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Roles of G-protein-coupled receptor dimerization

From ontogeny to signalling regulation

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The classical idea that G-protein-coupled receptors (GPCRs) function as monomeric entities has been unsettled by the emerging concept of GPCR dimerization. Recent findings have indicated not only that many GPCRs exist as homodimers and heterodimers, but also that their oligomeric assembly could have important functional roles. Several studies have shown that dimerization occurs early after biosynthesis, suggesting that it has a primary role in receptor maturation. G-protein coupling, downstream signalling and regulatory processes such as internalization have also been shown to be influenced by the dimeric nature of the receptors. In addition to raising fundamental questions about GPCR function, the concept of dimerization could be important in the development and screening of drugs that act through this receptor class. In particular, the changes in ligand-binding and signalling properties that accompany heterodimerization could give rise to an unexpected pharmacological diversity that would need to be considered.

Keywords: G-protein-coupled receptor function; heterodimerization; homodimerization; oligomerization; regulation
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Introduction

G-protein-coupled receptors (GPCRs) have classically been assumed to exist and function as monomeric entities, and the paradigms of ligand binding and signal transduction were based on this hypothesis. However, a growing body of biochemical and biophysical evidence indicates that some GPCRs can form both homodimers and heterodimers. Although their existence is now largely accepted (Angers *et al*, 2002; George *et al*, 2002), their functional importance remains more enigmatic and in some cases even controversial. The five stages of the GPCR life cycle that could be affected by dimerization are depicted in Fig 1. In this review, we focus our attention on the potential functional implications that have been proposed for GPCR dimerization at each of these stages, indicating, when possible, the aspects that will require additional studies before definitive conclusions can be drawn. Given that most current methods used do not strictly distinguish between dimers and larger oligomers, we refer systematically to the term 'dimer' because it represents the minimal oligomeric arrangement.

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Ontogeny

GPCR biosynthesis and transport along the secretory pathway are poorly characterized, but their exit from the endoplasmic reticulum (ER) is a crucial step that controls their cell surface expression (Petaja-Repo *et al*, 2000). Only correctly folded receptors escape the ER quality-control system and are allowed to exit, whereas incompletely folded or misfolded proteins are retained and eventually degraded (Petaja-Repo *et al*, 2001). For many proteins, oligomeric assembly has an important function in ER quality control because it masks specific retention signals or hydrophobic patches that would otherwise retain the proteins in the ER (Reddy & Corley, 1998). For GPCRs, the necessity of dimerization for correct transport to the plasma membrane has been clearly shown for the metabotropic γ-aminobutyric acid b receptor (GbR), which is composed of two subunits GbR_1 and GbR_2 (Marshall *et al*, 1999). When expressed alone, GbR_1 is retained intracellularly as an immature protein because it has a carboxy-terminal ER retention motif (Margeta-Mitrovic *et al*, 2000), whereas GbR_2 reaches the cell surface but is not functional. Following their co-expression, heterodimerization masks the GbR_1 ER retention signal, allowing the proper targeting of a functional heterodimeric GbR to the plasma membrane. Although a general role for heterodimerization and/or homodimerization in GPCR quality control and ER export has not yet been established, studies using cellular fractionation and fluorescence or bioluminescence resonance energy transfer (FRET and BRET, respectively) have revealed that several GPCRs dimerize in the ER (Issafras *et al*, 2002; Overton & Blumer, 2002; Terrillon *et al*, 2003; Floyd *et al*, 2003). Consistent with the idea that GPCR dimerization occurs early in the secretory pathway is the observation that truncated mutants of vasopressin V_2R (Zhu & Wess, 1998), D_3 dopamine (Karpa *et al*, 2000), GnRH gonadotropin-releasing hormone (Grosse *et al*, 1997) and CCR_5 chemokine (Benkirane *et al*, 1997; Shioda *et al*, 2001) receptors, as well as rhodopsin mutants (Colley *et al*, 1995) behave as dominant-negatives of their respective wild-type receptors by preventing their expression on the cell surface. As the physical interaction between wild-type and mutant receptors was confirmed by co-immunoprecipitation in some of these studies (Benkirane *et al*, 1997; Zhu & Wess, 1998; Karpa *et al*, 2000), the dominant-negative action was taken as evidence that early heterodimerization between wild-type and mutant receptors leads to their ER retention. For

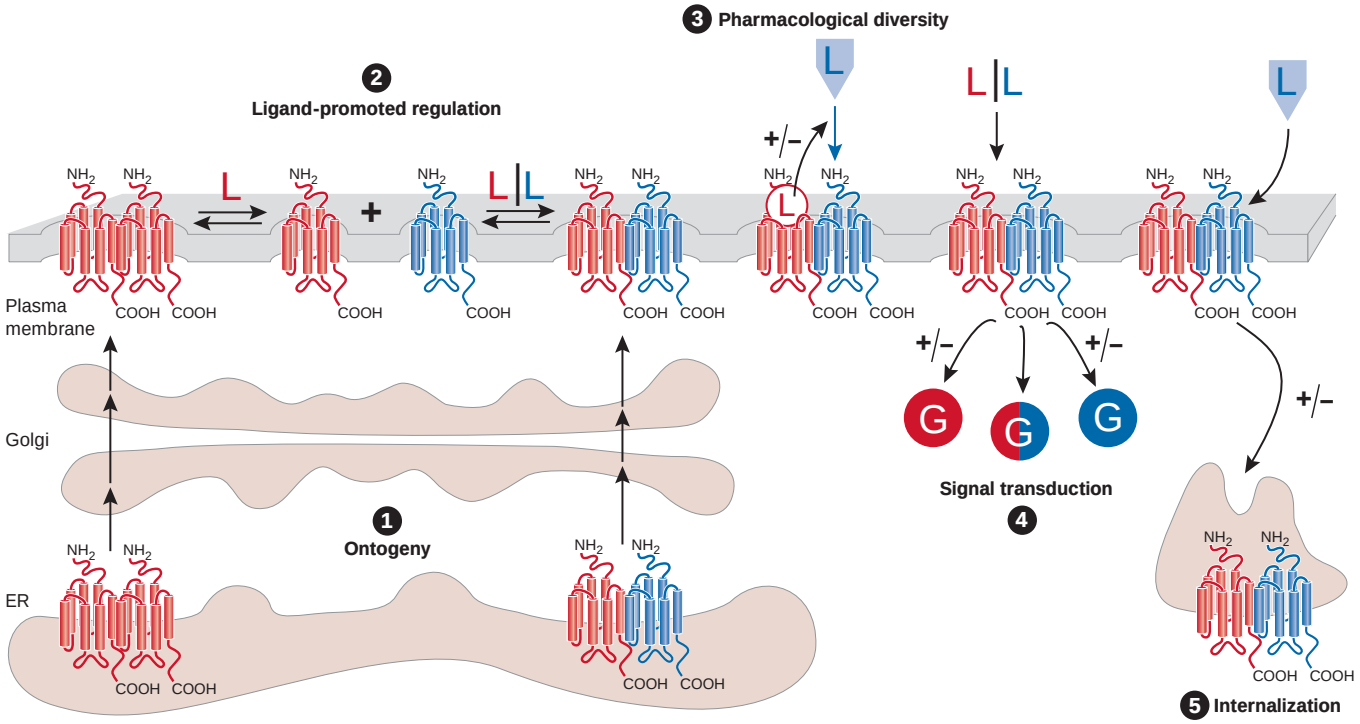

Fig 1 | Potential roles of G-protein-coupled receptor (GPCR) dimerization during the GPCR life cycle. (1) In some cases, dimerization has been shown to have a primary role in receptor maturation and allows the correct transport of GPCRs from the endoplasmic reticulum (ER) to the cell surface. (2) Once at the plasma membrane, dimers might become the target for dynamic regulation by ligand binding. (3) It has been proposed that GPCR heterodimerization leads to both positive (+) and negative (–) ligand binding cooperativity, as well as (4) potentiating (+)/attenuating (–) signalling or changing G-protein selectivity. (5) Heterodimerization can promote the co-internalization of two receptors after the stimulation of only one protomer. Alternatively, the presence of a protomer that is resistant to agonist-promoted endocytosis, within a heterodimer, can inhibit the internalization of the complex. G, G protein; L, ligand.

naturally occurring mutations this could have pathophysiological consequences. For example, it has been suggested that the loss of cell-surface expression of CCR_5 observed following its co-expression with the ER-retained $CCR_5\Delta32$ mutant contributes to the delayed onset of AIDS in HIV-infected patients that harbour a $CCR_5/CCR_5\Delta32$ genotype (Benkirane *et al*, 1997). A series of rhodopsin mutants was also proposed to cause retinal degeneration in *Drosophila* by interfering with the maturation of the wild-type photoreceptor (Colley *et al*, 1995).

Ligand-promoted regulation

A role for dimerization in GPCR ontogeny does not exclude the possibility that, once the receptor has reached the cell surface, its oligomeric state could be dynamically regulated. Whether receptor activation can promote or inhibit dimerization and/or favour exchanges between protomers is a central question with wide implications for the mechanisms of receptor activation and regulation. Unfortunately, no general consensus has yet been established. Whereas several studies suggest that ligand binding can regulate the dimer by either promoting (Rodriguez-Frade *et al*, 1999; Angers *et al*, 2000; Rocheville *et al*, 2000a,b; Cornea *et al*, 2001; Horvat *et al*, 2001; Kroeger *et al*, 2001; Wurch *et al*, 2001; Patel *et al*, 2002; Zhu *et al*, 2002; Hunzicker-Dunn *et al*, 2003; Roess & Smith, 2003) or inhibiting (Gines *et al*, 2000; Cheng & Miller, 2001; Latif *et al*, 2002) its formation, many others conclude that homodimerization and heterodimerization are constitutive processes that are not modulated by ligand binding (Overton & Blumer, 2000; Ayoub *et al*, 2002; Issafras *et al*, 2002; Jensen *et al*, 2002; Babcock *et al*, 2003; Canals *et al*, 2003; Dinger *et al*, 2003; Floyd *et al*, 2003; Guo *et al*, 2003; Stanasila *et al*, 2003; Terrillon *et al*, 2003; Trettel *et al*, 2003). Although such discrepancies might reflect intrinsic differences in the behaviour of the individual receptors considered in each study, they might also result from interpretational difficulties linked to the techniques used. In many cases, the extent of receptor co-immunoprecipitation, determined by western blot analysis, was used to assess the effects of ligands on the dimerization state. Unfortunately, such approaches are not highly quantitative, and changes in the immunoreactivity detected could result from agonist-induced conformational changes that alter epitope recognition rather than from varying amounts of receptor dimers. In other cases, ligand-induced increases or decreases in BRET or FRET were interpreted as ligand-regulated dimerization. However, given that the efficacy of BRET and FRET is highly dependent on the relative distance and orientation between the donor and acceptor fluorophores, ligand-promoted changes in energy transfer could reflect either a modulation of the dimerization process or changes in the conformation of pre-existing dimers. Additional experimental approaches will be required to determine unambiguously whether or not the dimerization process is subjected to ligand-promoted regulation.

In any case, the structural data available strongly suggest that at least some GPCRs can form dimers in the absence of ligand stimulation. For instance, crystallization of the extracellular amino-terminal ligand-binding domains of the metabotropic glutamate receptor, $mGluR_1$ (Kunishima *et al*, 2000), and the Wnt receptor, frizzled (Dann *et al*, 2001), revealed that they exist as dimers in the absence of their ligands. More recently, Palczewski and co-workers used

atomic force microscopy to show that rhodopsin and opsin form constitutive dimers in dark-adapted native retinal membranes (Fotiadis *et al*, 2003; Liang *et al*, 2003).

Pharmacological diversity

The concept that GPCR heterodimerization could have a role in pharmacological diversity was first indicated by studies on the δ- and κ-opioid receptors (Jordan & Devi, 1999). Co-expression of both receptors led to the formation of a stable heterodimer with a very low affinity for either the δ- or the κ-selective ligand alone. However, high affinity was restored following the combination of the two ligands, suggesting the occurrence of positive cooperativity. Although the direct link between heterodimerization itself and the changes in pharmacological properties has not been formally established, positive or negative ligand-binding cooperativity that occurs after receptor co-expression has been interpreted as resulting from receptor heterodimerization for many other GPCRs. These include the metabotropic GbR GbR_1/GbR_2 (Galvez *et al*, 2001), opioid δ/μ (Gomes *et al*, 2000), muscarinic m_2/m_3 (Maggio *et al*, 1999), somatostatin $SSTR_5$/dopamine D_2 (Rocheville *et al*, 2000a) and adenosine A_{2A}/dopamine D_1 (Franco *et al*, 2000) receptors. If this is a general phenomenon, such heterodimerization between pharmacologically distinct receptors could underlie a level of pharmacological diversity that would have far-reaching implications for drug development. In particular, it could provide new opportunities for the development of more selective compounds that would target specific heterodimers without affecting the individual protomers (George *et al*, 2002). Obviously, testing all possible combinations from existing GPCRs represents a daunting task that cannot be achieved with the current methods. So, establishing the rules that dictate the selectivity of interactions between receptors and determining their occurrence in native tissues will be essential before heterodimerization can be systematically incorporated into drug screening campaigns.

Signal transduction

The first convincing evidence that GPCR dimerization has a crucial function in signal transduction came from studies on the GbR. Even though GbR_1 harbours the binding site for γ-aminobutyric acid (GABA), its co-expression with GbR_2 was found to be essential for the formation of a receptor that can couple functionally to the G-protein signalling cascade (Margeta-Mitrovic *et al*, 2000; Galvez *et al*, 2001). This is not just because GbR_2 is required for the expression of GbR_1 on the cell surface (see above), as a mutant form of GbR_1 that lacks its ER retention signal and can reach the cell surface on its own still requires GbR_2 for functional activity (Margeta-Mitrovic *et al*, 2000). Using a combination of chimeric receptor constructs of GbR_1 and GbR_2, Pin and colleagues proposed a transactivation model in which GbR_1 binds GABA while GbR_2 activates the G protein (Galvez *et al*, 2001). More recently, it has also been proposed that heterodimerization is obligatory for the formation of functional taste receptors. Indeed, sweet (Nelson *et al*, 2001) and L-amino-acid (Nelson *et al*, 2002) taste responses were strictly dependent on the co-expression of T_1R_3 and either T_1R_2 or T_1R_1, respectively. Even if these three cases are the only ones for which obligatory heterodimerization has been firmly established, such oligomerization has often been invoked to explain the changes in signalling properties that result from the co-expression of distinct receptors. Signalling potentiation resulting from heterodimerization has been suggested for the opioid δ/κ (Jordan & Devi, 1999), opioid δ/μ (Gomes *et al*, 2000), chemokine CCR_5/CCR_2 (Mellado *et al*, 2001), somatostatin $SSTR_5$/dopamine D_2 (Rocheville *et al*, 2000a), angiotensin AT_1/bradykinin B_2 (AbdAlla *et al*, 2000) and metabotropic glutamate 1α/adenosine A_1 (Ciruela *et al*, 2001) receptors, whereas signal attenuation has been described for the somatostatin SST_{2a}/SST_3 (Pfeiffer *et al*, 2001), adenosine A_1/dopamine D_1 (Gines *et al*, 2000), angiotensin AT_1/bradykinin B_2 (AbdAlla *et al*, 2000) and yeast α-mating factor wild-type/mutant (Overton & Blumer, 2000) receptors. Heterodimerization has also been proposed to promote changes in the selectivity of some GPCRs towards the different G-protein subfamilies (G_s, G_i, G_q and G_{12}). In particular, a loss of G_i coupling has been reported following co-expression of μ- and δ-opioid receptors (George *et al*, 2000; Charles *et al*, 2003) and in cells that co-express CCR_5 and CCR_2 chemokine receptors (Mellado *et al*, 2001). Although the formation of heterodimers was confirmed in all the above studies by either co-immunoprecipitation or resonance-energy-transfer approaches, cross-talk regulation involving downstream components of the individual signalling pathways activated by each receptor cannot be formally excluded. So the contribution of heterodimerization to the observed phenotypes cannot be proved unambiguously.

The classical model of G-protein activation is based on the premise that one receptor interacts with one heterotrimeric G protein at a time. However, this concept should be re-examined in the context of GPCR dimerization. Does one dimer activate one or two G proteins? Structural studies of the receptor–G-protein interface have led to the identification of several points of contact between the G protein and the receptor on both α- and βγ-subunits (Hamm, 2001). However, the crystallographic structure of rhodopsin revealed that its cytoplasmic surface is too small to accommodate these points of contact simultaneously. This led to the proposition that two receptor molecules might be necessary to satisfy the binding requirements of a single G protein (Hamm, 2001; Liang *et al*, 2003). An elegant reconstitution study recently supported this idea. Chemical cross-linking followed by size-exclusion chromatography, mass spectroscopy and neutron scattering measurements demonstrated clearly that the complex formed between the purified, activated leukotriene B_4 receptor BLT_1 and $G\alpha_{i2}\beta_1\gamma_2$ corresponds to a pentameric assembly of one heterotrimeric G protein and one dimeric receptor (Baneres & Parello, 2003). It remains to be determined whether this arrangement of one GPCR dimer interacting with a single heterotrimeric G protein is the functional complex in cells.

Internalization

Several recent studies have suggested that heterodimerization could affect agonist-promoted GPCR endocytosis, a well-characterized process classically involved in signal attenuation. For many documented heterodimers, stimulation of only one of the protomers was sufficient to promote co-internalization of the two receptors. These include: $SSTR_1/SSTR_5$ somatostatin (Rocheville *et al*, 2000b), δ-opioid/β_2 adrenergic (β_2AR; Jordan *et al*, 2001), α_{2A}/β_1 adrenergic (Xu *et al*, 2003), α_{1A}/α_{1b} adrenergic (Stanasila *et al*, 2003), $SSTR_{2A}$ somatostatin/μ opioid (Pfeiffer *et al*, 2002) and A_{2A} adenosine/D_2 dopamine (Hillion *et al*, 2002) receptors. In the last two cases, the co-internalization was also associated with a cross-desensitization of the signalling activities. By contrast, receptors that do not undergo efficient agonist-promoted endocytosis were found to act as dominant-negatives for endocytosis-prone receptors after heterodimerizaton. For example, the κ-opioid receptor inhibited the endocytosis of both δ-opioid receptors (Jordan & Devi, 1999) and the β_2AR (Jordan *et al*, 2001), whereas the β_1AR

prevented the agonist-promoted internalization of the β_2AR (Lavoie *et al*, 2002). In some of these studies, a non-specific effect on the endocytosis of all GPCRs was ruled out by showing that the internalization of non-related GPCRs was not affected (Stanasila *et al*, 2003). Although of significant potential interest, the physiological consequences of these observations on the regulation of GPCR desensitization/resensitization cycles remain to be determined.

Physiological relevance

Until now, heterologous expression systems have been the preferred models to study the functional consequences of GPCR dimerization, which raises the question of physiological relevance. For instance, the artificial co-expression of receptors that are never expressed together in the same cells *in vivo* could lead to erroneous conclusions. In addition, although great care has been taken in many studies to maintain expression levels within physiological ranges, the high expression levels that are sometimes achieved with these systems could lead to spurious interactions. This possibility has been elegantly illustrated in a recent study, which reported that δ-opioid receptors and β_2AR could form heterodimers but only when expressed at high levels (Ramsay *et al*, 2002). Nevertheless, the potential physiological importance of heterodimerization is supported by studies in cells that endogenously co-express the GPCRs under consideration. For example, the synergistic binding or signalling documented in heterologous expression systems between the δ- and μ-opioid (Gomes *et al*, 2000), the metabotropic glutamate$_{1\alpha}$ and adenosine A_1 (Ciruela *et al*, 2001) as well as the angiotensin AT_1 and the bradykinin B_2 (AbdAlla *et al*, 2000) receptors were also observed in neuroblastoma cells, cortical neurons and smooth muscle cells, respectively. More recently, the blockade of either the AT_1 receptor or the β_2AR with selective antagonists was found to inhibit the signalling of both receptors simultaneously in freshly isolated mouse cardiomyocytes (Barki-Harrington *et al*, 2003), a phenomenon linked to the ability of the two receptors to heterodimerize.

Conclusion

Although first met with healthy scepticism, the concept that GPCRs can exist as dimeric entities is now largely accepted. As summarized here, mounting evidence now suggests that the homodimerization and heterodimerization of GPCRs could be important in aspects of their biology that range from ontogeny to the regulation of their pharmacological and signalling properties. However, many more studies will be required for the following: (1) to establish the general physiological importance of dimerization in native systems, (2) to identify the dimerization interface and elucidate the three-dimensional organization of the dimers, (3) to find out the rules that dictate the selectivity of interactions between receptors, (4) to determine the stoichiometry of interaction between receptors and their partner proteins, and (5) to assess whether higher-order oligomeric complexes that include more than two receptors exist. Gathering this information will be a challenging task that will require the further development of innovative approaches permitting both the study of protein–protein interactions in living cells and the determination of the three-dimensional structures of transmembrane proteins.

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