MINIREVIEW—EXPLORING THE BIOLOGY OF GPCRs: FROM IN VITRO TO IN VIVO

G Protein–Coupled Receptor Multimers: A Question Still Open Despite the Use of Novel Approaches

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ABSTRACT

Heteromerization of G protein–coupled receptors (GPCRs) can significantly change the functional properties of involved receptors. Various biochemical and biophysical methodologies have been developed in the last two decades to identify and functionally evaluate GPCR heteromers in heterologous cells, with recent approaches focusing on GPCR complex stoichiometry and stability. Yet validation of these observations in native tissues is still lagging behind for the majority of GPCR heteromers. Remarkably, recent studies, particularly some involving advanced fluorescence microscopy techniques, are contributing to our current knowledge of aspects that were not well known until now, such as GPCR complex stoichiometry and stability. In parallel, a growing effort is being applied to move the field forward into native systems. This short review will highlight recent developments to study the stoichiometry and stability of GPCR complexes and methodologies to detect native GPCR dimers.

Introduction

G protein–coupled receptors (GPCRs) play a key role in the regulation of cells in our body by activating intracellular signaling in response to a wide variety of specific agonists (Lagerström and Schiöth, 2008; Alexander et al., 2011). Most cells express multiple GPCR subtypes and are consequently able to respond to at least a corresponding number of different agonists (Vassilatis et al., 2003; Regard et al., 2008; Insel et al., 2012; Fève et al., 2014). Distinct GPCRs can affect each other’s functions to come to an integrative cellular response through direct physical interactions in heteromeric complexes. Over the last 20 years, dimerization and/or oligomerization was reported for nearly all tested GPCR subtypes using mostly engineered GPCR constructs expressed in heterologous systems (Fig. 1) (Khelashvili et al., 2010; Cottet et al., 2012).

Class C GPCRs require dimerization to transduce transmembrane signaling in response to agonists (Kniazeff et al., 2011; El Moustaine et al., 2012). The most studied example is the GABA<sub>B</sub> receptor, for which GABA<sub>B1</sub> and GABA<sub>B2</sub> functionally complement each other by forming the receptor heterodimer. Binding of GABA to the N-terminal extracellular domain (NTED) of GABA<sub>B1</sub> results in allosteric transactivation of the GABA<sub>B2</sub> protomer and subsequent G protein coupling to the activated 7 transmembrane domain (7TMD) of GABA<sub>B2</sub> (Galvez et al., 2001; Duthey et al., 2002; Kniazeff et al., 2002). Moreover, GABA<sub>B1</sub> requires heteromerization with GABA<sub>B2</sub> to traffic to the cell surface (Marjeta-Mitrovic et al., 2000; Paganò et al., 2001; Brock et al., 2005). Heteromerization of the taste 1
receptor (T1R) 3 with T1R1 or T1R2 results in the sensation of umami taste or sweeteners, respectively (Zhao et al., 2003). Indeed, knockout of the individual T1R1-, T1R2-, or T1R3-encoding genes in mice largely attenuates umami, sweet, or both flavors, respectively (Damak et al., 2003; Zhao et al., 2003). Dimerization of purified metabotropic glutamate (mGlu) 2 receptors in nanodiscs is required for G protein activation in response to the endogenous agonist glutamate (El Moustaine et al., 2012). In contrast, refolding of purified class A GPCRs in detergent micelles or nanodiscs revealed that monomers are fully able to bind their cognate ligand, activate G proteins, and/or recruit G protein–coupled receptor kinases and arrestins (Bayburt et al., 2007; Hanson et al., 2007; White et al., 2007; Whorton et al., 2007, 2008; Kuszak et al., 2009; Arcemisbéhère et al., 2010; Tsukamoto et al., 2010; Bayburt et al., 2011). In addition, the class B parathyroid hormone receptor activates G proteins when expressed as monomers, as observed using dimer-disrupting mutations (Pioszak et al., 2010).

Hence, dimerization is not required for tested class A and B GPCRs to transduce agonist-induced intracellular signaling. The question of why do these GPCRs dimerize and oligomerize obviously rises. Physically interacting GPCRs may modulate each other’s activities. However, unambiguous discrimination between GPCR crosstalk as a consequence of receptor heteromerization and that resulting from their intracellular signaling events (Schmidlin et al., 2002; Vázquez-Prado et al., 2003; Natarajan et al., 2006; Kelly et al., 2008; Rives et al., 2009; Nijmeijer et al., 2010) is experimentally challenging and requires experimental perturbation of these

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**Fig. 1.** Detection of GPCR dimers. Antibodies targeting epitope-tagged (depicted) or native GPCRs specifically label receptor populations to subsequently allow detection of physical interaction by communoprecipitation (co-IP) (A) or close proximity using in situ proximity ligation of DNA-conjugated secondary antibodies (B) and time-resolved (TR) FRET between fluorophore-conjugated antibodies (C). Time-resolved FRET between fluorescent ligands allows detection of receptor complexes in native tissue (D). Genetic fusion of luminescent or fluorescent donor and acceptor proteins or nonfunctional fragments of these proteins to the C-terminal tail of GPCRs allows detection of close proximity by RET (E) and bimolecular fluorescence (BIFC) or luminescence (BiLC) complementation (F). Heteromer identification technology (HIT): RET between β-arrestin and one GPCR subtype that is fused to donor or acceptor proteins upon agonist activation of another untagged GPCR subtype indicates that both GPCRs are in close proximity (G). CODA-RET detects the interaction of GPCR dimers with intracellular signaling partners using the combination of bimolecular fluorescence complementation with sequential FRET (H).
GPCRs to form heteromers. Moreover, in situ validation of GPCR heteromerization and their specific functional properties in native tissues is difficult and consequently often lacking (Pin et al., 2007). Hence, the physiologic relevance of many identified GPCR heteromers remains a topic of debate, not least because recent methodologies shed distinct light on the size and stability of GPCR heteromers (Lambert and Javitch, 2014). In this review, we will first focus on recent developments to determine the size, stability, and proximal signaling of GPCR complexes and secondly on evidence for GPCR heteromerization in native tissue.

**Proportion, Size, and Stability of GPCR Dimers and Oligomers**

GPCR oligomerization has been the subject of significant research over the last two decades by using a number of biochemical and biophysical approaches mostly involving engineered GPCR constructs, among other approaches. Hence, receptors harboring N-terminal epitope (e.g., hemagglutinin, FLAG, or cMyc) and/or SNAP/CLIP/Halo tags can be detected using specific high-affinity antibodies and covalent labeling, respectively. Subsequent coimmunoprecipitation and time-resolved FRET reveals the physical association and close proximity of GPCRs in heterologous cells (Fig. 1, A–C) (Milligan and Bouvier, 2005; Maurel et al., 2008; Faklaris et al., 2015). Moreover, fusion of bioluminescent, fluorescent proteins, or nonfunctional fragments of these proteins to the C-terminal tail of GPCRs allows close-proximity detection of GPCR dimers and/or oligomers in living cells using bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), or bimolecular complementation, respectively (Fig. 1, E and F), and approaches such as the heteromer identification technology or complemented donor-acceptor resonance energy transfer (CODA-RRET) have also been implemented for the investigation of complexes involving three interacting partners (Fig. 1, G and H) (Ciruela et al., 2010; Cottet et al., 2012; Kaczor et al., 2014).

Although most of these techniques have retrieved qualitative information on the formation of GPCR dimers, the proportion of GPCRs that are engaged in dimers and/or oligomers as well as the stoichiometry and stability of such complexes are generally not well characterized and require carefully controlled quantitative measurements. Saturation FRET between purified β2-adrenergic receptors (β2-ARs) site-specifically labeled with fluorophores and reconstituted in lipid bilayers suggested the predominant formation of tetramers (Fung et al., 2009). Three-color sequential BRET-FRET and bimolecular luminescence fluorescence complementation in combination with resonance energy transfer (RET) revealed the formation of GPCR hetero-multimers, consisting of at least three or four individual GPCRs, when expressed at physiologic levels (Lopez-Gimenez et al., 2007; Carriba et al., 2008; Guo et al., 2008; Nijmeijer et al., 2010; Armando et al., 2014). However, the interpretation of quantitative RET approaches between membrane-associated proteins has been challenged (James et al., 2006; Lambert and Javitch, 2014; Lan et al., 2015), and revised experimental designs (Szalai et al., 2014) as well as third-party RET approaches (Kuravi et al., 2010) have been proposed to improve the interpretation of results from RET experiments.

In this context, intensity-based FRET approaches (i.e., those based on measurements of emission intensity of the fluorophores rather than of their fluorescence lifetimes), where spectral datasets are acquired, can provide quantitative information not only of the apparent FRET efficiency of a sample, but also on donor-acceptor stoichiometry of their interactions. These methods can be combined with spectral imaging microscopy for spatial resolution and have allowed investigation of the quaternary structure of GPCRs in a more quantitative manner than other previously employed non-spectral intensity-based FRET approaches based on average measurements of apparent FRET efficiency (Zeug et al., 2012; Raicu and Singh, 2013). This is possible because spectrally resolved FRET approaches allow the accurate measurement of concentrations of donors and acceptors with overlapping emission spectra. Contaminations of the FRET signal as a consequence of the donor’s bleedthrough or direct acceptor excitation are corrected in spectrally resolved FRET, and possible contributions of an unpaired donor and acceptors in the sample are taken into account by applying specific algorithms. Some drawbacks of these techniques can be the instrumentation requirements, such as spectral imaging detectors for certain applications, or the requirement of donor and acceptor reference samples of a known concentration (i.e., purified fluorescent proteins) for spectral unmixing. Different spectrally resolved FRET approaches revealed the proportion of serotonin 5-hydroxytryptamine (5-HT) 1A receptors that were engaged in oligomers in transfected cells (Gorinski et al., 2012) as well as the formation of transient tetramers by stable M₃ muscarinic acetylcholine receptor dimers at the cell surface, with minimal interference from a bystander RET signal coming from nearby noninteracting partners (Patowary et al., 2013).

Fluorescence correlation spectroscopy (FCS) allows the detection of fluctuations in fluorescent intensity that result from the diffusion of fluorescent molecules in and out of an open, diffraction-limited, observation volume. Suitable for extracting two-dimensional information on membrane protein dynamics with submicrosecond temporal resolution, it constitutes a powerful approach to monitor the diffusion of GPCRs in the plasma membrane of single living cells in real time. To this end, receptors fused to a particular fluorescent protein are heterologously expressed in cells (Herrick-Davis et al., 2012, 2013; Teichmann et al., 2014). As a variant, the expression of receptors fused to complementary fragments of a fluorescent protein will allow the exclusive detection of diffusing receptor complexes that become fluorescent upon bimolecular fluorescence complementation (Briddon et al., 2008). FCS measurements achieve single molecule sensitivity and are more accurately performed in samples with very low expression levels, such as those found for some GPCRs in native cells. Hence, endogenous GPCRs can be labeled with fluorescent ligands (Cordeaux et al., 2008; Corrden et al., 2014) or specific antireceptor fragment antigen binding proteins fused to fluorescent moieties (Herrick-Davis et al., 2015) for FCS measurements (Fig. 2). In the latter case and due to the monovalent nature of the fragment antigen binding fragments opposite to antibodies, potential artifacts due to antibody-induced clustering of receptors are avoided. FCS can give information on a protein that is likely to be in the same complex based on the changes in the diffusion coefficients by the formation of larger codiffusing entities and on the brightness of the diffusing particles. However, it should be kept in mind that FCS measures codiffusing proteins that are
the intensity of the two fluorophores will correlate as they separately registered by two different detectors, and when the labeled diffusing particles resulting from their interaction. In applications that allows discrimination of single-labeled (Teichmann et al., 2014), a development of FCS for dual-color possible dimeric species was found, with a low proportion of and B GPCRs, an equilibrium between monomeric and complexes in the apical membrane of living choroid plexus existence of native serotonin 5-HT 2C receptor homomeric et al., 2012, 2013). These combined techniques suggest the oligomerization state of proteins of interest (Herrick-Davis et al., 1999). By doing this, the molecular brightness of the diffusing number of fluorescent protomers within diffusing complexes. Combining FCS with a photon counting histogram analysis enables quantification of the average number of molecules within the observation volume and informs on the number of diffusing entities but not on the number of fluorescent protomers within diffusing complexes. Situated within the same microdomain but do not provide proof for direct physical protein–protein interaction. In addition, due to the detection limit, FCS is not able to discriminate between diffusion times of molecules with similar masses. At least a difference by a factor of four in the molecular mass of the particles is required to resolve their diffusion times by FCS (Meseth et al., 1999). This poses a limitation of FCS to distinguish among GPCR monomers, dimers, or tetramers on the basis of diffusion times alone. Moreover, factors such as heterogeneity of membrane viscosity in microdomains or the interaction of receptors with other signaling or scaffolding proteins might affect diffusion times. Autocorrelation analysis of FCS measurements reveals the average number of molecules within the observation volume and informs on the number of diffusing entities but not on the number of fluorescent protomers within diffusing complexes. Diffusion times by FCS (Meseth et al., 1999). This poses a limitation of FCS to distinguish among GPCR monomers, dimers, or tetramers on the basis of diffusion times alone. Moreover, factors such as heterogeneity of membrane viscosity in microdomains or the interaction of receptors with other signaling or scaffolding proteins might affect diffusion times. Autocorrelation analysis of FCS measurements reveals the average number of molecules within the observation volume and informs on the number of diffusing entities but not on the number of fluorescent protomers within diffusing complexes. Combining FCS with a photon counting histogram analysis enables quantification of the intensity of fluorescence fluctuations, which provides information with regards to the stoichiometry of protein complexes (Fig. 2C) (Chen et al., 1999). By doing this, the molecular brightness of the diffusing particles and consequently the number of fluorophores codiffusing in each particle can be determined. Subsequent comparison with appropriate reference proteins with a known monomeric/oligomeric status allows quantification of the oligomerization state of proteins of interest (Herrick-Davis et al., 2012, 2013). These combined techniques suggest the existence of native serotonin 5-HT 2C receptor homomeric complexes in the apical membrane of living choroid plexus epithelial cells (Herrick-Davis et al., 2015). For some class A and B GPCRs, an equilibrium between monomeric and possible dimeric species was found, with a low proportion of dimers, by using fluorescence crosscorrelation spectroscopy (Teichmann et al., 2014), a development of FCS for dual-color applications that allows discrimination of single-labeled entities carrying two different fluorophores from the dual-labeled diffusing particles resulting from their interaction. In this approach, the emission of the two fluorophores are separately registered by two different detectors, and when the two differently labeled partners comigrate, the fluctuations in the intensity of the two fluorophores will correlate as they diffuse into and out of the observation volume together. The crosscorrelation function provides more sensitive information than the diffusion coefficient alone for the detection of interactions. The information gathered in the work of Teichmann et al. (2014) confirmed single molecule total internal reflection fluorescence microscopy (TIRFM) studies at β1-AR or M 1 muscarinic acetylcholine receptors (M1Rs) (Hern et al., 2010; Calebiro et al., 2013). However, a homogeneous population of dimers without evidence of the coexistence of the monomeric species in equilibrium was found in the case of serotonin 5-HT 2C and also β1-AR or M 1R in other FCS/photon-counting histogram–based studies (Herrick-Davis et al., 2012, 2013, 2015). These discrepancies might be related to different receptor expression levels in the cellular models employed, where very low expression levels of the receptors compatible with single molecule resolution might favor the occurrence of monomeric species in dynamic equilibrium with the dimer population.

Recently, super-resolution dual-color photoactivation localization microscopy using photoactivatable dye–photoactivated localization microscopy allowed imaging of the spatial arrangement of individual GPCR molecules in dimers and oligomers at the plasma membrane with a resolution of ~8 nm. To this end, hemagglutinin- and FLAG-tagged luteinizing hormone receptor (LHR) mutants that were either ligand binding (LHR 50) or signaling (LHR 50) deficient (Rivero-Müller et al., 2010) were expressed in human embryonic kidney 293 cells and specifically labeled with antibodies conjugated with CAGE 552 and CAGE 500 photoswitchable dyes for dual-color visualization (Fig. 3A) (Jonas et al., 2015). The number and identity (either LHR 50 or LHR 50) of GPCRs within a 50-nm radius of each single GPCR are determined, and the irreversible activation and bleaching of the dyes allowed quantification of dimer and oligomer complexes (Fig. 3, B and C). Expression of wild-type LHR at levels in the physiologic range observed in the ovary and testis revealed a diverse organization in monomers, dimers (14.6%), and oligomers (26.8%, varying from trimers to oligomers consisting of more than nine protomers) (Fig. 3D).

In this study, agonist stimulation did not elicit any change in either the percentage of associated molecules or relative
proportions of dimers and oligomers (Jonas et al., 2015). Yet, in spite of the detailed information on the size and spatial arrangement of GPCRs in oligomeric complexes by photoactivatable dye–photoactivated localization microscopy, this technique involves the acquisition of a time series of images that needs cell fixation, and therefore it is not well suited for the investigation of complex stability and real-time dynamics.

The stability and dynamics of GPCR dimers and oligomers has been monitored by real-time single-molecule imaging and tracking using fluorescent ligands (Hern et al., 2010; Kasai et al., 2011) or SNAP-tagged GPCR constructs (Calebiro et al., 2013). In the former approach, one has to consider the number of ligands bound per dimer. If agonist binding of an agonist to one protomer prevents ligand binding to the associated protomer, then the dynamics of the dimer rather than monomers are observed (Albizu et al., 2010). Single-molecule TIRFM studies reported the existence of an equilibrium between monomeric and oligomeric species for different GPCRs, with fast dynamics of less than 1 second (Hern et al., 2010; Kasai et al., 2011; Calebiro et al., 2013). In these approaches and differently to PALM, the x-y spatial resolution is limited by the optical diffraction limit (~220 nm) so that the possibility of interpreting two monomers within the resolution limit as an apparent dimer must be considered. In particular, two-color TIRFM revealed the transient nature of M1R and N-formylpeptide receptor homodimers at the cell surface (Hern et al., 2010; Kasai et al., 2011). Yet, the stability of the interactions might differ between GPCR subtypes. For example, β1-AR displays more transient interactions than β2-AR, as revealed by both TIRFM and dual-color fluorescence recovery after photobleaching studies (Dorsch et al., 2009; Calebiro et al., 2013). Affinity-based corecruitment of differentially tagged mGlu receptors into forced microdomains on the cell surface confirmed the stable nature of class C GPCR dimerization, which is mediated by disulfide bridges between the NTED of the protomers (Gavalas et al., 2013). On the other hand, differentially tagged β2-AR or μ-opioid receptor (MOR) fully segregated into distinct artificial microdomains, suggesting that homodimerization between these class A GPCRs is not sufficiently stable (Gavalas et al., 2013). Similar segregation was observed as a reduced BRET signal between wild-type and binding-deficient β2-AR upon agonist-induced internalization of only active (wild-type) β2-AR, whereas the inactive mutant remained at the cell surface (Lan et al., 2011). In contrast, coexpression of the wild-type β2-AR with receptors activated solely by a synthetic ligand (i.e. RASSL) β2-AR mutant revealed that agonist stimulation of either protomer induced internalization of the dimer (Sartania et al., 2007). Altogether, a discrepancy in the transient nature of at least some GPCR dimers is observed between single molecule labeling strategies and studies demonstrating cointernalization and cotrafficking of receptors (Milligan, 2010).

Several GPCRs have arranged as dimers or oligomers in recent high-resolution crystal structures, whereas others have crystallized as monomers. These crystal structures suggest that GPCRs can assemble in multiple ways, which might explain the possible formation of complexes larger than dimers. Antagonist-bound MOR crystallizes as oligomers, with a large
contact interface involving transmembrane (TM) 5/TM6 and a smaller interface formed by TM1/TM2/helix 8 (Manglik et al., 2012), whereas a ligand-free β₁-AR is arranged as oligomers via TM4/TM5 and TM1/TM2/helix8 interfaces (Huang et al., 2013). The antagonist-bound κ-opioid receptor is arranged as a dimer via a TM1, TM2, and helix 8 interface (Wu et al., 2012). CXCR4 crystallizes as dimers via a TM5/TM6 interface when occupied by antagonistic chemokine vMIPII or small-molecule (ITI1) and cyclic peptide (CVX15) antagonists (Wu et al., 2010; Qin et al., 2015), whereas antagonist-bound histamine H₄ receptor has a TM4 dimerization interface (Shimamura et al., 2011). However, it should be kept in mind that receptor modifications, bound ligand, and crystal packing conditions might affect (forced) dimerization interfaces and require systematic experimental validation using site-directed mutagenesis and/or interfering peptides. Indeed, TM1 forms the dimer interface in the mGlu₃ 7TMD crystal structure, whereas a TM4/TM5 interface is actually observed in full-length mGlu receptors in cells (Xue et al., 2015).

Hence, observed discrepancies in both the quaternary structure and stability of GPCR complexes between different studies emphasize the need for further refinement and systematic comparison of methods to monitor GPCR interactions in time.

Proximal Evidence for GPCR Heteromer Specific Signaling

GPCR heteromerization can significantly affect signaling and/or trafficking characteristics of individual GPCR subtypes (Jordan and Devi, 1999). However, unambiguous separation of heteromer-specific signaling from downstream crosstalk is difficult and requires experimental disruption of dimer formation (Prezeau et al., 2010; Vischer et al., 2011). Supportive evidence for heteromer-specific signaling comes from RET-based detection of signaling events very proximal to GPCRs in transfected cells. For example, agonist stimulation of a given (unmodified) GPCR subtype changes BRET between another coexpressed GPCR subtype and specific G proteins and/or β-arrestins (Fig. 1G) (See et al., 2011; Mustafa et al., 2012; Watts et al., 2013; Jonas et al., 2015). In addition, fusion of GPCRs to bimolecular luminescence or fluorescence complementation protein fragments and subsequent coexpression with compatible G protein or β-arrestin RET fusion constructs (i.e., CODA-RET) allowed simultaneous detection of GPCR heteromerization and engagement of intracellular signaling partners upon agonist stimulation (Fig. 1H) (Urizar et al., 2011; Armando et al., 2014; Guitart et al., 2014; Bellot et al., 2015; Frederick et al., 2015). Heteromerization of Gα₁c-coupled D₂R and the Gα₁s-coupled dopamine D₂ receptor (D₂R) induces intracellular Ca²⁺ mobilization upon agonist activation, which could be impaired by the Gα₁₅ inhibitor YM254890 (Lee et al., 2004; Rashid et al., 2007). Moreover, agonist-induced recruitment of Gα₁₅-green fluorescent protein to D₁R–Renilla luciferase requires coexpression of D₂R and could be inhibited by a membrane-permeable peptide that disrupts D₁R-D₂R heteromerization (Hasbi et al., 2014). In contrast, however, a recent study reported the lack of both G₉ recruitment to D₁R-D₂R heteromers in a CODA-RET assay and Gα₁₅ activation, as measured by BRET, between Gα₁₅–Renilla luciferase 8 and Gγ₂-Venus (Frederick et al., 2015). Although these engineered biosensor-expressing cells can reveal the potential of GPCRs to modify each other’s signaling upon heteromerization, it should be kept in mind that protein expression levels should be kept to a minimum to avoid nonspecific interactions as a consequence of membrane (microdomain) overcrowding.

Pharmacological Evidence for GPCR Dimers and Oligomers in Native Tissues

Ligand binding to one GPCR within a dimer or oligomer can rapidly change the conformation of an associated GPCR, as shown by the inhibition of norepinephrine-induced intramolecular FRET in an engineered α₂A-AR upon stimulation of the MOR with morphine (Vilardaga et al., 2008). This transconformational change is slightly faster than the rate for G protein activation, indicating direct allosterism between both receptor subtypes within the heteromer (Xue et al., 2015). Negative-binding cooperativity was observed in the 1970s for native β-AR and the thyroid-stimulating hormone receptor (TSHR) in membrane preparations of frog erythrocytes and human thyroid samples, respectively, already suggesting the existence of GPCR dimers (Limbird et al., 1975; Limbird and Lefkowitz, 1976; Powell-Jones et al., 1979).

TSHR forms homomers in transfected cells (Urizar et al., 2005). Equilibrium and dissociation binding on these cells using both wild-type and engineered TSHR chimeras confirmed negative cooperativity between their orthosteric-binding sites, which is negatively correlated to the level of constitutive activity of the protomer (Zoenen et al., 2012). The chemokine receptors CCR2, CCR5, and CXCR4 form heteromers and display negative-binding cooperativity for their cognate chemokines in transfected cells (El-Asmar et al., 2005; Springael et al., 2006; Sohy et al., 2007, 2009). Importantly, similar negative-binding cooperativity between chemokines was observed on intact human CD4⁺ T lymphocytes and purified monocytes endogenously expressing CCR2, CCR5, and CXCR4, consistent with the existence of chemokine receptor heteromers on native cells. Moreover, the CCR2/CCR5 antagonist TAK779 [dimethyl-[(4-[(3-4-methyl-phenyl)-9,9-dihydro-7H-benzo[7]annulene-6-carbonyl]amino)phenyl][methyl]-(oxan-4-yl)azanium] reduced CXCR4-mediated immune cell recruitment toward CXCL12 in both ex vivo and in vivo models (Sohy et al., 2009). Similarly, the CXCR4 antagonist AMD3100 [1,1’-[1,4-phenylenebis-(methylene)]-bis(1,4,8,11-tetraazacyclotetradecane) octahydrochloride] reduced CCR2- and CCR5-mediated ex vivo chemotaxis of CD4⁺ T lymphocytes toward CCL2 and CCL4, respectively, by cross-inhibiting chemokine binding (Sohy et al., 2007, 2009). In contrast, the CXCR4 inverse agonist TC14012 [L-arginyl-L-arginyl-3-(2-naphthalenyl)-L-ala-L-lys-L-tyr-L-lys-N⁵-(aminocarbonyl)L-orn-L-lys-N⁵-(aminocarbonyl)-L-lys-N⁵-(aminocarbonyl)-L-lys-L-pro-L-lys-N⁵-(aminocarbonyl) L-lys-N⁵-(aminocarbonyl)-L-lys-N⁵-(aminocarbonyl)-L-lys-N⁵-(aminocarbonyl)-L-lys-N⁵-(aminocarbonyl)-L-lys-L-lys-L-lys-L-lys]-isoleucine was unable to crossantagonize CCL2-induced β-arrestin2 recruitment to CCR2-CXCR4 heteromers in a CODA-RET assay (Armando et al., 2014). Cannabinoid CB₁ and CB₂ receptor antagonists inhibited signaling in response to agonists that activate the opposite receptor within cannabinoid CB₁/CB₂ heteromers in both heterologous cells and globus pallidus slices from a rat brain (Callen et al., 2012).

This antagonist crossinhibition offers great therapeutic potential but is not observed for other dimers that only display binding cooperativity between agonists (Albizu et al., 2015).
ing CXCR3 and CXCR4. In addition, positive-binding cooperativity of CXCR3 agonist VUF10661 [(3S)-N-[(1S)-5-amino-1-[(2,2-diphenylethyl)amino]carbonyl] pentyll]-2-(1,4-dioxo-4-phenylbutyl)-1,2,3,4-tetrahydro-3-isouquinolinecarboxamide], but not the CXCR3 antagonist TAK779, attenuated CXCL12 binding to membranes expressing CXCR3 and CXCR4. In addition, positive-binding cooperativity has been observed between the antagonist and agonist on the D₃R and oxytocin receptor heteromers in nucleus accumbens membranes (Romero-Fernandez et al., 2013).

Hence, binding cooperativity between ligands acting at different GPCRs suggests the existence of GPCR heteromers in native tissues. However, concerns have been raised on the interpretation of binding cooperativity since agonist binding to GPCRs can be G protein dependent (Chabre et al., 2009; Birdsell, 2010). Consequently, competition for a shared pool of G proteins between GPCRs could result in the observation of apparent negative agonist binding cooperativity. This G protein depletion is, in particular, possible in equilibrium competition analysis since agonist binding with CXCR4 (de Poorter et al., 2013; Watts et al., 2013).

Importantly, differential spatiotemporal expression of GPCR subtypes in tissues or even disease states may result in distinct heteromer-specific pharmacology. This may be particularly challenging for drug discovery programs that generally measure drug activity at a single target, and such initial screens may consequently not be adequate predictors for the in vivo effectiveness of drugs.

Detection of GPCR Dimers and Oligomers in Native Tissues

Although recombinant technologies using engineered GPCRs provide supportive evidence that many GPCRs might exist as dimers and/or oligomers, or at least exist in close proximity, these approaches are not easily applicable to identify GPCR complexes in native tissues. However, in a recent study, double knock-in mice expressing the MOR and δ-opioid receptor (DOR) fused in frame to mCherry and enhanced green fluorescent protein (eGFP), respectively, showed colocalization of these receptors in the midbrain and hindbrain (Érbs et al., 2015). Coimmunoprecipitation using anti-mCherry and anti-eGFP antibodies revealed that MOR-mCherry and DOR-eGFP belong to the same complex in the hippocampus from these mice, which confirms earlier detection of these MOR-DOR complexes in this brain region using a heteromer-specific antibody (Gupta et al., 2010). Interestingly, this antibody revealed increasing MOR-DOR complex levels in the cortex of animals chronically treated with morphine, whereas no coexpression of MOR-mCherry and DOR-eGFP was observed in the cortex of double knock-in mice in the absence or presence of the DOR agonist (Érbs et al., 2015). This discrepancy might be related to the difference in the receptor expression level and/or detection sensitivity.

Hitherto, only a limited number of the GPCR dimers and oligomers that were identified in heterologous cells have been validated in native tissues due to the absence of highly specific antibodies for most GPCR subtypes or heteromers (Michel et al., 2009). Indeed, coimmunoprecipitation of GPCR complexes from solubilized native tissues has been used for nearly two decades (Fig. 1A) (Kaupmann et al., 1998; González-Maeso et al., 2008; Pei et al., 2010) but requires critical analysis to ensure that physically interacting GPCRs are detected rather than aggregation artifacts due to the hydrophobic nature of GPCRs (Milligan and Bouvier, 2005). More recently, in situ GPCR complexes have also been detected using immunohistochemical antibodies. Labeling of native GPCR subtypes with specific primary antibodies followed by matching secondary antibodies that are conjugated to unique oligonucleotide sequences allows enzymatic ligation of these DNA strands if secondary antibodies are in close proximity (<16 nm), which corresponds to a theoretical distance of <40 nm between the GPCR subtypes (i.e., epitopes). The formed circle DNA strand is subsequently amplified and hybridized with fluorescent complementary oligonucleotide probes for high-sensitivity fluorescence microscopy analysis (Fig. 1B) (Weibrecht et al., 2010). This so-called proximity ligation assay (PLA) confirmed the close proximity of various GPCR pairs in the central nervous system. In situ PLA between D₃R and adenosine A₂A receptor (A₂AR) in the striatum of mice, rats, and monkeys (Trifillo et al., 2011; Bonaventura et al., 2014; Fernández-Dueñas et al., 2015) confirmed D₂R-A₂A R heteromer coimmunoprecipitation from the rat striatum (Cabello et al., 2009) as well as detection by coimmunoprecipitation, pull-down, FRET, BRET, and sequential BRET-FRET in heterologous cells (Canals et al., 2003; Kamiya et al., 2003; Cabello et al., 2009). Interestingly, in the striatum of an experimental Parkinsonism rat model, this PLA signal was significantly decreased as a consequence of reduced codistribution and proximity between D₃R and A₂A R (Fernández-Dueñas et al., 2015). In addition, PLA was detected between native cannabinoid CB₁ and CB₂ receptors in the rat brain pineal gland and nucleus accumbens (Callén et al., 2012) and between D₃R and oxytocin receptors in the rat dorsal striatum and the neuropil of nucleus accumbens (Romero-Fernandez et al., 2013). Interestingly, PLA between the dopamine D₄ receptor and α₁B- or β₁-AR was observed in a rat pineal gland dissected 1 hour after sunset, but not when pineal glands were isolated at sunset, which reflects the circadian variation in dopamine D₄ receptor expression levels (González et al., 2012). Dopamine D₁ receptor (D₁R) and D₂R form heteromers in heterologous cells (So et al., 2005; Frederick et al., 2015). D₁R/D₂R heteromers have been proposed to play an important role in various neuropsychiatric disorders. However, PLA was absent in the shell of the nucleus accumbens, despite coexpression of native D₁R and D₂R in these cells, but readily observed upon D₁R/D₂R overexpression using viral gene transfer (Frederick et al., 2015). On the other hand, native D₁R/D₂R heteromers were detected in situ in rat striatal neurons using antibody-based confocal FRET analysis (Fig. 1C) (Hasbi et al., 2009, 2014; Perreault et al., 2010; Verma et al., 2010). The discrepancy between these antibody-based techniques requires further systematic comparison on similar regions. Importantly, specificity of D₁R- and D₂R-primary antibodies in these studies was confirmed in cells heterologously expressing dopamine receptor subtypes and in
D₁R or D₂R knockout mice in situ (Lee et al., 2004; Perreaud et al., 2010).

In addition to antibodies, fluorescent ligands have been used to detect native GPCR heteromers (Fig. 1D). For example, ghrelin receptor heteromerization with D₂R was shown in a mice hypothalamus by confocal FRET and time-resolved FRET between fluorescently labeled agonist ghrelin and D₂R antibody—secondary antibody complexes, with the latter conjugated to Cy3 fluorophore or cryptate, respectively (Kern et al., 2012). TrFRET between the D₂R and A₂AR antagonists that were conjugated to Lumi4-Terbium and a red acceptor (dy647), respectively, confirmed the PLA signal in the rat striatum (Fernández-Dueñas et al., 2015). Likewise, native oxytocin receptor dimers were detected in the mammary gland using fluorescent antagonists, but to a much lesser extent with fluorescent agonists due to negative-binding cooperativity between agonists (Albizu et al., 2010).

Functional Evidence for GPCR Dimers in Native Tissue

Coexpression of two nonfunctional GPCRs to form a functional receptor provides convincing evidence for GPCR heteromerization, as exemplified by the functional complementation of native class C GPCRs GABAₐ₁ and GABAₐ₂, which is strictly required for cell-surface expression of a functional GABAₐ₂ receptor in vitro and in vivo (Prosser et al., 2001; Gassmann et al., 2004). The α₁D-AR is retained in the endoplasmic reticulum when individually expressed in heterologous cells. Systematic coexpression with 28 other class A GPCRs revealed that heteromerization with α₁B- or β₁-AR is required for the cell surface of α₁D-AR (Hague et al., 2004; Uberti et al., 2005). However, α₁D-AR stimulates the contraction of carotid arteries in α₁B-AR knockout mice (Deighan et al., 2005), suggesting that effective trafficking of α₁D-AR to the cell surface is mediated by, for example, native β₁-AR (Pernomian et al., 2013). In contrast to the majority of class A GPCRs, the follicle-stimulating hormone receptor (FSHR), TSHR, and LHR are characterized by a large leucine-rich repeat-containing NTED, which is exclusively involved in the selective and high-affinity binding of their corresponding glycoprotein hormones (Osuga et al., 1997; Vischer et al., 2003a,b; Fan and Hendrickson, 2005). All three glycoprotein hormone receptors form homo- and heteromers in transfected cells (Urizar et al., 2005; Feng et al., 2013). However, heteromerization between FSHR-LHR might be physiologically relevant as both receptors are shortly coexpressed in granulosa cells during follicle maturation (Thiruppathi et al., 2001). Taking advantage of the modular architecture of these glycoprotein hormone receptors, nonfunctional LHR mutants were created to impair either hormone binding to the NTED (i.e., LHR⁵⁺) or G protein activation by the 7TMD (i.e., LHR⁵⁻) (Osuga et al., 1997; Ji et al., 2002; Lee et al., 2002). Coexpression of LHR⁵⁺ with LHR⁵⁻ in transfected cells rescued hormone-induced cAMP production, suggesting that both nonfunctional constructs are at least organized as dimers. Similar functional complementation was observed in transfected cells coexpressing FSHR⁵⁻ and FSHR⁵⁺ (Ji et al., 2004) and TSHR⁵⁻ and TSHR⁵⁺ (Urizar et al., 2005). Moreover, coexpression of LHR⁵⁻ with LHR⁵⁻ in LHR knockout mice using a bacterial artificial chromosome to preserve correct spatiotemporal expression rescued both gonadal development and full spermatogenesis (Rivero-Müller et al., 2010). In contrast, expression of the individual loss-of-function mutants was ineffective. However, the same LHR⁵⁻ mutant (i.e., C²⁵²A) was more recently reported to induce some cAMP signaling in transiently transfected human embryonic kidney 293 cells (Zhang et al., 2012), which contrasts with earlier in vitro and in vivo observations (Ji et al., 2002; Lee et al., 2002; Rivero-Müller et al., 2010).

Perturbation of GPCR Heteromers in Native Tissue

D₁R/D₂R heteromerization involves the C-terminus of D₁R and intracellular loop 3 (IL3) of the long D₂R isofrom. Membrane-permeable fusion constructs consisting of a TAT sequence and D₁R G³⁹⁸–L⁴¹⁵ (TAT-D₁C) or D₂R M²⁵⁷–E²⁷¹ (TAT-D₂LIL₃) peptides disrupted D₁R/D₂R complexes and heteromer-induced signaling in striatal neurons (Pei et al., 2010; Hasbi et al., 2014). D₁R/D₂R coimmunoprecipitation was enhanced from brain tissue derived from patients suffering major depression as compared with healthy persons (Pei et al., 2010). Importantly, TAT-D₂LIL₃ has antidepressant-like effects when injected in the brain of rats, as revealed by increased mobility in forced swim tests and reduced escape failures in learned helplessness tests, leading the authors to suggest a prominent role for D₁R/D₂R heteromers in this neurologic disorder (Pei et al., 2010). The class A angiotensin receptor type 1a (AT₁AR) and class B secretin receptor (SCTR) are coexpressed in osmoregulatory brain centers and form heteromeric complexes in heterologous cells. AT₁AR/SCTR heteromerization was specifically inhibited by peptides derived from AT₁AR-TM1 and SCTR-TM2, whereas both homomerization and heteromerization were inhibited by AT₁AR-TM4 and SCTR-TM4 (Lee et al., 2014). Injection of AT₁AR-TM1 in mice brains reduced hyperosmolality-induced drinking, confirming the physiologic role of this class A/class B GPCR heteromer in the regulation of water homeostasis.

Conclusions

Dimerization is essential for class C GPCR functioning, whereas class A and B GPCRs can activate G proteins and recruit β-arrestins as monomers in response to agonists. Nevertheless, most tested GPCRs form dimers and oligomers in heterologous cells, resulting in an apparent plethora of functional consequences. However, the stability as well as the stoichiometry of GPCR complexes appear to vary considerably, with only class C GPCRs forming stable complexes. Hitherto, only a small percentage of GPCR dimers and oligomers have been validated in native tissues, despite the guidelines proposed by the International Union of Basic and Clinical Pharmacology in 2007 (Pin et al., 2007). Ex vivo and in vivo detection of native GPCR dimers largely rely on the availability of specific antibodies and/or fluorescent ligands. Recent progress in the generation of llama-derived nanobodies targeting GPCRs might facilitate future detection of native GPCR dimers and oligomers in situ. The higher affinities of engineered bivalent and bimaparatopic nanobodies in comparison with their monovalent counterparts might suggest binding to receptor dimers, although experimental proof for these observations is still required (Jähnichen et al., 2010; Maussang et al., 2013). Binding cooperativity might be
a feasible pharmacological approach to detect GPCR homomers and heteromers in ex vivo samples; however, influence from signaling molecules should be considered if using agonists, whereas both the absence and presence of binding cooperativity has been observed for antagonists. In vivo functional complementation has so far been performed for one class A GPCR subtype and requires well-characterized mutants that are present at physiologic levels with correct functional complementation has so far been performed for a feasible pharmacological approach to detect GPCR homo- and heteromers in the caudate nucleus.

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Acknowledgments

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