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G protein-coupled receptor-effector macromolecular membrane assemblies (GEMMAs)

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ABSTRACT

G protein-coupled receptors (GPCRs) are the largest group of receptors involved in cellular signaling across the plasma membrane and a major class of drug targets. The canonical model for GPCR signaling involves three components — the GPCR, a heterotrimeric G protein and a proximal plasma membrane effector — that have been generally thought to be freely mobile molecules able to interact by 'collision coupling'. Here, we synthesize evidence that supports the existence of GPCR–effector macromolecular membrane assemblies (GEMMAs) comprised of specific GPCRs, G proteins, plasma membrane effector molecules and other associated transmembrane proteins that are pre-assembled prior to receptor activation by agonists, which then leads to subsequent rearrangement of the GEMMA components. The GEMMA concept offers an alternative and complementary model to the canonical collision-coupling model, allowing more efficient interactions between specific signaling components, as well as the integration of the concept of GPCR oligomerization as well as GPCR interactions with orphan receptors, truncated GPCRs and other membrane-localized GPCR-associated proteins. Collision-coupling and pre-assembled mechanisms are not exclusive and likely both operate in the cell, providing a spectrum of signaling modalities which explains the differential properties of a multitude of GPCRs in their different cellular environments. Here, we explore the unique pharmacological characteristics of individual GEMMAs, which could provide new opportunities to therapeutically modulate GPCR signaling.

Abbreviations[1]

[1]GPCR nomenclature used in the current review follows the guidelines of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (https://www.guidetopharmacology.org/nomenclature.jsp) and adds an 'R' at the end as an abbreviation of 'receptor'.: AC, adenylate cyclase; AKAP, A-kinase anchoring protein; AMPAR, ionotropic glutamate AMPA receptors; BiBC or BiFC, bimolecular bioluminescence or fluorescence complementation; BRET or FRET, bioluminescence or fluorescence resonance energy transfer; CT or NT, carboxy terminus or amino terminus; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; GEMMA, GPCR-effector macromolecular membrane assembly; GPCR, G protein-coupled receptor; Kir, inwardly rectifying potassium channel; PDZ, PSD-95/Discs large/Zona occludens-1; PKA, protein kinase A; PM-effector, plasma membrane-effector; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; RAMP, Receptor activity-modifying protein; Rluc, *Renilla* luciferase; TM, transmembrane; YFP, yellow fluorescence protein..

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[1] GPCR nomenclature used in the current review follows the guidelines of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (https://www.guidetopharmacology.org/nomenclature.jsp) and adds an 'R' at the end as an abbreviation of 'receptor'.

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Contents

1. Introduction: the GEMMA concept

G protein-coupled receptors (GPCRs) form the largest group of receptors involved in cellular signaling across the plasma membrane. Heterotrimeric (Gαβγ) guanine nucleotide-binding proteins (G proteins) are the initial transducers that convey information from agonist-occupied GPCRs to a variety of effector proteins, many of them localized to the plasma membrane (PM-effectors). The canonical model thus involves three components, the GPCR, a heterotrimeric G protein and a proximal PM-effector, thought initially to be freely mobile molecules able to interact by 'collision coupling'.

In 1978, Tolkovsky and Levitzki articulated the notion of the collision coupling for GPCR signaling from studies on β-adrenergic receptor (βAR)-mediated adenylyl cyclase (AC) activation in turkey erythrocytes (Tolkovsky & Levitzki, 1978a). That same year, they reported that collision coupling could not explain results obtained using the same system on activation of AC by adenosine receptors. Instead, they observed a first-order process of AC activation, that could be better explained by the existence of pre-coupled GPCR-AC complexes (Braun & Levitzki, 1979; Tolkovsky & Levitzki, 1978b). Collision coupling was consistent with the 'fluid mosaic' model of the plasma membrane proposed by Singer and Nicholson in the early 1970s, which described the lipid bilayer as an isotropic milieu, allowing membrane-embedded proteins to diffuse and interact with each other by random collision (Singer & Nicolson, 1972). Initial support for collision coupling in GPCR signaling came from early studies on rhodopsin and its G protein partner transducin (Gt) in rod photoreceptor cells and on purified βAR, G proteins and AC reconstituted in phospholipid vesicles (Levitzki & Klein, 2002; Neubig, 1994).

However, the cellular models used in these influential studies were not generally representative of the common cellular environment of GPCRs and their interacting membrane signaling molecules. The outer segment disks of photoreceptors have a unique lipid composition that provides rhodopsin and Gt with much greater lateral mobility than that of most mammalian membrane proteins. Similarly, receptors and G proteins reconstituted in lipid vesicles are likely to be relatively mobile without the constraints on lateral motion imposed in cellular plasma membranes, such as interactions with the cytoskeleton and membrane nanodomains with different protein and lipid compositions, such as 'lipid rafts' (see below). Furthermore, the collision coupling model is less compatible with the existence, in the same cell, of a large variety of different GPCRs, heterotrimeric G proteins and PM-effectors (Fig. 1). In this context, the binding of an agonist must promote a series of specific and sequential intermolecular interactions between the activated GPCR, one or more heterotrimeric G proteins comprised of particular Gα, Gβ and Gγ subunit combinations and, in many cases, multiple PM-effectors, including AC and phospholipase C (PLC) subtypes and a number of GPCR-modulated ion channels (Cabrera-Vera et al., 2002).

Here, we establish the concept of a *GPCR-effector macromolecular membrane assembly* or *GEMMA*. A GEMMA is defined as *a pre-assembled signaling complex composed of particular combinations of GPCRs, G proteins, effectors and other associated transmembrane (TM) proteins localized to the plasma membrane*. Further, GEMMAs possess emergent functional and pharmacological characteristics making them potentially unique drug targets. Inherent in the definition of GEMMA is that the interactions between different components are membrane-delimited, which means that all the components are either intrinsic membrane or membrane-associated proteins, with no diffusible cytosolic intermediates between core components.

We must distinguish between the three primary components of membrane-delimited GPCR-mediated signaling pathways (GPCRs, G proteins and PM-effectors), from additional components that modulate or scaffold these core components. Among others, additional elements include G protein-coupled receptor kinases (GRKs) and arrestins, which are primarily recruited to the plasma membrane upon GPCR activation and may not directly interact with PM-effectors (Gurevich & Gurevich, 2019; Gurevich, Tesmer, Mushegian, & Gurevich, 2012; Homan, Glukhova, & Tesmer, 2013).

This notion revises the classical model of sequential ligand-induced association-dissociation of GPCR, G protein subunits and PM-effector, and posits that ligands can induce rearrangements within elements of these pre-assembled macromolecular complexes. Metaphorically, we can imagine that GPCRs, G proteins and PM-effectors act as 'clock gears', instead of 'billiard balls', as described by collision coupling. It can nevertheless be surmised that both alternative modes of interactions coexist in the same cell, particularly for GPCR-G protein interactions.

Within the GEMMA concept, it is necessary to address the impact of GPCR oligomerization. GEMMAs can include a number of identical or different GPCRs as well as orphan GPCRs, truncated GPCRs and other associated TM proteins, to generate unique macromolecular complexes with distinct functional and pharmacological properties. The homomeric nature of class C GPCRs is not a matter of dispute. Their obligate dimeric nature depends on an inter-protomer disulfide bridge formed via a conserved cysteine in their characteristic large binding domain (Ellaithy, Gonzalez-Maeso, Logothetis, & Levitz, 2020). The cryo-EM structures of several metabotropic glutamate receptor (mGluR) homodimers have been recently reported. These include the inactive and active conformations of $mGlu_5R$ and $mGlu_2R$ homodimers (Du et al., 2021; Koehl et al., 2019; Seven et al., 2021) (Fig. 2A), the active conformation of $mGlu_2R$ and $mGlu_4R$ homodimers coupled to a heterotrimeric Gi protein (Lin et al., 2021) and the inactive conformation of the $mGlu_7R$ homomer and the $mGlu_2R$-$mGlu_7R$ heteromer (Du et al., 2021). Whether class A (rhodopsin-like family) and family B (secretin receptor family) GPCR dimers are also constitutively formed has been a more contentious issue, even though the crystal structures of several GPCRs revealed homo-oligomers, including the chemokine CXC_4R (Wu et al., 2010), the μ-opioid receptor (MOR) (Manglik et al., 2012) and the β_1-adrenoceptor (β_1AR) (Huang, Chen, Zhang, & Huang, 2013) (Fig. 2B–D).

Apart from the crystallographic evidence, during the last two decades, numerous studies using different technical approaches have provided strong support to the increasingly accepted notion that GPCR homomers and heteromers constitute primary functional GPCR

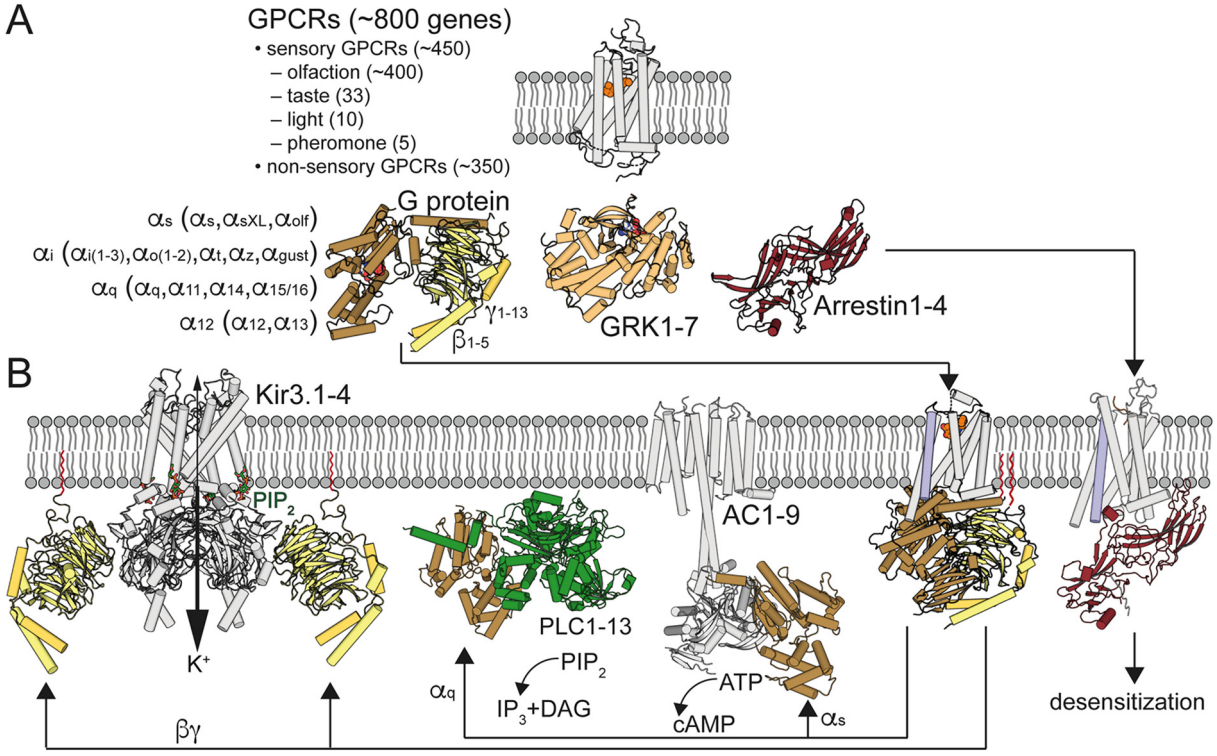

Fig. 1. G protein-coupled receptor signaling. (A) GPCRs are illustrated with the structure of retinal-bound rhodopsin in white (Palczewski et al., 2000). In humans, there are 16 Gα subunits (classified as Gαs, Gαi, Gαq and Gα12) 5 Gβ and 12 Gγ (Gγ1-13, but no Gγ6) subunits. Active GPCRs interact with Gαβγ proteins, illustrated with the structure of GDP-bound Gαi1β1γ2 in brown/yellow (Wall et al., 1995), G protein-coupled receptor kinases (GRKs), with 7 mammalian isoforms, illustrated with the structure of ATP-bound GRK1 in light orange (Singh, Wang, Maeda, Palczewski, & Tesmer, 2008), and arrestins, with 4 isoforms, illustrated with the structure of arrestin1 in red (Granzin et al., 1998). (B) Agonist binding triggers the movement of TM6 (in blue), opening an intracellular cavity for G protein arrangement, illustrated with the crystal structure of the agonist-β2AR-Gs complex (Rasmussen et al., 2011). Gαq activates PLC, with 13 mammalian isozymes, illustrated with the crystal structure of PLC-β3 bound to Gαq (Waldo et al., 2010), which catalyzes the hydrolysis of PIP_2 to the Ca^{2+}-mobilizing second messenger inositol 1,4,5-trisphosphate (IP_3) and the PKC-activating second messenger diacylglycerol (DAG). Gαs stimulates and Gαi inhibits the activity of AC, with 9 mammalian isoforms, illustrated with the cryo-EM structure of AC9 bound to Gαs (Qi et al., 2019). Gβγ subunits increase the activity of Kir3 channels, illustrated with the crystal structure of the Kir3.2-Gβγ (Whorton and MacKinnon, 2013). Desensitization is mediated by GRK-mediated GPCR phosphorylation and subsequent binding of arrestin, illustrated with the cryo-EM structure of neurotensin NT_1 receptor in complex with arrestin2 (Huang et al., 2020). Gγ isoprenylated group is shown in red.

signaling units (for reviews, see Ferré et al., 2014; Ferré, Ciruela, Casadó, & Pardo, 2020; Gomes et al., 2016; Sleno & Hébert, 2018; Gaitonde & González-Maeso, 2017; Bourque, Jones-Tabah, Devost, Clarke, & Hébert, 2020). GPCR oligomerization significantly broadens the allosteric mechanisms inherent in GPCRs, imposing conformational alterations and constraints of the individual GPCR units (Ferré et al., 2014, 2020). This gives the possibility of developing GEMMAs as targets for drug discovery, with the rationale of the existence of different GEMMAs with common components but with different oligomeric partners localized in different types of cells or cellular environments. Screening campaigns should look for molecules with the ability to promote distinct states of specific GEMMAs, using single agents or combinations of molecules with selectivity for their individual components.

2. Evidence for pre-assembly and ligand-induced rearrangement of specific GPCR, G proteins and effector complexes

2.1. Pre-assemblies of specific Gα, Gβ and Gγ subunits

Since Gilman's classic conceptualization (Gilman, 1987) it was generally assumed that upon binding to the ligand (a GPCR agonist), GPCRs undergo conformational changes activating heterotrimeric G proteins by promoting GTP binding to Gα subunits and triggering the release of Gβγ subunits. When dissociated, both α and βγ subunits modulate the activities of effector molecules directly responsible for generating cellular responses (Gilman, 1987). However, as we discuss, an alternative model suggests that G protein heterotrimers can be found in more stable complexes that rearrange rather than dissociate upon receptor activation.

In many cases, coupling between receptors and G proteins is pleiotropic such that a single receptor can activate more than one G protein heterotrimer (Flock et al., 2017; Okashah et al., 2019), which allows initiation of multiple signal transduction pathways. However, signaling *in vivo* most often results in regulation of selective effectors with high fidelity, suggesting that the cell is endowed with different modalities to allow segregation of specific connections between different GPCRs and their effectors. In humans, there are at least 16 different isoforms of Gα subunits, grouped into families- Gαs (Gαs, Gαolf and GαsXL), Gαi (Gαi1, Gαi2, Gαi3, Gαo1, Gαo2, Gαz, Gαt and Gαgust), Gαq (Gαq, Gα11, Gα14 and α15/16) and α12 (α12 and α13); and 5 different isoforms of Gβ (β1-5) and 12 Gγ (Gγ1-13, but no Gγ6) subunits (Downes & Gautam, 1999). The combinatorial association of different G protein subunits potentially provides the bandwidth necessary to generate a broad range of signals independently transduced from different GPCRs to different effectors transmitted by G proteins (Robishaw & Berlot, 2004).

Several seminal studies have provided evidence that specific combinations of Gα, Gβ and Gγ subunits are necessary to generate signal transduction from a given GPCR to a specific effector (Cabrera-Vera et al., 2002; Dupré, Robitaille, Rebois, & Hébert, 2009; Gudermann, Kalkbrenner, & Schultz, 1996; Rebois & Hébert, 2003). Studies in the early nineties using antisense oligonucleotides demonstrated that, even in the same cells, different combinations of specific Gα, Gβ and Gγ subunits allowed different receptors to regulate the same effector (Kleuss, Schultz, & Wittig, 1994). Somatostatin, muscarinic M_4

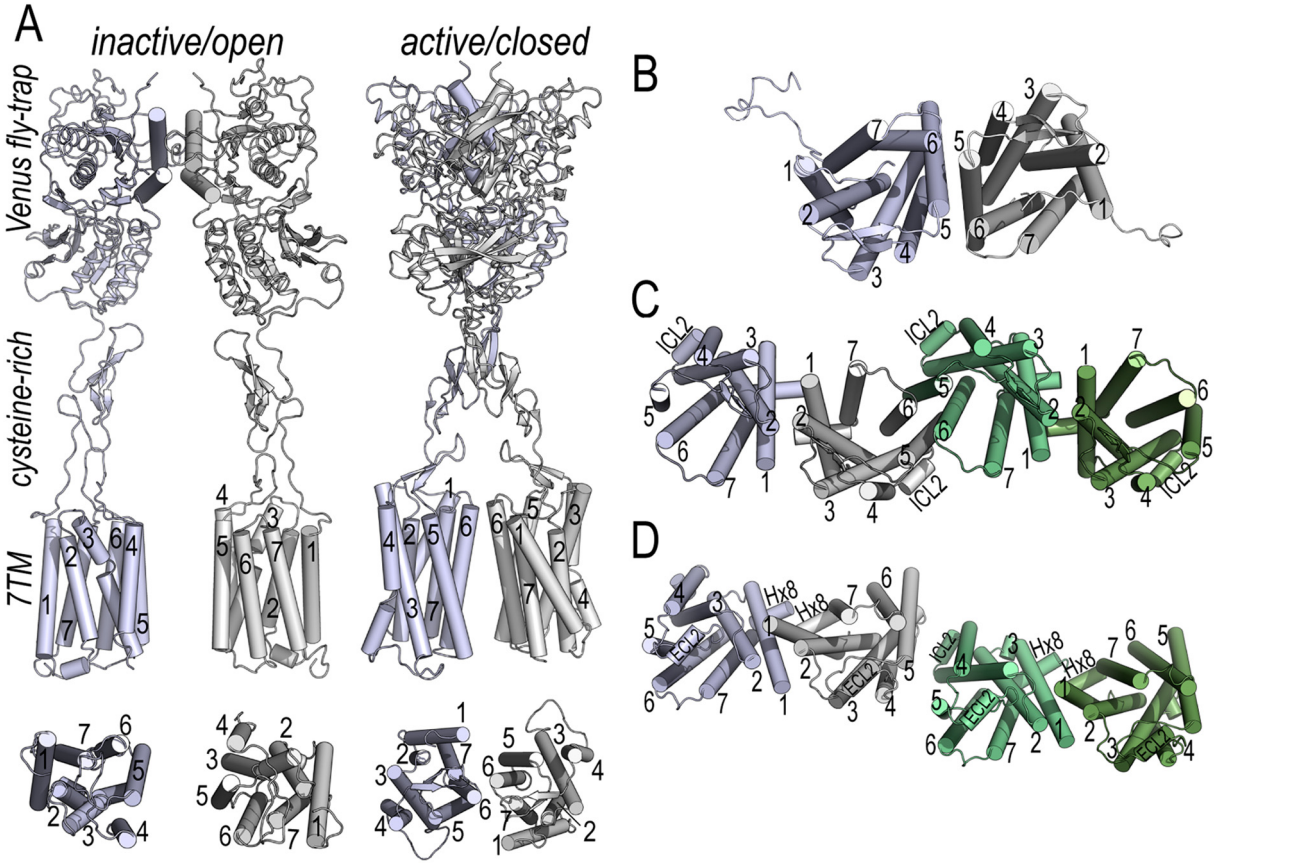

Fig. 2. Molecular structures of GPCR homomers. (A) Cryo-EM structures of the full length $mGlu_5R$ in the apo inactive/open and active/closed onformations (Koehl et al., 2019). Class C GPCRs are obligate cysteine-linked homomers and each protomer is formed by a large extracellular 'Venus fly-trap' domain that binds orthosteric ligands and promotes an inter-protomer disulfide bridge formed via a conserved cysteine, a cysteine rich domain and a seven TM domain (7TM). The helix interface between protomers of the Venus fly-trap domains is represented by cylinders. Activation leads to compacting of the $mGlu_5R$ homomer, bringing the cysteine rich domains into proximity and enabling the 7TM domains to approximate to initiate signaling. The inactive state shows a TM5-TM5 orientation but with substantial separation between the 7TM domains. Activation leads to a 20° rotation of each 7TM domain, resulting in a close contact TM6-TM6 homodimeric interface. Both protomers are colored in light blue and gray. (B) CXC_4R structure (Wu et al., 2010) reveals a homomer with an interface including TM5 and TM6. (C) MOR structure (Manglik et al., 2012) shows receptor protomers associated in pairs through two different interfaces. One interface is via TM1, TM2 and helix 8 (Hx8) (blue/white and light/dark green protomers) and the other interface comprises TM5 and TM6 (white/green protomers). (D) β_1AR structure (Huang et al., 2013) also displays two homomeric interfaces. One interface also involves TM1, TM2 and Hx8 (blue/white and light/dark green protomers), as with MOR. In contrast, the other interface engages residues from TM4, TM5 and intracellular loop 2 (ICL2) white/green protomers).

and galanin receptors inhibited voltage gated Ca^{2+} channels by specifically coupling to Gαo2β1γ3, Gαo1β3γ4 and Gαo1β2γ2, respectively (Kalkbrenner et al., 1995; Kleuss, Scherübl, Hescheler, Schultz, & Wittig, 1993). The same studies showed that galanin receptors selectively used the same combination of G protein subunits, Gαo1β2γ2, to inhibit voltage gated Ca^{2+} channels in a different cell type (Kalkbrenner et al., 1995).

However, there is also experimental evidence indicating that one GPCR couples to different combinations of G protein subunits in different cells. Particularly significant is the example of the α_{2A}-adrenoceptor ($\alpha_{2A}AR$) in the mouse brain, which, without considering the Gα subunit of the heterotrimer involved, was found to associate selectively to Gβ2, Gγ2 and Gγ3 in noradrenergic cells, but to Gβ4 and Gγ12 in non-noradrenergic cells (Yim et al., 2019). Another remarkable example for the need of specific Gαβγ subunit compositions comes from genetic studies, which demonstrate that, specifically in the striatum, the signaling of adenosine A_{2A} receptors ($A_{2A}R$) and dopamine D_1 receptor (D_1R) with AC, more specifically the AC5 isoform, is dependent on specific coupling to Gαolfβ2γ7 (Schwindinger et al., 2003, Schwindinger et al., 2010; Hervé, 2011; Xie et al., 2015). The involvement of specific asemblies of G protein subunits from a potential large number of combinations as components of specific complexes of GPCRs, G proteins and PM-effectors does not seem to depend on a specific preferential affinity between those subunits, but on their synchronized ER synthesis, allowing pre-assembly and delivery to the plasma membrane.

Several studies indicate that Gαβγ heterotrimers pre-assemble before delivery to the plasma membrane, although there remains controversy over the secretory pathway involved. One study suggested that fully processed Gβγ subunits form heterotrimers with Gα on the cytosolic face of the Golgi apparatus (Michaelson, Ahearn, Bergo, Young, & Philips, 2002). However, another study favored Golgi-independent trafficking of the Gαβγ heterotrimer, where formation was prevented by a dominant mutant of the small GTPase Sar1 (Takida & Wedegaertner, 2004), responsible for the assembly of coated vesicles at the endoplasmic reticulum (ER) membrane (Mizuno-Yamasaki, Rivera-Molina, & Novick, 2012). In any case, assembly of the heterotrimer precedes acylation of the Gα subunit, which is necessary for delivery of the heterotrimer to the plasma membrane (Dupré et al., 2009; Marrari, Crouthamel, Irannejad, & Wedegaertner, 2007). The evidence for pre-assembly of the full heterotrimeric G protein in its journey to the plasma membrane indicates that the different specific Gαβγ subunit combinations are not selected from a pool of subunits localized at the plasma membrane and sorted by random collision or another so far unidentified mechanism following receptor activation.

2.2. Stable interactions between specific GPCRs, G proteins and PM-effectors

Biophysical techniques have been widely used to evaluate the possible existence of intermolecular interactions between specific GPCRs, G proteins and PM-effectors in living cells (Bouvier, 2001; Milligan, 2001; Guo et al., 2017; El Khamlichi et al., 2019; Soave, Briddon, Hill, and Stoddart (2020). In most of these techniques, two chromophores that can change their biophysical properties when they are in contact, or in very close proximity, are separately fused to the two putative interacting proteins and transfected to mammalian cells in culture. These techniques include bioluminescence and Förster or fluorescence resonance energy transfer (BRET and FRET), time-resolved FRET (TR-FRET), bimolecular bioluminescence or fluorescence complementation (BiBC or BiFC), and fluorescence recovery after photobleaching (FRAP). A series of studies using combinations of these biophysical techniques with biochemical techniques (co-immunoprecipitation and pull-down strategies) has provided strong support for the existence of stable interactions between GPCRs and G proteins (Galés et al., 2005, 2006; Nobles, Benians, & Tinker, 2005; Dupré et al., 2006; Ayoub, Trinquet, Pfleger, & Pin, 2010; Qin, Dong, Wu, & Lambert, 2011; Damian et al., 2015; Andressen et al., 2018) and between G proteins and PM-effectors (Rebois et al., 2006; Riven, Iwanir, & Reuveny, 2006; Sadana, Dascal, & Dessauer, 2009; Yuan, Sato, Lanier, & Smrcka, 2007). Further evidence has also accumulated for the existence of direct interactions between GPCRs and PM-effectors, including L-type and N-type voltage-dependent Ca^{2+} channels (Beedle et al., 2004; Rebois et al., 2006), inwardly rectifying potassium Kir3 channels (David et al., 2006; Lavine et al., 2002) and ACs (Dupré, Baragli, Rebois, Ethier, & Hébert, 2007; Lavine et al., 2002; Navarro et al., 2018). These studies strongly support the existence of specific assemblies of GPCRs, G proteins and effectors localized in plasma membrane-delimited signaling complexes. Their specific make up raises the notion that components of such complexes could be pre-assembled before delivery to the plasma membrane. It could be demonstrated that dominant negative small GTPase isoforms promote retention of GPCR-G protein, G protein-PM-effector and GPCR-PM-effector assemblies. For instance, prevention of anterograde trafficking to the plasma membrane using mutants of the coat-recruitment small GTPase Sar1 or the also small GTPase Rab1, which guides transport vesicles to the plasma membrane (Mizuno-Yamasaki et al., 2012), reduced β_2AR plasma membrane localization but not interactions between β_2AR and Gβ1/Gγ2 subunits, AC2 and β_2AR or AC2 and β1 or Gγ2 fused to BRET chromophores (Dupré et al., 2006; Dupré et al., 2007), indirectly indicating the pre-assembly of β_2AR-β1γ2-AC2 complexes.

Several recent findings suggest plausible scenarios of GPCR-G protein interactions that are different from the fully engaged and agonist-promoted interactions in which helix 5 of the Ras-GTPase domain of the Gα protein interacts with the intracellular cavity of the GPCR formed by the agonist-promoted outward movement of TM6 (Weis & Kobilka, 2018). Whereas initial X-ray and cryo-EM structures of GPCR-G protein complexes were captured in their nucleotide-free state, recent structures based on time-resolved structural mass spectrometry techniques begin to observe alternative complexes and conformational transitions, all indicating the plasticity of interacting interfaces and dynamic nature of the GPCR-G protein complex (Du et al., 2019; Kato et al., 2019; Liu et al., 2019). Such techniques are expected to further reveal the molecular nature of GPCR-G protein pre-assemblies in the near future.

2.3. Association-dissociation of GPCRs and G proteins or ligand-induced rearrangement of GPCR-G protein complexes?

Biophysical techniques have also allowed the analysis of ligand-induced changes in the interactions between GEMMA components. The insertion of BRET chromophores in the α_{2A}AR and in different positions of the Gαi1, β1 and γ2 subunits was used to establish that during the early stages of receptor activation, G protein activation is associated with a relative movement between the Gα and Gβγ subunits that does not involve their complete dissociation (Galés et al., 2006). More precisely, these BRET experiments showed a ligand-induced separation of the Gα subunit helical domain *versus* the Ras-GTPase domain coupled to the Gβγ subunit (Galés et al., 2006). The relatively large separation of the helical and Ras-GTPase domains of the Gα subunit, which tightly sandwich the nucleotide in all nucleotide-bound G protein crystal structures, was later on confirmed by double electron-electron resonance spectroscopy (Van Eps et al., 2011), a crystal structure of a GPCR-G protein complex (Rasmussen et al., 2011), and deuterium-exchange and electron microscopy data (Chung et al., 2011; Westfield et al., 2011).

Subsequent studies confirmed the ability of different GPCRs to transit from inactive to active conformations by a rearrangement of a pre-assembled receptor-G protein complex, such as a study on monomeric ghrelin GHS_{1a} receptor ($GHS_{1a}R$) reconstituted in nanodiscs, using TR-FRET and normal mode (NM) analysis (Damian et al., 2015). On the other hand, a more recent study using single-molecule tracking (SMT) with total internal reflection fluorescence (TIRF) obtained results indicating the existence of a predominant population of short-lived (1 second) complexes of α_{2A}AR and Gαi1 labelled with two different organic fluorophores and expressed at low densities in mammalian transfected cells, in the absence of ligands (Sungkaworn et al., 2017). The authors proposed a model dependent on collision coupling, where agonists specifically regulate the kinetics of GPCR-G protein interactions by increasing their association rate (Sungkaworn et al., 2017).

Thus, the debate about a pre-assembly and rearrangement versus collision coupling and association-dissociation of GPCRs and G proteins remains open. Nevertheless, both mechanisms seem to operate and be dependent on protein density, the specific GPCR-G protein pair, and the cellular environment. With BRET and TR-FRET techniques, the GPCR protease-activated PAR_1 receptor was shown to specifically form assemblies with Gαi1 but not with Gα12 (Ayoub et al., 2010). Nevertheless, Gα12 was slowly recruited upon receptor activation, indicating the possibility of the two modes of interaction of the same receptor, pre-assembly and collision coupling, depending on the G protein partner (Ayoub et al., 2010). Similar conclusions were obtained from a study using FRAP and BRET techniques examining pre-assembly and agonist-induced rearrangement of the M_3R and Gαq (Qin et al., 2011). A six-amino-acid polybasic sequence distal to helix 8 was necessary for pre-assembly with Gq. Moreover, the polybasic epitope was also necessary for an efficient M_3R-mediated Gq activation, showing that GPCR-G protein pre-assembly significantly increased the rate of Gq-dependent signaling (Qin et al., 2011). Results obtained with FRAP and FRET techniques also showed a differential ability of 5-HT_7R and 5-HT_4R to preassemble with Gs proteins (Andressen et al., 2018). The results support a model where pre-associated 5-HT_7R-Gαsβ1γ2 complexes undergo agonist-induced rearrangement involving a rapid movement of Gα relative to the receptor, followed by a slower dissociation of Gβγ subunits from both the receptor and the Gα subunit. In contrast, 5-HT_4R displayed properties more consistent with collision coupling (Andressen et al., 2018). These studies (Andressen et al., 2018; Ayoub et al., 2010; Qin et al., 2011) therefore indicated the possibility of the coexistence of two alternative signaling modes in the same cell, with pre-assembly providing a means to regulate more specific signaling events as compared to the collision-coupling mode.

2.4. Association-dissociation of G proteins and PM-effectors or ligand-induced rearrangement of G protein-PM-effector assemblies?

A substantial number of studies provided evidence for pre-assembly (inactive state) and rearrangement (active state) of G proteins and PM-effectors, mainly dependent on interactions with Gβγ subunits. Gβγ subunits were initially thought to passively facilitate termination of intracellular information transfer by binding to the Gα subunit and

preventing its further spontaneous activation in the absence of receptor activation. This dogma was challenged when Gβγ was shown to directly activate a Kir3 channel in cardiac atrial cells (Logothetis, Kurachi, Galper, Neer, & Clapham, 1987). Gβγ subunits are now known to modulate different isoforms of ACs, PLCs, K^+ and Ca^{2+} channels and many other PM-effectors (Dupré et al., 2009; Khan et al., 2013; Khan, Sung, & Hébert, 2016; Smrcka & Fisher, 2019). The Gβγ structure includes the seven β-sheet WD40 repeat architecture of the Gβ subunit (the seven "blades" of the Gβ subunit "propeller") and a "hotspot" surface where the turns between blades intersect. The Gβγ hotspot is required for interactions with numerous effectors and it is concealed in the inactive state by its binding to the Ras-GTPase domain of the Gα subunit. Upon ligand-induced activation, the hotspot is exposed and interacts with available effectors (Cabrera-Vera et al., 2002; Davis, Bonacci, Sprang, & Smrcka, 2005). Distinct but overlapping motifs of the hotspot area are involved in the interactions with the Gα subunit and the different effectors (Cabrera-Vera et al., 2002; Davis et al., 2005; Mirshahi, Robillard, Zhang, Hébert, & Logothetis, 2002; Smrcka & Fisher, 2019). The sites required for agonist-induced activation of effectors by the Gβγ (or by the Gα) subunits are likely to be inaccessible in the GDP-bound, inactive, Gαβγ assembly, thus necessitating certain degree of dissociation of the assembly for activation of the effectors.

In addition to their key modulatory role in effector function, cumulative evidence supports an additional key role of Gβγ subunits in the pre-assembly of G proteins to PM-effectors in their inactive state. This has been addressed *in vitro* by demonstrating direct interactions between Gβγ subunits with the PM-effector when forming part of the GDP-bound Gαβγ assembly or without involving the hotspot area. Initial evidence was obtained from pull-down experiments with purified bovine Gβγ subunits and the cytosolic NT and CT of the Kir3.1 subunit of Kir3 channels (Huang, Slesinger, Casey, Jan, & Jan, 1995). Kir3 channels are prototypic PM-effectors of Gβγ subunits and are activated by direct interactions with Gβγ that follow GPCR-dependent activation of Gi/o proteins (Dascal & Kahanovitch, 2015; Logothetis et al., 1987, 2015). Kir3 channels are homo- or hetero-tetrameric and can be composed of four distinct subunits, Kir3.1-4. The basic architecture of the Kir3 channel subunits consists of two TM helices with the cytosolic NT and CT and an extracellular loop which folds back to form the pore-lining ion selectivity filter. Those initial pulldown experiments showed that Gβγ subunits bind to both cytosolic domains and that a GDP-bound Gαβγ assembly specifically binds to the NT of the Kir3.1 subunit, involving contacts mediated by both Gβγ and Gα subunits (Huang et al., 1995). The results also indicated that both cytoplasmic regions of the Kir3 channel subunit are involved in the activation of the Kir3 channel (Huang et al., 1995).

Subsequent studies, using different biochemical and biophysical techniques, provided additional evidence for the involvement of the Gβ subunit in the interaction with Kir3 channels, both in its active and inactive states. Key residues that constituted significant contact points with Kir3 channels and were not concealed by the non-activated Gα subunit were found to be involved in Gβγ-Kir3 channel interactions in the absence of agonist stimulation (Mirshahi et al., 2002). Experiments with internal reflection fluorescence (TIRF) microscopy combined with FRET in cells transfected with $mGlu_2R$, Kir3.1 or Kir3.4 fused at various positions of their cytosolic NT and CT with a FRET chromophore and Gβ1 fused to the other chromophore, indicated an interaction between the Gβγ subunits and the Kir3 channel in the resting state and a change in FRET in the presence of the endogenous ligand glutamate (Riven et al., 2006). The results suggested that receptor activation should promote an orientation switch of the Gβγ dimer, without affecting the position of Gα relative to the channel, which would allow the interaction of the Gβγ with the channel at a separate, independent site to promote opening (Riven et al., 2006).

Experiments with BRET in a cell line stably expressing β_2AR allowed further analysis of interactions between Gβγ and Kir3 channels and another PM-effector, AC2, in the absence and presence of ligands, again indicating both pre-assembly and agonist-induced rearrangement (Rebois et al., 2006). The same study provided evidence for pre-assembly and ligand-induced rearrangement of G proteins and Kir3 channels before trafficking to the plasma membrane, by fusing Kir3.1 and Gγ2 to BRET chromophores and measuring BRET changes in response to membrane-permeable and non-membrane agonists in the presence and absence of Kir3.4. In the absence of Kir3.4, the β1γ2-Kir3.1 complex did not traffic to the plasma membrane and changes in BRET were only evident using membrane-permeable agonists (Rebois et al., 2006). These results therefore indicated an intracellular localization of functional β_2AR as part of a GEMMA.

The subsequent publication of the crystal structure of the Kir3 channel with four Kir3.2 subunits in complex with Gβ1γ2 and PIP_2 confirmed the inferences made from many of the previous biochemical and biophysical studies (Glaaser & Slesinger, 2015). The biologically relevant complex consists of one channel tetramer, four Gβγ subunits, four PIP_2 molecules and four Na^+ ions bound to regulatory sites (Whorton & MacKinnon, 2013). Gβγ subunits interact directly with the cytosolic CT and they are oriented such that the CT of the Gγ subunit, which contains a covalent geranylgeranyl group, points directly to the membrane layer as if to function as an anchor (Whorton & MacKinnon, 2013) (Fig. 1B). Thus, the Kir3.2-Gβ1γ2 crystal structure is compatible with a physiological membrane-delimited Gβγ activation of Kir3. We are now waiting for structures of the Kir3 channel in complex with a GPCR and its heterotrimeric Gi protein in both inactive and active states. In the meantime, the studies described here provide evidence for pre-assembly and rearrangement of Gαβγ-Kir3 complexes in response to agonist. Both Gα and Gβγ subunits appear to be directly associated with Kir3 under both ligand-free inactive and agonist-induced active states. Agonist-induced rearrangement of the complex suggests separation of the Gβγ subunits from Gα subunit, but *within a metastable complex*, with Gβγ subunits moving away from the initial Kir3 NT- and CT-bound Gαβγ assembly.

The existence of G proteins and AC as a stable complex was first proposed by Levitzki based upon co-purification of AC and G proteins from turkey erythrocyte membranes independent of the activation state of the G proteins (Bar-Sinai, Marbach, Shorr, & Levitzki, 1992; Levitzki, 1988; Levitzki & Klein, 2002). A role of Gβγ subunits in the pre-assembly and scaffolding of ACs has also been supported by *in vitro* and *in cellulo* experiments (see below). Further, we also know that Gβγ subunits can directly interact with AC facilitating or inhibiting its function depending on the AC isoform. This modulatory role of Gβγ is also conditional on Gαs-mediated activation. ACs are generally classified into four different categories based on their regulatory properties. All isoforms of TM ACs are stimulated by GTP-bound Gαs. Group I includes Gβγ-inhibited and Ca^{2+}-stimulated AC1, AC3 and AC8; group II includes Gβγ-stimulated AC2, AC4 and AC7; group III includes Gαi and Ca^{2+}-inhibited and Gβγ-stimulated AC5 and AC6; and group IV includes AC9, which is not regulated by Gαi or Ca^{2+} and was initially believed to be forskolin-insensitive (Baldwin, Li, Brand, Watts, & Dessauer, 2019; Dessauer et al., 2017; Qi, Sorrentino, Medalia, & Korkhov, 2019). The topology of all ACs includes a long NT, two membrane-spanning domains, M1 and M2, each with six TM domains and two large cytoplasmic catalytic domains, C1 and C2. The C1 and C2 domains are homologous and dimerize to form the catalytic core at their interface, where ATP is converted to cAMP. When a GTP-bound Gαs subunit binds to C2, it increases the affinity between C1 and C2, promoting catalysis, while for group III, Gαi subunits bind to C1 and have the opposite effect (Baldwin et al., 2019; Dessauer et al., 2017; Sadana & Dessauer, 2009).

FRET experiments using truncated AC5 provided compelling evidence for a significant role of the NT of AC5 in scaffolding inactive, GDP-bound Gαβγ (Sadana et al., 2009). It was also shown that AC5 NT interacts with its catalytic domains to enhance Gαs- or forskolin-stimulated AC5 activity. These results support a model of Gαβγ-AC5 interactions where the AC5 NT brings the inactive heterotrimeric G

protein and AC5 catalytic core in close proximity for efficient GPCR activation (Sadana et al., 2009). A subsequent study showed that Gαβγ binds to the NT of most AC isoforms (Brand, Sadana, Malik, Smrcka, & Dessauer, 2015). Mutational analysis indicated that the Gβγ hotspot does not interact with the AC5 NT scaffolding side, although it remains necessary for conditional Gβγ-mediated stimulation of AC5. On the other hand, the Gβγ hotspot was required for both stimulating AC6 and interacting with its NT domain, indicating that Gβγ regulation of AC involves multiple binding events, and that the AC NT plays unique isotype-specific roles (Brand et al., 2015).

The recently reported structure of the active state (GTPγS-bound) of AC9 in complex with Gαs, using cryo-electron microscopy (cryo-EM) and single-molecule analysis, revealed for the first time the twelve TM domains of a TM AC isoform, as well as a helical domain that spans between the membrane and the catalytic domains of AC9 (Qi et al., 2019) (Fig. 1B). The helical domain, in fact, corresponds to a 40-residue-long cytosolic extension of TM6 and TM12 (Qi et al., 2019). This implies the necessity of dissociation of the Gα subunit from the pre-assembled GPCR-Gαβγ complex allowing the concealed domain of the Ras-GTPase domain of the Gα subunit to interact with the corresponding AC catalytic domain. Similarly to Kir3 channel-Gαβγ assembly, this suggests that ligand-induced rearrangements of the components of such complexes are associated with a separation of the Gα subunit from the Gβγ subunits, but *within the framework of the assembly*, guided by the modifications in the interactions between the Gα and Gβγ subunits with the AC NT.

3. Compartmentalization of signaling molecules at the plasma membrane by membrane nanodomains and scaffold proteins

3.1. Membrane nanodomains

Apart from pre-assembly of receptor-based signaling complexes, to achieve fidelity and maintain the efficiency of signaling across the plasma membrane, the cell uses two additional complementary mechanisms that promote co-localization or association of specific components of distinct GPCR signaling complexes: i) membrane nanodomains, such as lipid rafts and ii) scaffold proteins. Lipid rafts are defined as heterogeneous, dynamic, cholesterol- and sphingolipid-enriched membrane nanodomains (10–200 nm), which have the potential to form microscopic domains (>300 nm) upon clustering induced by protein-protein and protein-lipid interactions (Pike, 2006). Enriched hydrophobic components and phospholipids that contain saturated fatty acyl chains increase lipid packing and order and subsequent decreased fluidity (Ahmed, Brown, & London, 1997; Pike, 2003; Sezgin, Levental, Mayor, & Eggeling, 2017). Certain structural proteins enriched in lipid rafts serve as scaffolds or anchors for other proteins, including caveolins, glycosylphosphatidylinositol (GPI)-linked proteins and cortical actin, which also play an active part in the maintenance and remodeling of lipid rafts (Pike, 2003; Sezgin et al., 2017).

Since lipid rafts and other membrane nanodomains are not resolvable on a conventional optical microscope, super-resolution optical microscopy approaches such as photoactivated localization microscopy (PALM), stimulated emission depletion (STED) or stochastic optical reconstruction microscopy (STORM) have played a significant role in the visualization of lipid-mediated membrane protein clustering (for reviews, see Curthoys et al., 2015; Sezgin et al., 2017). For more dynamic measurements, the addition of SMT analysis has allowed to evaluate the diffusion of membrane molecules and relate it to models of heterogeneous organization of the membrane (Curthoys et al., 2015; Sezgin et al., 2017). Those include the membrane "hot spots" of interacting α_{2A}AR and Gαi1 described by Sungkaworn et al. (2017).

The presence within lipid rafts of a variety of membrane proteins involved in cell signaling led to the consensus that membrane nanodomains play an important role in facilitating rapid and specific signal transduction events (Simons & Toomre, 2000; Smart et al., 1999). Many GPCRs and their cognate signaling partners, including G protein subunits and AC isoforms, are enriched in lipid rafts (Ostrom, Violin, Coleman, & Insel, 2000). In the simplest scenario, lipid rafts could serve to co-localize specific GPCRs, G proteins and PM-effectors. Alternatively, rafts could contain a much larger array of signaling molecules activated when a receptor or other required molecule is recruited into the raft (Pike, 2003). There is also evidence that the targeting of GPCRs and their proximal signaling partners to lipid rafts can be cell specific (Ostrom et al., 2000; Ostrom et al., 2002). Finally, there is evidence indicating that not all lipid rafts are equivalent and may have distinctly different protein and/or lipid components that coexist in cells (Pike, 2003). However, there is no evidence for mechanisms to isolate specific GPCRs, G protein subunits and PM-effectors by segregating them into distinct lipid rafts or other membrane nanodomains and, given the large number of different signaling molecules that they can harbor, membrane nanodomains cannot be the primary means for generating signaling specificity.

3.2. Plasma membrane scaffold proteins and A kinase anchoring proteins

The most extensively studied plasma membrane scaffold proteins are those localized in and around the postsynaptic density of the glutamatergic synapse. PSD-95/Discs large/Zona occludens-1 (PDZ) domain-containing proteins (PDZ proteins) are the most abundant and PDZ domains represent the most common protein-protein interaction domain (Dunn & Ferguson, 2015). PDZ proteins directly bind to the PDZ-binding ligand motifs found in the CT region of many adhesion molecules and receptors and often contain additional modular interacting domains, which can bind to each other and to various signaling proteins encompassing both sides of the synapse (Dunn & Ferguson, 2015; Funke, Dakoji, & Bredt, 2005; Sheng & Sala, 2001; Zheng, Seabold, Horak, & Petralia, 2011). PSD-95 family PDZ domains bind to the CT of several GPCRs, including β_1AR and β_2AR (Dunn & Ferguson, 2015; He et al., 2006; Hu et al., 2000; Joiner et al., 2010; Li, Nooh, & Bahouth, 2013; Valentine & Haggie, 2011; Xiang, Devic, & Kobilka, 2002) and serotonin 5-HT_{2A} and 5-HT_{2C} receptors (Xia, Gray, Compton-Toth, & Roth, 2003). The PDZ domain of another PDZ protein family, Shank, interacts indirectly with GPCRs, including group I mGluRs through the additional scaffold protein Homer, which binds to the Shank proline-rich domain (Fig. 3A) (Bécamel et al., 2004; Dunn & Ferguson, 2015; Zheng et al., 2011). At the postsynaptic density, a key scaffolding link is determined by indirect interactions between PSD-95 and Shank proteins established by guanylate kinase-associated protein (GKAP), which refers to a family of four scaffold proteins which connect the guanylate kinase domain of PSD-95 with Shank PDZ domain (Shin et al., 2012) (Fig. 3A). Apart from specific interactions with GPCRs, direct interactions have been reported between PDZ proteins and the G protein subunit Gγ13, particularly expressed in taste and olfactory cells (Li, Benard, & Margolskee, 2006; Liu et al., 2012) and between Shank and the CT of the central pore subunit of the L-type voltage-dependent Cav1.3 Ca^{2+} channels (α1.3) (Olson et al., 2005). However, to our knowledge, there is no evidence to date for other direct interactions between PSD-95 and Shank families of PDZ proteins and G protein subunits or other PM-effectors.

A-kinase anchoring proteins (AKAPs) are a family of structural diverse proteins which share a common motif that binds to the regulatory subunits of the cyclic AMP-dependent protein kinase A (PKA) (Wong & Scott, 2004). More than 50 AKAPs have been identified which can be found in most cellular organelles, as well as the plasma membrane. The scaffolding of unique signaling elements by different types of AKAPs provides a framework for integration and modulation of distinct AC-cAMP-PKA-mediated cell signals (Dessauer, 2009). For instance, the plasma membrane AKAP5 (also known as AKAP79/150) targets not only PKA, but also PKC and protein phosphatase 2B (PP2B), to the inner face of the plasma membrane (Patriarchi, Buonarati, & Hell, 2018; Scott, Dessauer, & Taskén, 2013). In addition, AKAP5 binds to the intracellular

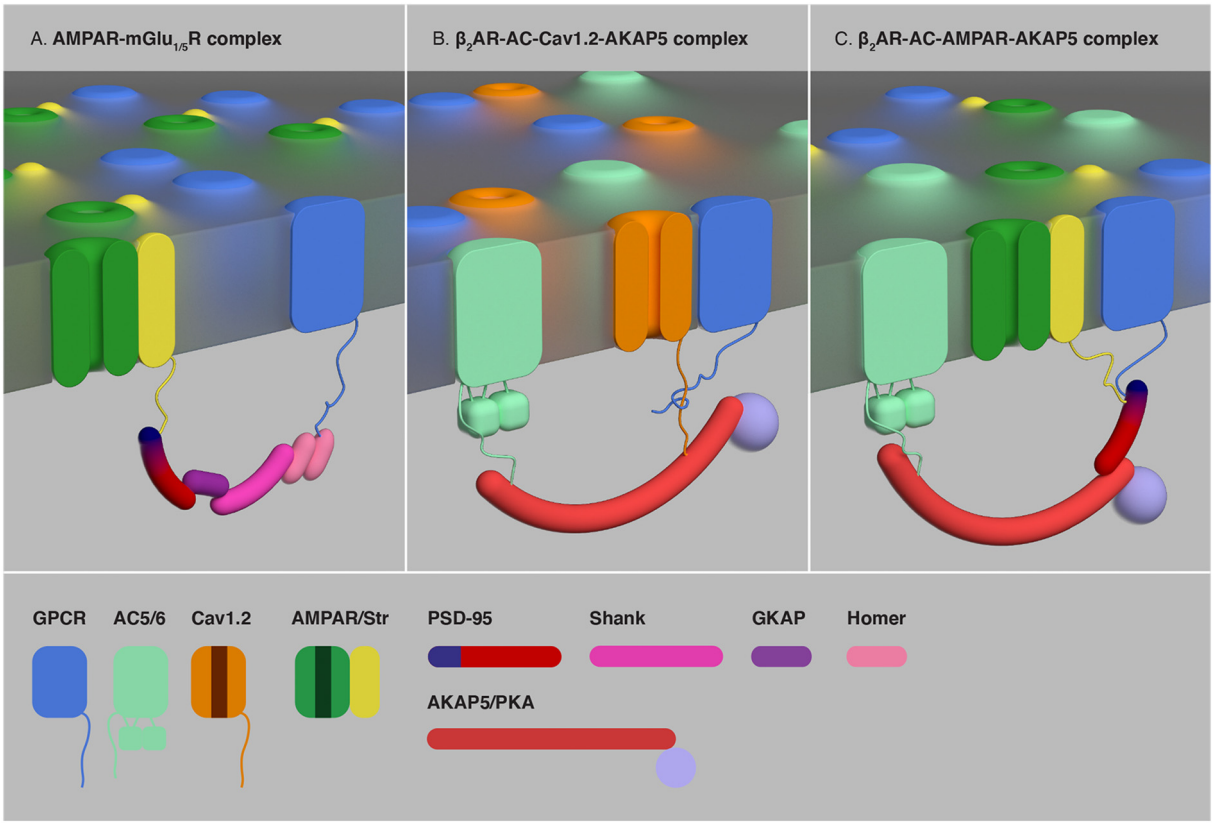

Fig. 3. Scaffolding of GPCRs, ligand-gated ion channels and PM-effectors mediated by PDZ proteins and AKAPs. (A) Complexes of ionotropic glutamate AMPAR and group I mGluRs ($mGlu_1R$ or $mGlu_5R$) established by a chain of interactions of the scaffold proteins PSD-95, GKAP, Shank and Homer. Homer directly interacts with the CT of $mGlu_1R$ or mGl_5R, while AMPAR indirectly binds to PSD-95 by oligomerization with the TM AMPAR regulatory protein stargazing (Str). (B) Complexes of β_2AR, AC and L-type voltage-dependent Cav1.2 Ca^{2+} channels mediated by AKAP5. (C) Complexes of β_2AR, AC and AMPAR mediated by AKAP5 and PSD-95. By bringing together β_2AR, AC and PKA, AKAP5-mediated complexes provide the frame for an efficient β_2AR-dependent, PKA-mediated phosphorylation of the α1.2 subunit of Cav1.2 channels (B) and the GluA1 subunit of AMPAR (C).

domains of several membrane-bound signaling molecules, including β_1AR and β_2AR (Fraser et al., 2000; Gardner, Tavalin, Goehring, Scott, & Bahouth, 2006; Li et al., 2013; Valentine & Haggie, 2011) and the PM-effectors AC5 and AC6 (Bauman et al., 2006; Efendiev et al., 2010) and L-type voltage-dependent Cav1.2 Ca^{2+} channels (Hall et al., 2007; Oliveria, Dell'Acqua, & Sather, 2007; Patriarchi et al., 2018). In addition, PSD-95 family proteins can interact through their SH3 and GK domains with a broad surface in the central region of AKAP5. PDZ proteins and AKAPs can therefore combine their interactions with different molecules to scaffold distinct macromolecular signaling complexes that include GPCRs. In this way, AKAP5 can promote the interaction of PKA with its plasma membrane-localized phosphorylation targets, either directly, such as for β_1AR and β_2AR (Fraser et al., 2000; Li et al., 2013; Valentine & Haggie, 2011), AC5 and AC6 (Bauman et al., 2006; Zhang et al., 2013) and the α1.2 subunit of Cav1.2 channels (Oliveria et al., 2007), or via binding to PSD-95 family proteins, such as for the GluA1 subunit of ionotropic glutamate AMPA receptors (AMPARs) (Colledge et al., 2000; Diering, Gustina, & Huganir, 2014; Joiner et al., 2010).

Interestingly, the β_2AR has been shown to be involved in AKAP5-dependent PKA-mediated phosphorylation of both the α1.2 subunit of Cav1.2 channels (Patriarchi et al., 2018) and the GluA1 subunit of AMPAR (Joiner et al., 2010); both events occur in dendritic spines of excitatory synapses (Patriarchi et al., 2018). Thus, at the postsynaptic density, there is evidence for the existence of different AKAP5-scaffolded macromolecular β_2AR-AC-AKAP5 complexes that include either Cav1.2 channels or AMPAR (Figs. 3B and 3C). In both complexes, the β_2AR does not seem to be directly connected to AKAP5. In the β_2AR-AC-Cav1.2-AKAP5 complex, β_2AR oligomerizes with Cav1.2 (Davare et al., 2001), which directly binds to AKAP5 (Hall et al., 2007; Oliveria et al., 2007) (Fig. 3B). In the β_2AR-AC-AMPAR-AKAP5 complex, β_2AR binds directly and GluA1 binds indirectly to PSD-95, through its oligomerization with the TM AMPAR regulatory protein stargazin; PSD-95 then couples to AKAP5 bringing AC5 and PKA to form the larger complex (Joiner et al., 2010; Zhang et al., 2013) (Fig. 3C). The AMPA-stargazin complex can also indirectly interact with group I mGluRs by means of a PSD-95-GKAP-Shank-Homer link (Dunn & Ferguson, 2015; Tu et al., 1999; Zheng et al., 2011) (Fig. 3A). In summary, there is evidence for specificity in the interactions of structurally similar GPCRs for some PDZ domains (He et al., 2006; Li et al., 2013; Valentine & Haggie, 2011) and different AKAPs (Fan, Shumay, Wang, & Malbon, 2001; Gardner et al., 2006; Valentine & Haggie, 2011). However, those involve relatively few GPCRs, which likely does not account for the multiplicity of specific combinations of GPCRs, G protein subunits and PM-effectors expressed in different cells or even the same cell. In summary, although complementary, neither membrane nanodomains nor scaffold proteins and AKAPs can generate the specificity of combinations of GPCRs, G protein subunits and PM-effectors provided by GEMMAs.

4. GPCR oligomerization

4.1. Homomers and heteromers as common functional GPCR units

Initial evidence for GPCR homomerization came from radioligand binding studies, with the demonstration of ligand binding cooperativity. This phenomenon has been recognized for many years and reproduced in many experimental preparations, including experiments with membrane extracts from multiple native tissues, mammalian transfected cells and other artificial systems, including GPCRs reconstituted in detergent micelles, liposomes (phospholipid vesicles with or without different proportions of cholesterol and integral membrane proteins) or

nanodiscs (phospholipid bilayer preparations held together by membrane scaffold proteins) (reviewed in Ferré et al., 2014, 2020). Additional evidence for GPCR homomerization came with pioneering studies with BRET and FRET techniques (reviewed in Bouvier, 2001; Milligan, 2001; Guo et al., 2017; El Khamlichi et al., 2019; Soave et al., 2020). This was followed by a large number of studies using these and other biophysical techniques in living cells, such as FRAP (Dorsch, Klotz, Engelhardt, Lohse, & Bünemann, 2009), TR-FRET (Cottet et al., 2012) and with single molecule-based methods, with the analysis of single fluorescence-labeled receptor molecules by fluorescence autocorrelation and cross-correlation spectroscopy (FCS and FCCS; Herrick-Davis, Grinde, Cowan, & Mazurkiewicz, 2013; Teichmann et al., 2014) and SMT analysis by TIRF (Calebiro et al., 2013; Scarselli et al., 2016). Single molecule-based methods in transfected cells also provided evidence for a differential degree of oligomerization and stoichiometry of GPCR oligomers *versus* monomers, depending on the type and density of GPCR (Calebiro et al., 2013; Scarselli et al., 2016; Teichmann et al., 2014). GPCR type-dependent extent of oligomerization was also recently reproduced using fluorescence-microscopy-based techniques in liposome preparations (Walsh et al., 2018).

Biophysical techniques have also been fundamental in the study of GPCR heteromers (Bourque et al., 2020; Ferré et al., 2014, 2020; Gaitonde & González-Maeso, 2017; Gomes et al., 2016; Sleno & Hébert, 2018). Furthermore, several studies also using biophysical techniques, dominant negative mutant GTPases and other complementary strategies, strongly supported the pre-assembly of not only family C (Margeta-Mitrovic, Jan, & Jan, 2000), but also family A GPCR homomers and heteromers in the ER (Décaillot, Rozenfeld, Gupta, & Devi, 2008; Dupré et al., 2006; Herrick-Davis, Weaver, Grinde, & Mazurkiewicz, 2006; Salahpour et al., 2004).

The application of techniques that selectively disrupt specific oligomeric interfaces have not only provided the means to establish the quaternary structure of GPCR oligomers, but also their emergent functional and pharmacological properties, as compared with parent monomers. Those techniques include mutating key residues, using chimeras, cysteine cross-linking techniques, or using synthetic peptides with the amino acid sequence of putative interacting domains, for instance of specific TM domains (TM peptides) (Ferré et al., 2014). Disrupting TM peptides were initially introduced to investigate the TM interface of the β_2AR homodimer in co-immunoprecipitation experiments, suggesting the involvement of TM6 (Hébert et al., 1996). More recently, the TM-peptide strategy has been applied to BiFC experiments to identify the interfaces of several GPCR homodimers and heteromers (Guitart et al., 2014; Guitart et al., 2019; Köfalvi et al., 2020; Navarro et al., 2018; Rivera-Oliver et al., 2019). With this approach, TM6 was also found to be involved in the interface of adenosine A_{2A} receptor (A_{2A}R) and dopamine D_2R (D_2R) homodimers (Navarro et al., 2018). The involvement of TM6 in D_2R homomerization was further substantiated by selective ability of peptides derived from TM6 of D_2R to completely counteract the decrease in binding of a bivalent ligand (see below) with selective picomolar affinity for the D_2R homodimer (Pulido et al., 2018). TM6 has also been shown to be part of the homomeric interface of high-resolution crystallographic structures of several GPCRs, including CXC_4R and MOR (Manglik et al., 2012; Wu et al., 2010) (Fig. 2B and C). In addition, using the TM-peptide-BiFC approach, TM6 was found to be involved in several interfaces of GPCR heteromers, including adenosine A_1R-dopamine D_1R, dopamine D_1R-D_3R and adenosine A_{2A}R-cannabinoid CB_1R heteromers (Guitart et al., 2014; Guitart et al., 2019; Köfalvi et al., 2020; Rivera-Oliver et al., 2019).

The fact that rearrangement of TM6 constitutes a critical ligand-induced conformational change that determines G protein activation and modulation of ligand affinity (Dupré et al., 2007), provides a framework for understanding allosteric communication through protomers of GPCR homomers or heteromers with a TM6 interface (Ferré et al., 2020; Navarro et al., 2018). Nevertheless, as reviewed elsewhere (Ferré et al., 2014), TM6 is not always involved in GPCR homomer or heteromer interfaces (see for instance the alternative interfaces obtained with the β_1AR and MOR crystal structures, shown in Fig. 2C and D). In some cases, the homomeric interface can change in the presence of an agonist, as shown for mGluRs. Recent results obtained with the cryo-EM structures of the full-length $mGlu_5$R shows a TM5-TM5 orientation in its inactive state, but with substantial separation between the seven TM domains. Activation of the receptor leads to a 20° rotation of each seven TM domain which results in a close contact TM6-TM6 homodimeric interface (Fig. 2A) (Koehl et al., 2019). Nevertheless, the disulfide bridge is not the only determinant of homodimerization in the inactive state of mGlu2R, which shows extensive interactions along the whole length of TM4 (Du et al., 2021). Agonist binding to the mGlu2R is then associated with a TM interface change mainly contributed by TM6 (Du et al., 2021). Finally, the same GPCR can utilize different TMs in their heteromeric interactions with different GPCRs and it can also display different homomeric interfaces when forming heteromers with different GPCRs (heteromers of homodimers; see below and Köfalvi et al., 2020).

4.2. Allosteric interactions of GPCR heteromers

There is now a long list of putative GPCR receptor heteromers (Gaitonde & González-Maeso, 2017; Gomes et al., 2016), discussion of which is beyond the scope of this review. Here, we will only summarize findings for the A_{2A}R-D_2R heteromer, a paradigmatic example that demonstrates the different types of allosteric interactions in the context of a GPCR heteromer which constitutes the core of a prototype GEMMA. Allosteric interactions constitute a common property of GPCR heteromers (Ferré et al., 2009, 2014, 2020). Importantly, these allosteric properties provide a framework for translational significance, for their potential role as therapeutic targets.

Allosterism is currently defined as "the process by which the interaction of a chemical or protein at one location on a protein or *macromolecular complex* (the allosteric site) influences the binding or function of the same or another chemical or protein at a topographically distinct site" (Smith & Milligan, 2010). Here we suggest three types of allosterism that can be identified in GPCR heteromers: type I allosterism, or allosterism between ligands, which entails the ability of an orthosteric ligand of one protomer in the GPCR heteromer to modify the affinity or efficacy of an orthosteric ligand of the other molecularly distinct protomer (Fig. 4A); type II allosterism, or ligand-independent allosterism, where heteromerization *per se* modifies the properties of a specific orthosteric ligand for one of the protomers of the GPCR heteromer, independent of ligand binding to the other molecularly distinct protomer (Fig. 4B); and type III allosterism, or allosterism through the effector, where the effector (PM-effector of a GEMMA) acts as an interface for the interactions between orthosteric ligands of the molecularly distinct protomers of a GPCR heteromer (Fig. 4C). Type I and III allosterisms also include the possibility of transactivation or transinhibition, by which an orthosteric ligand of one of the protomers leads to an increase or decrease in the intrinsic activity of the other molecularly different protomer.

The most described type I allosteric interaction in GPCR heteromers involves an agonist or an antagonist of one of the protomers that inhibit the affinity or efficacy of an agonist of the other molecularly distinct protomer. Such interactions are usually referred as 'negative crosstalk' or 'cross-antagonism', respectively, and their molecular mechanisms are beginning to be understood (Ferré et al., 2014, 2020) (Fig. 5). Since the first report in 1991 (Ferré, von Euler, Johansson, Fredholm, & Fuxe, 1991), A_{2A}R ligands have been repeatedly reported to antagonistically modulate the binding properties of D_2R ligands in membrane preparations from transfected mammalian cells and native tissues (reviewed in Ferré et al., 2018). Demonstration of the dependence of these ligand interactions on the integrity of the A_{2A}R-D_2R heteromer (with disruptive synthetic peptides or mutations of key residues of the heteromeric interface) (Bonaventura et al., 2015;

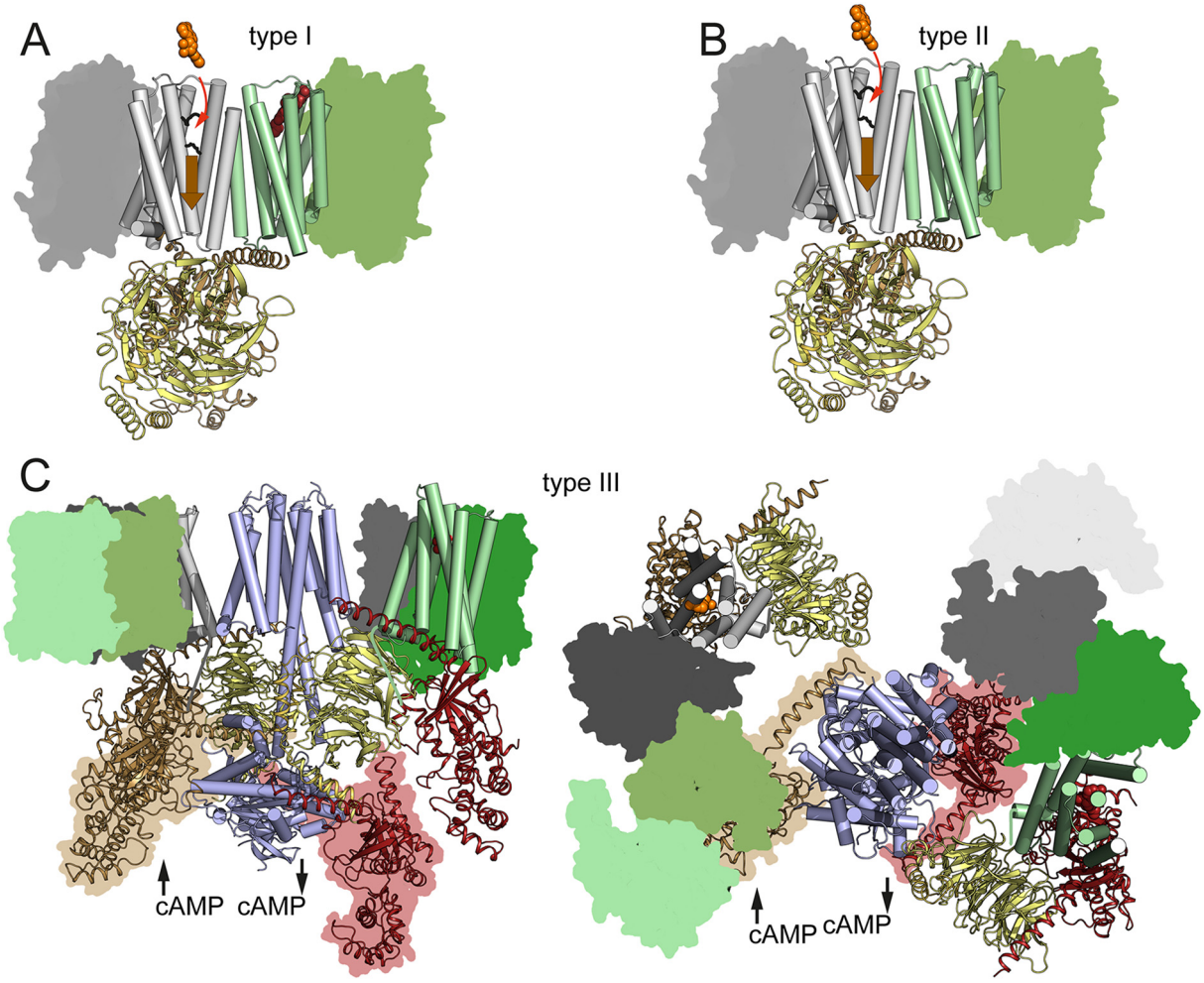

Fig. 4. Allosterism in GPCR heteromers. (A) Type I allosterism, in which a ligand (red spheres) binding to one protomer (green cylinders) in the GPCR heteromer can modify the affinity (ligand binding depicted by the red arrow) or the efficacy (receptor activation depicted by the wide orange arrow) of another ligand (orange spheres) binding to the partner receptor (gray cylinders) via their TM helices (see Fig. 5). (B) Type II allosterism, or ligand-independent allosterism, in which the affinity or efficacy of a ligand (orange spheres) for a GPCR (gray cylinders) can be modified just by heteromerization with a molecularly different GPCR (green cylinders). (C) Lateral view (left) and extracellular view (right) of a computer model of a GEMMA including two heterotetramers composed of two different GPCR homomers (one represented by white cylinders and grey surfaces and the other represented by green cylinders and surfaces), AC (light blue cylinders; based on the cryo-EM structure of AC9; Qi et al., 2019), Gs (brown for Gαs and yellow for Gβγ) and Gi (red for Gαi and yellow for Gβγ). This type of GEMMA, based on the $A_{2A}R$-D_2R-AC5 GEMMA described in Navarro et al. (2018), can explain the ability of a Gi-coupled GPCR to counteract AC activation mediated by a Gs-coupled GPCR (type III allosterism). The proposed contact between the CT domain of the receptor and the Gβ subunit in the inactive state (Tsai et al., 2019) is shown by a colored line. To facilitate visualization of all protomers of the GPCR oligomers, one of the protomers is represented by cylinders and the other protomers are represented as colored surfaces (with different shades of the same color for the same GPCR). Also for the purpose of simplification, only both possible positions of the Gαs and Gαi subunits are shown: in their Gβγ-associated and receptor-bound state (without surface) and in their Gβγ-dissociated and AC-bound state (with surface). The agonist-induced dissociation of Gαs and Gαi from their respective Gβγ takes place within the framework of the GEMMA. As explained in the text, the active state of this type of GEMMA, where both Gαs and Gαi subunits are bound to the respective catalytic domains of AC, is associated with a rearrangement of Gβγ subunits and a change in the interfaces of the heterotetramers with AC.

Borroto-Escuela et al., 2010; Borroto-Escuela et al., 2010) implied a type I allosterism in the $A_{2A}R$-D_2R heteromer. This allosterism determines the therapeutic effects of $A_{2A}R$ antagonists in Parkinson's disease, which target $A_{2A}R$-D_2R heteromers localized in striatopallidal neurons (for recent review, see Ferré et al., 2018). Significantly, the selective $A_{2A}R$ antagonist istradefylline, in combination with L-DOPA, is the first non-dopaminergic drug approved by FDA for the treatment of Parkinson's Disease (Chen & Cunha, 2020).

Type II allosterism has been described in the $A_{2A}R$-D_2R heteromer involving a specific $A_{2A}R$ antagonist (Fig. 4B). When comparing the binding properties of several selective orthosteric $A_{2A}R$ antagonists (including istradefylline) in mammalian transfected cells, only one compound, SCH442416, showed a selective low affinity for $A_{2A}R$ when co-expressed with D_2R, as compared with $A_{2A}R$ when expressed alone or co-expressed with A_1R (Orrú et al., 2011). The specific behavior of SCH442416 in the $A_{2A}R$-D_2R heteromer could also be demonstrated in the mouse striatum, but not in mice with conditional genetic deletion of striatal D_2R (Ferré et al., 2018). These results represented proof of concept for the possibility of using GPCR heteromers as targets for specific ligands. Thus, it could be demonstrated that SCH442416 is a preferential presynaptic striatal $A_{2A}R$ antagonist, because of the preferential pre-synaptic localization of A_1R-$A_{2A}R$ heteromers in striatal glutamatergic terminals, as compared with the post-synaptic striatal localization of $A_{2A}R$-D_2R heteromers in striatopallidal neurons (Ferré et al., 2018). The preferential striatal presynaptic effect of SCH442416 was suggested to determine its ability to reduce cannabinoid self-administration in monkeys and its possible application to cannabinoid use disorder (Justinová, Redhi, Goldberg, & Ferré, 2014).

Apart from the ability of $A_{2A}R$ ligands to allosterically modulate D_2R ligands, a reciprocal canonical Gs-Gi protein-dependent antagonistic interaction was discovered early on, where D_2R agonists, by activating Gi-coupled D_2R, could oppose Gs-coupled, $A_{2A}R$-mediated activation of AC-cAMP-PKA signaling (Kull et al., 1999). The balance between antagonistic allosteric and Gs-Gi-AC interactions determines the

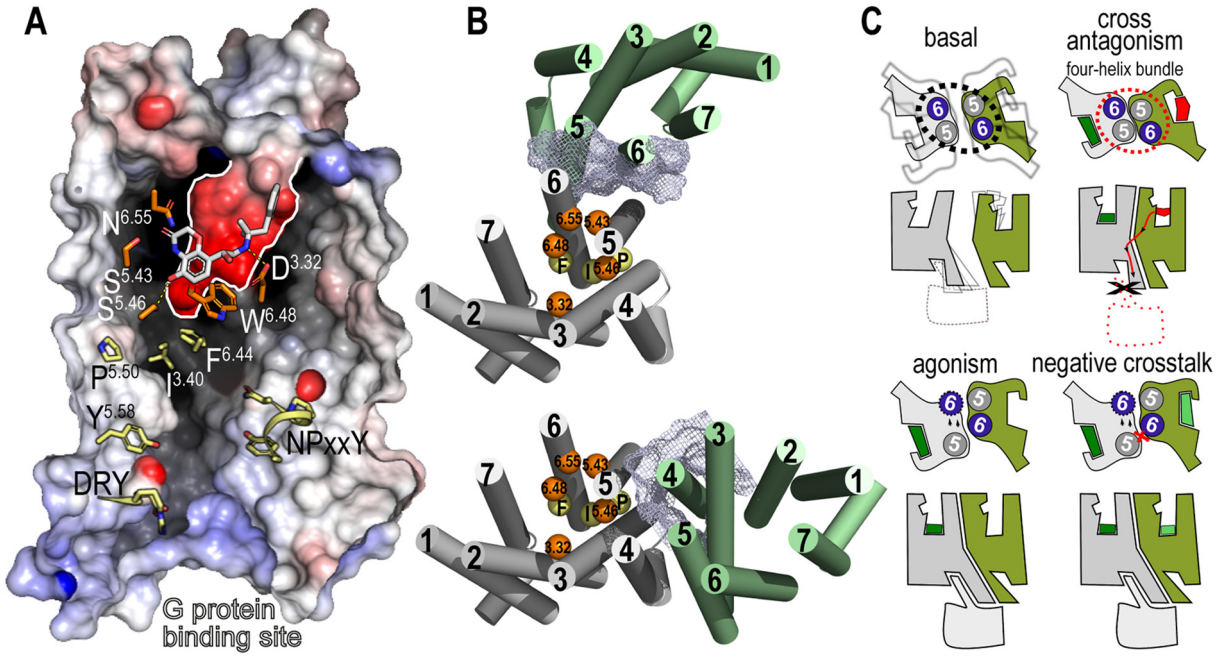

Fig. 5. Mechanisms of type I allosterism in GPCR oligomers heteromers. (A) Cross-section through a prototypical class A GPCR, highlighting the agonist (white sticks), the amino acids of the orthosteric site (delineated by a white line) involved in ligand binding (orange), the conserved PIF motif (yellow), and the NPxxY and DRY motifs and Y, (yellow) that transmit the signal from the PIF motif (transmission switch) to the G protein site (Weis & Kobilka, 2018). (B) Position of these amino acids involved in ligand binding (orange spheres) and signal transmission (yellow) in models of GPCR dimers via TM5 and TM6 (top) and TM4 and TM5 and intracellular loop 2 (bottom). Clearly, a ligand-bound (type I allosterism) or ligand-free (type II allosterism) or orphan GPCR (green cylinders) can modify the affinity (orange spheres) or efficacy (yellow spheres) of the partner receptor (gray) via the protein-protein interface (blue mesh). (C) GPCRs are dynamic proteins that adopt, in a ligand-free form, a number of conformations (shades in gray) that not only involve the extracellular and intracellular sites (Weis & Kobilka, 2018), but also a potential TM oligomeric interface. An inverse agonist (red polygon) binding to one of the protomers of a GPCR heteromer triggers a high surface complementarity between TM5 and TM6 of the two molecularly different protomers, via the four-helix bundle (dashed red circle) (Manglik et al., 2012), which blocks the opening of the intracellular cavity for G protein binding at the other protomer (cross-antagonism) (Viñals et al., 2015). An agonist (green polygon) binding to one of the protomers triggers the outward movement of TM 6 (see arrows), opening the intracellular cavity for G protein binding (Rasmussen et al., 2011), whereas the TM5 and TM6 heteromeric interface impedes simultaneous agonist-induced movement of both TM6 (negative crosstalk) due to a steric clash (red cross) (Guinart et al., 2020).

final output of $A_{2A}R$-D_2R heteromer-dependent signaling in the striatopallidal neuron (Ferré et al., 2018). Importantly, the Gs-Gi-AC interaction depends on the integrity of the $A_{2A}R$-D_2R heteromer, since the Gi-dependent regulation of Gs-mediated AC activation could also be disrupted by TM peptides that disrupt the heteromer (Navarro et al., 2018). This discovery represents a significant shift in our understanding of crosstalk between GPCR signaling pathways, since such interactions at the effector level were previously understood as related to independent changes in second messenger levels (Zoli et al., 1993). GPCR heteromer-dependent Gs-Gi-AC interactions have been demonstrated for several other GPCR heteromers, including A_1R-D_1R, D_1R-D_3R and $A_{2A}R$-CB_1R heteromers (Guitart et al., 2019; Köfalvi et al., 2020; Rivera-Oliver et al., 2019). In addition to GPCR heteromerization, the Gs-Gi-AC interaction suggests simultaneous respective interaction of Gαs and Gαi subunits with the C2 and C1 catalytic domains of the same molecule of AC, more specifically with group III ACs, the AC5 and AC6 isoforms. This depends on a pseudo-symmetrical arrangement of the C1 and C2 domains (Dessauer, Tesmer, Sprang, & Gilman, 1998). Another mechanism for antagonistic interactions between Gs- and Gi-coupled receptors can also occur with group I ACs, AC1, AC3 and AC8, involving interactions with Gβγ rather than the Gαi subunits of an activated Gi protein (Steiner, Saya, Schallmach, Simonds, & Vogel, 2006; Taussig, Quarmby, & Gilman, 1993).

The need for heteromerization of a Gs-coupled and a Gi-coupled GPCR, for oligomerization of those GPCRs with AC, and for the simultaneous binding of Gs and Gi to the C2 and C1 catalytic domains of AC, strongly suggests that a canonical antagonistic Gs-Gi-AC interaction should most commonly occur within GEMMAs that include a GPCR heteromer, Gs and Gi proteins and the PM-effector AC. Following the definition of allosterism, we can consider the antagonistic Gs-Gi-AC interaction as an allosteric interaction of a GPCR heteromer, as part of a macromolecular complex that includes a PM-effector, which we labeled type III allosterism (Fig. 4C).

4.3. GPCR heterotetramers

The relatively large size of the heterotrimeric G protein makes simultaneous binding of more than one G protein to a GPCR unlikely. Therefore, the pentameric structure consisting of a GPCR homodimer and one heterotrimeric G protein is likely the primary functional unit, as initially supported by studies with the detergent-solubilized leukotriene B_4 receptor BLT1 (BLT_1R) (Banères & Parello, 2003). The minimal functional quaternary structure of a GPCR heteromer able to sustain a Gs-Gi-AC interaction would then be tetrameric, including Gs- and Gi-coupled homomers, or a Gs-Gi-coupled heterotetramer, as previously hypothesized (Ferré, 2015). With computational analysis of information provided from TM-peptide-BiFC experiments and several reported crystallographic structures of GPCRs alone or bound to G protein partners, it was possible to infer an optimal quaternary structure of the $A_{2A}R$-D_2R heterotetramer: a linear structure with the two internal protomers involved in the heteromer interaction (via symmetrical TM4-TM5/TM5-TM4 interfaces) and the two external protomers of each homodimer (with a TM6/TM6 interface) coupled to their respective G proteins (Navarro et al., 2018) (Fig. 6A). Since AC5 and Golf are the major striatal AC and Gs family isoforms (Schwindinger et al., 2003, 2010; Hervé, 2011; Xie et al., 2015), evidence for Gαβγ-AC5 pre-assembly (see above) suggests that Gα(olf)βγ can indirectly pre-assemble AC5 with the $A_{2A}R$-D_2R heterotetramer, forming a $A_{2A}R$-D_2R-AC5 GEMMA, providing an example of an allosteric machine that integrates types I, II and III allosteric mechanisms (Fig. 6A).

Following the definition of GEMMA, the same study (Navarro et al., 2018) also provides, to our knowledge, the first non-equivocal demonstration of a ligand-dependent rearrangement of a complex including a GPCR or a GPCR oligomer and PM-effector. Using TM-peptide-BiFC experiments it could be demonstrated that specific TM domains of the $A_{2A}R$ and the D_2R directly interact with the TM domains of AC5, and that those interfaces changed upon the binding of agonists (Navarro et al., 2018). It could also be shown that disruption of the GPCRs-AC5 interfaces also disrupted the Gs-Gi-AC type III allosteric interaction within the $A_{2A}R$-D_2R-AC5 GEMMA (Navarro et al., 2018). The quaternary structure of the $A_{2A}R$-D_2R-AC5 GEMMA obtained by computer modeling suggested that two molecules of AC5 would be needed to interact simultaneously with $A_{2A}R$ and D_2R protomers in a single heterotetramer, while two heterotetramers are needed to allow the simultaneous interaction of $A_{2A}R$ and D_2R with a single AC5 molecule, to allow full Gs-Gi-AC interactions (Fig. 6A). According to this model, the $A_{2A}R$-D_2R heterotetramer is the more rigid part of the complex and the model also predicts the possible extension of this basic unit into a higher-order GEMMA (Navarro et al., 2018) (Fig. 6A).

The $mGlu_2R$-5-$HT_{2A}R$ heteromer is another example of a well-studied GPCR heteromer with evidence of both type I and II allosteric mechanisms. It is localized in cortical glutamatergic pyramidal neurons and represents an important target for the hallucinogenic effects of 5-$HT_{2A}R$ agonists and the antipsychotic effect of both 5-$HT_{2A}R$ antagonists/inverse agonists and $mGlu_2R$ agonists (Baki et al., 2016; Fribourg et al., 2011; González-Maeso et al., 2008; Moreno et al., 2012; Moreno et al., 2016). Its quaternary structure also seems to be tetrameric, constituted of $mGlu_2R$ and 5-$HT_{2A}R$ homodimers respectively coupled to Gi and Gq, with a clear involvement of TM4 in the heteromeric interface (Moreno et al., 2016; Shah, Toneatti, Gaitonde, Shin, & González-Maeso, 2020). In transfected mammalian cells expressing $mGlu_2R$-5-$HT_{2A}R$ heteromers, a high-efficacy orthosteric $mGlu_2R$ agonist promoted Gq protein-mediated increases in intracellular Ca^{2+} concentration in the absence of 5-$HT_{2A}R$ ligands (Moreno et al., 2016). By using different $mGlu_2R$-$mGlu_3R$ chimeras and mutant receptors unable to couple to their respective G proteins, it was shown that this pharmacological response involved $mGlu_2R$ agonist-induced transactivation of 5-$HT_{2A}R$-Gq-mediated signaling in the $mGlu_2R$-5-$HT_{2A}R$ heteromer (Moreno et al., 2016). It was also shown that transactivation required not only obligatory homomerization of $mGlu_2R$ and the Gq protein coupling to the 5-$HT_{2A}R$, but also Gαi coupling to the $mGlu_2R$ (Moreno et al., 2016).

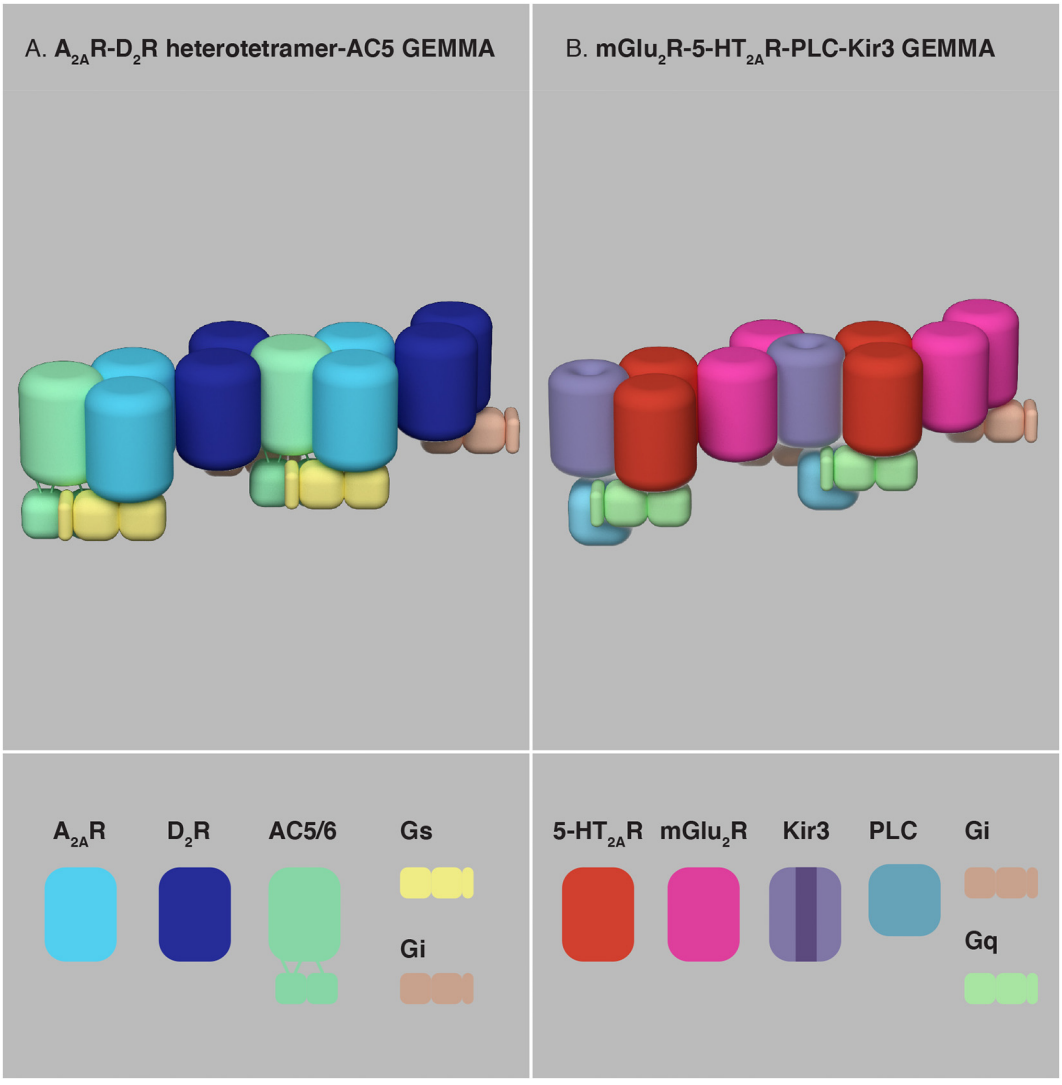

Fig. 6. The $A_{2A}R$-D_2R heterotetramer-AC5 and $mGlu_2R$-5-$HT_{2A}R$-PLC-Kir3 GEMMAs. (A) The $A_{2A}R$-D_2R heterotetramer-AC5 GEMMA, constituted by $A_{2A}R$ homomers coupled to Gs, D_2R homomers coupled to Gi and AC5, can be considered as a prototype of GEMMA that integrates canonical antagonistic Gs-Gi-AC interactions. (B) The $mGlu_2R$-5-$HT_{2A}R$-PLC-Kir3 GEMMA, constituted by $mGlu_2R$ homomers coupled to Gi, 5-$HT_{2A}R$ homomers coupled to Gq, PLC and Kir3, can be considered as a prototype of GEMMA that integrates canonical antagonistic Gi-Gq-Kir3 interactions.

Kir3 channels were also identified in transfected mammalian cells as a common PM-effector for both Gi and Gq protein-mediated signaling of the $mGlu_2R$-5-$HT_{2A}R$ heteromer (Baki et al., 2016). As mentioned above, Kir3 channels are activated by direct interactions with Gβγ subunits following activation of Gi-coupled receptors (Dascal & Kahanovitch, 2015; Logothetis et al., 1987; Logothetis et al., 2015; Riven et al., 2006). However, stimulation of Kir3 channels is critically dependent on phosphatidylinositol 4,5-bisphosphate (PIP_2) (Huang, Feng, & Hilgemann, 1998; Sui, Petit-Jacques, & Logothetis, 1998). A canonical effect of Gq activation is PLC activation, which leads to PIP_2 hydrolysis and opposes Gβγ-mediated activation (Logothetis et al., 2015). When added to evidence for pre-assembly of Gαβγ with Kir3 (see above) and with PLC (Smrcka & Fisher, 2019; Yuan et al., 2007), we suggest the existence of a $mGlu_2R$-5-$HT_{2A}R$-PLC-Kir3 GEMMA (Fig. 6B). This GEMMA would represent an allosteric machine that controls neuronal excitability by integrating antagonistic effects of serotonin and glutamate on Kir3 activity. As with the $A_{2A}R$-D_2R-AC5 GEMMA, it might be predicted that the $mGlu_2R$-5-$HT_{2A}R$-PLC-Kir3 GEMMA could be the basic unit of a higher-order GEMMA (Fig. 6B).

4.4. Oligomerization of GPCRs with orphan GPCRs, truncated GPCRs and other TM proteins

From the 800 GPCR genes identified, more than 100 GPCRs remain orphans, including 86 family A GPCRs (Davenport et al., 2013; Ngo et al., 2016). There is evidence indicating that some GPCRs will not be deorphanized because they may not have a natural ligand. Rather, they can oligomerize with other GPCRs and change their functional and pharmacological properties via allosteric interactions. This concept was originally suggested from studies with melatonin receptors and the orphan receptor GPR50 (Levoye, Dam, Ayoub, Guillaume, & Jockers, 2006). The melatonin receptor subfamily consists of MT_1R and MT_2R, with melatonin as their natural ligand, and the orphan receptor GPR50, which does not bind to melatonin or any other known endogenous ligand to date (Cecon, Oishi, & Jockers, 2018). MT_1R and MT_2R signal preferentially through Gi/o protein coupling, while GPR50 does not couple to G proteins and has a characteristic very long CT (~300 amino acids) (Oishi, Cecon, & Jockers, 2018). Several studies support the ability of the melatonin receptor family to oligomerize, including heteromerization of GPR50 with MT_1R and MT_2R (Levoye et al., 2006; Oishi et al., 2018) (Fig. 7A). Experiments using BRET in transfected mammalian cells suggested that MT_1R and MT_2R form constitutive homomers as well as MT_1R-MT_2R heteromers (Ayoub et al., 2002). In transfected cells the propensity for homomer and heteromer formation differs between both receptor subtypes, with a lower propensity of MT_2R to form homomers as compared with the MT_1R and a higher propensity of forming MT_1R-MT_2R heteromers, suggesting that the MT_2R preferentially exists as a heteromeric complex with MT_1R (Ayoub, Levoye, Delagrange, & Jockers, 2004). In fact, by inducing expression of tagged MT_1R and MT_2R in genetically modified mice, it was possible to demonstrate the existence of MT_1R-MT_2R complexes in the retina, where they mediate the effect of melatonin on light sensitivity of rod photoreceptors (Baba et al., 2013). Significantly, in transfected mammalian cells, MT_1R-MT_2R heteromers seem to preferentially couple to Gq and promote Gq-PLC-PKC signaling, a primary signaling pathway involved in the functional effects of melatonin in rod photoreceptors (Baba et al., 2013) (Fig. 7A). GPR50 promotes a decreased ability of melatonin to bind and signal though the MT_1R, which depends on the ability of the long GPR50 CT to alter the pre-coupling or rearrangement of G proteins to MT_1R in the MT_1R-GPR50 heteromer (Levoye, Dam, Ayoub, Guillaume, Couturier, et al., 2006; Oishi et al., 2018) (Fig. 7A).

Some GPCRs exist in different splice variants, which can be expressed by the same cell and, therefore potentially oligomerize leading to distinct functional outcomes. Some of these variants represent truncated versions of receptors, missing several TM domains, often resulting in their inability to bind to endogenous ligands *per* se (Wise, 2012). Truncated GPCR isoforms may be expressed at the plasma membrane and constitute components of GEMMAs. Those include D_3nf and GHS_{1b}, truncated splice variants of dopamine D_3 receptor (D_3R) and ghrelin GHS_{1a} receptor $GHS_{1a}R$, respectively. Both isoforms lack TM6 and TM7 domains, do not bind ligands or promote signaling themselves, but oligomerize with their respective functional isoforms (Chow et al., 2012; Elmhurst, Xie, O'Dowd, & George, 2000; Karpa, Lin, Kabbani, & Levenson, 2000; Leung et al., 2007; Mary et al., 2013; Navarro et al., 2016). Although under some experimental conditions both isoforms seem able to promote intracellular localization of their functional GPCR partners, there is evidence for the ability of D_3R-D_3nf and $GHS_{1a}R$-GHS_{1b} oligomers to localize to the plasma membrane, where the truncated partner exerts ligand-independent negative allosteric modulation (type II allosterism) of D_3R and $GHS_{1a}R$ ligands (Elmhurst et al., 2000; Mary et al., 2013; Navarro et al., 2016). In addition, BRET and signaling experiments in transfected mammalian cells indicate that $GHS_{1a}R$-$GHS_{1b}R$ can be part of a GEMMA that includes D_1R, in which ghrelin, instead of promoting its preferred $GHS_{1a}R$-dependent Gq-PLC signaling, activates a Gs-AC signaling, which is opposed by D_1R antagonists (Navarro et al., 2016) (Fig. 7B).

Receptor activity-modifying proteins (RAMPs) are an example of membrane-spanning accessory proteins that can alter the function of GPCRs. A small family of three proteins (RAMP1, RAMP2 and RAMP3) has substantial capacity for introducing functional diversity by directly interacting with GPCRs (Hay & Pioszak, 2016; Serafin, Harris, Nielsen, Mackie, & Caron, 2020). Structurally, RAMPs comprise a single TM domain with a long extracellular NT (90–100 amino acids) and a short intracellular CT (9 amino acids). The interactions of RAMPs with the family B GPCR calcitonin-like receptor (CLR) and the calcitonin receptor (CTR) are the most extensively studied and provide a picture of the broad influence that a single TM-domain protein can have when interacting with a GPCR (Fig. 7C). RAMPs are required to chaperone CLR to the cell surface, where RAMP-CLR complexes act as receptors for the peptide hormones calcitonin gene-related peptide (CGRP), adrenomedullin (AM) or adrenomedullin 2/intermedin (AM2), depending on the subtype of RAMP co-expressed (McLatchie et al., 1998; Serafin et al., 2020). Thus, the RAMP subtype determines which endogenous ligands preferentially bind to CLR and CLR-RAMP1, CLR-RAMP2 and CLR-RAMP3 constitute CGRP, AM and AM2 receptors, respectively (McLatchie et al., 1998; Poyner et al., 2002; Serafin et al., 2020) (Fig. 7C). CTR is the closest relative to CLR and it can reach the cell surface in the absence of RAMP, but CTR can drive RAMP translocation to the cell surface and CTR pharmacology is altered in the presence of RAMP, which increases the affinity of the endocrine hormone amylin (AMY), such that three amylin receptor subtypes (AMY_{1-3} receptors) result from each respective RAMP-CTR complex (Poyner et al., 2002).

5. Targeting specific GEMMAs

5.1. Specific GPCR ligands for individual GEMMAs

The main properties of GPCR oligomers (allosterism), orphan receptors, truncated GPCRs and other membrane-localized GPCR-associated proteins (such as RAMPs) can potentially provide significant functional and pharmacological properties to the GEMMAs that include them. The remarkable varied influence of TM RAMPs in modifying the properties of ligand binding to CLR and CTR, provides a clear demonstration for exploiting GEMMAs as targets for drug discovery. Namely, that different components of GEMMAs directly interacting with a particular GPCR can potentially modify the effects of specific ligands for this GPCR. Another reason is that localization of the same GPCR with additional components in different cells likely creates a new and unique GEMMA that can be targeted in a way distinct from the same receptor in a different complex. This raises the possibility of obtaining ligands with selectivity for the GPCRs in distinct GEMMAs which could mediate desired

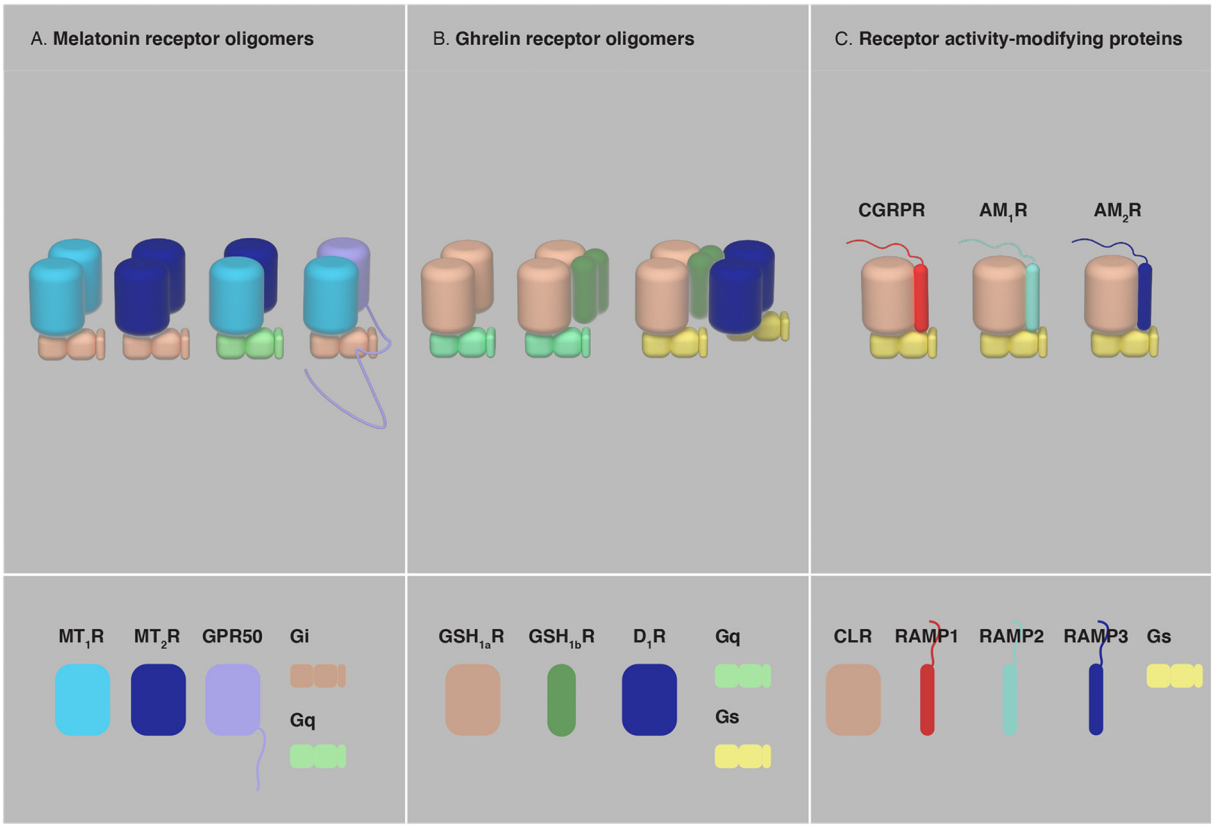

Fig. 7. Oligomerization of GPCRs with orphan GPCRs, truncated GPCRs and RAMPs. (A) Homomers and heteromers of MT_1R, MT_2R and GPR50. MT_2R preferentially exists as a heteromeric complex with MT_1R and MT_1R-MT_2R heteromerization drives a change in the preference of G protein subtype coupling, from Gi to Gq. The long CT of GPR50 significantly alters MT_1R-Gi protein signaling in the MT_1R-GPR50 heteromer (see text). (B) GHS1aR forms homomers and oligomerizes with its non-functional truncated isoform $GHS_{1b}R$ to form homomers and heteromers. One of the properties of the $GHS_{1a}R$-$GHS_{1b}R$ oligomers is the facilitation of an additional interaction with the D_1R, leading to a change in the preference of G protein subtype coupling of $GHS_{1a}R$, from Gq to Gs. (C) The single TM domain proteins RAMP1, RAMP2 and RAMP3 associate with CLR and form the Gs protein-coupled receptors CGRP, AM_1R and AM_2R, respectively, determining the ligands potentially binding to CLR.

therapeutic effects of the putative ligand, while avoiding targeting GEMMAs that mediate the adverse side effects.

Type II allosterism of GPCR heteromers and the changes it brings to pharmacological properties is noted with the example of $A_{2A}R$ heteromers discussed above. A significant clinically translational type II allosterism of GPCR heteromers for substance use disorders has been generated by the study of MOR heteromers. Since the pioneering studies from Lakshmi Devi's research group, MOR oligomerization has been the focus of a significant amount of experimental work, providing substantial evidence for MOR-δ opioid receptor (DOR) heteromerization and their role in the analgesic effects of opioids (Fujita, Gomes, & Devi, 2015). Although the initially proposed localization of MOR-DOR heteromers in the nociceptive sensory neurons of the dorsal root ganglia and dorsal horn of spinal cord was challenged (Scherrer et al., 2009), subsequent studies provided strong confirmatory evidence (Tiwari et al., 2020; Wang et al., 2010; Yekkirala et al., 2012). More recently, a major population of MOR localized in the mesencephalon, in the ventral tegmental area, has been shown to form heteromers with galanin Gal_1 receptors (Gal_1R) and to mediate the ability of opioids to activate the dopaminergic system and therefore abuse liability (Cai et al., 2019; Moreno et al., 2017). These studies support the rationale of selectively targeting MOR-DOR heteromers in the search for new effective analgesic drugs devoid of addictive properties.

Different approaches have been shown to be effective pre-clinically, including high-throughput screening of small-molecule libraries in cells expressing MOR-DOR heteromers, leading to the discovery of CYM51010, which showed a significant increase in its potency and efficacy to promote G protein activation mediated by the MOR in heteromers with DOR (Gomes et al., 2013). CYM51010 exhibited a potent antinociceptive activity with reduced tolerance potential compared to morphine in mice (Gomes et al., 2013) and, more recently, CYM51010 was also found effective in a rat model of neuropathic pain (Tiwari et al., 2020). Because of structural similarities of CYM51010 with carfentanyl, structure-activity relationship (SAR) analysis was used to develop several carfentanyl derivatives (Faouzi et al., 2020). One of the derivatives, MP135, showed even higher relative efficacy at the MOR-DOR heteromer versus MOR alone, as compared with CYM51010 (Faouzi et al., 2020). As expected, in rodents, it produced pronounced analgesic effects, but still showed rewarding properties and was readily self-administered (Faouzi et al., 2020). Although not tested yet, MP135 might also exhibit significant potency and efficacy at the MOR-Gal1R heteromer, which determines the dopaminergic/euphoric effects of opioids (Cai et al., 2019).

The effect of morphine, methadone and fentanyl, representatives of the three principal opioid structures, has been separately analyzed for both MOR-DOR and MOR-Gal_1R heteromers (Cai et al., 2019; Yekkirala et al., 2012; Yekkirala, Kalyuzhny, & Portoghese, 2010). The research group of Philip Portoghese found evidence for a significantly higher efficacy of the three opioids for MOR-DOR versus MOR alone (Yekkirala et al., 2010; Yekkirala et al., 2012). In a more recent study, for the first time, differential pharmacodynamic effects were observed for methadone versus morphine or fentanyl, a significant decrease in potency which depended on heteromerization of MOR with Gal_1R (Cai et al., 2019). As predicted from the predominant role of MOR-Gal_1R heteromers in the modulation of the dopaminergic system,

methadone was a much weaker activator of the dopaminergic system as compared with morphine and fentanyl and, it could be demonstrated clinically that it was also much weaker at producing euphoric effects (Cai et al., 2019). Elucidation of the molecular mechanisms of this type II allosterism in the MOR-Gal_1R heteromer should facilitate discovery of methadone-like compounds, with strong analgesic and reduced addictive properties. An additional challenge is to determine specific components of the MOR-containing GEMMAs responsible for the other major unwanted effects of opioids, such as respiratory depression and constipation.

5.2. Simultaneous targeting of different components of specific GEMMAs

Another approach to obtain opioid analgesia with low abuse liability would be using effective MOR-DOR ligands while selectively blocking the unwanted MOR signaling via the MOR-Gal_1R heteromer. This could be achieved by administering Gal_1R ligands, based on recently described type I allosterism in the MOR-Gal_1R heteromer, by which those ligands significantly reduce the affinity and efficacy of MOR agonists (Cai et al., 2019). Gal_1R are also localized in the spinal cord, where previous studies indicate their activation produces analgesic effects synergistic to those of opioids (Hua et al., 2004). Since the only available selective and potent Gal_1R ligands are galanin-derived peptides (Freimann, Kurrikoff, & Langel, 2015), the discovery of small molecules with significant brain penetrability targeting Gal_1R could provide an important new approach for the treatment of pain. Gal_1R agonists could be co-administered with lower therapeutic doses of MOR agonists, promoting analgesia and significantly reducing side effects, particularly the dopaminergic effects mediated by MOR-Gal_1R heteromers.

Type I allosterism in GPCR heteromers could then be used as a therapeutic strategy to target specific GEMMAs, either by specifically increasing the therapeutic effects or decreasing the unwanted effects of GPCR ligands. This is illustrated by the increase in the therapeutic index of L-DOPA by A_{2A}R antagonists in Parkinson's Disease (see above), which decreases the effective dose of L-DOPA required and, therefore, both short- and long-term side effects (Chen & Cunha, 2020).

A further step in drug discovery would be to obtain bivalent compounds that can simultaneously bind both protomers in the GPCR heteromer, defined as single chemical entities composed of two pharmacophore units covalently linked by an appropriate spacer. These ligands are designed to interact simultaneously within a GPCR homo- or heterooligomer, to enhance affinity and subtype selectivity and, in case of a GPCR heteromer, promote type I allosteric modulation (Daniels et al., 2005; Pulido et al., 2018; Soriano et al., 2009). Although it could be argued that the pharmacologic effects of bivalent ligands are related to the separate binding of each pharmacophore to two different GPCR oligomers, true simultaneous bivalent binding to the same GPCR oligomer can be demonstrated by dependence on the integrity of the GPCR oligomer. For instance, in a recent study, the dramatic increase of affinity of a D_2R ligand (from nanomolar to picomolar affinity) obtained when two molecules of the same ligand were attached using an appropriate spacer length was reversed upon disruption of the D_2R homomeric interface (see above) (Pulido et al., 2018).

Portoghese's group pioneered studies with bivalent ligands and opioid receptor heteromers. Their studies revealed additional implications with potential therapeutic importance. Thus, MOR-DOR bivalent ligands with specific length spacers and a MOR agonist and a DOR antagonist as pharmacophores produced analgesia without the development of tolerance (Daniels et al., 2005). This effect was attributed to the bivalent ligand-induced stabilization ("bridging") of the MOR-DOR heteromer with concomitant blockade of DOR signaling (Daniels et al., 2005), which facilitates internalization of the MOR-DOR heteromer and, therefore, subsequent development of tolerance to MOR agonist-mediated analgesia (Gomes et al., 2013).

Finally, apart from simultaneously targeting two different GPCRs, there are also possibilities of simultaneously targeting a GPCR protomer and another component of a specific GEMMA. This is best exemplified by the recent discovery of the specific activator of Kir3.1-Kir3.2 channels, GAT1508 (Xu et al., 2020). Kir3 channels are widely expressed in the brain and in the heart, although the localization of the Kir3.1-Kir3.4 channel is predominantly in the heart, whereas Kir3.1-Kir3.2 channels are more restricted to the central nervous system (CNS) (Dascal & Kahanovitch, 2015). GAT1508 represents the first specific activator of Kir3.1-Kir3.2 channels, making them CNS-selective. The mechanism of GAT1508-induced channel activation is related to strengthening channel-PIP_2 interactions (Xu et al., 2020). Importantly, at lower concentrations that are insufficient to produce significant activation of Kir3.1-Kir3.2 channels, GAT1508 significantly potentiated the ligand-induced activating effect of an associated GPCR (such as baclofen-induced activation of the $GABA_B$ receptor), suggesting synergism in the allosteric effects of GAT1508 and Gβγ subunits in fostering channel-PIP_2 interactions (Xu et al., 2020). This provides further selectivity of the effects of GAT1508 in the brain and evidence was provided by a specific effect of the baclofen-GAT1508 in the basolateral amygdala, with possible translational implications for post-traumatic stress disorder (Xu et al., 2020).

5.3. G protein subtype-dependent functional selectivity

The properties of a GPCR ligand can also depend on specific pre-assembled G protein subtypes, as recently shown in BRET-based experiments. This also extends the notion of functional selectivity, where a selective ligand regulates a subset G protein-dependent or independent signaling to biased G protein subtype-dependent signaling (Violin & Lefkowitz, 2007). For instance, the effect of several D_1R ligands were compared in their ability to modify BRET in cells transfected with D_1R and Gαs or Gαolf fused to BRET chromophores. The D_1R agonist dihydrexidine (DHX) was found to be a full D_1R agonist with Gαs but a weak partial agonist with Gαolf (Yano et al., 2018). It was then expected that DHX would be more effective in the cerebral cortex, where Gαs is the predominant isoform, than in the striatum, where Gαolf predominates. In fact, with electrophysiological and behavioral experiments, it was demonstrated that DHX had greater efficacy in cortical *versus* striatal neurons. It was then suggested that DHX or other functionally Gαs subtype-functionally selective D_1R agonists could be used as pro-cognitive drugs with reduced extrapyramidal side effects (Yano et al., 2018).

The same phenomenon had been previously observed for other GPCRs and G protein subtypes, although it was not conceptualized as functional selectivity. For instance, the selective CB_1R agonist WIN55,212-2 facilitated Gq-mediated signaling, while other classes of CB_1R agonists (including Δ^9-tetrahydrocannabinol and the endocannabinoids 2-arachidonoylglycerol and methanandamide) preferentially signaled through Gi/o proteins (Lauckner, Hille, & Mackie, 2005). Another example is the selective ability of the A_1R agonist 5′-*N*-cyclopentyl-carboxyamidoadenosine, as compared with a series of adenosine analogues, to facilitate Gs-mediated signaling, although A_1R preferentially signals through Gi/o proteins (Cordeaux, Ijzerman, & Hill, 2004). In those studies, the results were interpreted as dependent on a ligand-induced stabilization of a GPCR conformation that favored preferential coupling with a specific G protein subtype. This interpretation would be in line with collision-coupling. The GEMMAs concept provides another interpretation, the ability of ligands to differentially activate GPCRs pre-assembled with specific G protein subtypes.

6. Concluding remarks and future directions

The idea that GPCRs can serve as signaling platforms for assembly of macromolecular complexes of G proteins and other related signaling proteins to provide highly efficient and spatially restricted signaling events, with no requirement for G protein subunit dissociation and lateral diffusion within the membrane, has been recurrently considered

over the years (Neubig, 1994; Levitzki & Klein, 2002; Rebois & Hébert, 2003; Dupré et al., 2006, 2009; Hepler, 2014; Navarro et al., 2018; Sleno and Hébert, 2019). The GEMMA concept restricts the components of these macromolecular assemblies to GPCRs, G proteins, PM-effectors and other associated transmembrane proteins putatively pre-assembled prior to receptor activation by agonists, although we cannot ignore the idea that these core components of transmembrane signaling complexes will interact with additional cytosolic elements that will be recruited by the assembly upon GPCR activation, including GRKs and arrestins.

The GEMMA concept represents a divergent mechanism from collision coupling and both signaling modes are likely used by the cell, depending on the GPCRs and effectors involved and their need for providing more efficient and restricted signaling (GEMMA) or less efficient but more amplified signaling (collision-coupling mode). There is also evidence suggesting that these two poles are part of a spectrum of signaling mechanisms. Mixed models have been proposed where some of the components of the macromolecular membrane complex would still dissociate (Levitzki, 1986; Neubig, 1994). Such models can still explain the catalytic behavior of GPCRs observed in some cell systems, such as the amplification of signaling seen when one receptor activates numerous AC molecules, a phenomenon that, in fact, led to the formulation of the collision-coupling model (Tolkovsky & Levitzki, 1978a). On the other hand, amplification of signaling may not be expected with a ligand-induced rearrangement without dissociation of the GEMMA components. Nevertheless, as discussed, agonist-induced rearrangement of the G protein subunits of GEMMAs including the PM-effectors Kir3 or AC might necessarily imply limited dissociation between G protein subunits within the framework of the macromolecular assembly.

Many questions about GEMMAs remain to be answered, such as whether they can form higher-order macromolecular structures, as here suggested for the A_{2A}R-D_2R-AC5 GEMMA and the mGlu_2R-5-HT_{2A}R-PLC-Kir3 GEMMA. In that case, it will be important to resolve which basic units are pre-assembled in the ER and what determines and modulates the assembly and disassembly of the final macromolecular complex. It will also be important to establish the fate of GEMMAs and whether they can internalize as intact signaling complexes. In fact, internalization of GEMMAs would provide a very plausible framework for the now well established endosomal signaling mediated by internalized GPCRs (Calebiro, Nikolaev, Persani, & Lohse, 2010; Ferrandon et al., 2009; von Zastrow & Sorkin, 2021), although current evidence suggests that β_2AR and AC may traffic independently and potentially reform in endosomes (Lazar et al., 2020). Furthermore, it will be important to determine what is the role of arrestins and other cytosolic proteins that interact with GPCRs, G proteins and PM-effectors in the modulation of internalization and both G protein-dependent and independent signaling of GEMMAs.

Apart from their relevance as functional signaling units, the GEMMA concept will be important for further exploration of GPCR pharmacology. The localization in specific cellular environments, together with the unique properties of each GEMMA, determined by the large variety of potential components, should promote new directions in the search for new therapeutic agents. Screens should focus on molecules with the ability to promote changes in the function of a specific GEMMA, using single agents or combination of molecules with selectivity for the different components in particular GEMMAs.

Our primary recommendation is the further discovery and characterization of GEMMAs in their native environments with the identification of their primary components. This can only be achieved by combining the same approaches used for identification of GPCR oligomers with proteomic, biochemical or antibody-based techniques, such as proximity ligation or AlphaLISA assays (see, for instance, Moreno-Delgado et al., 2020; Valle-León et al., 2021). Such studies should be performed in parallel with reconstitution of the putative GEMMA in an artificial system, which should allow determination of interaction sites and interfaces between its putative components, using, for instance, the TM-peptide strategy. This should allow the establishment of unique pharmacological properties of the GEMMA that could be used for its identification in native tissue and potentially targeted for drug discovery.

We should also aim at elucidating the precise molecular structure of GEMMAs in the presence and absence of ligands that stabilize active and inactive states, which should already be possible with cryo-EM techniques. Dynamic transitions between active and inactive states could be pursued by molecular dynamics simulations. The elucidation of the molecular structures of GEMMAs and their dynamic interrelationships should provide a deeper understanding of the intricate mechanisms of membrane-delimited GPCR-mediated cell signaling and an evaluation of their potential as therapeutic targets.

Declaration of Competing Interest

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