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GPCR – G protein complexes – the fundamental signaling assembly

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Abstract

G protein coupled receptors (GPCR) constitute the largest group of cell surface receptors that transmit various signals across biological membranes through the binding and activation of heterotrimeric G proteins, which amplify the signal and activate downstream effectors leading to the biological responses. Thus, the first critical step in this signaling cascade is the interaction between receptor and its cognate G protein. Understanding this critical event at the molecular level is of high importance because abnormal function of GPCRs is associated with many diseases. Thus, these receptors are targets for drug development.

Keywords

G protein-coupled receptors; rhodopsin-transducin complex; binding interface; structural asymmetry

1. Structures of GPCRs and G proteins

GPCRs

G protein coupled receptors all share the same structural organization of seven transmembrane spanning domains connected by three short extracellular (ECL) and intracellular (ICL) loops. The GPCR family is divided into four subfamilies according to their sequence homology and pharmacological properties: rhodopsin-like family A, secretin-like family B, metabotropic/glutamate family C and frizzled receptors. The first crystal structure of any GPCR was solved from family A: inactive, dark state bovine rhodopsin in 2000. To date it is the only structure of GPCR in its native, genetically non-modified conformation (Palczewski et al. 2000). It took seven more years and a number of technological advances to solve a structure of another family A GPCR. In 2007 the first crystal structure of a GPCR activated by diffusible ligand, the β_2 adrenergic receptor (β_2AR), was solved (Cherezov et al. 2007; Rasmussen et al. 2007). Since then structures of many other family A GPCRs bound to different ligands, in different activation states have been determined. These revealed the overall structural architecture of these receptors is highly homologous despite differences in the sequence and ligand sensitivity. All of them feature general seven transmembrane architecture with cytoplasmic helix H8 laying parallel to the plasma membrane. They all contain conserved structural elements important in receptor activation: a) a disulfide bond linking ECL2 with TM III; b) the D[E]RY motif

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Conflict of interest
The author declares that she has no conflict of interst.

located on top of helix TM III that is a part of a hydrogen bonding network called the TM III - TM VI ionic lock; c) the WxP motif in helix VI; and d) the NPxxY motif in helix VII. The length of connecting loops differs among specific GPCRs which may be attributed to the binding specificity of G proteins. The largest diversity concerns the extracellular part of the receptor, specifically, the ligand binding region that usually is located in a cavity between TM helices. However, some GPCRs bind their diffusible ligands in their amino terminal region that form a distinct domain with a more open conformation.

Despite large diversity of the GPCR family, these membrane receptors interact with a relatively small number of G proteins.

Heterotrimeric G proteins

Heterotrimeric G proteins belong to a superfamily of regulatory GTP hydrolyses that are divided into main four classes based on the sequence similarity of Gα: namely $G\alpha_s$, $G\alpha_i$/$G\alpha_o$, $G\alpha_q$/$G\alpha_{11}$, and $G\alpha_{12}$/$G\alpha_{13}$. Each G protein is composed of three subunits α, β and γ with a nucleotide binding pocket located in the α subunit. In the GDP-bound conformation, nucleotide release is inhibited by the βγ dimer, which stabilizes an inactive conformation of the αβγ heterotrimer. G proteins are activated by binding to activated GPCR which causes rapid GDP→GTP nucleotide exchange. Binding of GTP results in dissociation of the GPCR-G-protein complex as well as the G-protein into free, active subunits of Gα and Gβγ that subsequently activate different downstream effectors leading to specific physiological responses (Fig. 1). The crystal structures of the heterotrimeric G proteins G_t and G_i revealed the overall topology to be highly similar and likely typical for all heterotrimeric G proteins (Sondek et al. 1996; Lambright et al. 1996; Noel et al. 1993). The Gα subunit consists of two conserved domains: a GTP-ase domain and helical domain. The GTP-ase domain mediates hydrolysis of GTP and provides the binding surface for the βγ dimer, GPCR, and effector proteins. Three flexible loops known as switch I, II and III were identified in the crystal structure of the GTP-ase domain. These loops most likely play a significant role in GDP/GTP exchange during activation process because they display the most significant conformational differences in the active and inactive structures of Gα. The Gα helical domain is composed of six α-helices that cover up the nucleotide binding pocket located within GTP-ase domain, retaining the nucleotide in the core of protein. In all classes, the N-terminus of the Gα subunit is posttranslationally modified by fatty acid palmitate. The only exceptions are $G_t\alpha$ and $G\alpha_i$ which are modified by myristoyl group. The Gβ subunit features a seven-bladed β propeller composed of seven W40 repeats and it forms strong affinity functional unit with Gγ. The βγ complex is assembled from a repertoire of five β subunits and twelve γ subunits (Wettschureck and Offermanns 2005). All of the Gγ subunits are modified at their C-terminus by either a farnesyl or a gerenylgeranyl moiety. Although Gα and Gγ do not form direct contacts, their modified N- and C-termini are in close proximity and are responsible for membrane association.

2. Mechanism of GPCR and G protein activation

The first insights into GPCR activation were gained after the crystal structures of the photoactivated rhodopsin intermediate (Salom et al. 2006), opsin (Standfuss et al. 2007), and the complex of opsin (Scheerer et al. 2008) with the C-terminal peptide of $G_t\alpha$ (Park et al. 2008) were obtained. The differences between dark state rhodopsin and its photoactivated intermediate were mainly associated with reorganization of the receptor's cytoplasmic surface within ECL2, ECL3, and the C-terminus. However, the structure of opsin revealed both rearrangement of extracellular loops ECL2 and ECL3 and the movement of TM V and TM VI which broke the ionic lock, accompanied by a previously predicted ~ 6 Å outward movement of helix VI (Fig. 2). This reorganization at the cytoplasmic surface opens a groove enabling the binding of heterotimeric G protein, where the C-terminus of $G_t\alpha$

interacts with Arg135 from the D[E]RY motif. Smaller energy movements of TM VII lead to distortion of the NPxxY motif. Similar overall structural rearrangements have been observed in structures of other GPCRs crystallized in their activated states (Rasmussen et al. 2011b; Rasmussen et al. 2011a; Lebon et al. 2011; Xu et al. 2011; White et al. 2012), indicating a common activation mechanism for the whole family of GPCRs. Availability of GPCR crystal structures in different activation stages agrees with the existence of multiple conformations in the signaling cycle and structural dynamics of these receptors. Moreover, a multistage GPCR activation mechanism has been proposed by solid state H_2 NMR studies of rhodopsin which found that retinal isomerization initiates fluctuation of the helices and cytoplasmic loops during transition to its active Meta II state (Struts et al. 2011). Changes in receptor flexibility upon activation were derived also from HDX exchange studies that suggested structural rigidity of dark state rhodopsin, its relaxation upon transition to Meta II and a following increase in structural rigidity upon binding of G_t (Orban et al. 2012). Conserved internal water molecules were identified in the core of the GPCR structures and their involvement in receptor activation and mediation of the signal across biological membranes has been proposed as well (Angel et al. 2009). Monitoring of dynamic changes and reorganization of these waters by radiolytic footprintng in combination with mass spectrometry revealed significant, water mediated modification of residues localized close to the chromophore binding pocket and cytoplasmic face of the receptor. Striking differences in the modification rates between dark state rhodopsin, rhodopsin activated by light, and rhodopsin in complex with G_t could be attributed to the differences in the arrangement of internal waters in all three rhodopsin states confirming their unique structural properties. Superposition of the dark state rhodopsin with its light activated conformer demonstrated a different arrangement of these internal water molecules. Moreover the importance of this internal water network was emphasized by the M257Y rhodopsin mutant that causes disruption of water mediating bonding network, affecting chromophore induced helix movement and resulting in receptor constitutive activity (Deupi et al. 2012). Therefore, along with changes in the protein structure stimulated by ligand, internal water molecules, involved in local changes can act as a prosthetic group by transmitting the signal from the extracellular part of the receptor to its intracellular, cytoplasmic surface that must be rearranged to allow G protein binding and activation.

Dating from the initial crystal structure of a G protein in 1993 (Noel et al. 1993), it was recognized that during activation release of nucleotide would probably require an opening between the GTP-ase and helical domain to form a nucleotide-free complex. But no experimental evidence supporting this idea was available. Flexibility of the Gα subunit upon binding of activated GPCR and possible opening of the interface between Gα and Gβγ required for GDP release was reported in 2006 by several groups (Gales et al. 2006; Van Eps et al. 2006; Oldham et al. 2006; Abdulaev et al. 2006). However, a true separation of the GTP-ase and helical domain was not demonstrated for the next several years. In 2011, Van Eps et al. by using site-directed spin labeling (SDSL) and double electron-electron resonance spectroscopy (DEER) demonstrated a significant increase ~ 20 Å in the distance between nitroxide probes positioned on the GTP-ase and helical domains during formation of the nucleotide free complex between $G\alpha_i$ and the activated GPCR, rhodopsin (Van Eps et al. 2011). Soon thereafter, a ~130 Å sideways rotation of the GTP-ase domain relative to the helical domain was found in the crystal structure of the nucleotide-free T4L-β_2AR-G_s-nanobody complex, confirming the necessity of movement of these two domains to allow nucleotide exchange (Rasmussen et al. 2011b). However, the domain opening detected in the last crystal structure was unexpectedly large, possibly enforced by crystallization conditions and/or bound nanobodies. Therefore, this is rather unlikely to occur under physiological conditions where rapid G protein turnover is essential for fast biological responses. At the same time, at low resolution EM-derived 3D map of the photoactivated rhodopsin-G_t (Rho*-G_t) complex in the free-nucleotide state revealed its overall shape (Fig.

3). To achieve a 'best fitting' of the G_t structure into the obtained density map, an opening of Gα subunit at the nucleotide binding cleft by ~ 30 Å was applied, providing another demonstration of Gα flexibility and necessity of domain movements to allow GDP to GTP exchange (Fig. 3a) and (Jastrzebska et al. 2011). Moreover, HDX experiments confirmed that both nucleotide-free G_t and G_s in complexes with their respective receptors exhibited a significantly greater deuterium uptake (Chung et al. 2011; Orban et al. 2012)

3. Binding interface in GPCR-G protein complexes

A first glimpse of the contacting surfaces in the activated GPCR-G protein complex was delivered by using site directed cysteine mutagenesis in combination with crosslinking. These studies demonstrated that intracellular loops ICL2 and ICL3, and helix H8 in photoactivated rhodopsin, and the C-terminus of $G_t\alpha$ (residues 340–350) are important for the assembly of this complex (Itoh et al. 2001; Cai et al. 2001). Besides the α subunit, the C-terminus of $G_t\gamma$ also interacts with the receptor's helix H8 (Kisselev and Downs 2006). Because these two regions of G_t are located quite far apart, it is unlikely that a single receptor could provide a surface large enough to satisfy an interaction with both structural regions. Therefore, it is highly possible that a rhodopsin dimer provides an interacting platform to accommodate the G_t heterotrimer.

GPCR activation initiates structural rearrangements of ICL2 and ICL3 and opens the crevice at the cytoplasmic surface. This enables G protein binding and has been found in the crystal structures of both opsin and Meta II-like rhodopsin with bound C-terminal peptide of $G_t\alpha$, confirming early predictions (Scheerer et al. 2008; Choe et al. 2011). However, the most detailed molecular determinants involved in the binding of GPCR and G protein were revealed by solving the crystal structure of the T4L-β_2AR-G_s-nanobody complex (Rasmussen et al. 2011b). Interestingly, extensive interaction was found only between the receptor and GTP-ase domain of the α subunit. This interface is formed between ICL2, TM V and TM VI of the receptor and the C-terminal α5-helix, αN-b1 junction, the top of β3-strand and α4-helix of the G_s GTP-ase domain. Conformational changes in TM V and TM VI are smaller than in the Meta II-G_t peptide structure, and the position of the α5-helix of G_t is tilted ~ 30° relative to the position of the same region of G_s, which may be a consequence of more extensive contacts in the case of intact G protein. Surprisingly, no interaction between receptor and either the beta or gamma subunits have been detected raising the possibility that these interactions could be observed only in the dimerized receptor, whereas in the structure of the T4L-β_2AR-G_s-nanobody complex just a single receptor is bound to the G protein. Nevertheless, the β_2AR has a propensity to self-associate upon reconstitution into lipid vesicles, and it also forms oligomers in the native membranes (Fung et al. 2009; Vobornik et al. 2009). Therefore, its monomeric form found in the crystal structure of the complex with G_s could result from its selection during protein purification or due to crystallization conditions.

4. GPCR dimerization

All GPCRs including rhodopsin were initially assumed to be single receptors freely diffusing in the lipid bilayer that, upon activation, bind to heterotrimeric G proteins in a 1:1 stoichiometry. However, this view was challenged by growing evidence that GPCRs exist and function as dimers and/or higher oligomers. For most GPCRs evidence for oligomerization comes from indirect immunoprecipitation, crosslinking, fluorescence, or bioluminescence energy transfer experiments. However, the development of high resolution imaging techniques allowed direct visualization of some GPCRs in their native membranes. For example, existence of densely packed rows of rhodopsin dimers in native disk membranes (Fotiadis et al. 2004, 2003; Filipek et al. 2004; Liang et al. 2003) was revealed

by atomic force microscopy (AFM). Interestingly, these oligomers could be extracted as separate dimers or entire rows of dimers in mild detergents (Jastrzebska et al. 2006). Clusters of β_1AR and β_2AR receptor molecules on the surface of cardiac myocytes were visualized by near field scanning optical microscopy (NSOM) (Ianoul et al. 2005; Vobornik et al. 2009). Dimers of CXCR4 were detected by using tetrametylrhodamine-labeled bivalent ligands conjugated with a short, rigid linker on the surface of cancerous cells expressing elevated levels of this receptor (Tanaka et al. 2010). Dimers of vasopressin receptors were found on the surface of mammary glands of lactating mice by using fluorescently labeled specific ligands and fluorescence resonance energy transfer (FRET) assays (Albizu et al. 2010). Therefore, the simplified view of GPCR as a single mobile signaling molecule must be revised.

Several crystal structures provide more evidence of GPCRs dimerization. The dimer of rhodopsin, although in an anti-parallel orientation, was detected in the first crystal structure of dark state bovine rhodopsin (Palczewski et al. 2000). Its dimeric organization was later observed in 2D crystals of rhodopsin Meta I (Ruprecht et al. 2004). These dimers were in a 'head to head' orientation, the same as in the membrane with the interface formed by TM I of each molecule. The short H8 helix located by the C-terminus formed a close interaction between subunits as well. Similar dimers were observed in 2D crystals of bovine and frog dark state rhodopsin (Schertler et al. 1993; Schertler and Hargrave 1995). The crystal structure of photoactivated rhodopsin also implicated a parallel dimer with contacts between TM I and H8 as well as TM II (Salom et al. 2006). Similar dimers in a 'membrane-like' orientation were also reported in more recently solved crystals of other family A GPCRs, including CXCR4, μ-opioid, κ-opioid receptors (Audet and Bouvier 2012) and β1-arenergic receptor (Huang et al. 2013). Significant protein-protein interfaces were found between TM V and TM VI in CXCR4 (Wu et al. 2010) and between TM II and H8 in κ-opioid receptor (Wu et al. 2012). In the μ-opioid receptor, contact surfaces were seen between TM V and TM VI and also between TM I, TM II and H8 (Manglik et al. 2012). Two contacting surfaces were identified also in β_1AR where one employs TM I and H8 and the second involves TM IV, TM V, ICL2 and ECL2 (Huang et al. 2013). The presence of two distinct interfaces found on opposite sides of the μ-opioid and β_1AR receptor raises the possibility of forming higher-ordered oligomers in a fashion similar to the packing observed by AFM for rhodopsin oligomers in native disk membranes (Fotiadis et al. 2003). Thus a 'native-like' dimeric organization observed in crystals of multiple family A GPCRs strongly suggests that dimerization of these receptors is the rule rather than an exception. Moreover, structures of GPCRs, G proteins and other GPCR-interacting proteins are highly homologous, implying conservation of their mechanisms. If oligomers serve as functional units for some class A GPCRs (Rivero-Muller et al. 2010), they likely do the same for most if not all GPCRs.

5. Binding stoichiometry of GPCR-G protein complexes

Cumulative evidence indicates that GPCRs can traffic and signal as multimeric complexes (Milligan 2010; Gurevich and Gurevich 2008a, b). The requirement of quaternary structure for cell surface delivery was initially noted for the family C GPCR, $GABA_B$ but further studies demonstrated that this also applies to family A GPCRs. For example, dimerization of β_2AR was found to be obligatory for cell surface targeting and mutations at the dimeric interface led to receptor arrest in the ER (Salahpour et al. 2004; Kobayashi et al. 2009). GPCR dimerization also influences G protein binding. Even though activation of a single receptor suffices to trigger a physiological response *in vitro* (Baylor et al. 1979; Whorton et al. 2007; Ernst et al. 2007; Whorton et al. 2008; Bayburt et al. 2007), such experiments do not provide information about the actual stoichiometry of the receptor-G protein complex in the native membrane. In fact, binding of the receptor dimer to a single G protein and formation of pentameric complexes are supported by reconstitution experiments involving

the leukotriene B4 receptor BLT1, serotonin 5HT4 receptor, and dopamine D2 receptor, and metabotropic receptor- each with their respective G proteins (Baneres and Parello 2003; Han et al. 2009; El Moustaine et al. 2012). Convincing evidence supporting the functional significance of a GPCR dimer in signal mediation can also be derived from *in vivo* studies on the luteinizing hormone receptor, which revealed intermolecular cooperation between a ligand-binding deficient GPCR and G protein activation deficient mutants (Rivero-Muller et al. 2010). Moreover, studies of rhodopsin lateral diffusion in photoreceptor membranes (Govardovskii et al. 2009) as well as Monte Carlo simulations of stochastic encounters between photoactivated rhodopsin and G_t in disk membranes (Dell'Orco and Schmidt 2008), also proposed the binding of one G_t to at least two receptors. These findings greatly support the concept of a GPCR dimer as the smallest functional unit, organized in higher ordered structures, to provide a kinetic advantage in rapidly responding to an appropriate stimulus (Dell'Orco and Schmidt 2008; Dell'Orco et al. 2009). Using transmission electron microscopy (TEM) and single particle reconstruction, we visualized and solved the three dimensional envelope of the rhodopsin-G_t complex which contained one rhodopsin dimer and one G_t heterotrimer (Jastrzebska et al. 2011) and (Fig. 3). The composition of this has been recently confirmed by labeling of rhodopsin molecules within the rhodopsin-G_t complex with succinylated concanavalin A (sConA), a lectin that binds mannose, one of two sugars decorating the rhodopsin structure (Jastrzebska et al. 2013b). In contrast, a crystal structure of T4L-β_2AR complexed to G_s and nanobody provides the most detailed insight into GPCR-G protein interaction and was solved with a 1:1 binding stoichiometry (Rasmussen et al. 2011b). Interestingly, the crystal structure of T4L-β_2AR-G_s-nanobody fits quite well into the EM density of the rhodopsin-G_t complex with the exception of the extremely opened conformation of helical domain and a significant unoccupied space corresponding to the position of the second rhodopsin (Fig. 3b). Despite substantial progress, the role of GPCR oligomerization in the molecular mechanism of activation is still not fully understood and these apparently conflicting observations could result from the dynamic nature of receptor oligomers that form and dissociate depending on the receptor cell cycle (Hern et al. 2010; Lambert 2010).

6. Functional and structural asymmetry of the GPCR dimer

Convincing evidence indicates that GPCR dimerization and activation are intricately associated. The functional consequences of GPCR dimerization are revealed by studies showing inter-communication between two protomers in the dimer. This communication can occur at the level of: i) agonist binding (Ilien et al. 2009), ii) ligand-induced allosteric modulation between receptor protomers (Vilardaga et al. 2008; Han et al. 2009; Albizu et al. 2010), and/or iii) binding of GPCR-associated proteins such as specific G proteins (Baneres and Parello 2003; Damian et al. 2006; Pellissier et al. 2011). Such cross-communication supports the asymmetric nature of GPCR dimers wherein each protomer performs a specific task. Analysis of the relationship between subunits of the leukotriene B4 receptor BLT1 revealed that ligand-induced activation of a single receptor can trigger G protein activation but that bound G protein restricts conformational changes in the second receptor protomer; thus only one subunit in the dimer could achieve the fully activated state (Damian et al. 2006). Moreover, dissociation of the dimer with TM VI peptide resulted in lower stability of the receptor-G protein complex. Similarly, activation of one protomer in the serotonin type 4 receptor (5-HT4R) dimer sufficed to stimulate G protein signaling but the binding affinity was two times greater when both protomers were activated (Pellissier et al. 2011). From this perspective, the dimeric assembly of GPCRs is required for more efficient coupling of different regions of the G protein to the receptor, thereby assuring rapid signal transduction (Jastrzebska et al. 2006). Dimer functional asymmetry agrees with studies of trans-complementation between a receptor defective in ligand binding and a receptor defective in G protein activation demonstrated for family A GPCRs, both *in vitro* (Pellissier et al. 2011)

and, more importantly, *in vivo* (Rivero-Muller et al. 2010; Moepps et al. 2006; Struts et al. 2011). Recently structural asymmetry of the rhodopsin dimer was identified in the pentameric complex formed with heterotrimeric G protein (Jastrzebska et al. 2013a). Each rhodopsin molecule was found to be in a different activation state contributing unevenly to the rhodopsin-transducin complex (Fig. 4). Only one rhodopsin monomer in this complex, most likely this interacting with the C-terminus of $G_t\alpha$, is stabilized in its Meta II conformation, whereas the second decays eventually to opsin and free retinal. Interestingly, this observation greatly supports the results obtained for the rhodopsin-arrestin complex, where again 50% of the receptor was stabilized in the Meta II state and the other 50% evolved toward an opsin conformation, suggesting a dimeric nature of the receptor bound to arrestin (Sommer et al. 2012). This opsin monomer within the rhodopsin-G_t complex can be regenerated *in vitro* with the iso-chromophore 9-*cis*-retinal. Subsequently, all-*trans*-retinal can be removed from the Meta II rhodopsin monomer by treatment with the strong nucleophile NH_2OH, without influencing complex stability. Regeneration of such a derived complex with 11-*cis*-retinal results in formation of the rhodopsin-G_t complex containing one rhodopsin in a 9-*cis*-retinal bound conformation and the other surprisingly in an all-*trans*-retinal bound conformation (Fig. 4a). This result indicates that G_t stabilizes one rhodopsin in Meta II like state even after chromophore release and increases its structural rigidity. Thus, the receptor forces isomerization of unstable 11-*cis*-retinal to the all-*trans* configuration in the rigorously defined space of the chromophore binding pocket. This observation is in agreement with HDX exchange studies of rhodopsin-G_t that demonstrated tightening of the more relaxed Meta II structure upon G_t binding (Orban et al. 2012). Moreover treatment of rhodopsin-G_t with NH_2OH can produce a chromophore depleted complex, which unlike free opsin (Heck et al. 2003) can take up all-*trans*-retinal and reform a Schiff base linkage (Jastrzebska et al. 2013a). Interestingly, only 50% of the complex can be regenerated with all-*trans*-retinal (Fig. 4b). Taking into account that precoupled rhodopsin-G_t complexes in the native membranes most likely exist in the dark and the fact that only one rhodopsin molecule provides an interface for G_t binding the possibility of asymmetric rhodopsin activation arises (Cangiano and Dell'Orco 2013). In such a scenario, photoexcitation of chromophore and its isomerization in the rhodopsin monomer, which does not physically interact with the G protein, induces conformational changes in the precoupled rhodopsin monomer leading to structural evolution toward the Meta II state (Neri et al. 2010). Thus, this tandem mechanism could be critical to prevent loss of energy, enhance the efficiency of receptor activation, and possibly its desensitization. Receptor inactivation is initiated by its phosphorylation with a specific kinase. Cross-phosphorylation between activated and non-activated receptors has been reported for rhodopsin expressed in mouse rod outer segments (Shi et al. 2005) and for chemokine CCR5 and C5a receptors as well (Huttenrauch et al. 2005). Following phosphorylation, the receptor is inactivated by coupling with visual arrestin. The bimodal structure of arrestin, which displays two clefts on its receptor binding surface with the size of individual rhodopsin molecules (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Palczewski et al. 2000) suggests that a rhodopsin dimer could be accommodated by a single arrestin (Modzelewska et al. 2006).

7. Future directions

Constant improvements and great development and of crystallization technologies applicable to membrane binding proteins has already resulted in more than 75 structures of 18 different family A GPCRs in different activation states. Solving the crystal structure of β_2AR in complex with its cognate G protein delivered an eagerly awaited "holy grail", greatly furthering our understanding of the molecular mechanism of GPCRs/G protein activation. However, solving more high resolution structures of other GPCRs with their partner G proteins is required to evaluate the first one in adequate detail and learn about possible differences in the activation of specific G proteins and oligomeric organization of

GPCRs within these complexes. Although the structure of T4L-β_2AR-G_s-nanobody revealed 1:1 GPCR:G protein stoichiometry, an EM-derived, low resolution 3D structure of native rhodopsin-G_t complex indicated rhodopsin dimerization is important in binding with its cognate G protein, transducin. Moreover, the rhodopsin dimer within this complex displays asymmetric properties suggesting the possibility of a tandem asymmetric activation mechanism. Nevertheless, this concept needs to be tested experimentally by using electrophysiological approaches. Oligomerization of GPCRs is now a highly accepted phenomenon. If GPCR oligomerization is compromised, signal transduction could be impaired as well. Improper oligomerization therefore could be an underlying cause of some disease states. Thus, accurate identification of residues that interact at the dimer interface would further our understanding of GPCR signal transmission across the membranes and the mechanisms of GPCR-linked diseases. For example, missense and nonsense mutations of rhodopsin can cause at least three types of human visual disorders: autosomal dominant retinitis pigmentosa (adRP) (Farrar et al. 2011), congenital stationary night blindness (CSNB) (McAlear et al. 2010), and autosomal recessive retinitis pigmentosa (arRP) (Wen et al. 2011) and more than 60 rhodopsin mutations can cause RP. Interestingly, some of these mutations are located at the rhodopsin dimer interface, suggesting that improper rhodopsin oligomerization could contribute to this disease phenotype.

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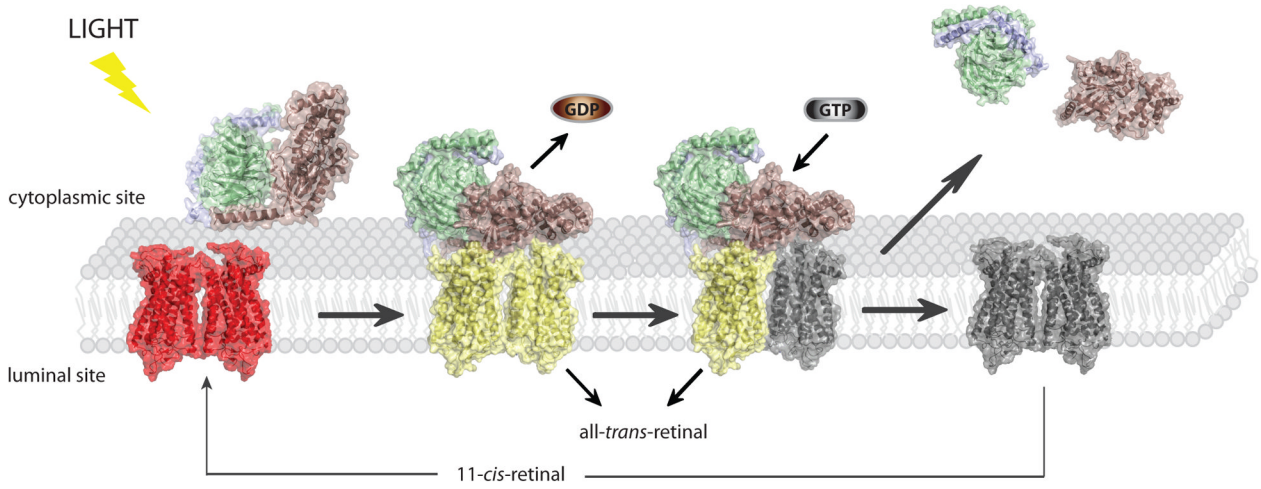

Fig. 1.

Rhodopsin signaling. Light activation of rhodopsin enables binding of its cognate G protein, transducin and triggers GDP→GTP exchange in its α subunit. Transiently, high affinity, nucleotide free rhodopsin-G_t complex is formed. Binding of GTP causes complex dissociation as well as dissociation of $G_t\alpha$ subunit from $G_t\beta\gamma$ dimer and they activate different downstream pathways leading in effect to neuronal response in the brain. Dissociation of transducin from activated rhodopsin enables dissociation of is chromophore all-*trans*-retinal. Accumulated free opsin has to be regenerated with 11-*cis*-retinal in order to restore its function.

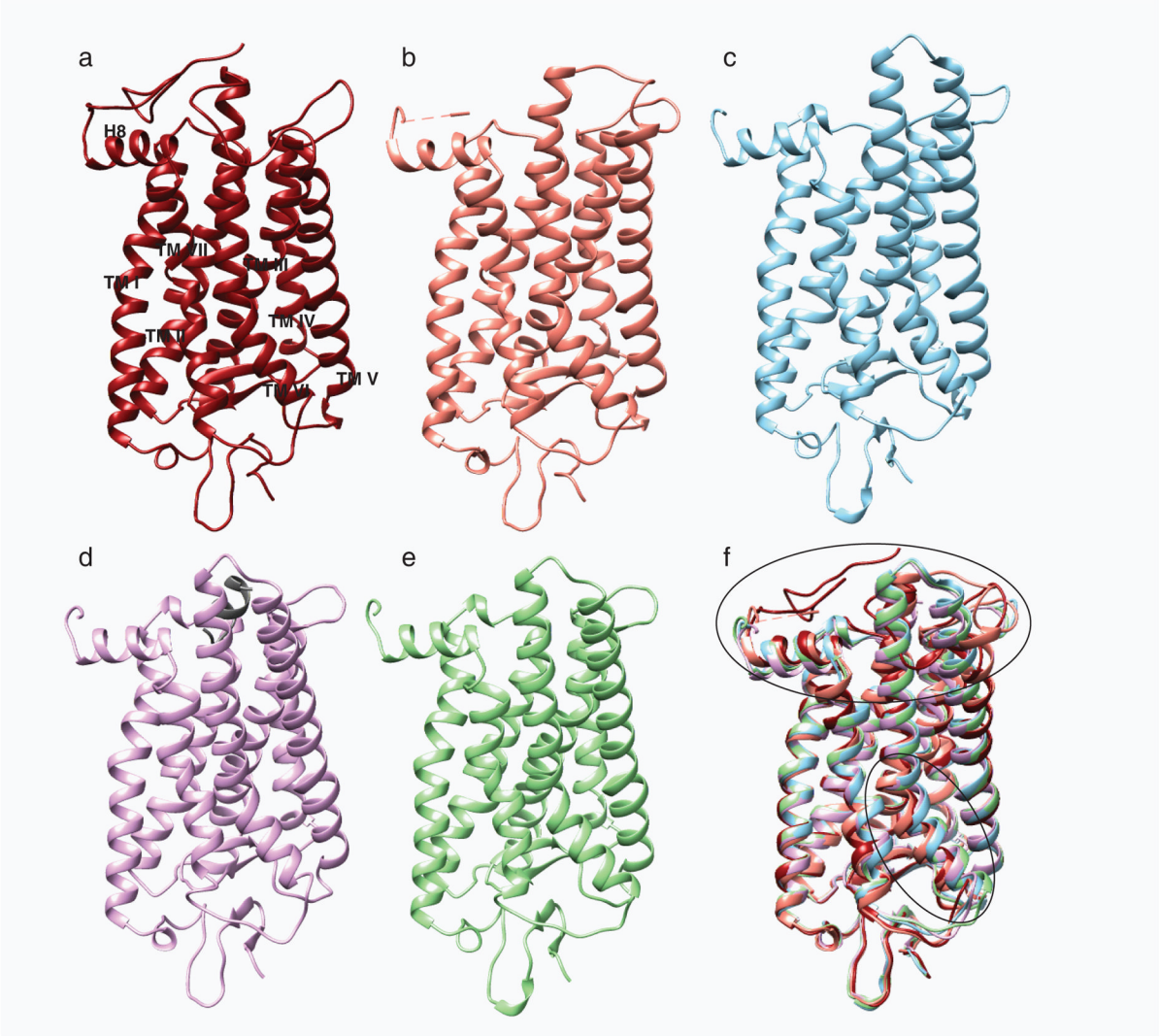

Fig. 2.

Structural comparison of different photo-states of rhodopsin. **a**, Structures of ground state rhodopsin (1u19) (Pescitelli et al. 2008); **b**, photoactivated rhodopsin (2i35) (Salom et al. 2006); **c**, opsin (3cap) (Park et al. 2008); **d**, opsin with bound C-terminal peptide of G_t (3dqb) (Scheerer et al. 2008) and **e**, Meta II (3pxo) (Choe et al. 2011) are shown in dark red, salmon, blue, purple and green, respectively. **f**, All activated rhodopsin states were superposed on its ground state structure. Superposition of these structures show rearrangement of the cytoplasmic loops and movement of helix TM VI depicted with black ovals.

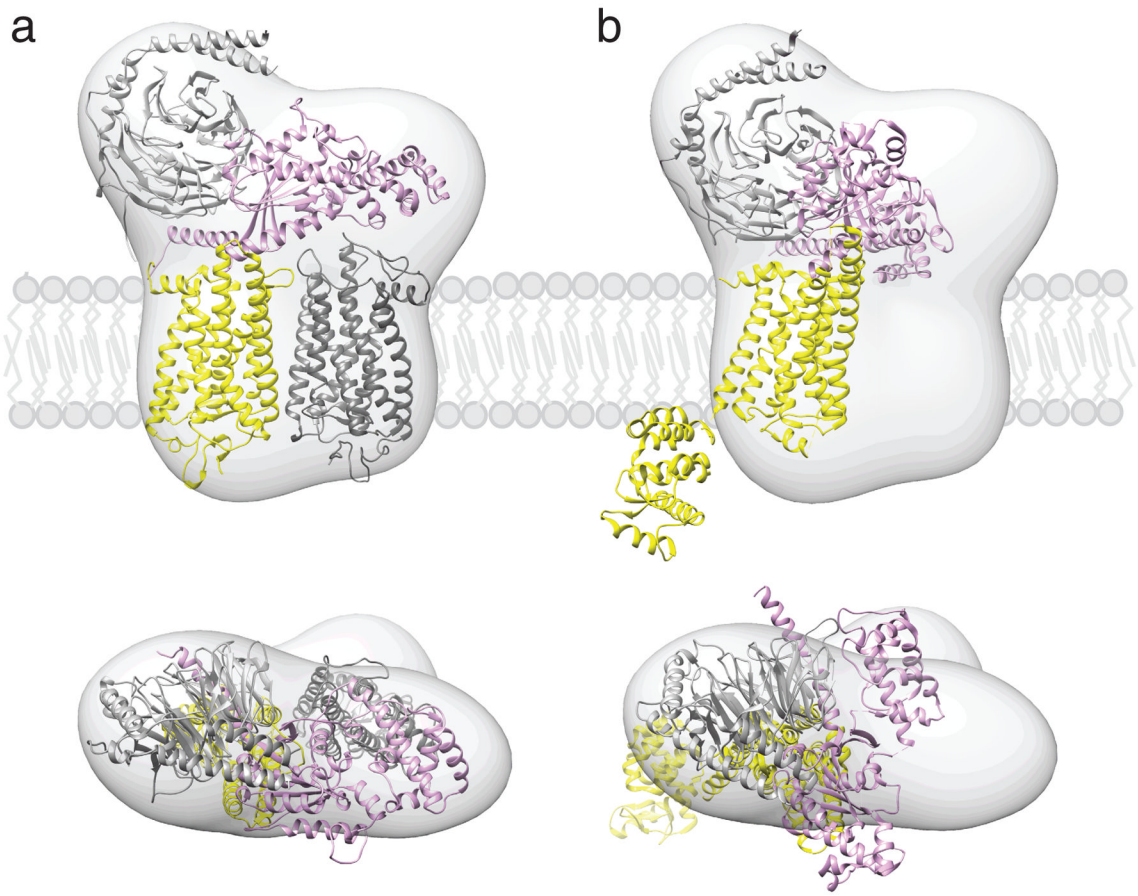

Fig. 3.

Proposed location of GPCR-G protein complexes in a phospholipid bilayer. Semi-empirical model of the complex formed between light-activated rhodopsin dimer and a G_t heterotrimer (**a**) (Jastrzebska et al. 2011) and a structure of T4L-β_2AR-Gs complex (**b**) (Rasmussen et al. 2011b) were fitted into a 3D molecular map derived from EM-single particle reconstructions of negatively stained, crosslinked rhodopsin-G_t complexes purified in LMNG. Best fitting of the rhodopsin-G_t model was achieved for rhodopsin dimer and G_t hereotrimer (GOT1) after 30° hinge-like motion of the α-helical domain of G_t was applied. Fitting the T4L-β_2AR-Gs-nanobody structure into our EM 3D map leaves enough unoccupied space to accommodate a second molecule of this receptor, conformation of $G_s\alpha$ helical domain is inconsistent with our EM-density. The photoactivated rhodopsin that binds C-terminus of $G_t\alpha$ and molecule of T4L-β_2AR is depicted with yellow. The second rhodopsin molecule is shown in dark grey. Gα is colored pink; Gβ and Gγ are colored light grey.

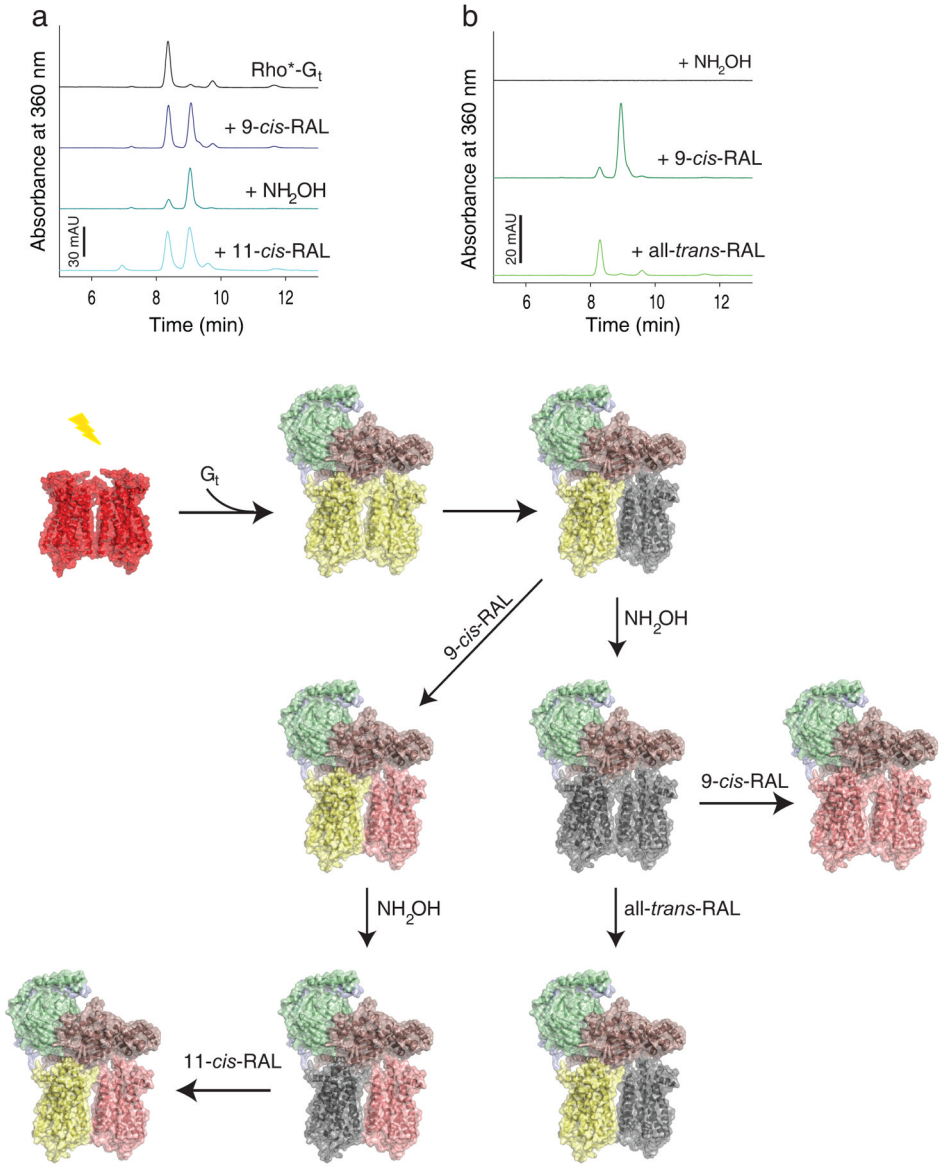

Fig. 4.

Asymmetry of activated rhodopsin (Rho*) dimer within rhodopsin-G_t (Rho*-G_t) complex. G_t binds to fully light activated rhodopsin (Rho) forming the Rho*-G_t complex. After light illumination, 11-*cis*-retinal (11-*cis*-RAL) of ground state rhodopsin (shown as red-red dimer) isomerizes to all-*trans*-retinal (all-*trans*-RAL) (yellow-yellow dimer) allowing binding of the G_t heterotrimer ($G_t\alpha$ is depicted in salmon, $G_t\beta$ in green and $G_t\gamma$ in light blue). This binding prevents chromophore release from one rhodopsin in the dimer, introducing dimer asymmetry in the Rho*-G_t complex (shown as a yellow-grey dimer bound to G_t and black trace on the chromatogram). **a**, retinal-free rhodopsin molecule of the Rho*-G_t complex can be regenerated with 9-*cis*-retinal (9-*cis*-RAL) (shown as yellow-pink rhodopsin dimer bound to G_t and blue trays on the chromatogram). Release of chromophore from the G_t-protected rhodopsin molecule can be promoted by NH_2OH, resulting then in formation of an asymmetric retinal free and 9-*cis*-retinal regenerated rhodopsin dimer complexed to G_t (shown as grey-pink rhodopsin dimer bound to G_t and dark cyan trace on the chromatogram). Regeneration of this complex with 11-*cis*-retinal surprisingly results in

formation of all-*trans*-retinal bound and 9-*cis*-retinal bound asymmetric rhodopsin dimer in the complex with G_t (shown as yellow-pink rhodopsin dimer bound to G_t and cyan trace on the chromatogram). **b**, the chromophore-free Rho*-G_t complex (modeled as a grey-grey rhodopsin dimer bound to G_t and black lane on the chromatogram) can be generated by treatment of the Rho*-G_t complex with NH_2OH. Then this chromophore-free Rho*-G_t complex can be regenerated with 9-*cis*-retinal which binds to both rhodopsin monomers within the dimer (shown as a pink-pink dimer bound to G_t and dark green trace on the chromatogram). Alternatively, it can be regenerated with all-*trans*-retinal but only one rhodopsin molecule, the one directly coupled to G_t binds all-*trans*-retinal (shown as a yellow-grey dimer bound to G_t and light green trace on the chromatogram).