G protein-coupled receptor multimers: a question still open despite the use of novel approaches

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ABBREVIATIONS – AR, adrenergic receptor; AT1aR, angiotensin receptor type 1a; A2aR, adenosine A2a receptor; BRET, bioluminescence resonance energy transfer; CODA-RET, complemented donor-acceptor resonance energy transfer; DOR, δ-opioid receptor; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; D4R, dopamine D4 receptor; eGFP, enhanced green-fluorescent protein; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; FSHR, follicle-stimulating hormone receptor; GPCR, G protein-coupled receptor; mGlu, metabotropic glutamate; HIT, heteromer identification technology; IL3, intracellular loop 3; LHR, luteinizing hormone receptor; MOR, μ-opioid receptor; M1R, M1 muscarinic acetylcholine receptor M3R, M3 muscarinic acetylcholine receptor; NTED,
N-terminal extracellular domain; PCH, photon counting histogram; PD-PALM, photoactivatable dye-photoactivated localization microscopy; PLA, proximity ligation assay; RASSL, receptors activated solely by synthetic ligands; RET, resonance energy transfer; Rluc, Renilla luciferase; SCTR, secretin receptor; SRET, sequential BRET-FRET; TIRFM, total internal reflection fluorescence microscopy; TM, transmembrane; trFRET, time-resolved fluorescence energy transfer; TSHR, thyroid-stimulating hormone receptor; T1R, taste 1 receptor; 7TMD, transmembrane domain
ABSTRACT

Heteromerization of G protein-coupled receptors (GPCR) can significantly change the functional properties of involved receptors. Various biochemical and biophysical methodologies have been developed 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 lacking behind for the majority of GPCR heteromers. Remarkably, recent studies, particularly some involving advanced fluorescence microscopy techniques, are contributing to our current knowledge on aspects that were not well known till 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 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 functions to come to an integrative cellular response through direct physical interactions in heteromeric complexes. Over the last 20 years, di- and/or oligimerization 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; 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 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 (Margeta-Mitrovic et al., 2000; Pagano et al., 2001; Brock et al., 2005). Heteromerization of the taste 1 receptor 3 (T1R3) with the 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 (Zhao et al., 2003; Damak et al., 2003). Dimerization of purified metabotropic glutamate 2 (mGlu2) receptors in nanodiscs is required for G protein activation in response to the endogenous agonist glutamate (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 (Hanson et al., 2007; White et al., 2007; Whorton et al., 2007; Bayburt et al., 2007; Whorton et al., 2008; Kuszak et al., 2009; Arcemisbéhère et al., 2010; Tsukamoto et al., 2010; Bayburt et al., 2011). In addition, 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 obviously rises why do these GPCRs di- and oligomerize? Physically interacting GPCRs may modulate each other activities. However, unambiguous discrimination between GPCR crosstalk as a consequence of receptor heteromerization and those 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 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 physiological 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 GPCRs heteromers (Lambert and Javitch, 2014). In this review, we will first focus on recent developments to determine 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 fluorescence energy transfer (trFRET) reveals physical association and close proximity of GPCRs in heterologous cells (Fig. 1A-C) (Milligan and Bouvier, 2005; Maurel et al., 2008; Faklaris et al., 2015). Moreover, fusion of bioluminescent or fluorescent proteins or non-functional 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 1E-F), and approaches such as the heteromer identification technology (HIT) or complemented donor-acceptor resonance energy transfer (CODA-RET) have also been implemented for the investigation of complexes involving three interacting partners (Fig 1G-H) (Ciruela et al., 2010; Cottet et al., 2012; Kaczor et al., 2014).

While 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-AR) site-specifically labeled with fluorophores and reconstituted in lipid bilayers suggested the formation of predominantly tetramers (Fung et al., 2009). Three-color sequential-BRET-FRET (SRET) and bimolecular luminescence/fluorescence complementation (BiLC and BiFC) in combination with RET revealed the formation of GPCR heteromultimers, consisting of at least 3 or 4 individual GPCRs, when expressed at physiological 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 in order 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 life-times) 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 they 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 donor’s bleedthrough or direct acceptor excitation are corrected in spectrally-resolved FRET, and possible contributions of 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 known concentration (i.e. purified fluorescent proteins) for spectral unmixing. Different spectrally-resolved FRET approaches revealed the proportion of serotonin 5-HT\textsubscript{1A} receptors that were engaged in oligomers in transfected cells (Gorinski et al., 2012), as well as the formation of transient tetramers by stable M\textsubscript{3} muscarinic acetylcholine receptor (M\textsubscript{3}R) dimers at the cell surface with minimal interference from bystander RET signal coming from nearby non-interacting partners (Patowary et al., 2013).

Fluorescence Correlation Spectroscopy (FCS) allows the detection of the 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 sub-microsecond 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,
expression of receptors fused to complementary fragments of a fluorescent protein will allow the
detection exclusively 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; Corriden et al., 2014) or specific anti-
receptor fragment antigen binding (Fab) 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 Fab fragments opposite to antibodies, potential artifacts due to antibody-induced
clustering of receptors are avoided. FCS can give information on protein that are likely to be in
the same complex based on the changes in the diffusion coefficients by the formation of larger
co-diffusing entities and on brightness of the diffusing particles. However, it should be kept in
mind that FCS measures co-diffusing proteins that are 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 4 in the molecular mass of the particles is required
in order 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
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 photon counting
histogram (PCH) analysis enables quantification of the intensity of fluorescence fluctuations,
which provides information as regards the stoichiometry of protein complexes (Fig. 2C) (Chen et
al., 1999). By doing this, the molecular brightness of the diffusing particles can be determined
and consequently the number of fluorophores co-diffusing in each particle. Subsequent
comparison with appropriate reference proteins with known mono/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 nature 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 class B GPCRs, an equilibrium between monomeric and possible dimeric species was found, with a low proportion of dimers by using fluorescence cross-correlation 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 co-migrate, the fluctuations in the intensity of the two fluorophores will correlate as they diffuse into and out of the observation volume together. The cross-correlation function provides more sensitive information than the diffusion coefficient alone for detection of interactions. The information gathered in the work of Teichmann et al. confirmed single molecule total internal reflection fluorescence microscopy (TIRFM) studies at β₁-AR or M₁ muscarinic acetylcholine receptors (M₁R; see below) (Hern et al., 2010; Calebiro et al., 2013). However, a homogeneous population of dimers without evidence of the coexistence of monomeric species in equilibrium was found in the case of serotonin 5-HT₂C but also β₁-AR or M₁R in other FCS/PCH-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 dyes (PD-PALM) 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, HA- and FLAG-tagged luteinizing hormone receptor (LHR) mutants that were either ligand binding (LHR⁻) or signaling (LHR⁺) deficient (see below (Rivero-Müller et al., 2010)) were expressed in HEK293 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⁻ or LHR⁺) 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. 3B-C). Expression of wild type LHR at
levels in the physiological range observed in 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 9 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 PD-PALM, this technique involves acquisition of 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 dynamics of dimer rather than monomers are observed (see section below) (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, the $\beta_1$-AR displays more transient interactions than $\beta_2$-AR as revealed by both TIRFM and dual-color fluorescence recovery after photobleaching (FRAP) studies (Dorsch et al., 2009; Calebiro et al., 2013). Affinity-based co-recruitment 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 $\beta_2$-AR or $\mu$-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 reduced BRET
signal between wild type and binding deficient β2-AR upon agonist-induced internalization of only the active (wild type) β2-AR, while the inactive mutant remained at the cell surface (Lan et al., 2011). In contrast, co-expression of the wild type β2-AR with a RASSL (i.e. Receptors Activated Solely by Synthetic Ligands) 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 co-internalization and co-trafficking of receptors (Milligan, 2010).

Several GPCRs are arranged as dimers or oligomers in recent high-resolution crystal structures, whereas others crystalizes 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 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 dimer via a TM1, TM2, and helix 8 interface (Wu et al., 2012). CXCR4 crystallizes as dimers via TM5/TM6 interface when occupied by antagonistic chemokine vMIPII or small-molecule (IT1t) and cyclic peptide (CVX15) antagonists (Wu et al., 2010; Qin et al., 2015), whereas antagonist-bound histamine H1 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 requires systematic experimental validation using site-directed mutagenesis and/or interfering peptides. Indeed, TM1 forms the dimer interface in the mGlu1 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 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 HETEROIMER 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 co-expressed 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 BiLC or BiFC protein fragments and subsequent co-expression with compatible G protein or β-arrestin RET fusion constructs (i.e. CODA-RET: complemented donor-acceptor resonance energy transfer), allowed simultaneous detection of GPCR heteromerization and engagement of intracellular signaling partners upon agonist stimulation (Fig. 1H) (Urizar et al., 2011; Guitart et al., 2014; Armando et al., 2014; Frederick et al., 2015; Bellot et al., 2015). Heteromerization of Gs-coupled D1R and the Gi/o-coupled D2R induces intracellular Ca2+ mobilization upon agonist activation, which could be impaired by Gq11 inhibitor YM254890 (Lee et al., 2004; Rashid et al., 2007). Moreover, agonist-induced recruitment of Gq-green fluorescent protein (GFP) to D1R-Renilla luciferase (Rluc) requires co-expression of D2R, and could be inhibited by a membrane-permeable peptide that disrupts D1R-D2R heteromerization (see below) (Hasbi et al., 2014). In contrast, however, a recent study reported the lack of both Gq recruitment to D1R-D2R heteromers in a CODA-RET assay and Gq activation as measured by BRET between Gq-Rluc8 and Gγ2-Venus (Frederick et al., 2015). Although these engineered can reveal the potential of GPCRs to modify each other 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 allosteryism between both receptor subtypes within the heteromer (Xue et al., 2015). Negative binding cooperativity was observed in the 70s for native β-AR and 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). 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 reduced CXCR4-mediated immune cell recruitment towards CXCL12 in both ex vivo and in vivo models (Sohy et al., 2009). Similarly, the CXCR4 antagonist AMD3100 reduced CCR2- and CCR5-mediated ex vivo chemotaxis of CD4+ T lymphocytes towards CCL2 and CCL4, respectively, by cross-inhibiting chemokine binding (Sohy et al., 2007; 2009). In contrast, the CXCR4 inverse agonist TC14012 was unable to cross-antagonize CCL2-induced β-arrestin2 recruitment to CCR2-CXCR4 heteromers in a CODA-RET assay (see section below) (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 rat brain (Callén et al., 2012). This antagonist cross-inhibition offers great therapeutical potential, but is not observed for other dimers that only display binding cooperativity between agonists (Albizu et al., 2006; 2010). For example, the CXCR4 antagonist AMD3100 did not cross-inhibit binding of chemerin and CXCL10 to ChemR23 and CXCR3, respectively, that form heteromers with CXCR4 (de Poorter et al., 2013; Watts et al., 2013). However, the small molecule CXCR3 agonist VUF10661, but not CXCR3 antagonist TAK779, attenuated CXCL12 binding to membranes expressing CXCR3
and CXCR4. In addition, positive binding cooperativity has been observed between antagonist and agonist on the D2R and oxytocin receptor heteromers in nucleus accumbens membranes (Romero-Fernandez et al., 2013).

Hence, binding cooperativity between ligands acting at different GPCR 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; Birdsall, 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 binding on membrane preparations as G protein coupling to activated GPCRs might be almost irreversible in the absence of free GTP (Chabre et al., 2009), but can be prevented by overexpression of G proteins (Nijmeijer et al., 2010).

Importantly, differential spatiotemporal expression of GPCR subtypes in tissues or even disease states may result in distinct heteromer-specific pharmacology. This may be in particular challenging for drug discovery programs that generally measures 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 co-localization of these receptors in mid- and hindbrain (Erbs et al., 2015). Co-immunoprecipitation using anti-mCherry and anti-eGFP antibodies revealed that MOR-mCherry and DOR-eGFP belong to the same complex in 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 co-expression of MOR-
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mCherry and DOR-eGFP was observed in the cortex of double knock-in mice in the absence or presence of DOR agonist (Erbs et al., 2015). This discrepancy might be related to difference in 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, co-immunoprecipitation 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 <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 close proximity of various GPCR pairs in the central nervous system. In situ PLA between dopamine D2 receptor (D2R) and adenosine A2a receptor (A2aR) in striatum of mice, rat, and monkeys (Trifilieff et al., 2011; Bonaventura et al., 2014; Fernández-Dueñas et al., 2015), confirmed D2R-A2aR heteromer co-immunoprecipitation from rat striatum (Cabello et al., 2009), as well as, detection by co-immunoprecipitation, pull-down, FRET, BRET, and SRET in heterologous cells (Kamiya et al., 2003; Canals et al., 2003; Cabello et al., 2009). Interestingly, in striatum of an experimental parkinsonism rat model this PLA signal was significantly decreased as a consequence of reduced co-distribution and proximity between D2R and A2aR (Fernández-Dueñas et al., 2015). In addition, PLA was detected between native cannabinoid CB1 and CB2 receptor in rat brain pineal gland and nucleus accumbens (Callén et al., 2012), and between D2R and oxytocin receptors in rat dorsal striatum and the neuropil of nucleus accumbens (Romero-Fernandez et al., 2013). Interestingly, PLA between the dopamine
D₄ receptor (D₄R) and α₁β- or β₁-AR was observed in rat pineal gland dissected 1 hour after sunrise, but not when pineal glands were isolated at sunset, which reflects the circadian variation in D₄R expression levels (González et al., 2012). Dopamine inhibits adrenergic receptor signaling within these heteromers and consequently limits serotonin and melatonin synthesis and release in the pineal gland. 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 (see below).

However, PLA was absent in the shell of the nucleus accumbens despite co-expression 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; Verma et al., 2010; Perreault et al., 2010; Hasbi et al., 2014). 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; Perreault 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 mice hypothalamus by confocal FRET and trFRET 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 (see above) in rat striatum (Fernández-Dueñas et al., 2015). Likewise, native oxytocin receptor dimers were detected in 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 – Co-expression of two non-functional GPCRs to form a functional receptor provides convincing evidence for GPCR heteromerization, as exemplified by the functional complementation of native class C GPCRs
GABA\textsubscript{B\textsubscript{1}} and GABA\textsubscript{B\textsubscript{2}}, which is strictly required for cell-surface expression of a functional GABA\textsubscript{B} receptor in vitro (see above) and in vivo (Prosser et al., 2001; Gassmann et al., 2004). The \(\alpha_{1D}\)-AR is retained in the endoplasmic reticulum when individually expressed in heterologous cells. Systematic co-expression with 28 other class A GPCRs revealed that heteromerization with \(\alpha_{1B}\)- or \(\beta_{2}\)-AR is required for the cell surface of \(\alpha_{1D}\)-AR (Hague et al., 2004; Uberti et al., 2005). However, the \(\alpha_{1D}\)-AR stimulates contraction of carotid arteries in \(\alpha_{1B}\)-AR knockout mice (Deighan et al., 2005), suggesting that effective trafficking of \(\alpha_{1D}\)-AR to the cell surface is mediated by for example native \(\beta_{2}\)-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 N-terminal extracellular domain (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, only heteromerization between FSHR-LHR might be physiological relevant as both receptors are shortly co-expressed in granulosa cells during follicle maturation (Thiruppathi et al., 2001).

Taking advantage of the modular architecture of these glycoprotein hormone receptors, non-functional LHR mutants were created to impair either hormone binding to the NTED (i.e. LHR\textsuperscript{B\textsubscript{1}}) or G protein activation by the 7TMD (i.e. LHR\textsuperscript{S\textsubscript{1}}) (Osuga et al., 1997; C Lee et al., 2002; Ji et al., 2002). Co-expression of LHR\textsuperscript{B\textsubscript{1}} with LHR\textsuperscript{S\textsubscript{1}} in transfected cells rescued hormone-induced cAMP production, suggesting that both non-functional constructs are at least organized as dimers. Similar functional complementation was observed in transfected cells co-expressing FSHR\textsuperscript{B\textsubscript{1}} and FSHR\textsuperscript{S\textsubscript{1}} (Ji et al., 2004), and TSHR\textsuperscript{B\textsubscript{1}} and TSHR\textsuperscript{S\textsubscript{1}} (Urizar et al., 2005). Moreover, co-expression of LHR\textsuperscript{B\textsubscript{1}} with LHR\textsuperscript{S\textsubscript{1}} in LHR knockout mice using 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 LHR\textsuperscript{B\textsubscript{1}} mutant (i.e. C\textsuperscript{22A}) was more recently reported to induce some cAMP signaling in transiently transfected HEK293 cells (Zhang et al., 2012), which contrasts the earlier in vitro and in vivo observations (Lee et al., 2002; Ji et al., 2002; Rivero-Müller et al., 2010).
PERTURBATION OF GPCR HETEROMERS IN NATIVE TISSUE - D_{1}R/D_{2}R heteromerization involves the C-tail of D_{1}R and intracellular loop 3 (IL3) of the long D_{2}R isoform. Membrane-permeable fusion constructs consisting of a TAT sequence and D_{1}R G^{396-413} (TAT-D_{1}C) or D_{2}R M^{257-271} (TAT-D_{2}L_{IL3}) peptides disrupted D_{1}R/D_{2}R complexes and heteromer-induced signaling in striatal neurons (Pei et al., 2010; Hasbi et al., 2014). D_{1}R/D_{2}R coimmunoprecipitation was enhanced from brain tissue derived from patients suffering major depression as compared to healthy persons (Pei et al., 2010). Importantly, TAT-D_{2}L_{IL3} has antidepressant-like effects when injected in brain of rats as revealed by increased mobility in a forced swim tests and reduced escape failures in learned helplessness tests, leading the authors to suggest a prominent role for D_{1}R/D_{2}R heteromers in this neurological disorder (Pei et al., 2010). The class A angiotensin receptor type 1a (AT1aR) and class B secretin receptor (SCTR) are co-expressed 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 homo- and heteromerization were inhibited by AT1aR-TM4 and SCTR-TM4 (Lee et al., 2014). Injection of AT1aR-TM1 in mice brain reduced hyperosmolality-induced drinking, confirming the physiological 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 dimer and oligomers in heterologous cells, resulting in an apparent plethora of functional consequences. However, the stability as well as the stoichiometry of GPCR complexes appears to vary considerably, with only class C GPCR 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 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 biparatopic nanobodies in comparison to 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 homo- and heteromers in ex vivo samples, however, influence from signaling molecules should be considered if using agonists, whereas both 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 physiological levels with correct spatio-temporal expression patterns. Finally, confirmed disruption of heteromerization using interfering peptides followed by changes in phenotypical response provided evidence for the presence as well as (patho)-physiological function of some GPCR heteromers. Translation of in vitro observations for more GPCR heteromers to native tissues is required in the near future to confirm that GPCR dimer and oligomers exist beyond engineered model systems.
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AUTHORSHIP CONTRIBUTIONS

Henry F. Vischer, Marián Castro, and Jean-Philippe Pin wrote the manuscript.
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FOOTNOTES
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FIGURE LEGENDS

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 co-immunoprecipitation (A) or close-proximity using in situ proximity ligation of DNA-conjugated secondary antibodies (B) and (tr)FRET between fluorophore-conjugated antibodies (C). (tr)FRET between fluorescent ligands allows detection of receptor complexes in native tissue (D). Genetic fusion of luminescent or fluorescent donor and acceptor proteins or non-functional fragments of these proteins to the C-terminal tail of GPCRs allows detection of close-proximity by RET (E) and bimolecular fluorescence or luminescence complementation (F). 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 BiFC with sequential FRET (H).

Fig. 2. Fluorescence correlation spectroscopy combined with photon counting histogram analysis to investigate oligomerization status of native serotonin 5-HT\textsubscript{2C} receptors in living cells. Native serotonin 5-HT\textsubscript{2C} receptors in choroid plexus epithelial cells were labeled with monoclonal anti-5-HT\textsubscript{2C} Fabs fused to GFP (A). FCS measurements were made on the apical surface of living cells using an one-photon excitation microscope equipped with a sensitive photon counting detector, creating an observation volume of less than 1 fL (< 1 \textmu m\textsuperscript{3}), and GFP fluorescence emission was registered for 100 seconds, as 10 consecutive 10-second intervals (B). Autocorrelation analysis (ACA) of the recorded fluorescence intensity traces (FIT) from 10-second observation periods informs on the number of molecules in the observation volume and diffusion time (C), whereas photon counting histogram (PCH) of the FCS recordings provides a quantification of the photons emitted from the individual fluorescent molecules. This allows to determine the average molecular brightness of the sample and to identify the number of GFP-labeled protomers that take part of a co-diffusing complex (FCS traces and autocorrelation/PCH analysis taken from (Herrick-Davis \textit{et al.}, 2015), with permission).
Fig. 3. Dual-color photoactivatable dyes and localization microscopy (PD-PALM) to investigate spatial and structural organization of GPCR complexes. Epitope-tagged binding-deficient (LHRB−) and signaling-deficient (LHRS−) receptors were labeled with specific antibodies conjugated with CAGE 552 or 500 dyes, and cells were subsequently fixed (A). PD-PALM images were acquired using a TIRF-equipped microscope by iterative cycles of photoconversion of the CAGE dyes upon UV illumination and simultaneous dual-channel single-molecule imaging and irreversibly photo-bleaching (B). Localization coordinates (x-y) were assigned to individual fluorescent particles detected in each imaged channels. Analysis of the number of associated protomers was conducted based on a recursively search for neighborhood particles within a radius of 50 nm from each individual protomer (C). In combination with molecular modeling studies, this approach allowed to resolve the composition and spatial arrangement of each associated group of molecules (di/oligomers) visualized by PD-PALM (D). The structural models, depicted from the extracellular side, are taken from (Jonas et al., 2015) under Creative Commons Attribution Unported License to Author Choice articles.
**Fig. 1**

A. co-IP

B. PLA

C. RET/TR-FRET

D. RET/TR-FRET (native)

E. RET

F. BiFC/BiLC

G. HIT

H. CODA-RET

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Fig. 2
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Fig. 3