Most cells express a panel of different G protein-coupled receptors (GPCRs) allowing them to respond to at least a corresponding variety of extracellular ligands. In order to come to an integrative well-balanced functional response these ligand–receptor pairs can often cross-regulate each other. Although most GPCRs are fully capable to induce intracellular signalling upon agonist binding on their own, many GPCRs, if not all, appear to exist and function in homomeric and/or heteromeric assemblies for at least some time. Such heteromeric organization offers unique allosteric control of receptor pharmacology and function between the protomers and might even unmask ‘new’ features. However, it is important to realize that some functional consequences that are proposed to originate from heteromeric receptor interactions may also be observed due to intracellular crosstalk between signalling pathways of non-associated GPCRs.

Abbreviations
BRET, bioluminescence resonance energy transfer; CaMKIIα, calcium/calmodulin-dependent protein kinase IIα; EL, extracellular loop; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GABA, γ-amino butyric acid; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, GPCR kinase; IL, intracellular loop; NTED, N-terminal extracellular domain; PFC, protein-fragment complementation; RASSL, receptor activated solely by synthetic ligands; RET, resonance energy transfer; Rluc, Renilla reniformis luciferase; TM, transmembrane

G protein-coupled receptors and cellular communication
A sophisticated biochemical communication network regulates coordinated functioning of individual cells within the human body. An important part of this network consists of extracellular messenger molecules (i.e. ligands) and cognate receptor proteins that are present on the cellular surface (Ben-Shlomo et al., 2003). The G protein-coupled receptors (GPCRs) are by far the largest family of membrane-associated receptors, and are characterized by the presence of seven transmembrane (TM) α-helices that are connected by alternating intracellular and extracellular loops (i.e. ILs and ELs respectively). The human genome encodes approximately 800 different GPCRs that are responsive to a plethora of endogenous (e.g. ions, lipids, biogenic amines, peptides and glycoproteins) and exogenous (e.g. odorants, tastants, photons and therapeutic drugs) ligands (Lagerstrom and Schioth, 2008). Not surprisingly, GPCRs are involved in the regulation of nearly all processes in our body and their dysfunction contributes to numerous human pathologies (Dorsam and Gutkind, 2007; Smit et al., 2007). Hence, GPCRs are today’s favourite drug targets with ~40% of all current therapeutic molecules acting on members of this protein family.

Binding of an agonist to the extracellular site of the GPCR (i.e. N-terminus, ELs and/or pocket that is formed by the 7TM
helices) induces conformational changes in the 7TM and intracellular domains of the receptor, allowing coupling and activation of specific heterotrimeric G proteins (Oldham and Hamm, 2008; Nygaard et al., 2009). Activated G proteins dissociate from the GPCR to relay the signal to downstream effector proteins. Subsequent phosphorylation of the intracellular domains of activated GPCRs by GPCR kinases (GRKs) promotes the recruitment of β-arrestins (Kelly et al., 2008; Tobin, 2008). Bound β-arrestin inhibits G protein signalling by hindering GPCR–G protein coupling and by recruiting proteins involved in receptor endocytosis. However, β-arrestin can also scaffold new signalling cascade components to the activated GPCR, thereby initiating a second wave of intracellular signalling (Lefkowitz and Shenoy, 2005).

Most of our cells express several dozen different GPCR subtypes, which can be variably mixed and matched in different cell types, and are consequently responsive to at least a corresponding number of ligands (Vassilatis et al., 2003; Gurevich and Gurevich, 2008; Regard et al., 2008). Importantly, individual ligand–receptor combinations do generally not operate in isolation, but may rather ‘talk’ to each other to come to a balanced cellular response to two or more simultaneous stimuli. This crosstalk can occur at (a combination of) various levels along the GPCR signal transduction pathway. First of all, GPCRs can allosterically interact with each other by forming homomeric or heteromeric (i.e. between similar or different receptor subtypes respectively) assemblies (Figure 1A). Second, GPCRs can desensitize other GPCRs via second messenger-dependent protein kinases (PK) A or C (Figure 1B) (Vazquez-Prado et al., 2003; Kelly et al., 2008). Third, GPCRs may impair other GPCRs by scavenging shared signalling and/or scaffolding proteins (e.g. G proteins and β-arrestins) that are limiting for receptor signalling (Figure 1C) (Schmidlin et al., 2002; Nijmeijer et al., 2010). And finally, GPCRs can activate distinct signal transduction pathways that may converge at downstream signalling hubs, as for example the opposite regulation of adenylate cyclase by G<sub>qi</sub> and G<sub>gs</sub>-coupled GPCRs or regulation of intracellular Ca<sup>2+</sup> levels by G<sub>qi</sub> and G<sub>q</sub>-coupled GPCRs (Figure 1D) (Natarajan et al., 2006).

Heteromerization between different GPCR subtypes can significantly modify functional characteristics of the individual protomers, including subcellular localization, ligand binding cooperativity and proximal signalling (Levoye et al., 2006b; Springael et al., 2007; Milligan, 2009). However, GPCR heteromer-induced changes in biochemical GPCR signalling properties are often difficult to distinguish unambiguously from downstream crosstalk between non-associated GPCR pairs. In this review, we will focus on the question ‘do GPCRs that walk hand-in-hand, also talk hand-in-hand?’.

**GPCR oligomerization**

Dimerization and/or higher order oligomerization of otherwise non-functional protomers is a common phenomenon for most cell surface receptor families. Oligomerization of three to five subunits is required to form a ligand-gated ion channel, whereas ligand-induced dimerization is mandatory for activation and signalling of 1TM-domain receptors such as cytokine receptors, receptor tyrosine and serine/threonine kinases (Heldin, 1995; Marianayagam et al., 2004). Also class C GPCRs exist and function as obligate dimers (Pin et al., 2003; 2009). For example, the γ-amino butyric acid (GABA) B receptor consists of two different 7TM subunits GABA<sub>B1</sub> and GABA<sub>B2</sub> that are non-functional when expressed on their own. The GABA<sub>B1</sub> subunit is retained in the endoplasmatic reticulum as export through the Golgi is prevented by binding of coat protein I complex (COP1) to the RXR retention motif in its C-tail (Brock et al., 2005). However, the GABA<sub>B2</sub> subunit masks this COP1 binding site through a
coiled–coil interaction of their C-tails, allowing trafficking of the heteromeric GABA\textsubscript{B} receptor to the cell surface (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). Moreover, the GABA\textsubscript{B} subunits have complementary roles in GABA-induced signalling, with GABA binding exclusively to the N-terminal extracellular domain (NTED) of GABA\textsubscript{B}\textsubscript{A}, and G proteins exclusively being activated by GABA\textsubscript{B}\textsubscript{A} upon transactivation of this subunit by the agonist-occupied GABA\textsubscript{B}\textsubscript{A} (Galvez et al., 2001; Duthey et al., 2002; Havlickova et al., 2002; Kniazef et al., 2002). Similarly, the umami and sweet taste receptors are heterodimeric assemblies of T1R, in combination with T1R or T1R, respectively (Zhao et al., 2003). Indeed, T1R, knockout mice show diminished detection of both umami and sweet taste, whereas only the umami or sweet sensation was affected in T1R, and T1R, knockout mice respectively (Damak et al., 2003; Zhao et al., 2003). Finally, the calcium sensing receptor and eight metabotropic glutamate receptors (mGlu) are homodimers in which the two NTEDs are linked by disulfide bonds (Romano et al., 1996; Ray et al., 1999). Agonist-induced movement of these NTEDs relative to each other results in activation of these receptor dimers (Pin et al., 2005). Interestingly, constitutive homodimerization of class B secretin receptors was found to facilitate G protein coupling, which is mandatory for high affinity secretin binding (Harikumar et al., 2006; Gao et al., 2009). Hence, functional class C (and possibly class B) receptors (i.e. capable to induce intracellular signalling in response to agonist stimulation) are macromolecular assemblies of two 7TM subunits that are non-functional on their own.

In contrast to class C (and possibly class B) GPCR 7TM subunits, most class A GPCRs are fully capable to interact as single 7TM units with their ligands and intracellular protein partners (i.e. heterotrimeric G protein, GRK or \(\beta\)-arrestin) in a 1:1 stoichiometry, as observed in recent studies using purified GPCR monomers that were refolded in small lipid bilayer nanodiscs or detergent micelles (Bayburt et al., 2007; Hanson et al., 2007; White et al., 2007; Whorton et al., 2007; 2008; Kuzak et al., 2009; Arcemisbehere et al., 2010; Bayburt et al., 2010; Tsukamoto et al., 2010). Nonetheless, increasing experimental evidence suggests that most if not all class A GPCRs can form homomers and/or heteromers (Ferre and Franco, 2010). Already in 1975, negative cooperativity in radioligand binding studies with 5-HT that homodimers might be assembled as homodimers in erythrocyte membrane preparations (Limbird et al., 1975). Even though biochemical evidence for the existence of class A GPCR dimers (e.g. cross-linking, co-immunoprecipitation, photo-affinity labelling and radiation inactivation experiments) was also reported in the following two decades (Bouvier, 2001), the concept that GPCRs can physically interact with each other became only more widely accepted after the identification of the aforementioned, obligatory GABA\textsubscript{A} heterodimer in the mid-to late-1990s (Romano et al., 1996; Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). New experimental approaches, including protein-fragment complementation (PFC) techniques and resonance energy transfer (RET)-based methods (see below), have catalysed the identification of GPCR homomers and heteromers in heterologous expression systems during the last decade (Ciruela et al., 2010; Khelashvili et al., 2010). Hitherto, however, confirming the presence of GPCR assemblies in native cells is still technically challeng-
2008). While reconstituted Rluc fragments can unfold and separate upon dissociation of interacting protein complexes, the refolding of GFP variants is irreversible resulting in an artificial stabilization of transient interactions between GPCR–PFC fusion proteins (Michnick et al., 2007). Combining the PFC method with BRET measurements allows detection of close proximity between four GPCRs (Guo et al., 2008; Nijmeijer et al., 2010). Fusion of a SNAP (or CLIP) tag to the N-terminus of GPCRs allows covalent labelling of surface expressed GPCRs with membrane-impermeant time-resolved FRET (trFRET) compatible donor (e.g. Eu³⁺ or Tb²⁺ cryptate) and acceptor (e.g. D2, Red) fluophores (Maurel et al., 2008; Alvarez-Curto et al., 2010). trFRET relies on long-lived lanthanide (donor) emission versus short acceptor emission lifetime. Acceptor emission due to direct acceptor excitation decays rapidly, allowing detection of long-lived (indirect) energy transfer-mediated acceptor emission. These trFRET fluophores can also be conjugated to antibodies and even more interesting to GPCR ligands (Maurel et al., 2004; Albizu et al., 2010). Ligand (antagonist)-based trFRET has very recently successfully been used to detect endogenous oxytocin receptor oligomers in mammary gland (Albizu et al., 2010). Even though well-designed RET- and/or PFC-based experiments may provide compelling evidence for specific GPCR interactions, one has to keep in mind that close proximity rather than physical interactions between proteins is detected.

Convincing evidence for direct physical interactions between GPCRs is provided by functional complementation experiments in which two non-functional receptor protomers are engineered and co-expressed to obtain a functional (quasi)-heteromeric receptor complex (Figure 2D). The best-known example of functional receptor complementation is provided by nature herself: the obligatory heteromeric GABA_B receptor in which the NTED of GABA_B1 is required for ligand binding, whereas the 7TM domain of GABA_B2 activated

Figure 2
Detection of G protein-coupled receptor (GPCR) oligomers. (A) The ‘blue’-tagged GPCR is only co-immunoprecipitated if physically associated with the ‘red’-tagged receptor (i.e. bottom-right panel). (B) Resonance energy transfer between donor and acceptor molecules that are fused to GPCR occurs when they are brought in close proximity (<10 nm) by interacting GPCR. (C) Protein-fragment complementation of non-functional biosensor protein fragments occurs when they are brought in close proximity (<2–5 nm) by interacting GPCRs as fusion proteins. (D) Two non-functional GPCRs are functionally reconstituted upon co-expression in the same cell, for example by domain-swap dimerization.
the G protein (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). The glycoprotein hormone receptors distinguish themselves from other class A GPCRs by having an extended NTED, which is exclusively involved in hormone binding (Osuga et al., 1997; Fan and Hendrickson, 2005). Taking advantage of this modular nature of hormone–receptor and receptor–G protein interactions, luteinizing hormone receptor (LHR) and follicle-stimulating hormone (FSHR) mutants were engineered in which either hormone binding to the NTED or G protein activation by the 7TM domain was disrupted (Ji et al., 2002; 2004; Lee et al., 2002). Interestingly, co-expression of these LHR or FSHR mutants restored hormone-induced cAMP production, indicating that these loss-of-function mutants can form functional dimers. Moreover, transgenic co-expression of binding- and signalling-deficient LHR mutants in Leydig cells of male hypogonadal LHR knockout mice at physiological levels, restored LH-induced Leydig cell differentiation, testosterone production, gonadal development to sexual maturation and spermatogenesis, confirming for the first time the significance of intermolecular interactions between co-expressed GPCRs in a physiological context (Rivero-Muller et al., 2010).

The mode of action of this functional complementation remains somewhat puzzling, however, as some of these binding-deficient receptor mutants can also be transactivated by NTEDs tethered to a glycosyl phosphatidylinositol moiety or CD8 TM domain. This suggests that (dimeric) interactions between two 7TM domains are not required for this functional rescue (Ji et al., 2002; 2004). In contrast to the modular glycoprotein hormone receptors, the majority of class A GPCRs bind their ligands within or near the pocket formed by the 7TM domain (Kristiansen, 2004). Consequently, ligand binding and receptor activation domains of these GPCRs cannot be easily separated. However, co-expression of two binding-deficient histamine H1 receptors (H1R) with a single mutation in TM3 or TM6 (i.e. H1R-D13.12-A and H1R-F5.52-A respectively) restored ligand binding, revealing a physical interaction between the two receptor mutants (Bakker et al., 2004b). As only TM1–5 of H1R-D13.12-A and TM6–7 of H1R-D5.52-A can contribute to a functional H1R binding pocket, these data suggest that these dimers are organized in a reciprocal domain-swap configuration. A similar domain-swap arrangement was shown by rescued ligand binding upon co-expression of M1 muscarinic receptor/α2C-adrenoceptor chimeras in which TM6–7 domains were reciprocally exchanged (Figure 2D) (Maggio et al., 1993). Interestingly, co-expression of binding-deficient angiotensin II type 1 receptor (AT1R) constructs with a single mutation in TM3 or TM5 also restored binding of angiotensin II and related analogues (Monnot et al., 1996).

**Do GPCRs walk hand-in-hand?**

A large number of class A and C GPCR subtypes are not delivered to the cell surface when transfected in heterologous cells, and it has been proposed that heteromerization of GPCRs that share similar spatiotemporal expression profile in native cells might be required for proper folding and export of these from the endoplasmic reticulum (ER) to the cell surface (Figure 3A) (Minneman, 2007; 2010; Achour et al., 2008). Indeed, the coiled–coil interaction between the C-tails of GABAB1 and GABAB2 is required for cell surface targeting of the heteromeric GABA receptor, confirming that GPCR homomers and heteromers are formed during early biosynthesis and protein maturation in the ER. Heteromerization of the β2- or α1B-adrenoceptor with the α1D-adrenoceptor is essential for cell surface targeting of the latter receptor in heterologous cells, whereas co-expression with 26 other related class A GPCRs did not promote surface

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**Figure 3**

G protein-coupled receptors (GPCRs) are walking hand-in-hand. (A) GPCR oligomerization in the endoplasmic reticulum (ER) is required for cell surface delivery; however, oligomerization may also retain GPCRs in the ER. (B and C) GPCRs exist at the cell surface in an equilibrium between oligomers and monomers. The transition between these configurations may be affected by ligands. (D) Agonist stimulation of one protomer results in internalization of the heteromeric assembly.

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expression of the $\alpha_{1B}$-adrenoceptor (Hague et al., 2004; Uberti et al., 2005). On the contrary, various naturally occurring GPCR splice variants and mutants have been reported to trap co-expressed wild-type counterparts in the ER by forming heteromeric assemblies (Figure 3A) (Zhu and Wess, 1998; Coge et al., 1999; Karpa et al., 2000; Seck et al., 2003; Calebiro et al., 2005; Pidashева et al., 2006; van Bijin et al., 2008). For example, natural occurring rat histamine H₃ receptor (H₃R) splice variants that lack TM7 impair cell surface targeting of wild-type H₃R (Bakker et al., 2006). Interestingly, the expression levels of these truncated isoforms and wild-type H₃R in rat brain are oppositely modulated by the convulsant pentylentetrazol, resulting in increased H₃R activity, whereas high-fat diet induced down-regulation of the dominant negative GIP receptor splice variant resulting in an up-regulation of wild-type GIP receptors in obese mice (Harada et al., 2008). In addition, dominant negative receptor mutants have been engineered by introducing ER-retention signals into the C-tail of GPCRs or site-directed mutation of ER-export motifs. Substitution of the C-tail of the $\beta_{2}$-adrenoceptor with the C-tail ER-retention motif of GABAB₁ resulted in an ER-trapped receptor mutant, which also prevented cell surface targeting of wild-type $\beta_{2}$-adrenoceptor (Salahpour et al., 2004). Likewise, fusion of the ER-retention motif of the $\alpha_{1C}$-adrenoceptor to the C-tail of the CXC-chemokine receptor 1 (CXC1R) impaired its trafficking to the cell surface (Wilson et al., 2005). Moreover, this ER-retained CXC1R mutant inhibited cell surface trafficking of wild-type CXC1R and the closely related CXC2R, by forming homomers and heteromers respectively. In contrast, cell surface delivery of co-expressed $\alpha_{1A}$-adrenoceptor was not affected, which correlated with the observation that CXC1R and $\alpha_{1C}$-adrenoceptor do not form heteromers. The importance of a correct quaternary structure for cell surface delivery was furthermore demonstrated by a $\alpha_{1A}$-adrenoceptor mutant in which hydrophobic residues in TM1 and TM4 were Ala-substituted (Lopez-Gimenez et al., 2005). This TM1–TM4 mutant was trapped in the ER and displayed an altered oligomeric organization in comparison with wild-type receptors as indicated by sequential three-colour FRET analysis. Interestingly, the cell-permeant $\alpha_{1C}$-adrenoceptor-antagonist prazosin changed the quaternary structure of TM1–TM4 mutants to an oligomeric organization that resembles wild-type $\alpha_{1A}$-adrenoceptor. In addition, prazosin acted as pharmacological chaperone by promoting terminal N-glycosylation and maturation, resulting in cell surface delivery of this TM1–TM4 mutant (Canals et al., 2009). Several other examples of pharmacological chaperones that restore cell surface delivery of disease-linked receptor mutants have been reported (Bernier et al., 2004). However, whether this pharmacological rescue involves changes in quaternary receptor organization remains to be investigated.

Collectively, most data suggest that receptor oligomers are preassembled in the ER and ‘walk hand-in-hand’ to the cell surface. Obviously, the covalent disulfide-bonded homomeric class C GPCRs keep on walking hand-in-hand at the cell surface, whereas the heteromeric GABAB₁ receptor is stabilized by a coiled-coil configuration of the C-tails and direct interactions of the NTEDs (Pin et al., 2005). For long, the stability of class A GPCR oligomers has been an enigma, although RET data suggest that most GPCRs remain organized as oligomers (Figure 3B). In addition, mutational analysis revealed that heteromers between the dopamine D₁ receptor, adenosine A₂ receptors and the cannabinoid CB₁ receptors are stabilized by electrostatic interactions between Arg-rich motifs in IL3 of D₁ and A₂ receptors and phosphorylated caspase kinase 1/2 sites in IL3 and C-tail of the CB₁ receptor, and the C-terminus of the A₂A receptor (Borroto-Escuela et al., 2010; Navarro et al., 2010). In addition, the Arg-rich motif in IL3 of the dopamine D₂ receptor is involved in a stabilizing electrostatic interaction with a di-glutamate motif in the C-terminus of the serotonin 5-HT₃a and D₁ receptor (Lukasiewicz et al., 2009; 2010). Recently, however, the lateral mobility of one protomer was monitored using dual fluorescence recovery after photobleaching (FRAP) microscopy upon antibody immobilization of the other protomer at the cell surface (Dorsch et al., 2009; Fonseca and Lambert, 2009). To this end, different GFP variants were fused to the N- or C-terminus of the GPCRs. Immobilization of one YFP-$\beta_{2}$-adrenoceptor almost completely impaired lateral diffusion of at least four co-expressed $\beta_{2}$-adrenoceptor-CFP fusion proteins into the bleached region of the cell membrane, suggesting that receptors form stable higher order homomers (Dorsch et al., 2009). In contrast, the mobility of $\beta_{2}$-adrenoceptor and D₁ receptor was only modestly affected by the antibody immobilization of their homomeric counterparts, suggesting that these receptors form rather transient homomers (Figure 3C) (Dorsch et al., 2009; Fonseca and Lambert, 2009). Intensity imaging of M₁ muscarinic receptors that were labelled with a slowly dissociating fluorescent antagonist using total internal reflection fluorescence microscopy indicates that transient M₁ receptor homodimers are being formed and/or fall apart within seconds. This short-lived nature of M₁ receptor homodimers was confirmed by recording the lateral mobility trajectory of dual-colour labelled receptors. Approximately 30% of the M₁ receptors was engaged in homodimers at any given moment, whereas higher order oligomers were never detected for this receptor (Hern et al., 2010). Interestingly, the apparent transient nature of $\beta_{2}$-adrenoceptor, D₁ and M₁ receptor homodimers as observed in these imaging-based studies is in contrast with earlier BRET studies for these receptors, which indicated that the vast majority of these receptors exist as constitutive dimers at nearly physiological expression levels (Mercier et al., 2002; Goin and Nathanson, 2006; Guo et al., 2008). In addition, BRET data indicated that $\beta_{2}$- and $\beta_{2}$-adrenoceptors have equal propensities to form homodimers and heterodimers (Mercier et al., 2002), whereas D₁ receptor homodimerization was confirmed in cross-linking and functional complementation experiments (Guo et al., 2008; Han et al., 2009). In fact, higher order D₂ receptor oligomers have been detected using PFC in combination with BRET analysis; however, it should be kept in mind that the transient nature of D₂ receptor interactions might be obscured the permanent reconstitution of the sensor proteins from their split fragments. Higher order homomers and heteromers (i.e. trimeric and tetrameric assemblies) have also been detected for other class A GPCRs using this PFC-BRET analysis (Gandía et al., 2008; Guo et al., 2008; Sohy et al., 2009; Nijmeijer et al., 2010), but also using three-colour FRET (Lopez-Gimenez et al., 2007; Canals et al., 2009).
Do GPCRs talk hand-in-hand?

Clear evidence that GPCRs ‘talk hand-in-hand’ comes from the aforementioned obligatory heterodimer GABA<sub>B</sub> in which one protomer (i.e. GABA<sub>B2</sub>) is exclusively responsible for protein binding, whereas only the other protomer (i.e. GABA<sub>B1</sub>) can activate heterotrimetric G proteins. Such functional asymmetry in protomer functioning has also been observed in class A GPCR oligomers. Despite the fact that GPCR monomers can efficiently couple to G proteins and β-arrestins in response to agonist stimulation (see below), functional asymmetry is often apparent once they are engaged in homomeric and/or heteromeric assemblies. For instance, ligand binding to one protomer can affect the associated protomer through intermolecular allosteric interactions. Propagation of conformational changes from one to the other protomer has been directly shown within the α<sub>2A</sub>-adrenoceptor/µ-opioid receptor (µOR) heterodimer (Vilardaga et al., 2008). Binding of morphine to the µOR triggers a conformational change in the associated norpinephrine-occupied α<sub>2C</sub>-adrenoceptor, as detected by a decrease in the norpinephrine-induced FRET efficiency between two fluorophores in IL3 and C-tail of α<sub>2C</sub>-adrenoceptor, which is translated within milliseconds in reduced G protein activation by the α<sub>2C</sub>-adrenoceptor protomer (Figure 4A). Well-designed molecular engineering also revealed functional allosterism in dopamine D<sub>2</sub> receptor homodimers (Han et al., 2009). To this end, the D<sub>2</sub> receptor was fused without a linker to the chimeric G protein Ge<sub>q,i5</sub>. This fusion protein (i.e. D<sub>2</sub>–Ge<sub>q,i5</sub>) was non-functional when expressed on its own; however, co-expression of wild-type D<sub>2</sub> receptor resulted in agonist-induced coupling of the latter to the Ge<sub>q,i5</sub> protein of the non-functional D<sub>2</sub>–Ge<sub>q,i5</sub> by forming dimers. In contrast, binding- or coupling-deficient D<sub>2</sub> receptor mutants were unable to signal through the fused Ge<sub>q,i5</sub> when co-expressed with D<sub>2</sub>–Ge<sub>q,i5</sub>. Moreover, also the capacity of the non-functional D<sub>2</sub>–Ge<sub>q,i5</sub> to interact with G proteins appeared to be essential for dimer-induced signalling. Interestingly, this D<sub>2</sub> receptor dimer is fully activated by agonist binding to one protomer, confirming the asymmetric nature between dimer protomers. In fact, binding of an additional agonist or inverse agonist to the second protomer disrupted or increased dimmer signalling respectively. Importantly, as an ‘artificial’ Ge<sub>q,i5</sub>-mediated response is measured, observed dimer signalling must result from physical interactions between the protomers and G protein rather than from downstream crosstalk in signalling pathways. Indeed, differential cross-linking of D<sub>2</sub> dimers in inverse agonist versus agonist-bound state suggests that conformational changes at the dimer interface is part of the receptor activation mechanism (Guo et al., 2005).

Similar to the engineered D<sub>2</sub> receptor dimer with the single fused G protein, the leukotriene B<sub>4</sub> receptor BLT<sub>1</sub> homodimer only couples to one heterotrimetric G protein at a time (Baneres and Parello, 2003). Agonist-induced activation of one of the BLT<sub>1</sub> protomers is sufficient to promote G protein coupling and activation (Damian et al., 2006). Moreover, fluorescence spectroscopy analysis revealed that the other protomer adopts a distinct conformation than the activated protomer. However, this difference in protomer conformation was not observed in the absence of G proteins, suggesting that the G protein confers asymmetry to the BLT<sub>1</sub>
GPCR oligomerization and crosstalk

Figure 4

G protein-coupled receptors (GPCRs) are talking hand-in-hand. (A) Agonist (red triangle)-induced conformational change in one (white) protomer is transferred to the second agonist (blue triangle)-occupied protomer, resulting in a changed conformation as detected by decreased intramolecular fluorescence resonance energy transfer. (B) Negative binding cooperativity between ligand on a GPCR heteromer. (C) Positive binding cooperativity between ligands on a GPCR heteromer. (D) Change in G protein coupling and downstream signalling upon heteromerization of the $G_{\alpha}\iota$-coupled D1 and the $G_{\alpha}\omega$-coupled D2 receptor, resulting in $G_{q}$-mediated $Ca^{2+}$ signalling.

homodimer by restricting conformational changes in the second protomer.

Intermolecular crosstalk within receptor oligomers can result in allosterism between the orthosteric binding pockets of the individual protomers. Negative binding cooperativity has been observed for both GPCR homomers and heteromers using equilibrium binding and/or radioligand dissociation experiments (Springael et al., 2007). The latter is in particular interesting in light of GPCR crosstalk as mutual exclusive binding of one ligand to receptor heteromers results in a decreased responsiveness to the ligand of the other protomer (Figure 4B). Detection of negative binding cooperativity using equilibrium binding assays on membrane preparations co-expressing the receptors of interest has been questioned on the merit that G protein coupling to agonist-occupied receptors might be irreversible in the absence of free GTP to substitute the released GDP (Chabre et al., 2009). G protein scavenging by the agonist-occupied GPCR may deplete a shared pool of G proteins from interacting with other (perhaps non-associated) receptors, often resulting in decreased apparent affinities of the latter receptors for their agonists (Chabre et al., 2009; Birdsall, 2010). This may be easily misinterpreted as being negative binding cooperativity between two interacting protomers. However, co-expression of additional G protein may shed light on this matter by preventing depletion (Nijmeijer et al., 2010). Moreover, this proposed G protein-stealing hypothesis is not compatible with an increased dissociation rate of pre-bound agonist from one protomer upon agonist binding to the second protomer if there is negative cooperativity between the two binding sites. For instance, negative cooperative has been detected within CCR2, CCR5 and CXCR4 heteromeric complexes in both recombinant cells and native immune cells (El-Asmar et al., 2005; Springael et al., 2006; Sohy et al., 2007; 2009). Cross-competition was detected between their cognate chemokines in equilibrium binding experiments on both membrane preparations and intact cells, with the extent of cross-inhibition corresponding roughly to the anticipated proportion of cognate receptors involved in heteromeric complexes. Acceleration of each other’s dissociation rates in ‘infinite’ tracer dilution experiments confirmed the allosteric nature of this cross-inhibition rather than steric hindrance between these chemokines at the extracellular surface of the receptor heteromers. Interestingly, negative cooperativity within CCR2, CCR5 and CXCR4 heteromers is not limited to agonist (i.e. chemokines) but was also observed for low molecular weight antagonists of these receptors, suggesting that downstream signalling is not per se involved in this cross-regulation. Moreover, cross-inhibition of chemokine-induced immune cell recruitment both in vitro and in vivo by antagonists that interact with other chemokine receptor subtypes within the heteromer, confirmed the functional relevance of the observed binding cooperativity between these receptors. Interestingly, D2 receptor homodimers and vasopressin $V_{1a}$/oxytocin (OT) receptor heterodimers were readily detected by trFRET upon binding of fluorophore-conjugated antagonists to each of the protomers, whereas incubation of similar samples with an excess of fluorescent agonist resulted in very weak FRET signals (Albizu et al., 2010). Similar discrepancy in cooperativity between agonists and antagonists was observed in radioligand binding experiments on membranes that express $V_{1a}$, oxytocin or D2 receptors (Albizu et al., 2006; Kara et al., 2010). Hence, the apparent absence of binding cooperativity between antagonists on $V_{1a}$, OT or D2 receptor homomers and/or heteromers is different from the observations in chemokine receptor heteromers (Sohy et al., 2009). Interestingly, opposite binding cooperativity was observed within 5-HT$_{2a}$/mGlu$_{R}$ receptor heteromers in mouse somatosensory cortex membranes (Gonzalez-Maeso et al., 2008). The mGlu$_{R}$ agonist LY379268 increases the affinity...
of hallucinogenic agonists such as 1-(2,5)-dimethoxy-4- indophenyl)-2-aminopropane (DOI) for the 5-HT2A receptor, whereas DOI decreases the affinity of LY379268 for the mGlulR. On the contrary, however, the sensitized Gαi signalling of 5-HT2A/mGlulR receptor heteromers in response to hallucinogenic 5-HT2A receptor agonists was reversed upon activation of mGlulR by LY379268.

Besides intermolecular inhibitory crosstalk between protomer binding pockets, also specific interactions between their intracellular domains may affect the ligand binding properties of GPCR heteromers. For example, the orphan receptor GPR50 forms heteromer with the Gαi-coupled melatonin receptors MT1 and MT2 (Levoye et al., 2006a). GPR50 inhibited melatonin binding to the associated MT1, but not MT2, protomer, suggesting that downstream G protein stealing per se was not the underlying mode of action. Inhibition of MT1 protomer function appeared to be attributed to the long C-tail of GPR50, which apparently interact differently with the MT1 as compared with MT2 protomer, thereby hindering G protein coupling to the MT1 protomer (Levoye et al., 2006a).

Stimulation of the δOR/Mas-related GPCR member X (MRGPRX1, a.k.a. SNSR-4) heteromer with selective δOR or MRGPRX1 agonists triggered Gαi or Gαq signalling respectively. However, simultaneous binding of selective δOR and MRGPRX1 agonists to the δOR/ MRGPRX1 heteromer led exclusively to Gαi activation, suggesting a dominant negative effect of the activated MRGPRX1 protomer on δOR-specific signalling (Breit et al., 2006).

On the other hand, agonist binding and activation of both receptor protomers is required for efficient signalling of some other homomers and heteromers. Although a single glutamate molecule is sufficient to promote mGlul homodimer signalling, the binding of two glutamate molecules per homodimer is required for full activation (Kniazeff et al., 2004). Heteromerization of the δ- with κ-opioid receptor (δOR and κOR respectively) resulted in a loss of binding affinity for either δOR- or κOR-selective ligands, whereas partially selective ligands preserved or increased their affinity for the δOR–κOR heteromer (Jordan and Devi, 1999). However, positive binding cooperativity was observed when either δOR- or κOR-selective agonists or a combination of selective antagonists were incubated with a non-selective radiolabelled antagonist and δOR-κOR heteromer-expressing membranes, resulting in at least a 50-fold increase in affinity (Figure 4C).

Surprisingly, only a 10- to 20-fold potentiation in signalling was seen in cells co-expressing δOR and κOR upon co-stimulation with the selective agonists. Even though intermolecular interactions between δOR and κOR are apparent and give rise to a distinctive ligand binding profile, the exact quality and quantity of allosterism within this heteromer seems puzzling (Birdsall, 2010). Interestingly, no positive cooperativity was observed between δOR-selective antagonist and κOR-selective agonist on δOR–κOR heteromers. In contrast, δOR-selective antagonists enhance agonist binding and signalling to the μOR protomer within δOR–μOR heteromers (Gomes et al., 2000). Activated δOR or μOR preferentially activates Gαi proteins as determined by 35S-GTPγS incorporation in selectively immunoprecipitated G proteins, whereas activated δOR–μOR heteromers interact selectively with Gαi proteins (Fan et al., 2005). In addition and in contrast to its homomeric constituents, the δOR–μOR heteromer constitutively recruits β-arrestin2 and is primed to signal through non-G protein-activated pathways (Rozendfeld and Devi, 2007). Activation of the Gαi-coupled dopamine D1 receptor increases the agonist affinity of Gαi-coupled D1 receptors. This positive binding cooperativity within D1–D2 receptor heteromers results in increased Gαi-mediated locomotor activity, which can be inhibited by D1 receptor antagonists (Marcelino et al., 2008). On the other hand, heteromerization of the D1 receptor with the Gαi-coupled histamine H3 receptor triggered Gαi-dependent but Gαi-independent MAPK signalling pathway activation in response to dopaminergic or histaminergic agonists, which could be (cross-)blocked by selective antagonists acting at either of the two protomers (Ferrada et al., 2009). This acquired capacity of histaminergic agonists to induce MAPK signalling through the H3R was strictly dependent on the presence of the D1 receptor. The D1 and D2 receptors activate of Gαi and Gαq proteins, respectively, resulting in an opposite regulation of cAMP production by adenyl cyclase (Figure 4D) (Lee et al., 2004). However, D1–D2 receptor heteromers can couple to Gαq,11 proteins upon agonist binding to both protomers, resulting in intracellular calcium release from the ER and subsequent activation of calcium/calmodulin-dependent protein kinase Iα (CaMKIα) (Lee et al., 2004; Rashid et al., 2007). Importantly, the Gαq,11 inhibitor YM254890 could fully inhibit D1–D2 receptor heteromer-induced intracellular Ca2+ mobilization, revealing that Gαq,11 coupling rather than downstream crosstalk initiates this signalling pathway. D1–D2 receptor heteromers have been detected in various brain regions and their capacity to activate CaMKIα can be inhibited by pre-administration of D1 or D2 receptor antagonists, and is disrupted in D1 + D2 receptor knockout mice (Rashid et al., 2007). D1–D2 receptor heteromer signalling has been linked to synaptic plasticity as well as behavioural sensitization to psychostimulants, while reduced D1–D2 receptor heteromer activity has been linked to schizophrenia as disturbed calcium homeostasis is thought to underlie this neuropsychiatric disease (Rashid et al., 2007). Hence, the intracellular surface of GPCR heteromers has likely a distinctive conformation as compared with their constituent mono- and/or homo-oligomers, which may result in the recognition of different signalling partners.

Agonist-induced cross-linking of AT1R homodimers by intracellular factor XIIIa transglutaminase increased Gαq,i activation and the formation of inositol 1,4,5-trisphosphates as compared with non-cross-linked AT1R. Noteworthy, factor XIIIa activity and cross-linked AT1R homodimers were increased in hypertensive patients, resulting in enhanced monocyte adhesion to vascular endothelial cells (Abd Alla et al., 2004). On the other hand, monomers of purified rhodopsin, μOR, neurotensin receptor NTs, β-adrrenecceptor and leukotiennie B4 receptor BLT2, reconstituted in nanodiscs or liposomes were shown to bind and activate G proteins and/or (β)-arrestin equally or often more efficiently than their respective homomers (White et al., 2007; Whorton et al., 2007; 2008; Kussak et al., 2009; Arcemisbehere et al., 2010; Tsukamoto et al., 2010). In line, higher order GABAB receptor oligomers (i.e. homomers of the obligate heterodimer GABA A2/GABA A3) had a lower efficacy to activate G proteins than non-associated GABA A receptors (Maurel et al., 2008). Hence, homomerization may control cellular.
responsiveness by limiting G protein coupling efficacy when receptor levels and consequently homomer numbers are elevated to avoid hyperstimulation. In short, convincing evidence shows intramolecular communication within GPCR oligomers, which may result in both positive and negative ligand binding cooperativity, as well as differential coupling to G protein subtypes and/or β-arrestins in comparison with their monomeric counterparts.

Do GPCRs (oligomers) shout from a distance?

Crosstalk between co-expressed GPCRs is not limited to physical receptor–receptor interactions, but can also occur along intracellular signalling pathways that may be interconnected in integrative networks or share limiting components. Consequently, it may be difficult to distinguish whether one receptor affects the signalling properties of an associated GPCR causally due to oligomerization or perhaps due to downstream crosstalk in signalling pathways.

G protein-coupled receptors can dampen each other's agonist responsiveness if they are competing for the same G protein subtype. This crosstalk becomes particularly apparent when one of the competing GPCRs is constitutive active and effectively depletes the cellular pool of available G proteins. For example, cannabinoid CB<sub>1</sub> and μ-opioid receptors activate predominantly G<sub>a1</sub>-coupled signalling pathways and are co-expressed in individual neurons in the striatum, caudate nucleus and dorsal horn. However, the CB<sub>1</sub> receptor constitutively inhibits agonist-induced μOR signalling, which can be restored by co-incubation with a CB<sub>1</sub> receptor inverse agonist or silencing of the ligand-independent CB<sub>1</sub> receptor signalling by site-directed mutagenesis (Canals and Milligan, 2008). Although BRET experiments suggested that CB<sub>1</sub> receptor and μOR exist as heteromers, microscopy studies revealed distinct subcellular localization patterns of both GPCR proteins (Canals and Milligan, 2008). The latter implies that CB<sub>1</sub> receptor and μOR are not assembled as heteromers and cross-regulation of μOR signalling by the constitutive active CB<sub>1</sub> receptor is downstream, presumably via G protein scavenging.

The Epstein-Barr virus-encoded GPCR BILF1 forms heteromers with the human chemokine receptor CXCR4 (Vischer et al., 2008; Nijmeijer et al., 2010). The constitutive active BILF1 also inhibits binding of CXCL12 to CXCR4, whereas a BILF1 mutant, deficient in G protein coupling had a much lesser effect on CXCR4 functioning. Importantly, CXCL12 binding to CXCR4 is highly dependent on the availability of G<sub>a1</sub> proteins, and co-expression of additional G<sub>a1</sub> proteins with BILF1 and CXCR4 restored normal functioning of the latter (Nijmeijer et al., 2010). Although intermolecular inhibition of CXCR4 by BILF1 within a heteromeric complex cannot be ruled out, the rescue of CXCR4 functioning by additional G proteins supports the hypothesis that BILF1 inhibits co-expressed G<sub>a1</sub>-coupled GPCRs by constitutive scavenging of a shared pool of available G<sub>a1</sub> proteins.

In addition, GPCRs can impair each other's agonist's responsiveness by activating second messenger-dependent protein kinases A or C. These protein kinases can phosphorylate both inactive and active receptors but also G proteins, resulting in reduced responsiveness of multiple GPCR subtypes to their cognate agonists (Kelly et al., 2008; Chu et al., 2010).

Although examples of G<sub>q</sub>- and G<sub>i</sub>-coupled receptors that modulate each other's activity through heteromerization are available, compelling evidence for downstream crosstalk between these (constitutively active) GPCRs have been reported as well. Constitutive signalling of the G<sub>q</sub><sub>11</sub>-coupled histamine H<sub>1</sub> receptor is increased in cells co-expressing G<sub>i</sub><sub>11</sub>-coupled serotonin 5-HT<sub>1A</sub>, adenosine A<sub>1</sub>, or M<sub>3</sub> muscarinic receptors, in a Pertussis toxin-sensitive manner (Bakker et al., 2004a). This H<sub>1</sub>R-mediated signalling can be further increased by stimulation with agonists of the co-expressed receptors. On the other hand, the 5-HT<sub>1B</sub> receptor inverse agonist inhibited the Pertussis toxin-sensitive increase in signalling in H<sub>1</sub>R and 5-HT<sub>1A</sub> receptor co-expressing cells, whereas the H<sub>1</sub>R inverse agonist mepyramine inhibited all signalling. Importantly, GPCR-independent stimulation of G<sub>i</sub> proteins by using mastoparan-7 resulted in a similar potentiation of H<sub>1</sub>R signalling indicating unambiguously that the observed crosstalk is on the level of intracellular signalling pathways rather than through receptor heteromerization (Bakker et al., 2004a). Similar downstream crosstalk mechanism was observed between the constitutively active G<sub>q</sub><sub>11</sub>-coupled human cytomegalovirus-encoded receptor US28 and G<sub>i</sub><sub>11</sub>-coupled CCR1 chemokine receptors, the constitutive active G<sub>q</sub><sub>11</sub>-coupled mGlur<sub>1</sub>, and G<sub>i</sub><sub>11</sub>-coupled GABA<sub>B</sub> receptor (Rives et al., 2009), and might also apply for the sensitization of G<sub>q</sub><sub>11</sub>-coupled orexin-1 receptor by the constitutively active G<sub>i</sub><sub>11</sub>-coupled CB<sub>1</sub> receptor in a Pertussis toxin-sensitive manner, which was suggested by the authors to be a direct consequence of orexin 1/CB<sub>1</sub> receptor heteromerization (Hilairet et al., 2003). Heteromerization between CB<sub>1</sub> and orexin-1 receptor was indeed confirmed in distinct cells and was accompanied with a change in cellular distribution of the orexin-1 receptor (Ellis et al., 2006). However, in this study CB<sub>1</sub> had only marginal effect on agonist-induced orexin-1 receptor signalling, which was explained as a difference in cellular background (Ellis et al., 2006).

Conclusions

Increasing evidence suggest that GPCR oligomerization is essential for cell surface targeting of GPCRs. Whether GPCRs keep on walking hand-in-hand on the cell surface is currently under investigation. Some GPCRs appear to form stable oligomeric complexes, while other spend most of their time wandering around alone. In fact, purified and reconstituted class A GPCR monomers are fully capable to mediate agonist-induced signalling. On the other hand, compelling evidence is available that GPCR oligomers do talk differently hand-in-hand than when they are on their own, for example by shifting from G protein class or from G protein to β-arrestin coupling. However, apparent crosstalk between GPCRs may as well originate more distal from GPCRs by interacting or limiting intracellular signalling network constituents, which may actually affect GPCR properties like agonist binding. Showing that physical GPCR interactions are absolutely

required for unique agonist-induced signalling, by actually disrupting them, might therefore be helpful to unambiguously distinguish crosstalk within GPCR heteromers from crosstalk events (far) below these heteromers.

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Conflict of interest

The authors state no conflict of interest.

References


