa) receptor constructs are shown. The immunoblots shown are representative of three independent experiments.

Myc agarose, and subjected to immunoblot analysis with FLAG antibodies. In this case, the FLAG-$5HT_{1B}$ receptor was not detected in the precipitated material (Fig. 3*A*). Similarly, immunoprecipitation of the mixed extract with anti-FLAG agarose followed by immunoblot analysis with c-Myc antibodies did not detect the c-Myc-$5HT_{1A}$ receptor (Fig. 3*B*). This confirmed that successful immunoprecipitation of $5HT_{1A}/5HT_{1B}$ hetero-oligomers requires co-expression within the same cells before the time of lysis. Thus, the oligomerization of the c-Myc-tagged $5HT_{1A}$ and FLAG-tagged $5HT_{1B}$ receptors in the present study is not likely to be caused by nonspecific aggregation. The same results were found for all of the other GPCR/GPCR hetero-oligomers tested in this study (data not shown).

Sequential Immunoprecipitation/Immunoblot Analysis of HEK-293 Cells Transfected with Varying Amounts of $5HT_{1A}$ and EDG_1 Receptors—To determine whether the hetero-oligomers observed in this study are comprised of GPCRs at discrete protein/protein ratios, HEK-293 cells were co-transfected with a fixed amount (25 μg) of the c-Myc-tagged $5HT_{1A}$ receptor and varying amounts (25, 5, 1, and 0.2 μg) of the FLAG-tagged EDG_1 receptor. The relative amounts of the c-Myc-$5HT_{1A}$ receptor that were associated with the decreasing levels of the FLAG-EDG_1 receptor were determined by sequential immunoprecipitation and immunoblot analysis. Immunoprecipitation of the FLAG-EDG_1 receptor with anti-FLAG-agarose resulted in co-precipitation of similar decreasing levels of c-Myc-$5HT_{1A}$ receptor (Fig. 4*B*) as shown by immunoblot analysis with c-Myc antibodies. Similarly, immunoprecipitation of the c-Myc-$5HT_{1A}$ receptor resulted in co-precipitation of decreasing levels of the FLAG-EDG_1 receptor (Fig. 4*D*). Sequential immunoprecipitation/immunoblot analysis of the lysates with c-Myc/c-Myc or FLAG/FLAG confirmed that the c-Myc-$5HT_{1A}$ receptor was actually expressed in the cells at relatively constant levels and that expression of the FLAG-EDG_1 receptor decreased accordingly with the amounts of cDNA transfected into the cells (Fig. 4, *A* and *C*). Similar results were obtained for all of the other receptors under study (data not shown).

DISCUSSION

We have used a co-immunoprecipitation approach to provide direct evidence that GPCRs from different subfamilies are capable of forming hetero-oligomers when co-expressed in HEK-293 cells. Cells were co-transfected with GPCR constructs containing different epitope tags followed by immunoprecipitation of one receptor and detection of co-precipitated receptors by immunoblot analysis. The $5HT_{1A}$ receptor formed a homo-oligomer when expressed alone and formed hetero-oligomers when co-expressed with the closely related $5HT_{1B}$ and $5HT_{1D}$ receptors (~36% amino acid homology) and also with the more distantly related EDG_1, EDG_3, GPR_{26}, and $GABA_{B2}$ receptors (4–16% amino acid homology). Interestingly, hetero-oligomer formation was shown for all other GPCR combinations tested including the $5HT_{1D}$ receptor with the $5HT_{1B}$ and EDG_1 receptors. The heterodimerization of $5HT_{1B}$ and $5HT_{1D}$ receptors has been shown previously in insect SF9 cells using a viral expression system followed by Western blot analysis (14).

The results of this study suggest that the receptor complexes were pre-existing in cells at the time of lysis and were not caused by nonspecific aggregation. Oligomerization was observed only in lysates from co-transfected cells and not in mixed lysates from singly transfected cells. Also, the observed receptor/receptor interactions appeared to occur at defined stoichiometries in co-transfected cells because decreased expression of one receptor resulted in a similar incremental decrease in the amount of the second receptor immunoprecipitated. The appearance of high molecular weight immunoreactive bands corresponding in size to $5HT_{1A}$ and $5HT_{1D}$ receptor homo-oligomers appeared to require intact disulfide bonds because these were only detected when gels were run under non-reducing conditions. Little or no $5HT_{1A}$ and $5HT_{1D}$ receptor homo-oligomers could be detected when gels were run under reducing conditions. In contrast, a significant proportion of the $5HT_{1B}$ receptor was detected as high molecular weight oligomers when gels were run under reducing conditions with increased levels of the oligomer and reciprocal loss of the monomer under non-reducing conditions. This indicates that the mechanism of $5HT_{1B}$ receptor homo-oligomer formation differs from that of the $5HT_{1A}$ and $5HT_{1D}$ receptors. Also, the observed immunoisolation of the putative $5HT_{1B}$ receptor dimer by immunoprecipitation of the $5HT_{1A}$ receptor indicates that these receptors may be present as higher order hetero-oligomers such as trimers or tetramers.

A major prerequisite for the physiological assembly of hetero-oligomeric GPCRs is co-expression in the same cells. To demonstrate this would require detailed immunological or specific binding experiments that localize the receptors under study to the same cells combined with co-precipitation studies using receptor-specific antibodies or ligands. The GPCRs tested in this study show a wide range of tissue distributions in the brain. Within the 5HT receptor subfamily $5HT_{1A}$, $5HT_{1B}$, and $5HT_{1D}$ receptors all show pre- and postsynaptic localizations and can be found together in some of the same brain regions including the cortex and dorsal raphe nucleus (20–22). The $5HT_{1B}$ receptor shows co-expression with the $5HT_{1A}$ receptor in

the hippocampus and with the $5HT_{1D}$ receptor in the olfactory tubercle although the $5HT_{1B}$ receptor is expressed separately in the anterior caudate putamen, hypothalamus, and thalamus, and the $5HT_{1D}$ receptor is localized separately in the trigeminal nucleus and in parts of the cerebellum (21). Hetero-oligomerization of the $5HT_{1A}$ with the $GABA_{B2}$ and GPR26 receptors may be of interest because all of these receptors are widely distributed throughout the cortex and may therefore share some regions of overlap (22–24). The physiological relevance of the hetero-oligomerization of $5HT_{1A}$ receptor with the EDG_1 and EDG_3 receptors is not clear because no detailed studies have been carried out on the distribution of the latter receptors within the brain. However, the interactions observed between the $5HT_{1A}$ and EDG_3 receptors are not likely to occur *in vivo* because the relative expression levels of the latter are relatively low in the brain with the highest levels observed in peripheral tissues such as lung and heart (25, 26).

Supporting evidence for oligomerization of endogenously expressed GPCRs comes from Western blot studies of brain tissues revealing the presence of high molecular weight immunoreactive forms of the dopamine D2 receptor (27) and the A1 adenosine receptor (28). More recently an immunoelectron microscopy study showed that CCR5 and CXCR4 receptors are clustered and closely apposed on microvilli and in trans-Golgi vesicles of human macrophages and T cells (29). The strongest evidence comes from studies showing that $GABA_BR1$ and $GABA_BR2$ receptors could be co-immunoprecipitated from rat cortex (9).

This is the first study in which the phenomenon of GPCR oligomerization has been investigated using a large number of diverse receptor subtypes. Interestingly, all of the receptors tested were capable of forming hetero-oligomers when co-expressed in tissue culture cells suggesting that this might be a general characteristic of these receptors. These findings highlight the importance for further studies to investigate the occurrence of GPCR hetero-oligomerization in tissues and cells where they are endogenously expressed. Such studies will help to establish the physiological role of such complexes in GPCR function.

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