Published in final edited form as:

Trends Pharmacol Sci. 2018 July ; 39(7): 672–684. doi:10.1016/j.tips.2018.04.002.

GPCRs and signal transducers: interaction stoichiometry

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Abstract

Until the late 1990s class A G protein-coupled receptors (GPCRs) were believed to function as monomers. Then indirect evidence that they might internalize or even signal as dimers has emerged, along with proof that class C GPCRs are obligatory dimers. Crystal structures of GPCRs and their much larger binding partners were consistent with the idea that two receptors might engage a single G protein, GRK, or arrestin. However, recent biophysical, biochemical, and structural evidence invariably suggested that a single GPCR binds G proteins, GRKs, and arrestins. Here we review existing evidence of the stoichiometry of GPCR interactions with signal transducers and discuss potential biological roles of class A GPCR oligomers, including proposed homo- and heterodimers.

Keywords

GPCRs; oligomerization; G proteins; GRKs; arrestins

A Bit of History

Ever since single photon sensitivity of mammalian rod photoreceptors was demonstrated [1], the visual field was convinced that the activation of just one rhodopsin molecule was necessary and sufficient for signaling. The similarity to rhodopsin in sequence and membrane topology of the first hormone receptor cloned, the β 2-adrenergic receptor (β 2AR) [2], revealed the existence of a family of rhodopsin-like receptors, which was termed G protein-coupled receptors (**GPCRs**, see Glossary). This homology suggested functional similarity, so the field believed that all class A (rhodopsin-like) GPCRs signal as monomers (reviewed in [3]). However, indirect evidence that the process of internalization [4] and signaling [5] of some class A receptors can be explained by their dimerization accumulated. Unraveling the mechanisms of function of class C GPCRs, which are obligatory homo- or heterodimers (recently reviewed in [6]), inspired many to believe that class A receptors can also function as dimers (see [7,8] for review). The first GPCR crystal structure, that of rhodopsin [9], revealed a relatively small cytoplasmic tip with a diameter of no more than 44Å (Figure 1A). The structure of another prototypical GPCR, β 2AR [10], appeared

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remarkably similar (Figure 1C). The cytoplasmic surfaces of activated rhodopsin [11] (Figure 1B) and β 2AR [12] (Figure 1D) were also relatively small. In contrast, the structures of potential signal transducers, such as **heterotrimeric G proteins** [13,14] (Figure 1E), **GRKs** [15–19] (Figure 1F), and **arrestins** [20–23] (Figure 1G), showed a much greater potential footprint of these proteins (90–92Å), which specifically bind active GPCRs. Thus, it was fairly easy to structurally fit a G protein [8] or an arrestin [24] to a GPCR dimer. The debate regarding what constitutes the functional unit of class A GPCRs continues unabated to this day [25–28].

GPCR Superfamily

G protein-coupled receptors are the most numerous signaling proteins in animals, with 800–900 subtypes in primates (including humans) and bats, and a significantly greater variety in most mammalian species (<http://sevens.cbrc.jp/>). GPCRs are membrane proteins responding to a wide variety of stimuli: hormones, neurotransmitters, peptides, proteins, small molecule odorants, pheromones, light, extracellular calcium, protease activity, etc [29]. A large number of GPCRs are odorant/pheromone receptors, but every mammalian species expresses ~400 non-odorant GPCRs. Structurally, the only common part in all these receptors is an arrangement of seven trans-membrane helices (Figure 1A–D) (often termed the heptahelical domain, or HD), whereas the extracellular N-terminus and loops, as well as intracellular loops and the C-terminus widely vary in size and function, so that GPCRs range from fewer than 350 to more than 5,900 residues [30]. It appears that in all cases receptor activation requires the movement of the trans-membrane helices relative to each other, leading to the emergence of the cavity between them on the cytoplasmic side of the receptor [31,12].

The majority of the GPCRs belong to the three classes. Class A, or rhodopsin-like receptors, is by far the largest (more than 700 in humans) [32]. These GPCRs bind orthosteric ligands (those that bind to the same site as the native ligands)(or light-sensitive 11-cis-retinal in case of photopigments) in the HD between the helices closer to the extracellular surface. The binding of activating ligands (agonists) or light-induced isomerization of retinal directly causes the shift of the α -helices, which is the hallmark of receptor activation. Humans have 15 class B (secretin receptor-like) GPCRs [32]. These receptors have a large extracellular N-terminal domain, which contains the high-affinity binding site for their peptide ligands, with the lower affinity site localized between the HD helices, pretty much where the orthosteric ligands of class A receptors bind [30]. We have 15 class C GPCRs, which are obligatory dimers (reviewed in [6]). These receptors also have a large N-terminal domain, but it is different from class B: all class C GPCRs have a bilobal Venus flytrap domain (VFT), homologous to the periplasmic bacterial proteins that bind amino acids and ions, and most have a cysteine-rich domain between the VFT and HD. Class C dimers in most cases are stabilized by covalent sulfhydryl bonds, although separated VFTs form dimers even without S-S bonds, just like their bacterial ancestors [6]. Orthosteric ligands of class C receptors bind between the lobes of their VFT domains, inducing closed conformation of the VFT. Many of their allosteric modulators bind within the HD, pretty much where the orthosteric ligands of class A receptors bind [30]. In fact, when the HD of class C GPCR is expressed without its extracellular N-terminal elements, it can be activated by positive allosteric

modulators binding within the HD just like class A GPCR are activated by their orthosteric agonists [33,34]. Some class C receptors are homodimers (e.g., calcium-sensing receptor and metabotropic glutamate receptors (mGluRs)), whereas others are heterodimers consisting of two different protomers (GABA_B receptors [35,36], sweet and umami taste receptors [37]; mGluRs can also apparently form heterodimers within subfamilies [38,39]). Interestingly, it has been established that in the GABA_B receptor, the best studied heterodimer, only one VFT binds the ligand, whereas the HD of the other protomer couples to a G protein [35,36], suggesting that allosteric interactions between VFT and HD domains are necessary for receptor activation [6]. Inter-domain allosteric interactions and functional asymmetry, where only one protomer couples to the G protein, appear to apply to all class C receptors, although there are nuances. In homodimeric mGluRs, agonist binding to one VFT activates the receptor, but its binding to both VFTs further enhances the activity [6]. To the best of our knowledge, dimeric class C receptors can engage only one G protein molecule at a time.

Class A GPCRs in the Plasma Membrane

When the monomer-dimer equilibrium of class A GPCRs was studied in near-native conditions of the cellular plasma membrane, both states were shown to be transient [40]. The dimers of M1 muscarinic [40] and N-formylpeptide receptor [41] had very short lifetimes, of 0.5 s and 91 ms, respectively. However, the lifetime of the N-formylpeptide receptor monomer was also short, ~150 ms. Thus, at near-physiological expression level of ~6,000 receptors of this subtype per cell, at any given moment ~2,500 receptors existed as dimers and ~3,500 receptors as monomers [41]. Two recent studies addressed the dimerization of D2 dopamine receptors [42,43]. One found the half-life of a dimer being ~0.5 s at 24°C, and showed that the agonist increases the fraction of the D2 receptors present as dimers [42]. The other measured a much shorter dimer half-life, ~68 ms, at the physiological temperature of 37 °C [43]. The addition of an antagonist UH-232 did not change it, whereas the addition of agonists, dopamine or quinpirole, significantly increased the half-life of D2 receptor dimers to ~99 and ~104 ms, respectively [43]. Thus, the dimers of class A GPCRs do exist, even though they are fairly transient. At near-physiological receptor expression levels, GPCR monomers and dimers co-exist in equilibrium. These data suggest that dimerization of class A GPCRs has functional significance.

One group reported specific orthosteric ligand binding properties of the M2 muscarinic receptor in liposomes, where it can form oligomers, that were not reproduced in high density lipoprotein particles (nanodiscs) containing monomeric M2 receptor [44]. Similarly, a unique interplay between the binding of orthosteric and allosteric ligands was found in the M2 receptors in liposomes, that was absent in the case of nanodisc-reconstituted monomers [45]. Interpretation of ligand binding studies is far from straightforward, and is sometimes complicated by the fact that orthosteric ligands also bind to the allosteric site, as was shown by careful studies of the M2 muscarinic receptors [46]. In general, the ligand binding data essentially confirm that class A GPCR oligomers exist, as shown by single-molecule studies [40,43,41,42], but does not shed light on their functional role. Below we review potential functional roles of GPCR homodimers in signal transduction, focusing on biochemical and

structural evidence that has emerged in the last 10 years, and then discuss the data used to suggest class A GPCR heterodimerization.

G proteins

The first question asked experimentally regarding GPCR-G protein interaction was whether a single monomeric GPCR could activate a heterotrimeric G protein. Both rhodopsin [47,48] and β 2AR [49] reconstituted as monomers into the lipid bilayer in nanodiscs were shown to effectively couple to their cognate G proteins, transducin and Gs, respectively. Monomeric receptors facilitated G protein activation catalyzing the exchange of GDP bound to the inactive G protein for GTP. In the case of β 2AR, high agonist affinity receptor-G protein complex was shown to be formed [49], just like in cell membranes or liposomes [50], where the receptor could have potentially oligomerized. Moreover, this complex demonstrated the same high sensitivity to GTP analog as a similar high agonist affinity complex formed by the β 2AR in native membranes [49]. Interestingly, rhodopsin reconstituted at two molecules per nanodisc was only half as active per rhodopsin molecule, suggesting that only one of the two was activating G proteins [47], and is in agreement with a previous report that dimerization reduces G protein coupling of another GPCR, the neurotensin NTS1 receptor [51]. The crystal structure of the β 2AR complex with Gs revealed a single receptor coupled to the heterotrimeric G protein, with the interaction largely mediated by the $G\alpha$ subunit [52] (Figure 2A). The structure of another class A receptor, the adenosine A2A, with an engineered single-subunit G protein revealed the same 1:1 complex [53], although in this case one could argue that this artificial G protein was engineered to fit a single GPCR. Crystallization of GPCR complexes with interacting proteins remains tricky, so that other methods that yield structural information, such as cryo-electron microscopy (cryo-EM), are becoming popular. Recently cryo-EM structures with near-atomic resolution of two different class B receptors in complex with G protein also revealed the same 1:1 stoichiometry [54,55]. In contrast, there were no follow-up structures after reports of the isolation of pentameric complexes containing two GPCR molecules and a single heterotrimeric G protein [56,57]. Of course, one can argue that in all available crystal and cryo-EM structures only one type of G protein, Gs, was used. Yet transducin that couples to rhodopsin belongs to the G_i subfamily. Generally speaking, protein structures in crystals, which are usually obtained under highly non-physiological conditions, could be misleading. However, when many structures show the same thing, and it fully agrees with existing biochemical evidence, it has to be taken seriously. Undoubtedly, the structure of full-length truly dimeric class C GPCR with coupled G protein would be very illuminating. Yet the evidence available today, biochemical as well as structural, suggests that a single GPCR is necessary and sufficient to activate its cognate G protein. If anything, clustering of two GPCRs appears to impede G protein coupling [47,51], suggesting that in a dimer likely only one protomer interacts with the G protein, like in dimeric class C GPCRs [6]. Interestingly, oligomerization of the naturally dimeric GABA_B receptor that belongs to class C also reduces its signaling via G proteins [58]. In fact, this phenomenon might reveal an underappreciated regulatory mechanism that needs to be further investigated.

GRKs

GRKs were shown to be directly activated by physical interaction with an active GPCR [59]. Incorporation of monomeric GPCRs into nanodiscs was used to test whether a monomer is sufficient to activate GRK and serve as its substrate at the same time. First, these experiments were performed with wild type (WT) native rhodopsin purified from bovine retinas. Monomeric light-activated rhodopsin was found to successfully activate GRK1 (historic name - rhodopsin kinase) and become phosphorylated by it to the extent slightly exceeding that of the rhodopsin in the native disc membrane, where it was free to form oligomers [60]. Later the same was found to be true for the constitutively active rhodopsin mutants, also purified and reconstituted into nanodiscs as monomers, which were effectively phosphorylated by purified GRK1 [61,62]. Non-visual GRKs 2 and 5 were shown to phosphorylate the monomeric agonist-activated NTS1 receptor [63], as well as the monomeric β 2AR [64], both purified and reconstituted into nanodiscs. Thus, biochemical experiments suggested that GPCR monomers effectively activate GRKs of all three subfamilies represented in vertebrates [65] and become phosphorylated by these GRKs. Structural studies later confirmed this 1:1 arrangement in case of β 2AR complex with GRK5 [19] (Figure 2B) and rhodopsin complex with both GRK1 and GRK5 [66]. It should be noted that these are not crystal structures, but a generalized view based on several lines of experimental evidence supported by modeling. Thus, all available evidence suggests that a single active class A GPCR binds a GRK molecule and is phosphorylated by it. In view of these data, earlier reports that upon light activation of a small fraction of rhodopsin GRK1 can phosphorylate multiple inactive rhodopsin molecules [67,68], as well as being co-expressed in the same membrane inactive cone opsin [69], suggests that the complex of activated GRK1 with active rhodopsin lives long enough to allow other molecules of visual pigment to diffuse into its vicinity and become phosphorylated. In fact, these findings are in excellent agreement with the observation that GRK1 bound to light-activated rhodopsin becomes active and can phosphorylate any available substrate, including exogenously added peptides [59].

Arrestins

The first attempt to gauge the stoichiometry of the arrestin-receptor interaction was made *in vivo*, using established translocation of visual arrestin-1 (please note that we use systematic names of arrestin proteins, where the number after the dash indicates the order of cloning: arrestin-1 [historic names S-antigen, 48 kDa protein, visual or rod arrestin], arrestin-2 [β -arrestin or β -arrestin1], arrestin-3 [β -arrestin2 or hTHY-ARRX], and arrestin-4 [cone or X-arrestin]) to the outer segment of rod photoreceptors in the light, where it remains due to rhodopsin binding [70]. WT mice express eight molecules of arrestin-1 per ten rhodopsins [71,72]. The arrestin-1 translocation in genetically modified mouse lines expressing arrestin-1 and rhodopsin at different ratios, from 0.4 to 2.4, was measured [73]. In bright light ensuring virtually 100% light activation of rhodopsin, up to 8 molecules of arrestin-1 per 10 rhodopsins present translocated to the outer segment, suggesting that two rhodopsins cannot be needed to bind a single arrestin-1 [73]. Of course, one could argue that there are many proteins in the rod outer segment, and we cannot be sure that every molecule of translocated arrestin-1 actually bound rhodopsin in this compartment. To exclude

participation of other proteins, the titration of fixed amount of purified phosphorylated rhodopsin with increasing amounts of purified arrestin-1 was performed. It revealed that the saturation is achieved at 0.99 ± 0.08 mol/mol, i.e., at about 1:1 ratio [73]. However, visual arrestin-1 self-associates, forming dimers and tetramers [74,75]. So, these data did not exclude the role of rhodopsin oligomerization, as the binding of an arrestin dimer to a rhodopsin dimer could have yielded the same 1:1 ratio. A biophysical study of arrestin-1 oligomerization showed that only arrestin-1 monomer binds rhodopsin, or, rather, that when bound to rhodopsin, arrestin-1 becomes monomeric [74], thereby excluding dimer-to-dimer binding mode. Naturally, the next step was reconstitution of single molecules of phosphorylated rhodopsin into nanodiscs and testing whether it still binds arrestin-1. These experiments, performed independently by two different labs, showed that it does [76,60]. Monomeric rhodopsin in nanodiscs also bound non-visual arrestin-2 [76]. Moreover, it was shown that arrestin-1 binds monomeric active phosphorylated rhodopsin with the K_D of 3–4 nM, i.e., with physiological high affinity [60]. Subsequent studies showed that arrestin-1 effectively binds monomeric constitutively active rhodopsin mutants as well [61,62]. Finally, a recent crystal structure of the arrestin-1-rhodopsin complex (Figure 2C) also revealed the 1:1 arrangement predicted by these studies [77,78]. The central arrestin “finger loop”, earlier implicated in direct receptor binding of arrestin-1 [79] and non-visual arrestin-2 [80], was inserted into the cavity between the trans-membrane helices that appears upon rhodopsin activation due to activation-induced movement of helices V and VI [31]. Importantly, the engagement of the inter-helical cavity by arrestin, which is an important part of the receptor-G protein interface [52], is consistent with earlier data that arrestin blocks G protein binding to the receptor via direct competition [81,82], and that arrestin-2 competes with GRK2 for the β_2 -adrenergic receptor [83]. The structure of the arrestin-rhodopsin complex was rigorously tested by H/D exchange, disulfide cross-linking between the two proteins, and distance measurements between selected points in rhodopsin and arrestin-1 using pulse EPR technique double electron-electron resonance [77,78]. It is also consistent with numerous lines of accumulated biological, biochemical and biophysical evidence (reviewed in [84]). Thus, similar to G proteins and GRKs, a single molecule of active phosphorylated GPCR is necessary and sufficient to bind arrestins.

New Twist: One Receptor, Two Signal Transducers

In full agreement with an old model positing that arrestin has two distinct receptor-binding elements, one engaging receptor-attached phosphates, and the other specifically recognizing active receptor conformation [85], two “flavors” of GPCR complexes with arrestin were visualized by cryo-EM [86]. One was “hanging”, with arrestin interacting solely with the phosphorylated receptor C-terminus, whereas the other appeared fully engaged, where this interaction was accompanied by the binding of the center of the arrestin molecule to the center of the cytoplasmic side of the receptor [86], closely resembling the crystal structure of the arrestin-1 complex with rhodopsin [77,78]. Clearly, in the hanging conformation arrestin does not block the inter-helical cavity necessary for the G protein binding [52]. Indeed, further studies revealed that a single GPCR molecule can bind G protein and arrestin simultaneously, which might explain sustained G protein signaling by internalized receptors [87]. Using arrestin lacking the finger loop, which can only bind GPCRs in hanging

configuration, it was recently shown that this partially engaged arrestin mediates receptor internalization and ERK activation, but does not preclude G protein coupling, and therefore does not mediate receptor desensitization [88]. Similar results were obtained with mutant vasopressin receptor that did not bind the finger loop of arrestin, which is considered a hallmark of core engagement [89,90]. Collectively, these data suggest something that even the most devout monomer believers did not envision: simultaneous interaction of a single GPCR molecule with two potential signal transducers. One obvious caveat of these studies is that in all of them engineered receptors, β 2AR with the C-terminus of V2 vasopressin receptor, β 2AR with the deletion of the 3rd cytoplasmic loop to preclude the engagement of arrestin finger loop, or vasopressin receptor with the deletion in the 3rd cytoplasmic loop, were used, rather than native WT GPCRs. The other important caveat is that in neither of these studies the activation of ERK1/2 was shown to be strictly arrestin-mediated, independent of G proteins. This is particularly important in view of a recent study with the WT forms of these receptors, and many other GPCRs, showing that in the absence of G protein activation no arrestin-mediated signaling can be detected [91]. This issue needs to be resolved experimentally before any general conclusions are made. These results also appear to contradict a large body of evidence that the key function of arrestins is to preclude GPCR coupling to G proteins, thereby mediating desensitization (reviewed in [92]). However, these recent data are rather suggestive, although it remains to be elucidated whether this partial arrestin engagement by any naturally occurring GPCR actually happens and yields stable complexes.

GPCR Heterodimers

Numerous studies claimed the existence of class A GPCR heterodimers with properties distinct from those of their protomer components (Figure 3). Several studies suggested the existence of heterodimers based on the ligand binding properties. For example, co-expression of κ - and δ -opioid receptors was found to generate ligand-binding sites with properties not found in cells expressing these receptor subtypes individually [5]. Many studies have reported positive or negative cooperativity of agonist binding upon activation of the other protomer [93–95]. Then there is a more rare phenomenon of antagonist cross-inhibition, when an antagonist of one protomer blocks signaling via the other [96]. Ligand binding data extended the observations of the existence of class A GPCR homodimers in the plasma membrane to heterodimers. The key question is, does GPCR heterodimerization affect signaling?

In some cases, co-expression of two different GPCRs was reported to result in a unique pattern of signaling or trafficking (Figure 3). The formation of the κ - and δ -opioid receptor heterodimers has been shown to potentiate the G protein-mediated signaling and MAP kinase activation [5]. The heterodimers of D1 and D2 dopamine receptors [97,98], angiotensin AT1 and α 2C-adrenergic receptors [99], and melatonin MT1–MT2 receptors [100] coupled to different G protein species as compared to the protomers: Gq instead of Gs or Gi in case of the D1–D2 dimer, Gq instead of Gi in case of melatonin receptors, and Gs instead of Gi or Gq in case of α 2c-AT1 dimer (Figure 3A). Altered signaling properties attributable to the heterodimer formation have been reported for numerous other GPCRs (for a comprehensive review, see [101]). The surface expression of the α 1D adrenergic receptor

in heterologous HEK293 cells requires its co-expression with the $\alpha 1B$ - or $\beta 2$ -adrenoreceptor, whereas it is retained in the endoplasmic reticulum when expressed alone [102,103] - a situation clearly reminiscent of that of the stably heterodimeric class C GABA_B receptor [104]. The removal of a GPCR from the cell membrane was also reported to be modified by heteromerization. For example, the presence of one protomer could suppress agonist-dependent internalization of the other [5] (Figure 3B). Alternatively, an agonist to one protomer could promote internalization of the partner protomer or both [105–108], or alter the fate of the internalized receptors [108,109] (Figure 3B). Unfortunately, studies of GPCR trafficking altered by coexpression of a different subtype usually do not take into account alternative ways one GPCR can affect the trafficking of another. These include documented competition of two GPCRs for a common pool of arrestins [110], the effects of GPCR phosphorylation by second messenger-activated kinases on arrestin binding [111], as well as the regulation of critical players in GPCR trafficking, GRKs and arrestins, by various protein kinases [65,112], the activity of which can be affected by the second GPCR.

It is important to keep in mind that many of these studies have been performed with GPCRs heterologously expressed in cultured cells. The field recognizes that GPCR over-expression in heterologous cultured cells can lead to artifacts due to crowding potentially exacerbated by the localization of certain GPCRs in membrane micro-domains that constitute only a small fraction of plasma membrane, as has been shown for neurotensin NTS1 receptors [113]. Therefore, lately the attention shifted to the demonstration of the existence of heteromers and the exploration of their potential functional role in native tissues. Three criteria were proposed for the proof of the existence of GPCR heterodimers: 1) heteromer components should colocalize and physically interact; 2) heteromers should exhibit properties distinct from those of the protomers; 3) heteromer disruption should lead to a loss of heteromer-specific properties [101].

To detect co-expression and, particularly, interaction of different GPCRs, numerous sophisticated methods have been used [114]. Although these techniques can demonstrate co-existence of the protomers within a limited space in cells, they generally do not have the ability to prove that the receptors are close enough to enable allosteric modulation, presumably via interaction of their HDs. Energy transfer-based methods show that the fluorescent or luminescent tags are within 40–45 Å of each other, which, depending of the structure of particular GPCRs and the positions of these moieties in engineered receptors, can reflect the formation of a true dimer with interacting HDs, or report that the HDs of the two receptors are up to 500 Å apart (discussed in [115]). Recently developed proximity ligation assay suffers from the same drawback: it only shows that the two receptors are within ~400 Å of each other [114], which is many times greater than the diameter of the HD of any GPCR (Figure 1). Complementation-based methods, where the two receptors are tagged with two parts of the same protein (e.g., GFP) that becomes functional only after the receptors come together were also used. A major flaw of these methods is that complementation is irreversible. Random encounter of the two diffusing proteins can yield complementation, forcing them to stay together regardless of the specificity of their interaction. Thus, all available methods can demonstrate that the two receptors are fairly

close to each other or at least were close at some point in time, but do not prove that they specifically interact and form a dimer.

A review of GPCR heterodimers reported to exist in native tissues concluded that in very few cases all three criteria have been met [101]. Furthermore, even when they apparently were, the evidence can still be insufficient or misleading (Figure 3). Putative dopamine D1 and D2 receptor heterodimer offers an instructive example. D1 receptors couple to Gs, whereas D2 couple to Gi/o. An interesting report suggested that the D1–D2 heterodimer has a unique ability to couple to Gq that neither individual subtype had [97,98]. The claim was supported by a significant amount of the *in vitro* and *in vivo* data obtained with the use of a wide array of experimental tools apparently demonstrating the existence, unique signaling properties, and the functional role of the heterodimer [98,97,116–119]. Although it is very hard to prove a negative, a recent comprehensive study using experiments in cell culture, brain slices, and genetically modified mice presented compelling evidence that while D1–D2 heterodimers can be formed in over-expressing cultured cells, they do not exist in the mouse brain, where the two receptor subtypes are not colocalized even in neurons co-expressing them [120]. That study showed that SKF83959-induced motor responses in mice, which were ascribed to the coupling of D1–D2 dimers to Gq, require the D1 receptor but not the D2, the dimer, or Gq-mediated signaling, since these responses were preserved in both D2 and Gq knockout animals [120]. This example shows how careful one has to be claiming that certain class A GPCR heterodimers detectable in over-expressing cells exist and possess unique signaling properties *in vivo* [121].

There are other ambiguities in methods used to demonstrate the existence of GPCR heterodimers. Often the data interpreted as the evidence of heterodimerization could be equally satisfactorily explained by signaling crosstalk (Figure 3). Unique ligand binding, in addition to the existence of transient heterodimers similar to the transient homodimers [40,43,41,42], can reflect that one receptor depletes the G protein necessary for the other. While this mechanism was discussed regarding agonist binding [114], G proteins were recently shown to affect the binding of antagonists, as well [122]. Unique trafficking properties of a GPCR co-expressed with another subtype can be the result of the activity of GRK bound to one receptor towards another brought close to it by lateral diffusion, as was demonstrated in case of photopigments [69,67,68], or even of phosphorylation of one GPCR by second messenger-activated protein kinases activated by another receptor [111]. A GPCR can also deplete proteins, such as arrestins, in the cell, thereby causing changes in the trafficking of another subtype [110]. In fact, the unique signaling pattern attributed to heterodimers has never been unequivocally proven to be the result of oligomerization of different GPCRs, rather than the interplay of signaling pathways in the cell [3]. Generally speaking, there is only one type of experiment that would prove beyond reasonable doubt the existence and unique functional characteristics of putative GPCR heterodimers. Two different native GPCRs must be purified and reconstituted into phospholipids (liposomes, nanodiscs, or bicelles) separately and together. It is important to mimic the lipid composition of the native membrane: dimerization of serotonin 5HT2A receptor was found to require cholesterol, whereas upon cholesterol depletion this receptor exists exclusively as a monomer [123]. If joint reconstitution leads to an activation of purified G protein type that individual receptors do not activate, or the binding of purified arrestin that individual

receptors do not bind, or the interactions with a particular G protein or arrestin in response to a ligand of one GPCR that does induce this interaction when this receptor is reconstituted alone, one can be certain that heterodimers with unique functional properties are actually formed. So far, this type of experiment was never performed, so the definitive proof of the existence of GPCR heterodimers is lacking.

Finally, it is important to keep in mind that specific antibodies, bivalent ligands, or any other means that can simply force the dimerization of two different GPCRs might have therapeutic value even if the heterodimer in question is never formed naturally. Thus, in and of itself therapeutic usefulness of the tools of this kind does not prove (or disprove) the existence of GPCR homo- or heterodimers.

Concluding Remarks

So far all available data with class A and class B GPCRs indicate that a single receptor is necessary and sufficient to bind all three classes of proteins that specifically recognize active receptors: G proteins, GRKs, and arrestins. This statement is based on a huge body of biological, biochemical, and biophysical evidence. It is illustrated by structures of the complexes in Figure 2 not because crystallography is considered to be the ultimate proof, but simply because the structures provide a very clear picture. Despite the profusion of indirect evidence, not a single case was reported where a dimer or higher order oligomer of class A or B GPCR is necessary for signaling. However, as single-molecule experiments invariably show the existence of transient GPCR homodimers in equilibrium with transient monomers, and these data are supported by complex ligand binding kinetics in some cases, the dimers likely have biological functions. Numerous cell culture experiments with heterologously expressed GPCRs, as well as studies in native tissues and living animals, have demonstrated that some class A GPCRs might heterodimerize producing signaling units with properties different from those of the protomers. However, heterodimerization of class A GPCRs has not been proved to the exclusion of alternative mechanisms that do not imply receptor dimerization. The lifecycle of a GPCR is complex: it includes maturation and trafficking to the plasma membrane from the endoplasmic reticulum through Golgi, signaling, as well as endocytosis and subsequent sorting of internalized receptors. Thus, as homo- or heterodimers and higher order oligomers do not appear to be required for signaling, they might serve as inactive “storage” forms in the plasma membrane, or play a role at other stages of the GPCR lifecycle. Elucidation of the biological role(s) of class A and B GPCR oligomers, which are formed but do not apparently participate in GPCR signaling, is the next challenge (see Outstanding questions).

Outstanding questions

- Is reduced signaling capability of class A GPCR dimers a general rule?
- Can GPCRs simultaneously engage more than one signal transducer?
- Do oligomeric forms of class A GPCRs play a role in receptor maturation?
- Do oligomeric forms of class A GPCRs play a role in receptor trafficking from ER via Golgi to the plasma membrane?

- Do oligomers of class A GPCRs play a role in receptor internalization?
- Do oligomers of class A GPCRs play a role in post-internalization sorting of receptors?
- Do oligomers of class A GPCRs play a role in receptor ubiquitination and/or deubiquitination?

Acknowledgments

Supported in part by NIH RO1 grants EY011500, GM077561 and GM109955 (the latter two ROIs were replaced by R35 GM122491) (VVG), NS065868 and DA030103 (EVG).

Glossary

GPCRs

G protein-coupled receptors. Common core of all GPCRs consists of seven trans-membrane helices (hence the synonym of GPCRs, seven trans-membrane domain receptors, or 7TMRs), whereas extracellular N-terminus and loops, as well as intracellular C-terminus and loops widely vary in size. Three classes of proteins preferentially bind active GPCRs: heterotrimeric G proteins, which were the first discovered signal transducers, GPCR kinases (GRKs), and arrestins.

Heterotrimeric G proteins

Guanyl nucleotide binding proteins that consist of three subunits, α , β , and γ . The α -subunit is ~42–44 kDa. It consists of a ~20 kDa Ras domain (homologous to small G proteins) and helical domain, with the nucleotide bound in the cleft between these two domains. G proteins selectively bind to active GPCRs. GPCRs serve as guanyl nucleotide exchange factors (GEFs) for heterotrimeric G proteins. Receptor binding facilitates the release of GDP bound to an inactive G protein, whereupon GTP, which is much more abundant in the cytoplasm binds. GTP binding induces G protein dissociation from GPCR, as well as the dissociation of $G\alpha$ -GTP and $G\beta\gamma$ dimer, both of which regulate various effectors. Mammals express ~20 different α -subunits and smaller numbers of β - and γ -subunits.

GRKs

G protein-coupled receptor kinases that preferentially phosphorylate active GPCRs. The reason for this specificity (in contrast to other protein kinases that recognize certain sequences around targeted residues) is believed to be that GRKs are activated by physical binding to active GPCRs. GRKs belong to the AGC kinase family (to which PKA, PKG, and PKC also belong). Most mammals express seven GRK subtypes (nocturnal rodents express six, lacking GRK7, which is specifically expressed in cone photoreceptors that function in relatively bright light).

Arrestins

44–48 kDa proteins that specifically bind active phosphorylated GPCRs. Vertebrates have four subtypes: arrestin-1 and -4 are specialized visual, and are predominantly expressed in photoreceptor cells in the retina, whereas arrestin-2 and -3 (a.k.a. β -arrestins 1 and 2) are

non-visual, and are expressed in virtually every cell. These two arrestin subtypes bind hundreds of different GPCRs and dozens of non-receptor protein partners, mediating signaling in numerous pathways.

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Highlights

- Class A GPCRS exist in monomer-dimer equilibrium
- Monomeric class A GPCRs effectively activate G proteins, are phosphorylated by GRKs, and bind arrestins
- GPCR oligomers may play a role at other stages of the receptor lifecycle that do not involve signaling

Figure 1. Crystal structures of GPCRs and signal transducers

A–D. Crystal structures of inactive (**A**)(PDB ID: 1F88)[9] and active (**B**)(PDB ID: 5W0P) [77] rhodopsin, as well as inactive (**C**)(PDB ID: 2RH1) [10] and active (**D**)(PDB ID: 3SN6) [52] β_2 -adrenergic receptor. Note that the diameter of the cytoplasmic tip does not exceed 49 Å. **E.** Crystal structure of arrestin-2 (PDB ID: 1G4M)[21] with the elements colored, as follows: the N-domain, gray; the C-domain, blue; the inter-domain hinge, green; the part of the C-terminus anchored to the N-domain and visible in the structure, violet. **F.** Crystal structure of heterotrimeric G protein transducin (PDB ID: 1GOT) [14], with the subunits colored, as follows: guanyl nucleotide binding α -subunit, violet; β -subunit, teal; γ -subunit, dark blue. **G.** Crystal structure of GRK2 (PDB ID: 1OMW) [17], with the elements colored, as follows: RGS homology domain, light violet; kinase domain, dark green; C-terminus containing plekstrin homology domain, brown.

Figure 2. Structures of GPCR complexes with signal transducers

A. Crystal structure of the complex of β 2-adrenergic receptor with cognate G protein Gs (PDB ID: 3SN6) [52]. **B.** Deduced structure of the complex of β 2-adrenergic receptor with GRK5 [124]. **C.** Crystal structure of the complex of constitutively active rhodopsin with "pre-activated" arrestin-1 mutant (PDB ID: 5W0P) [77].

Figure 3. GPCR heterodimerization and possible alternative explanations

A. Unique ligand binding characteristics and/or signaling, in cells co-expressing two different GPCRs was often explained by the formation of heterodimers (left panel). The ligand binding and signaling could be affected by simultaneous activation of both protomers or by ligand selective for the heterodimer (bi-color oval). Dimers could also display cross-antagonism when the binding of an antagonist (purple pentagon) to one protomer inhibits signaling via another. However, the observed phenomena can be explained equally well by signaling crosstalk (right panel). It can be at the level of G proteins as well as crosstalk of the signaling pathways downstream of G proteins. These processes can result in changes in ligand binding, second messenger (cAMP, Ca²⁺) concentrations, or signaling outcomes, such as ERK phosphorylation. Both Gs and Gi/o regulate the same pool of cAMP; the activity of various adenylyl cyclases can be modulated by intracellular Ca²⁺ and other factors. The level of Ca²⁺ can be regulated indirectly via downstream signaling affecting various Ca²⁺ channels. These phenomena are sometimes interpreted as a switch in GPCR coupling to a different G protein type. **B.** A change in trafficking pattern of a GPCR subtype co-expressed with another GPCR was also interpreted as evidence for the formation of heterodimers. Stimulation of one protomer could promote internalization of the other or both, or change the fate of the internalized receptor (left panel). However, alternative mechanisms that could mediate the effect of one GPCR on the trafficking of another without heterodimerization have been documented (right panel): for example, competition for the same pool of arrestins or arrestin binding due to receptor phosphorylation by second messenger-activated kinases. Moreover, both key actors in GPCR trafficking, GRKs and arrestins, are also regulated via phosphorylation by various kinases that can be affected by the signaling of another receptor. Thus, as it is virtually impossible to exclude all alternative explanations of the observations

in cell-based experiments, the experiments performed so far do not definitively prove GPCR heterodimerization.

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