Chapter 2

G protein-coupled receptors: walking hand-in-hand, talking hand-in-hand?

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Abstract

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 signaling upon agonist binding on their own, many GPCRs, if not all, appear to exist and function in homo- 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 signaling pathways of non-associated GPCRs.
**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. 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. Not surprisingly, GPCRs are involved in the regulation of nearly all processes in our body and their dysfunction contributes to numerous human pathologies. Hence, GPCRs are today’s favorite 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. 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. Bound β-arrestin inhibits G protein signaling by hindering GPCR-G protein coupling and by recruiting proteins involved in receptor endocytosis. However, β-arrestin can also scaffold new signaling cascade components to the activated GPCR, thereby initiating a second wave of intracellular signaling.

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. 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 (Fig. 1A). Secondly, GPCRs can desensitize other GPCRs via second messenger-dependent protein kinases A or C (Fig. 1B).\textsuperscript{7,13} Thirdly, GPCRs may impair other GPCRs by scavenging shared signaling and/or scaffolding proteins (e.g. G proteins and β- arrestins) that are limiting for receptor signaling (Fig. 1C).\textsuperscript{14,15} And finally, GPCRs can activate distinct signal transduction pathways that may converge at downstream signaling hubs, as for example the opposite regulation of adenylate cyclase by $G_{i/o}$- and $G_s$-coupled GPCRs or regulation of intracellular Ca$^{2+}$ levels by $G_{i/o}$- and $G_q$-coupled GPCRs (Fig. 1D).\textsuperscript{16}

Figure 1. Crosstalk between GPCRs. (A) Intermolecular communication between GPCR homo- and heteromers. (B) Agonist (red triangle)-induced signaling of one GPCR can desensitize other GPCRs via 2$^{nd}$ messenger-dependent protein kinases (PK) A or C. (C) Agonist-induced signaling of one GPCR depletes a shared pool of available G proteins, thereby inhibiting other GPCRs. (D) Signaling pathways of $G_{i/o}$- and $G_q$-coupled GPCRs converge on the regulation of adenyl cyclase (AC).
Heteromerization between different GPCR subtypes can significantly modify functional characteristics of the individual protomers, including subcellular localization, ligand binding co-operativity, and proximal signaling.\textsuperscript{17-19} However, GPCR heteromer-induced changes in biochemical GPCR signaling 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 signaling of 1TM-domain receptors such as cytokine receptors, receptor tyrosine and serine/threonine kinases.\textsuperscript{20,21} Also class C GPCRs exist and function as obligate dimers.\textsuperscript{22,23} For example, the γ-amino butyric acid (GABA) B receptor consists of two different 7TM subunits GABA\textsubscript{B1} and GABA\textsubscript{B2} that are non-functional when expressed on their own. The GABA\textsubscript{B1} 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.\textsuperscript{24} However, the GABA\textsubscript{B2} 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.\textsuperscript{25,26} Moreover, the GABA\textsubscript{B} subunits have complementary roles in GABA-induced signaling, with GABA binding exclusively to the N-terminal extracellular domain (NTED) of GABA\textsubscript{B1} and G proteins exclusively being activated by GABA\textsubscript{B2} upon trans-activation of this subunit by the agonist-occupied GABA\textsubscript{B1}.\textsuperscript{27-30} Similarly, the umami and sweet taste receptors are heterodimeric assemblies of T1R\textsubscript{3} in combination with T1R\textsubscript{1} or T1R\textsubscript{2}, respectively.\textsuperscript{31} Indeed, T1R\textsubscript{3} knockout mice show diminished detection of both umami and sweet taste, whereas only the umami or sweet sensation was affected in T1R\textsubscript{1} and T1R\textsubscript{2} knockout mice, respectively.\textsuperscript{31,32} Finally, the calcium sensing receptor and 8 metabotropic glutamate receptors
(mGlu) are homodimers in which the two NTEDs are linked by disulfide bonds.\textsuperscript{33,34} Agonist-induced movement of these NTEDs relative to each other results in activation of these receptor dimers.\textsuperscript{35} Interestingly, constitutive homodimerization of class B secretin receptors was found to facilitate G protein coupling which is mandatory for high affinity secretin binding.\textsuperscript{36,37} Hence, functional class C (and possibly class B) receptors (i.e. capable to induce intracellular signaling 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 β-arrestin) in a 1:1:1 stoichiometry, as observed in recent studies using purified GPCR monomers that were refolded in small lipid bilayer nanodiscs or detergent micelles.\textsuperscript{38-46} Nonetheless, increasing experimental evidence suggests that most if not all class A GPCRs can form homomers and/or heteromers.\textsuperscript{47} Already in 1975, negative cooperativity in radioligand binding studies suggested that β-adrenoceptors might be assembled as homodimers in erythrocyte membrane preparations.\textsuperscript{48} Even though biochemical evidence for the existence of class A GPCR dimers (e.g. cross-linking, co-immunoprecipitation, photo-affinity labeling, and radiation inactivation experiments) was also reported in the following two decades\textsuperscript{49}, 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.\textsuperscript{34,50-52} New experimental approaches, including protein fragment complementation (PFC) techniques and resonance energy transfer (RET)-based methods (see below), have catalyzed the identification of GPCR homo- and heteromers in heterologous expression systems during the last decade.\textsuperscript{53,54} Hitherto, however, confirming the presence of GPCR assemblies in native cells is still technically challenging and remains limited to only a few examples.\textsuperscript{56-58}
Evidence for GPCR oligomerization. Over the last thirty years, a wide variety of biochemical and biophysical methodologies have been applied to collect evidence for GPCR oligomerization, mostly using heterologously expressed GPCR constructs that are engineered to include epitope tags (e.g. HA, FLAG, or myc) or biosensors (e.g. green fluorescent protein variants or luciferase) to allow or facilitate their detection. In this respect, it is important to verify that receptor expression levels are physiologically relevant to avoid artificial aggregation. Moreover, GPCR interactions and functional consequences thereof that were identified in heterologous expression systems should be validated in native cells to confirm their physiological relevance.\textsuperscript{56,57}

The most widely used biochemical proof for physical interactions between GPCRs is by co-immunoprecipitation (co-IP) of these assemblies from solubilized cells using a specific antibody against one protomer, followed by immunoblotting of the SDS-PAGE-resolved samples using a specific antibody against the other protomer (Fig. 2A).\textsuperscript{58} Disruption of the cellular integrity may cause aggregation of non-associated receptors, which should be taken into account by comparing cells that co-express both receptors of interest, with cells that express the individual receptor subtypes and are mixed in an 1:1 ratio prior to solubilization. Epitope-tagged GPCRs are routinely used in co-IP experiments, which allow the use of highly specific antibodies that have high affinity for their respective tag. However, if high quality GPCR-specific antibodies are available this method is one of the few that can be used to detect endogenous GPCR oligomers in native cells.

The last decade, resonance energy transfer (RET) and protein-fragment complementation (PFC)-based methods are routinely used to detect GPCR oligomerization in living cells.\textsuperscript{53} RET between a fluorescent (FRET) or bioluminescent (BRET) donor and a suitable fluorescent acceptor only occurs if these molecules are brought in close proximity (i.e. \(< 10\) nm) by interacting proteins (Fig. 2B). To this end, FRET-compatible donor or acceptor variants of green fluorescent protein (GFP) are fused in frame to the C-terminus of the GPCRs of interest. Hitherto, up to three compatible fluorophores have been simultaneously
used to detect the close proximity of (at least) three GPCRs in a so-called sequential 3-color FRET. If the RET donor is the bioluminescent enzyme Renilla reniformis luciferase (Rluc), BRET can be measured if it is brought in close proximity to green (i.e. BRET² using DeepBlue C as substrate) or yellow fluorescent protein (i.e. BRET using Coelenterazine H as substrate) by interacting GPCRs. In addition, BRET can be combined with FRET between compatible fluorescent proteins in sequential RET to detect close proximity between three GPCRs. Variants of GFP and Rluc can be genetically split into two nonfunctional protein fragments, which are fused in frame to the C-terminus of GPCRs (Fig. 2C). If these split protein fragments are brought in close proximity by interacting GPCRs they will reconstitute into a functional biosensor. 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. Combining the PFC method with BRET measurements allows detection of close proximity between 4 GPCRs. Fusion of a SNAP (or CLIP) tag to the N-terminus of GPCRs allows covalent labeling of surface expressed GPCRs with membrane impermeant time-resolved FRET (trFRET) compatible donor (e.g. Eu³⁺ or Tb³⁺ cryptate) and acceptor (e.g. D2, Red) fluophores. Time-resolved FRET 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. Ligand (antagonist)-based trFRET has very recently successfully been used to detect endogenous oxytoxin receptor oligomers in mammary gland. 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 (Fig. 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 the G protein.$^{50-52}$ The glycoprotein hormone receptors distinguish themselves from other class A GPCRs by having an extended NTED, which is exclusively involved in hormone binding.$^{67,68}$ 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.$^{69-71}$ 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 signaling-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.$^{72}$ The mode of action of this functional complementation remains somewhat puzzling, however, since some of these binding-deficient receptor mutants can also be trans-activated by NTEDs tethered to a glycosyl phosphatidylinositol moiety or CD8 transmembrane domain. This suggests that (dimeric) interactions between two 7TM domains are not required for this functional rescue.$^{69,70}$ 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.$^{73}$ Consequently, ligand binding and receptor activation domains of these GPCRs cannot be easily separated. However, co-expression of two binding-deficient histamine H$_1$ receptors (H$_1$R) with a single mutation in TM3 or TM6 (i.e. H$_1$R-D$_{3.32}$A and H$_1$R-F$_{6.52}$A, respectively) restored ligand binding, revealing a physical interaction between the two receptor mutant.$^{74}$
Figure 2. Detection of 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) RET between Donor and Acceptor molecules that are fused to GPCR, occurs when they are brought in close proximity (<10 nm) by interacting GPCR. (C) PFC 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.

Since only TM1-5 of H1R-F6.52A and TM6-7 of H1R-D3.32A 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 $M_3$ muscarinic receptor/$\alpha_{2C}$-adrenoceptor chimeras in which TM6-7 domains were reciprocally exchanged (Fig. 2D). 

Interestingly, co-expression of binding-deficient angiotensin II type 1 receptor (AT$_1$R) constructs with a single mutation in TM3 or TM5 also restored binding of angiotensin II and related analogs.

**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 spatio-temporal expression profile in native cells, might be required for proper folding and export of these from the endoplasmic reticulum (ER) to the cell surface (Fig. 3A).

Indeed, the coiled-coil interaction between the C-tails of GABA$_{B1}$ and GABA$_{B2}$ is required for cell surface targeting of the heteromeric GABA$_B$ receptor, confirming that GPCR homo- and heteromers are formed during early biosynthesis and protein maturation in the ER. Heteromerization of the $\beta_2$- or $\alpha_{1B}$-adrenoceptor with the $\alpha_{1D}$-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 expression of the $\alpha_{1D}$-adrenoceptor.

On the contrary, various naturally occurring GPCR splice variants and mutants have been reported to trap co-expressed wildtype counterparts in the ER by forming heteromeric assemblies (Fig. 3A). For example, natural occurring rat histamine H$_3$ receptor (H$_3$R) splice variants that lack TM7 impair cell surface targeting of wildtype H$_3$R. Interestingly, the expression levels of these truncated isoforms and wildtype H$_3$R in rat brain are oppositely modulated by the convulsant pentyleneetetrazole, resulting in increased H$_3$R activity, whereas high-fat diet induced down-regulation of the dominant negative GIP receptor splice variant resulting in an up-regulation of wildtype GIP receptors in obese mice. 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 GABA$_{B1}$ resulted in an ER-
trapped receptor mutant, which also prevented cell surface targeting of wildtype β2-adrenoceptor. Likewise, fusion of the ER-retention motif of the α2C-adrenoceptor to the C-tail of the CXC-chemokine receptor 1 (CXR1) impaired its trafficking to the cell surface. Moreover, this ER-retained CXR1 mutant inhibited cell surface trafficking of wildtype CXR1 and the closely related CXR2, by forming homomers and heteromers, respectively. In contrast, cell surface delivery of co-expressed α1A-adrenoceptor was not affected, which correlated with the observation that CXR1 and α1A-adrenoceptor do not form heteromers. The importance of a correct quaternary structure for cell surface delivery was furthermore demonstrated by a α1B-adrenoceptor mutant in which hydrophobic residues in TM1 and TM4 were Ala-substituted. This TM1-TM4 mutant was trapped in the ER and displayed an altered oligomeric organization in comparison to wildtype receptors as indicated by sequential three-colour FRET analysis. Interestingly, the cell-permeant α1B-adrenoceptor-antagonist prazosin changed the quaternary structure of TM1-TM4 mutants to an oligomeric organization that resembles wild type α1B-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. Several other examples of pharmacological chaperones that restore cell surface delivery of disease-linked receptor mutants have been reported. However, whether this pharmacological rescue involves changes in quaternary receptor organization remain to be investigated.

Collectively, most data suggest that receptor oligomers are pre-assembled 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 GABA receptor is stabilized by a coiled-coil configuration of the C-tails and direct interactions of the NTEDs. For long, the stability of class A GPCR oligomers has been an enigma, although RET data suggest that most GPCRs remain organized as oligomers (Fig. 3B). In addition, mutational analysis revealed that heteromers between the dopamine D2, adenosine
A2A, and the cannabinoid CB1 receptors are stabilized by electrostatic interactions between Arg-rich motifs in IL3 of D2 and A2A receptors and phosphorylated casein kinase 1/2 sites in IL3 and C-tail of the CB1 receptor, and the C-terminus of the A2A receptor. 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-HT2A and D1 receptor. 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. To this end, different GFP variants were fused to the N- or C-terminus of the GPCRs. Immobilization of one YFP-β2-adrenoceptor almost completely impaired lateral diffusion of at least 4 co-expressed β2-adrenoceptor-CFP fusion proteins into the bleached region of the cell membrane, suggesting that receptors form stable higher order homomers. In contrast, the mobility of α1-adrenoceptor and D2 receptor was only modestly affected by the antibody immobilization of their homomeric counterparts, suggesting that these receptors form rather transient homomers (Fig. 3C). Intensity imaging of M1 muscarinic receptors that were labeled with a slowly dissociating fluorescent antagonist using total internal reflection fluorescence microscopy (TIRFM) indicate 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-color labeled receptors. Approximately 30 percent of the M1 receptors was engaged in homodimers at any given moment, whereas higher order oligomers were never detected for this receptor. Interestingly, the apparent transient nature of β1-adrenoceptor, D2 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. In addition, BRET data indicated that β1- and β2- adrenoceptors have equal propensities to form homo- and heterodimers, whereas D2 receptor homodimerization was confirmed in crosslinking and
functional complementation experiments.\textsuperscript{62,103} In fact, higher order D\textsubscript{2} receptor oligomers have been detected using PFC in combination with BRET analysis, however it should be kept in mind that the transient nature of D\textsubscript{2} receptor interactions might be obscured the permanent reconstitution of the sensor proteins from their split fragments. Higher order homo- and heteromers (i.e. tri- and tetrameric assemblies) have also been detected for other class A GPCRs using this PFC-BRET analysis\textsuperscript{14,62,104,105}, but also using 3-color FRET\textsuperscript{59,60}, and sequential RET studies.\textsuperscript{95,106} In addition, GABA\textsubscript{B} dimers (i.e. homodimers of heterodimers) have been detected by trFRET-SNAP\textsuperscript{65}.

Hitherto, regulation of GPCR oligomerization dynamics at the cell surface upon ligand binding remains still controversial (Fig. 3B,C). For example, the agonist isoproterenol and antagonist propranolol did not affect the stability or amount of β\textsubscript{1}- and β\textsubscript{2}-adrenoceptor homomers in FRAP experiments.\textsuperscript{98}

\textbf{Figure 3.} GPCRs are walking hand-in-hand. (A) GPCR oligomerization in the ER is required for cell surface delivery, however oligomerization may also retain GPCRs in the ER. (B-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.
Similarly, isoproterenol had little effect on FRET efficiency between purified and reconstituted $\beta_2$-adrenoceptor homomers, while the inverse agonist ICI 118,551 significantly increased the FRET efficiency.\(^{107}\) On the other hand, isoproterenol dose-dependently increased BRET efficiency between $\beta_2$-adrenergic receptors in living cells.\(^{108}\) Since RET efficiency is determined by both distance and orientation of donor and acceptor dipole moments, ligand-induced changes in RET intensity do not necessarily reflect de novo formation or dissociation of GPCR oligomers, but may also represent conformational changes in existing oligomers. In addition, Western blot analyses revealed that agonist or inverse agonist treatment of $\beta_2$-adrenoceptor-expressing membranes shifted the equilibrium towards the homomeric or monomeric state, respectively.\(^{109}\) Similar inconsistencies have been reported for other GPCRs, and are most presumably related to methodological limitations. Interestingly, agonists increased trFRET between SNAP-tagged M3 receptors but decreased FRET between M3 receptors that were C-terminally fused to GFP variants.\(^ {64}\) Since encaged lanthanides have no donor dipole constraint, the trFRET efficiency between covalently bound SNAP-tag fluorophores is minimally effected by orientation and the observed increase in trFRET may in fact reflect an agonist-induced increase in M3 receptor oligomerization.\(^{110}\)

If GPCRs exist as (relatively) stable oligomers on the cell surface or become so upon agonist stimulation, one might expect that they keep walking hand-in-hand during internalization (Fig. 3D). Engineering a “homodimer” between the wildtype and a RASSL (i.e. Receptor Activated Solely by Synthetic Ligands) $\beta_2$-adrenoceptor, it was demonstrated that agonist binding to one of the protomers induces internalization of these “homodimers”.\(^{111}\) Likewise, D1 and D2 receptor heteromers internalize upon selective activation of one of the two protomers.\(^ {112}\) Interestingly, the fate of the internalized vasopressin V1A and V2 receptor heteromers was found to be dependent on which protomer is being activated. Stimulation with V1A receptor-selective agonist confers a V1A receptor-like endocytosis/recycling profile to the V1A-V2 receptor heteromer, whereas stimulation with a nonselective agonist
results in \( V_2 \) receptor-like intracellular accumulation of the \( V_1A-V_2 \) receptor heteromer.\(^{113}\)

Taken together, oligomerization seems to be required for cell surface expression of most GPCRs. The fate of GPCR oligomers at the cell surface is GPCR subtype dependent, with some GPCRs forming short-lived, transient oligomers, whereas others are organized as long-lived, stable (higher-order) oligomers. GPCR oligomers internalize upon agonist stimulation of one of the protomer.

**Do GPCRs talk hand-in-hand?** Clear evidence that GPCRs “talk hand-in-hand” comes from the aforementioned obligatory heterodimer GABA\(_{\beta_1}\), in which one protomer (i.e. GABA\(_{\beta_1}\)) is exclusively responsible for protein binding, whereas only the other protomer (i.e. GABA\(_{\beta_2}\)) can activate heterotrimeric 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 \( \beta \)-arrestins in response to agonist stimulation (see below), functional asymmetry is often apparent once they are engaged in homo- 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 \( \alpha_{2A} \)-adrenoceptor/\( \mu \)-opioid receptor (\( \mu \)OR) heterodimer.\(^ {114}\) Binding of morphine to the \( \mu \)OR triggers a conformational change in the associated norepinephrine-occupied \( \alpha_{2A} \)-adrenoceptor, as detected by a decrease in the norepinephrine-induced FRET efficiency between two fluorophores in IL3 and C-tail of \( \alpha_{2A} \)-adrenoceptor, which is translated within milliseconds in reduced G protein activation by the \( \alpha_{2A} \)-adrenoceptor protomer (Fig. 4A). Well-designed molecular engineering also revealed functional allosterism in dopamine \( D_2 \) receptor homodimers.\(^ {103}\) To this end, the \( D_2 \) receptor was fused without a linker to the chimeric G protein Ga\(_{q5}\). This fusion protein (i.e. \( D_2 \)-Ga\(_{q5}\)) was nonfunctional when expressed on its own, however, co-expression of wildtype \( D_2 \) receptor resulted in agonist-induced coupling of the latter to the Ga\(_{q5}\) protein of the nonfunctional \( D_2 \)-
Ga\textsubscript{q5} by forming dimers. In contrast, binding- or coupling-deficient D\textsubscript{2} receptor mutants were unable to signal through the fused Ga\textsubscript{q5} when co-expressed with D\textsubscript{2}-Ga\textsubscript{q5}. Moreover, also the capacity of the nonfunctional D\textsubscript{2}-Ga\textsubscript{q5} to interact with G proteins appeared to be essential for dimer-induced signaling. Interestingly, this D\textsubscript{2} 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 dimer signaling, respectively. Importantly, since an “artificial” Ga\textsubscript{q5}-mediated response is measured, observed dimer signaling must result from physical interactions between the protomers and G protein rather than from downstream crosstalk in signaling pathways. Indeed, differential crosslinking of D\textsubscript{2} dimers in inverse agonist versus agonist-bound state suggests that conformational changes at the dimer interface is part of the receptor activation mechanism.\textsuperscript{115}

Similar to the engineered D\textsubscript{2} receptor dimer with the single fused G protein, the leukotriene B\textsubscript{4} receptor BLT\textsubscript{1} homodimer only couples to one heterotrimeric G protein at a time.\textsuperscript{116} Agonist-induced activation of one of the BLT\textsubscript{1} protomers is sufficient to promote G protein coupling and activation.\textsuperscript{117} 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\textsubscript{1} homodimer by restricting conformational changes in the second protomer.

Intermolecular crosstalk within receptor oligomers can result in allostery between the orthosteric binding pockets of the individual protomers. Negative binding co-operativity has been observed for both GPCR homo- and heteromers using equilibrium binding and/or radioligand dissociation experiments.\textsuperscript{19} 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 (Fig. 4B). Detection of negative binding co-operativity 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.\textsuperscript{118} 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.\textsuperscript{118,119} This may be easily misinterpreted as being negative binding co-operativity between two interacting protomers. However, co-expression of additional G protein may shed light in this matter by preventing depletion.\textsuperscript{14} 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 co-operativity between the two binding sites. For instance, negative co-operative has been detected within CCR2, CCR5, and CXCR4 heteromeric complexes in both recombinant cells and native immune cells.\textsuperscript{105,120-122} 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 co-operativity 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 signaling 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 co-operativity between these receptors. Interestingly, D\textsubscript{2} receptor homodimers and vasopressin V\textsubscript{1}A/oxytocin (OT) receptor heterodimers were readily detected by trFRET upon binding of fluophore-
conjugated antagonists to each of the protomers, whereas incubation of similar samples with an excess of fluorescent agonist resulted in very weak FRET signals. \(^{55}\) Similar discrepancy in co-operativity between agonists and antagonists was observed in radioligand binding experiments on membranes that express V\(_2\)A, oxytocin, or D\(_2\) receptors. \(^{123,124}\) Hence, the apparent absence of binding co-operativity between antagonists on V\(_2\)A, OT, or D\(_2\) receptor homo- and/or heteromers is different from the observations in chemokine receptor heteromers. \(^{105}\) Interestingly, opposite binding co-operativity was observed within 5-HT\(_{2A}\)/mGlu\(_2\) receptor heteromers in mouse somatosensory cortex membranes. \(^{125}\) The mGluR\(_2\) agonist LY379268 increases the affinity of hallucinogenic agonists such as 1-(2,5)-dimethoxy-4-indophenyl)-2-aminopropane (DOI) for the 5-HT\(_{2A}\) receptor, whereas DOI decreases the affinity of LY379268 for the mGluR\(_2\). On the contrary, however, the sensitized G\(_{i/o}\) signaling of 5-HT\(_{2A}\)/mGlu\(_2\) receptor heteromers in response to hallucinogenic 5-HT\(_{2A}\) receptor agonists was reversed upon activation of mGluR\(_2\) 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 heteromers with the G\(_{i/o}\)-coupled melatonin receptors MT\(_1\) and MT\(_2\). \(^{126}\) GPR50 inhibited melatonin binding to the associated MT\(_1\) but not MT\(_2\) protomer, suggesting that downstream G protein stealing per se was not the underlying mode of action. Inhibition of MT\(_1\) protomer function appeared to be attributed to the long C-tail of GPR50, which apparently interact differently with the MT\(_1\) as compared to MT\(_2\) protomer, thereby hindering G protein coupling to the MT\(_1\) protomer. \(^{126}\)

Stimulation of the δOR/Mas-related GPCR member X (MRGPRX1, a.k.a. SNSR-4) heteromer with selective δOR or MRGPRX1 agonists triggered G\(_{i/o}\) or G\(_q\) signaling, respectively. However, simultaneous binding of selective δOR and MRGPRX1 agonists to the δOR/ MRGPRX1 heteromer led to exclusively to G\(_q\) activation,
suggesting a dominant negative effect of the activated MRGPRX1 protomer on δOR-specific signaling.\textsuperscript{127}

Figure 4. 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 FRET. (B) Negative binding co-operativity between ligand on a GPCR heteromer. (C) Positive binding co-operativity between ligands on a GPCR heteromer. (D) Change in G protein coupling and downstream signaling upon heteromerization of the G\textsubscript{q} coupled D\textsubscript{1} and the G\textsubscript{i/o} coupled D\textsubscript{2} receptor, resulting in G\textsubscript{q}-mediated Ca\textsuperscript{2+} signaling.

On the other hand, agonist binding and activation of both receptor protomers is required for efficient signaling of some other homo- and heteromers. Although a single glutamate molecule is sufficient to promote mGlu\textsubscript{5} homodimer signaling, the binding of two glutamate molecules per homodimer is required for full activation.\textsuperscript{128} 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. However, positive binding co-operativity was observed when either δOR- and κOR-selective agonists or a combination of selective antagonists were incubated with a non-selective radiolabeled antagonist and δOR-κOR heteromer-expressing membranes, resulting in at least a 50-fold increase in affinity (Fig. 4C). Surprisingly, only a 10 to 20-fold potentiation in signaling 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 allostery within this heteromer seems puzzling. Interestingly, no positive co-operativity was observed between δOR-selective antagonist and κOR-selective agonist on δOR-κOR heteromers. In contrast, δOR-selective agonists enhance agonist binding and signaling to the μOR protomer within δOR-μOR heteromers. Activated δOR or μOR preferentially activate Gαi proteins as determined by 35S-GTPγS incorporation in selectively immunoprecipitated G proteins, whereas activated δOR-μOR heteromers interact selectively with Gαz proteins. 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. Activation of the Gαi/o-coupled dopamine D3 receptor increases the agonist affinity of Gs-coupled D1 receptors. This positive binding co-operativity within D1-D3 receptor heteromers results in increased Gs-mediated locomotor activity, which can be inhibited by D3 receptor antagonists. On the other hand, heteromerization of the D1 receptor with the Gαi/o-coupled histamine H3 receptor triggered Gt-dependent but Gt-independent MAPK signaling pathway activation in response to dopaminergic or histaminergic agonists, which could be (cross-)blocked by selective antagonists acting at either of the two protomers. This acquired capacity of histaminergic agonists to induce MAPK signaling through the H3R was strictly dependent on the presence of the D1 receptor. The D1 and D2 receptors activate of Gt and Gz proteins, respectively, resulting in an opposite regulation of cAMP production by adenylyl cyclase (Fig. 4D). However, D1-D2
receptor heteromers can couple to \( \text{G}_{q/11} \) proteins upon agonist binding to both protomers, resulting in intracellular calcium release from the ER and subsequent activation of calcium/calmodulin-dependent protein kinase IIα (CaMKIIα).\(^{135,136}\) Importantly, the \( \text{G}_{q/11} \) inhibitor YM254890 could fully inhibit D₁-D₂ receptor heteromer-induced intracellular \( \text{Ca}^{2+} \) mobilization, revealing that \( \text{G}_{q/11} \) coupling rather than downstream crosstalk initiates this signaling pathway. D₁-D₂ receptor heteromers have been detected in various brain regions and their capacity to activate CaMKIIα can be inhibited by pre-administration of D₁ or D₂ receptor antagonists, and is disrupted in D₁ or D₂ receptor knockout mice.\(^{136}\) D₁-D₂ receptor heteromer signaling has been linked to synaptic plasticity as well as behavioral 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.\(^{136}\) Hence, the intracellular surface of GPCR heteromers has likely a distinctive conformation as compared to their constituent mono- and/or homo-oligomers, which may result in the recognition of different signaling partners.

Agonist-induced crosslinking of AT₁R homodimers by intracellular factor XIIIa transglutaminase increased \( \text{G}_{q/11} \) activation and the formation of inositol 1,4,5-trisphosphates as compared to non-crosslinked AT₁R. Noteworthy, factor XIIIa activity and crosslinked AT₁R homodimers were increased in hypertensive patients, resulting in enhanced monocyte adhesion to vascular endothelial cells.\(^{137}\) On the other hand, monomers of purified rhodopsin, μOR, neurotensin receptor NTS1, β₂-adrenoceptor, and leukotriene 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.\(^{38,42,44-45,138}\) In line, higher order GABA₆ receptor oligomers (i.e. homomers of the obligate heterodimer GABA₆₁/GABA₆₂) had a lower efficacy to activate G proteins than non-associated GABA₆ receptors.\(^{65}\) 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 show 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 to 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 signaling pathways that may be interconnected in integrative networks or share limiting components. Consequently, it may be difficult to distinguish whether one receptor affects the signaling properties of an associated GPCR causally due to oligomerization or perhaps due to downstream crosstalk in signaling pathways.

GPCRs 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 CB₁ and µ-opioid receptors activate predominantly Gαi/o-coupled signaling pathways and are co-expressed in individual neurons in the striatum, caudate nucleus, and dorsal horn. However, the CB₁ receptor constitutively inhibits agonist-induced µOR signaling, which can be restored by co-incubation with a CB₁ receptor inverse agonist or silencing of the ligand-independent CB₁ receptor signaling by site-directed mutagenesis. Although BRET experiments suggested that CB₁ receptor and µOR exist as heteromers, microscopy studies revealed distinct subcellular localization patterns of both GPCR proteins. The latter implies that CB₁ receptor and µOR are not assembled as heteromers and cross-regulation of µOR signaling by the constitutive active CB₁ receptor is downstream, presumably via G protein scavenging.

The Epstein-Barr virus-encoded GPCR BILF1 forms heteromers with the human chemokine receptor CXCR4. 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 Ga1 proteins, and co-expression of additional Ga1 proteins with BILF1 and CXCR4 restored normal functioning of the latter. 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 Gq/11-coupled GPCRs by constitutive scavenging of a shared pool of available Gq/11 proteins.

In addition, GPCRs can impair each other 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. Although examples of Gq- and Gi-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 signaling of the Gq/11-coupled histamine H1 receptor is increased in cells co-expressing Gi/o-coupled serotonin 5-HT1B, adenosine A1, or M2 muscarinic receptors, in a pertussis toxin-sensitive manner. This H1R-mediated signaling can be further increased by stimulation with agonists of the co-expressed receptors. On the other hand, the 5-HT1B receptor inverse agonist inhibited the pertussis toxin-sensitive increase in signaling in H1R and 5-HT1B receptor co-expressing cells, whereas the H1R inverse agonist mepyramine inhibited all signaling. Importantly, GPCR-independent stimulation of Gi/o proteins by using mastoparan-7 resulted in a similar potentiation of H1R signaling indicating unambiguously that the observed crosstalk is on the level of intracellular signaling pathways rather than through receptor heteromerization. Similar downstream crosstalk mechanism was observed between the constitutively active Gq/11-coupled human cytomegalovirus-encoded receptor US28 and Gq/o-coupled CCR1 chemokine receptors, the constitutive active Gq/11-coupled mGlu1a and Gq/o-coupled GABAB receptor, and might also apply for the sensitization of Gq/11-coupled orexin-1 receptor by the constitutively active Gq/o-coupled CB1 receptor in a pertussis toxin-
Sensitive manner, which was suggested by the authors to be a direct consequence of orexin-1/CB₁ receptor heteromerization. Heteromerization between CB₁ and orexin-1 receptor was indeed confirmed in distinct cells and was accompanied with a change in cellular distribution of the orexin-1 receptor. However, in this study CB₁ had only marginal effect on agonist-induced orexin-1 receptor signaling, which was explained as a difference in cellular background.

Conclusions

Increasing evidence suggests 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 signaling. 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 signaling network constituents, which may actually affect GPCR properties like agonist binding. Showing that physical GPCR interactions are absolutely required for unique agonist-induced signaling, 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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