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G protein-coupled receptor–G protein interactions: a single-molecule perspective

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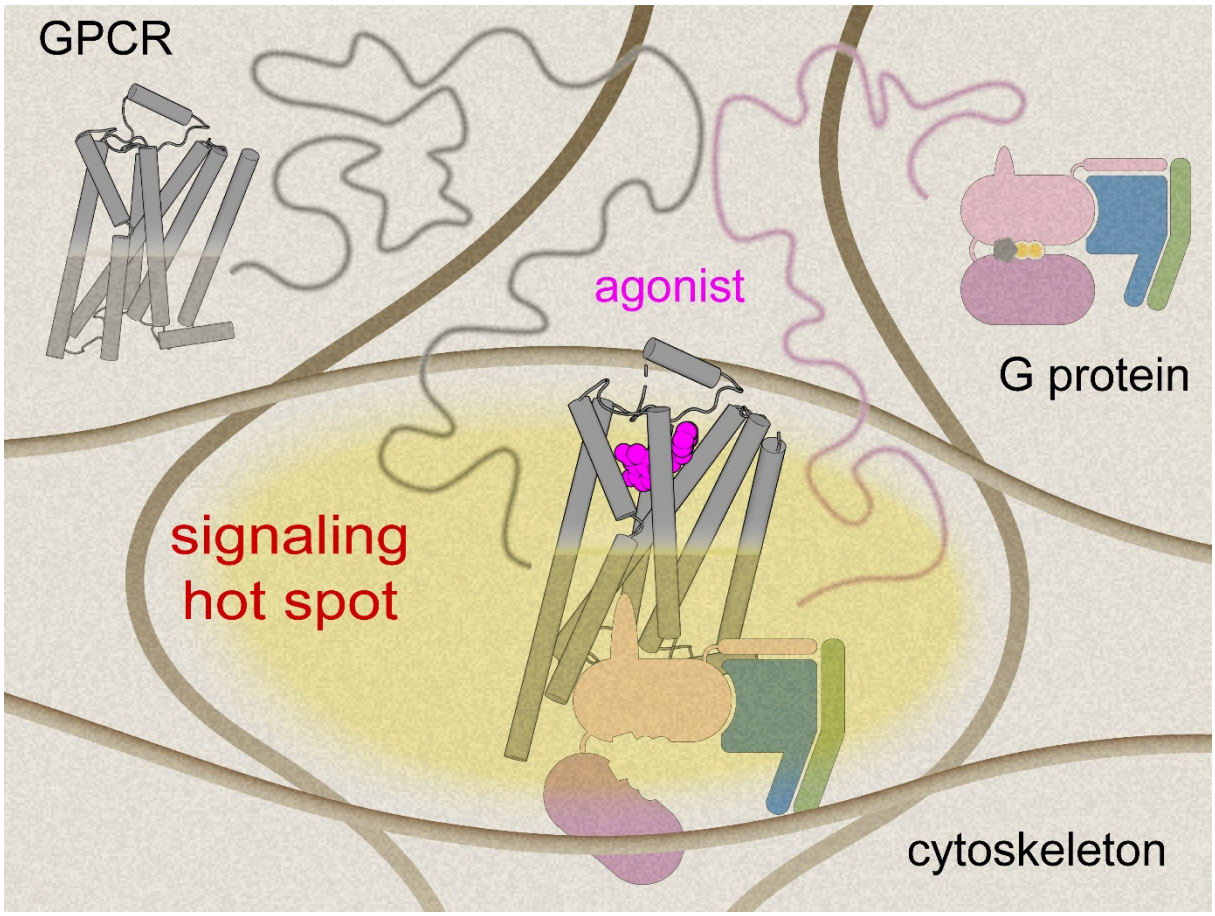
ABSTRACT

G protein-coupled receptors (GPCRs) regulate many cellular and physiological processes, responding to a diverse range of extracellular stimuli including hormones, neurotransmitters, odorants and light. Decades of biochemical and pharmacological studies have provided fundamental insights into the mechanisms of GPCR signaling. Thanks to recent advances in structural biology, we now possess an atomistic understanding of receptor activation and G protein coupling. However, how GPCRs and G proteins interact in living cells to confer signaling efficiency and specificity remains insufficiently understood. The development of advanced optical methods, including single-molecule microscopy, has provided the means to study receptors and G proteins in living cells with unprecedented spatio-temporal resolution. The results of these studies reveal an unexpected level of complexity, whereby GPCRs undergo transient interactions among themselves as well as with G proteins and structural elements of the plasma membrane to form short-lived signaling nanodomains that likely confer both rapidity and specificity to GPCR signaling. These findings may provide new strategies to pharmaceutically modulate GPCR function, which might eventually pave the way to innovative drugs for common diseases such as diabetes or heart failure.

Key words: GPCR, G protein, single-molecule microscopy, cell signaling, protein–protein interactions.

GRAPHICAL ABSTRACT



I. INTRODUCTION

G protein coupled receptors (GPCRs) represent the largest and most diverse family of cellular receptors in eukaryotes (249, 325). Of the approximately 800 GPCRs encoded in the human genome (149), about 460 recognize odorants and are mainly, although not exclusively, involved in olfaction (112). The remaining receptors mediate the effects of a wide range of both endogenous and exogenous cues, including light, ions, metabolites, hormones and neurotransmitters. Although the ligands of many orphan receptors have been identified, more than 100 GPCRs remain with no known endogenous ligand (244).

The main function of membrane receptors is to relay extracellular signals to the cell interior, allowing cells to communicate with each other and sense the extracellular environment. Other classes of membrane receptors are ion channels or possess an intrinsic enzymatic activity that generates an intracellular signal upon activation. In contrast, GPCRs rely on their interaction with G proteins to transmit signals to membrane-bound effectors including ion channels and enzymes. The involvement of G proteins as intermediate transducers plays a critical role in ensuring the high flexibility, sensitivity and specificity observed in GPCR signaling. A thorough understanding of how GPCRs and G proteins interact in our cells appears crucial to fully comprehend their role in physiology and disease as well as how to better control them for pharmacological purposes. In this review, we will discuss the mechanisms at the basis of receptor–G protein interactions and their physiological implications in the light of recent findings obtained using single-molecule microscopy and other innovative approaches.

II. ROLE OF GPCRs AND G PROTEINS IN PHYSIOLOGY

GPCRs play a fundamental role in human physiology, participating in the control of virtually all physiological functions, including neurotransmission, hormone release, heart contractility and immune responses. To accomplish such a broad range of functions, GPCRs have evolved from a common, ancestral seven transmembrane receptor to give rise to the large and highly diverse superfamily of membrane receptors found in humans and other vertebrates.

From an evolutionary perspective, the GPCR signaling module emerged very early in the development of eukaryotes, being conserved from excavates to mammals (82, 225). Although bacterial and metazoan rhodopsin show almost no sequence homology, they share some structural features, which may be indicative of an even older evolutionary relationship. Intriguingly, the appearance of GPCRs and their signaling machinery, such as G proteins, arrestins and regulators of G protein signaling (RGS) proteins, predates the development of the nervous system and even the evolution of multicellular organisms, having been found in simple eukaryotic life forms such as amoebae or yeast. Plants also have G proteins; however, plant G proteins possess a unique mechanism of activation, which does not rely upon GPCRs (10). Remarkably, it is believed that the last eukaryotic common ancestor (LECA) already contained a complete repertoire of GPCRs and heterotrimeric G proteins (82). Although the exact functional role of GPCRs at the beginning of eukaryotic evolution is unknown, ancestral GPCRs might have been involved in sensing the extracellular environment or allowing some primitive form of intercellular communication. Perhaps the best evidence for a role of GPCRs in cell–cell communication in unicellular eukaryotes comes from studies of colony formation in the social amoeba *Dictyostelium discoideum* (264) and mating in the baker's yeast *Saccharomyces cerevisiae* (8). Upon starvation, *Dictyostelium* uses cyclic AMP (cAMP) as a chemoattractant to induce colony aggregation and the formation of multicellular structures that can withstand harsh environmental conditions. The cAMP released into the extracellular medium activates a high affinity GPCR for cAMP, the cyclic AMP receptor 1 (cAR1), located on the plasma membrane of neighboring *Dictyostelium* cells, which, in turn, induces intracellular cAMP production and protein kinase A (PKA) activation. *Saccharomyces cerevisiae* possesses two pheromone receptors, Ste2 and Ste3, which belong to GPCR Family D (8). *Saccharomyces cerevisiae* exists in three distinct forms, two haploid forms termed 'a' and 'α' as well as a diploid 'a/α' form generated by pheromone-induced cell fusion or 'mating' of an a and an α cell. Studies with mating-deficient mutants led to the identification of Ste2 and Ste3, which mediate Yeast pheromone signaling via activation of a heterotrimeric G protein consisting of an α (Gpa1), β (Ste4) and γ (Ste18) subunit. Interestingly, Ste2 and Ste3 were the first agonist-binding GPCRs to be cloned

and characterized (358). Experiments investigating Ste2 signaling were also the first to show that G protein signaling does not necessarily require full G protein dissociation into Gα and Gβγ subunits, as a Gpa1-Ste2 fusion protein was shown to mediate comparable pheromone signaling as the two proteins expressed separately (207).

Although a small number of GPCRs are found in ancestral eukaryotes and G proteins are highly conserved between unicellular holozoans and metazoans, GPCRs underwent a dramatic expansion during the evolution of metazoans (82). This was accompanied by the acquisition of new and more sophisticated forms of cell-to-cell communication. It was indeed the development of specialized cell types devoted to cell communication – such as neurons and endocrine cells – that likely fostered the flourishing of the GPCR superfamily, ultimately placing it center stage in human physiology and disease. An illustrative example of such an expansion can be found in the model nematode *Caenorhabditis elegans*, where over 1,000 putative GPCR genes are present (425).

A prime example of GPCRs in physiology is their well-known involvement in the neurohumoral regulation of heart contractility. Upon sympathetic activation, adrenaline and noradrenaline bind to β-adrenergic receptors (βARs) on the surface of cardiomyocytes, where they exert positive inotropic and chronotropic effects. These effects are mediated via activation of the G_s protein, which, via stimulation of the cAMP/protein kinase A (PKA) pathway, promotes heart contractility (26). Moreover, the resulting increase in intracellular cAMP levels in the pacemaker cells of the sinoatrial node increases heart rate. These effects are at least partially mediated by the opening of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (20). The effects of adrenaline and noradrenaline are counteracted by acetylcholine, via activation of muscarinic M2 receptors (M2Rs), which are coupled to $G_{i/o}$ proteins and, thus, inhibit adenylyl cyclase. In addition, Gβγ subunits released upon $G_{i/o}$ activation promote the opening of G protein-coupled inwardly-rectifying potassium (GIRK) channels, increasing the membrane potential of pacemaker cells (166, 172, 261, 351, 462).

Another example is given by the fundamental role of GPCRs as receptors for several pituitary hormones and hypothalamic release factors (459). All major known hypothalamic releasing hormones – thyrotropin releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH) and growth hormone-releasing hormone (GHRH) – and inhibiting factors, including somatostatin and dopamine, act via specific GPCRs. Similarly, all anterior and posterior pituitary hormones, with the exception of the growth hormone and prolactin, signal through the activation of GPCRs. These include the thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), vasopressin and oxytocin. Many of the physiological effects of these hormones are mediated via activation of the G_s protein and the resulting stimulation of cAMP/PKA signaling. As a consequence, alterations of key elements of these signaling pathways, for instance caused by genetic mutations, are frequently found in endocrine disease (50, 239).

III. THE BASIC MECHANISMS OF GPCR SIGNALING

Given the fundamental role of GPCRs in physiology and disease, the mechanisms underlying GPCR signaling have been extensively investigated, mainly applying classical biochemical and pharmacological methods to crude membrane preparations or purified proteins. Pioneering studies initiated in the 1950's by Earl Sutherland and Ted Rall (415) and later continued by Alfred G. Gilman and Martin Rodbell (130, 356) elucidated the core machinery of GPCR signaling, which includes a receptor, a G protein acting as transducer and an effector, all associated with the plasma membrane.

GPCRs are integral membrane proteins, with a characteristic seven transmembrane domain (325). They are the largest superfamily of receptors, encoded by roughly 800 genes in the human genome (149). Based on structural similarities, GPCRs are generally subdivided into 6 major families, consisting of rhodopsin-like (Family A), secretin-like (Family B), metabotropic glutamate-like (Family C), fungal mating pheromone (Family D), cAMP (Family E) and frizzled/smoothened (Family F) receptors (11, 219, 234). Of these, Families D (fungal mating pheromone receptors) and E (cAMP receptors in

Dictyostelium) are not represented in mammals. Alternatively, GPCRs have been classified based on a phylogenetic analysis according to the GRAFS (glutamate, rhodopsin, adhesion, frizzled/taste2, secretin) system (112, 234). Family A is the largest, with approximately 670 receptors that share high homology with the visual receptor rhodopsin (234). This family contains receptors for a heterogeneous group of ligands, including biogenic amines, nucleotides, lipid-like substances, peptides and proteins. Small ligands bind within the receptor 7-transmembrane (TM) bundle, whereas peptides and protein hormones typically bind to the N-terminus and/or the extracellular loops (186, 234, 382).

G proteins are heterotrimeric guanine nucleotide binding proteins composed of a Gα, Gβ and Gγ subunit (37). They owe their name to the molecule that regulates their activity, the energy carrier guanosine triphosphate (GTP), and its inactive form guanosine diphosphate (GDP). Under resting conditions, the inactive Gα subunit is bound to one molecule of GDP as well as the Gβ and Gγ subunits, which form a tight heterodimer (130). Upon agonist binding, receptors undergo conformational rearrangements that ultimately increase their affinity for G proteins, leading to G protein recruitment. The interaction of a G protein with an active receptor stimulates the release of GDP from the Gα subunit. Since the concentration of GTP is approximately ten times higher than that of GDP in the cytosol (39), this eventually leads to the replacement of GDP by GTP on the Gα subunit. In this respect, the active receptor acts as a guanine nucleotide exchange factor (GEF) for G proteins. The active, GTP-bound Gα subunit is then thought to disassociate from the receptor and the Gβγ dimer – although the latter dissociation might be partial – allowing the resulting GTP-bound Gα subunit and Gβγ dimer to interact with various downstream effectors, including enzymes such adenylyl cyclase or phospholipase C-β (PLC-β) as well as both potassium (GIRK) and calcium channels. The signal is terminated by the intrinsic GTPase activity of the Gα subunit, which hydrolyses GTP to GDP, returning the Gα subunit to its inactive state, followed by reassociation of the G protein heterotrimer.

The human genome contains a total of 33 genes encoding G protein subunits, including 16 genes for Gα (TABLE 1) (151, 286, 368, 386, 459), 5 genes for Gβ and 12 genes the Gγ subunits (96). G proteins

are typically grouped into four families based on their constituent Gα subunit: $G_{s/olf}$, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$. Members of each of the four families activate distinct signaling pathways, enabling diverse physiological processes to be modulated in response to extracellular stimuli. The G_s protein was the first to be isolated based on its ability to activate adenylyl cyclases (130, 360). $G_{s/olf}$ proteins interact directly with adenylyl cyclases, stimulating their catalytic activity and, thus, cAMP production. Intracellular cAMP levels are reduced following activation of $G_{i/o}$ proteins, which inhibit several adenylyl cyclase isoforms. However, it should also be noted that not all adenylyl cyclase isoforms are inhibited by $G_{i/o}$ and, in some cases, prolonged $G_{i/o}$ stimulation may enhance cAMP production (44). Members of the $G_{q/11}$ family interact with and activate PLC-β, leading to the synthesis of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). IP3, in turn, stimulates the release of Ca^{2+} from intracellular stores, which, together with DAG, activates protein kinase C (PKC). Lastly, the $G_{12/13}$ family stimulates Rho guanine nucleotide exchange factors (RhoGEFs), leading to the subsequent activation of the Rho family of small GTPases, which are implicated in the regulation of actin dynamics. Although individual GPCRs were originally thought to primarily couple to only one type of G protein, it is now established that GPCRs are often promiscuous and can simultaneously signal via multiple G protein families.

Gα proteins are intrinsically weak GTPases, i.e. the transition of Gα proteins from their active to inactive state may take a matter of seconds to minutes (359). This has consequences on both their activation and deactivation kinetics. The low rate of GTP hydrolysis slows the signaling cascade as G proteins cannot enter a new cycle and integrate new signaling information before GTP hydrolysis has occurred. This process is accelerated by members of the RGS protein superfamily. First described in the desensitization of the yeast pheromone response (60, 61, 93, 94), RGS stimulate the intrinsic GTPase activity of Gα subunits by as much as 1,000-fold (3, 24, 25, 93, 94, 215). RGS proteins bind Gα subunits via their 'RGS-box' domain and stabilize the Gα in an intermediate transition-state during the GTPase reaction, thus lowering the energy barrier required for GTP hydrolysis and subsequent return to the GDP-bound state (24, 420). A total of 20 genes coding for canonical RGS proteins have been

identified in humans (407). Selected RGS proteins have been subsequently shown to exert additional functions such as the binding of the atypical $G\beta_5$ subunit (48), which does not engage in GPCR signaling but rather promotes RGS folding and stability (62, 362, 464). More recently, additional RGS family members have been discovered that share the conserved GoLoco motif. This motif allows them to form a stable complex with $G\alpha$-GDP that prevents the release of GDP. It has since become apparent that RGS proteins can additionally function as chaperones, *bona fide* effectors or even GEFs (19, 202, 404).

Conversely to RGS molecules, activators of G protein signaling (AGS) act as GEFs and prolong G protein signaling – independently of GPCR activation – through various mechanisms. There are at least 14 AGS proteins, divided into three main groups: AGS proteins that directly activate $G\alpha$ subunits, those that modulate $G\alpha$–$G\beta\gamma$ interactions by binding to $G\alpha$, and, lastly, those that modulate $G\alpha$–$G\beta\gamma$ interactions by binding to $G\beta\gamma$ (31). These non-classical GEFs are activated downstream of various intracellular signaling pathways, such as those initiated at the plasma membrane by receptor tyrosine kinases (118). A fourth group of AGS proteins was suggested to selectively activate $G\alpha_{15}$ and include the MiTF/TFE family of transcription factors (31).

Compared to $G\alpha$, there are fewer genes encoding $G\beta$ and $G\gamma$ subunits, and as a result, signaling pathways activated via $G\beta\gamma$ subunits have long been considered an accessory component of G protein signaling. However, over the last 30 years, $G\beta\gamma$ proteins have emerged as *bona fide* signal transducers that play an equally important role in GPCR signaling (398). It is now believed that following $G\alpha$ dissociation from its cognate $G\beta\gamma$ partner, multiple $G\beta\gamma$ surfaces are exposed, thus allowing $G\beta\gamma$ to interact with receptors, membrane proteins and various downstream effectors. An interesting feature of $G\beta\gamma$-mediated signaling is the sheer number of targets to which $G\beta\gamma$ subunits have been proposed to bind, which apparently occurs in the absence of a clearly conserved binding domain (380, 397). Among the many targets of $G\beta\gamma$ regulation are GIRK channels (261), Ca^{2+} channels (83, 169), GPCR kinases (GRKs) (142, 213, 331), mitogen-activated protein kinases (MAPK) (265),PLC-β (56, 171) and

phosphatidylinositol 3-kinase γ (438). Moreover, there is evidence that Gβγ subunits might translocate to sites of receptor activation, potentially allowing for a local regulation of GPCR signaling (4, 205, 370).

The coordinated actions of two families of proteins, GRKs and arrestins, are critical for GPCR down-regulation and the attenuation of G protein-dependent signaling. GRKs phosphorylate serine/threonine sites located on the C-terminal tail or intracellular loops of GPCRs (226, 329, 330, 408). This promotes the recruitment of arrestins from the cytosol and their binding to ligand-occupied and phosphorylated receptors. Via occupying the receptor core, arrestins sterically hinder G protein binding and, thus, mediate fast receptor desensitization (107). In parallel, arrestins serve as scaffolds for the adaptor protein 2 (AP-2) and clathrin heavy chain (135, 242, 243), thus promoting receptor accumulation in clathrin-coated pits (CCP) and their internalization via clathrin-mediated endocytosis (CME) (226, 330). In addition to these classical roles in rapid signal desensitization and receptor internalization, additional functions of arrestins have been subsequently described. Importantly, these include a role of β-arrestins as *bona fide* signal transducers capable of mediating G protein-independent activation of other signaling cascades, most notably the extracellular signal-regulated kinase (ERK) MAPK pathway (77, 250, 340).

Besides binding G proteins and arrestins, there is evidence that GPCRs can interact among themselves to form dimers as well as higher-order oligomers. This phenomenon has been implicated in various aspects of GPCR biology, including intracellular trafficking, cell surface expression and downstream signaling. For a thorough discussion, we refer the reader to previous reviews (284, 327, 328).

IV. STRUCTURAL INSIGHTS INTO GPCR AND G PROTEIN ACTIVATION

A. Structure and activation of GPCRs

The first structure of a visual GPCR, bovine rhodopsin, was determined 20 years ago by X-ray crystallography (318). Due to the much lower expression levels of non-visual GPCRs, it took a further seven years to purify, crystallize and resolve the first structure of a non-visual GPCR (66). These

pioneering studies paved the way for an explosion of structural information, fostered by important technical developments including the introduction of cryo-electron microscopy (cryo-EM) (423). As a result, we now possess a number of high-resolution structures of non-visual GPCRs bound to both agonists and antagonists, as well as in complex with G proteins or arrestins (58, 66, 95, 119, 143, 167, 181, 227, 246, 247, 270, 342-344, 409, 471, 475, 476). For an updated list of determined GPCR structures see GPCRdb (https://gpcrdb.org/structure/) (289).

The available structures confirm the previously predicted overall GPCR architecture featuring a transmembrane domain (TMD) of seven α-helices connected by three extracellular and three intracellular loops (ECLs and ICLs, respectively), an N-terminal extracellular domain (ECD) and a C-terminal intracellular domain (ICD) (FIGURE 1). Family A GPCRs possess an additional intracellular amphipathic α-helix (H8), which is tethered to the plasma membrane via palmitoylated cysteine residues (133). The seven α-helices of the TMD are inserted near perpendicular into the plasma membrane with an overall arrangement that is similar, albeit not identical, in all available GPCR structures. These α-helices form an inner pocket within the TMD where most small molecule ligands bind. In addition, they provide binding sites for membrane lipids, such as cholesterol or PIP2, which have been directly observed in some structures (146, 167). The ECD differs significantly in size and structure among receptors, ranging from a short flexible stretch of amino acids in most Family A GPCRs, to large and structured domains, as observed in Family B and C GPCRs. These large ECDs provide the binding sites for peptides and large protein hormones, as well as small ligands such as glutamate, γ-aminobutyric acid or Ca^{2+} ions. A series of post-translational modifications further contribute to the overall structure of GPCRs and influence their trafficking and signaling. These include the glycosylation of specific amino acid sequences within the ECD (441), the formation of a disulfide bridge between TM3 and ECL2 that confers structural stability (443) and the above-mentioned palmitoylation of intracellular cysteine residues that provide additional anchor points to the plasma membrane.

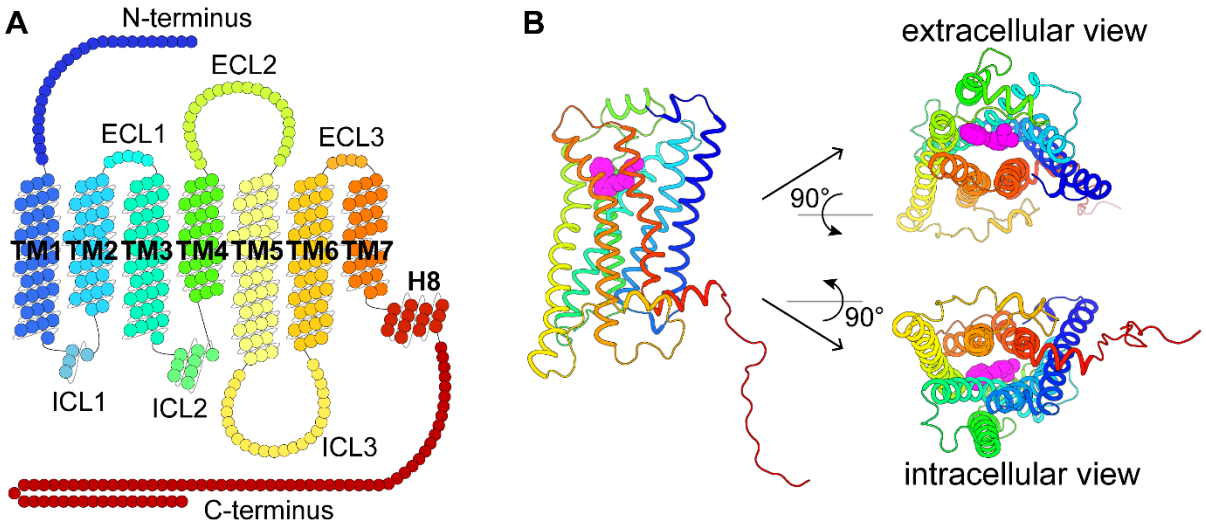


FIGURE 1. Common structural features of GPCRs. The β_2AR is used as a model. Shown are a snake plot (A) and a homology model (B) of the receptor, generated by GPCRM (https://gpcrm.biomodellab.eu/) based on the structure of β_2AR bound to the inverse agonist carazolol (PDB ID: 2RH1). GPCRs consist of seven transmembrane helices (TM1-TM7) connected by three intracellular (ICL) and three extracellular (ECL) loops, an extracellular N-terminal domain (ECD), an optional intracellular helix (H8) and an intracellular C-terminal domain (ICD). Carazolol is shown in magenta.

In the following description of the structural elements shared by GPCRs, we adopt the GPCRdb numbering scheme (179), where each residue in the TMD of a GPCR is identified by two numbers separated by the letter 'x'. The first number denotes the transmembrane α-helix in which the residue is located, whereas the second number corresponds to the position of the residue relative to the most conserved residue in that helix. The latter is arbitrarily assigned the position 50, with numbers increasing towards the C-terminus. Interestingly, three of the most conserved residues within the TMD, 5x50, 6x50 and 7x50, are prolines. These conserved prolines induce 'kinks', which are frequently found in transmembrane α-helixes and are stabilized by interactions with membrane lipids. Importantly, these kinks impart a unique shape to the corresponding α-helixes, allowing them to bend either towards or away from the receptor core (419).

It is widely accepted that agonist binding on the extracellular side of a GPCR induces a series of conformational changes within its TMD. These conformational changes culminate in the opening of a cavity on the intracellular side of the receptor, which provides the binding site for G proteins and arrestins (344). The comparison of inactive and active structures of Family A GPCRs (58, 66, 95, 119, 143, 181, 227, 246, 270, 342-344, 471) has provided important insights into the conformational changes that occur during this process. These include a slight rotation and upward movement of TM3, movement of TM1, TM5 and TM7 towards the receptor core, and a major rotation and outward movement (up to 14 Å) of the intracellular end of TM6, ultimately exposing the receptor core to G proteins and β-arrestins (FIGURE 2). These conformational changes result from a series of smaller rearrangements discussed below, most notably involving the NPxxY, DRY and CWxP motives, as well as highly conserved hydrophobic residues in TM3 and TM6, accompanied by the formation of a water molecule network within the inner cavity of the TMD (419, 477).

The DRY motif (E/D3x49-R3x50-Y/W3x51), which is present in several receptors at the cytoplasmic end of TM3, plays a key role in receptor activation and G protein coupling (6, 18). In the absence of an agonist, residues E/D3x49, R3x50 and E6x30 form an ionic interaction – known as 'ionic lock' – which restricts the receptor in a closed, inactive conformation. During receptor activation, this ionic lock breaks, allowing Y5x58/Y7x53 to stabilize R3x50 and making it available to interact with G proteins (344).

The NPxxY motif is comprised of residues N7x49, P7x50 and Y7x53. Residue Y7x53 interacts with N1x50 and D2x50, the two most conserved residues in TM1 and TM2, respectively. N1x50 contributes to helix packing through hydrogen bonding with the backbones of residues 1x46 and 7x46, and interacts with D2x50 through a conserved water molecule, connecting TM1 with TM2 and TM7 (344). Upon receptor activation, Y7x53 forms new contacts with residue R3x50 of the DRY motif and interacts with residues in the Gα subunit (442).

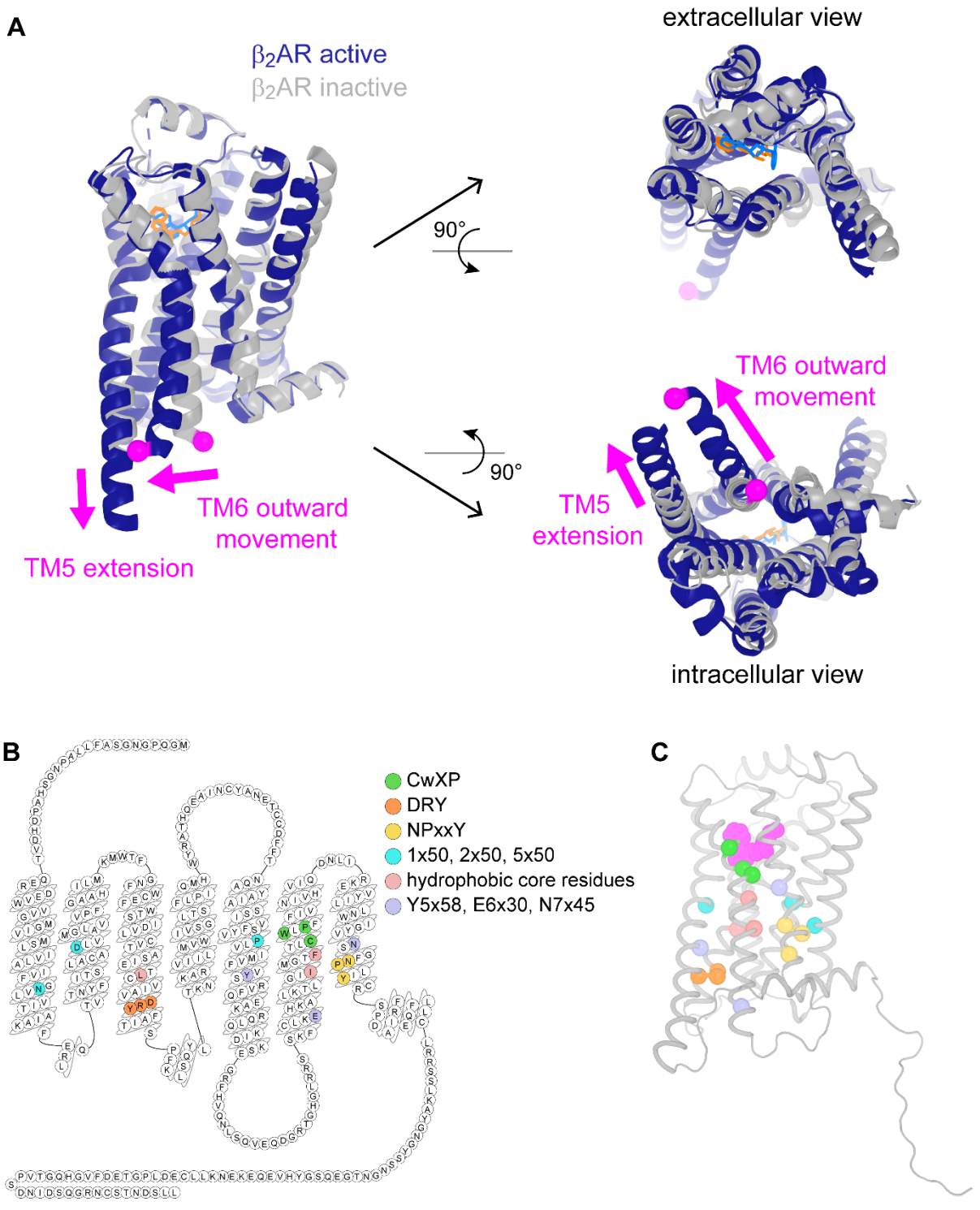


FIGURE 2. Conformational changes associated with GPCR activation. A: Comparison of β_2AR inactive (PDB ID: 2RH1, grey with orange ligand) and active structures (PDB ID: 3SN6, dark blue with blue ligand). Magenta spheres, location of the intracellular end of TM6. Magenta arrows, most prominent changes. B and C: Conserved domains involved in receptor activation. The domains are highlighted with different colors on a snake plot (B) or a model (C) of the β_2AR obtained as in FIGURE 1.

Another region thought to be important for the activation of GPCRs is the so-called CWxP motif (C/S/T6x47-W6x48-P6x50), residing deep within the ligand-binding pocket (163). While residue 6x47 assists in the reorganization of interactions between TM6 and TM7 during activation, the movement of W6x48 enables the intracellular end of TM6 to bend and rotate (315). In the inactive state, residue 6x47 interacts with residues 7x44/7x45, preventing residue N7x49 of the NPxxY motif from contacting D2x50. During activation, this interaction breaks, allowing N7x49 to interact with D2x50 (315).

In addition, the hydrophobic receptor core, which normally comprises of residues L3x43, F6x44 and I/L/V/M6x40, undergoes a series of conformational rearrangements that leads to the formation of a water channel within the core of the activated receptor (419). These rearrangements include the breaking of interactions within the hydrophobic receptor core, the formation of new interactions between L3x43 and L2x46, as well as the establishment of interactions of N7x49 and D2x50 with water molecules. This is accompanied by the formation of a new interaction between Y5x58 and R3x50 that prevents the reestablishment of the ionic lock.

Taken together, the aforementioned rearrangements ultimately result in an upward movement of TM3, inward movement of TM5, as well as an outward movement and rotation of TM6, all of which are stabilized by receptor interactions with helix 5 of the Gα subunit or the finger loop of β-arrestin. Interestingly, G protein binding has been shown to stabilize the receptor in an active or pre-active conformation in the absence of a bound agonist, leading, in some cases, to closure of the ligand binding pocket (89). This suggests a bidirectional allosteric coupling between the ligand binding pocket and the intracellular binding site.

Although these mechanisms have been thoroughly characterized for a few model receptors such as the β_2AR, there is evidence that they might be shared among a large number of GPCRs. For example, a recent comparison of the active structures of the muscarinic M1 receptor in complex with the G_{11} protein and that of the muscarinic M2 receptor in complex with the G_{oA} protein have revealed that

most residues important for receptor activation adopt similar conformations in both structures, despite the two receptors being bound to different G proteins (270).

B. GPCR conformational dynamics

While X-ray crystallography and cryo-EM have provided important snapshots of receptors in both inactive and active states, other biophysical approaches using purified proteins – such as nuclear magnetic resonance (NMR), double electron-electron resonance (DEER) and fluorescence spectroscopy – have revealed a highly dynamic picture, whereby receptors exist in an equilibrium among multiple states (FIGURE 3). For a comprehensive discussion see Manglik and Kobilka (274).

In the case of the β_2AR, at least four distinct transient states have been identified: two inactive states corresponding to the presence of an intact or broken ionic lock (306), an intermediate partially active state, and a fully active state (38, 201, 217, 259, 273, 307). Similarly, four distinct states have been observed for the angiotensin II type 1 receptor (AT_1R), including one inactive and three active-like states that are favored by G protein- or arrestin-biased agonists (463). Additionally, other biophysical studies have provided evidence that biased agonists may favor different receptor conformations (88), possibly coupled to distinct signaling pathways. The underlying mechanisms are not fully elucidated, with some studies suggesting selection from a conformational ensemble (312) and others promotion of distinct receptor conformations by different ligands (259, 338). For the β_1AR, an equilibrium between an inactive and a pre-active state has been observed, with exchange rates in the μs to ms timescale (401). Interestingly, agonists increase β_1AR conformational dynamics (401). Importantly, these and similar studies have shown that binding of a G protein or G protein-mimetic is required to fully stabilize GPCRs in their open, fully active conformation (180, 273, 307, 405, 473).

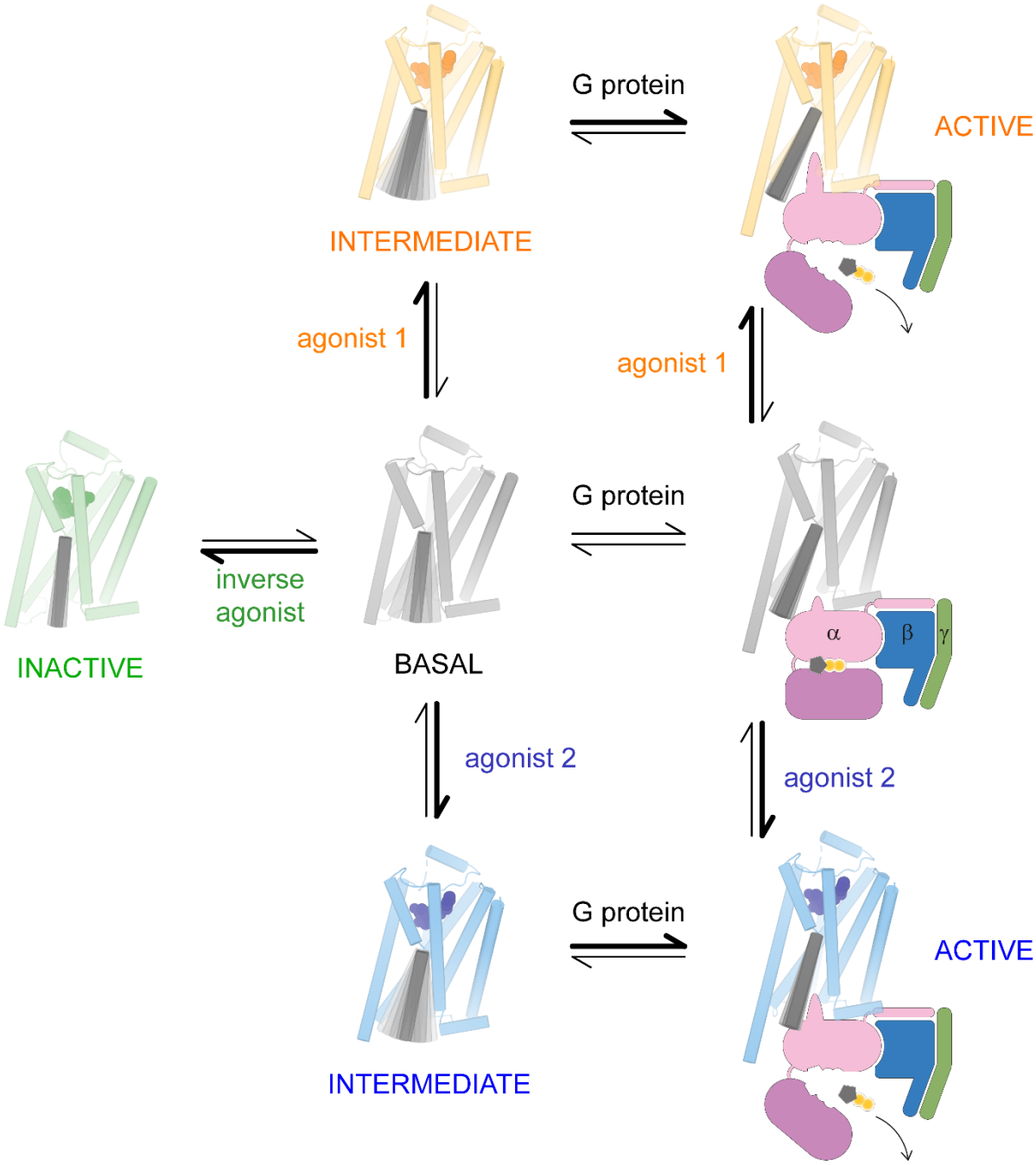


FIGURE 3. GPCR conformational dynamics. Shown is a schematic representation of the dynamic equilibrium found between inactive, intermediate and active receptor states, and how the equilibrium is affected by the binding of ligands and/or G proteins. Three pharmacologically different ligands are shown. An inverse agonist stabilizes the receptor in the inactive state. Agonists stabilize the receptor in dynamic intermediate states. G protein binding stabilizes the receptor in fully active states.

The existence of multiple active states might also help to explain the phenomenon of partial agonism. Spectroscopic data and MD stimulations suggest that partial agonists might stabilize receptors in intermediate states along the receptor activation pathway or, alternatively, in distinct active states (88, 99, 136, 189, 218, 259, 273, 312, 338, 401, 473). For instance, a study on the adenosine A_{2A} receptor ($A_{2A}R$) revealed the existence of a distinct active state that is selectively stabilized by both a partial agonist and an allosteric modulator (473). These results suggest that partial agonism might be achieved by selection of a distinct active state, possibly characterized by reduced G protein coupling.

Additional evidence for the existence of multiple active states comes from recent structural studies. A cryo-EM study examining the neurotensin receptor 1 (NTSR1) in complex with an agonist peptide and the G_{i1} protein identified two distinct conformations: a canonical conformation similar to the one observed in other receptor–G protein complexes and a non-canonical conformation in which the G protein is rotated by approximately 45° (194). The receptor in the non-canonical conformation shows features found in both active and inactive structures, suggesting that it might represent an intermediate state during receptor activation (194). Interestingly, a recent cryo-EM study compared the active-state structures of the β_1AR bound to conformation-specific nanobodies in the presence of four agonists with varying efficacy (452). The results indicate that the four agonists induce a different degree of ligand binding pocket closure, which correlates with their efficacy (452).

Another phenomenon that finds a possible explanation in the high conformational dynamics of GPCRs is their constitutive activity. It is well known that many GPCRs exhibit constitutive activity in the absence of ligands. This appears to be a consequence of GPCRs sampling a number of conformations in the absence of ligand, with one or more of these conformations capable of activating G proteins (87).

C. Structure and activation of G proteins

As already discussed, heterotrimeric G proteins are composed of a Gα, a Gβ and a Gγ subunit (FIGURE 4). The Gα subunit contains a unique helical domain and a GTPase domain that is conserved in all members of the G protein superfamily, including monomeric G proteins and elongation factors.

The GTPase domain hydrolyses GTP and provides the binding surfaces for the Gβγ dimer, GPCRs and effector proteins (204). The helical domain is the most variable domain across all existing Gα subunit isoforms, suggesting a possible role in determining receptor and/or effector specificity (260). The Gβ and Gγ subunits form a highly stable complex, which is believed to not dissociate under physiological conditions (378).

Heterotrimeric G proteins are anchored to the inner side of the plasma membrane through protein lipidation. The N-terminal region of the Gα and the C-terminal region of the Gγ subunits are both sites of lipid modification. With the exception of transducin, all Gα subunit isoforms are palmitoylated. Additionally, members of the $G\alpha_i$ family are myristoylated (350). Gγ subunits are isoprenylated with the addition of either a farnesyl ($G\gamma_1$, $G\gamma_8$ and $G\gamma_{11}$) or a geranylgeranyl (all remaining Gγ subunits) moiety (479). Since these lipid moieties are found in close proximity within the G protein heterotrimer, hydrophobic interactions among them might help stabilize the interaction between the Gα and Gβγ subunits (29, 168, 173, 256).

High-resolution structures of G proteins in different conformations, as well as bound to several GPCRs, have provided important insights into their organization (70, 78, 119, 120, 235, 236, 270, 285, 305, 334, 344, 422, 433, 449). For a complete list of determined complex structures we refer the reader to GPCRdb (https://gpcrdb.org/structure/) (289). In the following discussion, a common G protein numbering system is adopted (110), where each residue is identified by a superscript indicating the domain in which it is located (G for GTPase or H for helical), the secondary structure of the structural element in which it resides (H for α-helix or S for β-sheet) and its relative position within that structural element based on homology. Loops are identified with their two flanking structural elements in lower case.

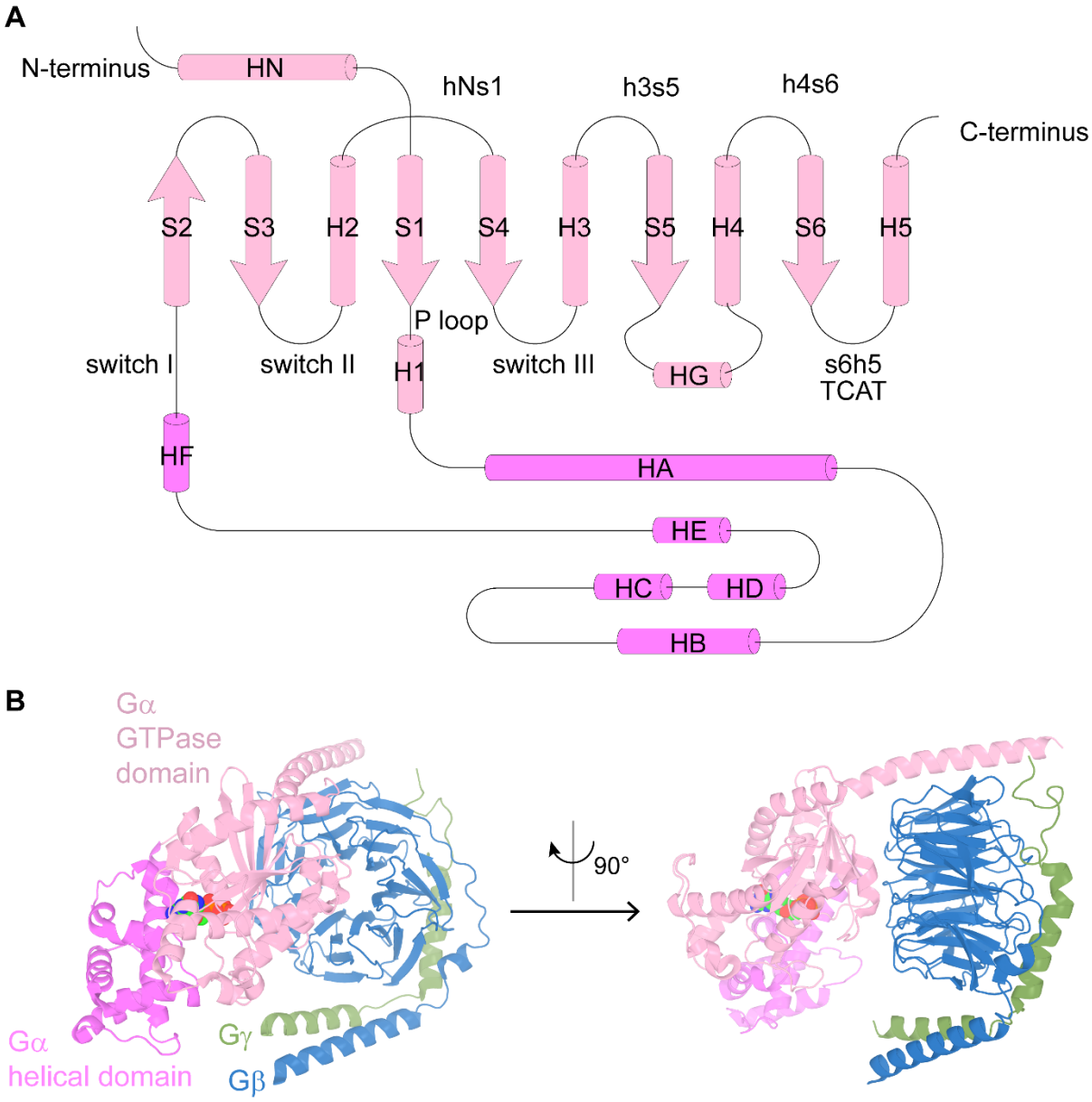


FIGURE 4. Structural characteristics of heterotrimeric G proteins. A: Schematic representation of the domain architecture of the Gα subunit. B: Structure of the GDP-bound G_s protein (PDB ID: 6EG8). The Gα subunit is composed of a GTPase (pink) and a helical (magenta) domain, with a GDP molecule (spheres, colored by atom type) bound between the two domains. The Gβ subunit (blue) is folded into a seven-bladed β-propeller structure, which makes extensive contacts with the small Gγ subunit (green). The latter consists of two α-helices connected by a short linker.

The GTPase domain is organized as a compact packing of seven α-helices (HN, H1 to H5, and HG) and six β-strands (S1 to S6) connected by flexible loops (FIGURE 4). Three loops, termed switches I, II and III, display significant structural differences between the inactive (GDP-bound) and active (GTP-bound) states, being more rigid and ordered in the active conformation (70, 235, 236, 285, 305, 449). The helical domain is composed of a six α-helix bundle (HA to HF) that forms a lid-like structure over the nucleotide-binding pocket, burying the bound nucleotides in the core of the Gα subunit (70, 235, 236, 285, 305, 449). In the inactive state, the helical and GTPase domains are held together by a series of interactions involving highly conserved residues within HA and HF of the helical domain and H1, H5 and the P-loop of the GTPase domain (110).

The Gβ subunit folds into a seven-bladed β-propeller structure, containing seven WD-40 repeats, which is connected to the N-terminal α-helix. The small Gγ subunit contains two α-helices connected by a short linker (FIGURE 4). The N-terminal α-helix of the Gβ subunit forms a coiled-coil interaction with the N-terminal α-helix of the Gγ subunit, providing the basis for the strong association observed between the Gβ and Gγ subunits (236, 402, 449). The primary contact between the Gα subunit and the Gβγ dimer involves a hydrophobic pocket in the Gα subunit formed by switches I and II with a contribution of the N-terminal HN helix of the GTPase domain (236, 449).

When the Gα subunit is in the inactive state, a GDP molecule is bound between its GTPase and helical domains. While the GTPase domain provides the main binding site for nucleotides and is responsible for GTP hydrolysis (276), the helical domain helps prevent nucleotide dissociation and assists in GTP hydrolysis (383).

The interaction of a G protein with an active receptor triggers a series of conformational changes in the Gα subunit, which include the repositioning of H5 and an approximately 127° rotation of the helical domain relative to the GTPase domain (344) (FIGURE 5). These conformational changes ultimately promote the release of GDP from the Gα subunit, GDP replacement by GTP and the dissociation of the active GTP-bound Gα subunit (Gα-GTP) from the Gβγ dimer (100, 314). Similar to

receptors, G proteins likely exist in a dynamic equilibrium among different sub-states in which the helical and GTPase domains adopt different orientations relative to each other, as supported by BRET (117), EM (69, 458) and DEER spectroscopy (440) studies.

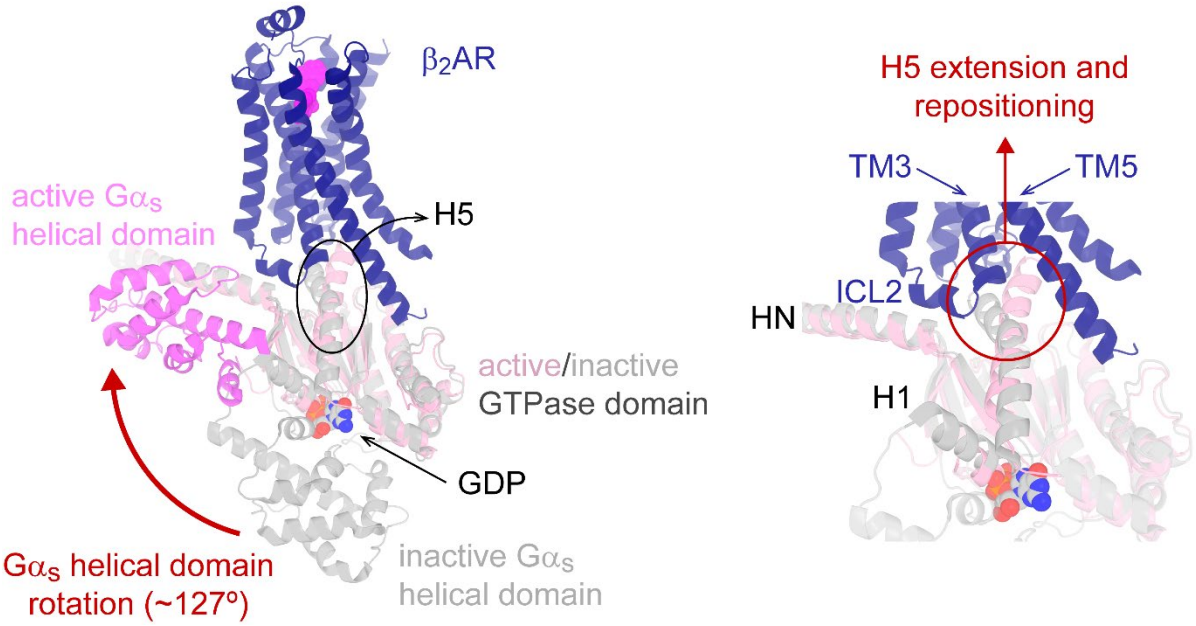


FIGURE 5. Conformational changes associated with G protein activation. Shown are superimposed structures of the inactive, GDP-bound heterotrimeric G_s protein (PDB ID: 6EG8; gray) and the active, nucleotide-free G_s protein in complex with β_2AR (PDB ID: 3SN6). Magenta, helical domain; pink, GTPase domain. The G$\beta\gamma$ dimer is omitted for clarity. The main conformational changes are highlighted in red.

Several G protein regions are involved in receptor interaction, as revealed by the structure of the active β_2AR–Gα_s complex (344). These include the α-helixes H4, H5 and HN, the β-strand S6, and the loops s2s3, hNs1, h4hG and h4s6 (FIGURE 6). A crucial role is played by H5, which comprises of the 26 most C-terminal amino acids of the Gα subunit (H5.01-26), with the last five residues folded to form a hook-like structure (434). H5 provides a large part of the Gα_s interaction surface, contacting residues in TM3, TM5, TM6, ICL2 and ICL3 of β_2AR.

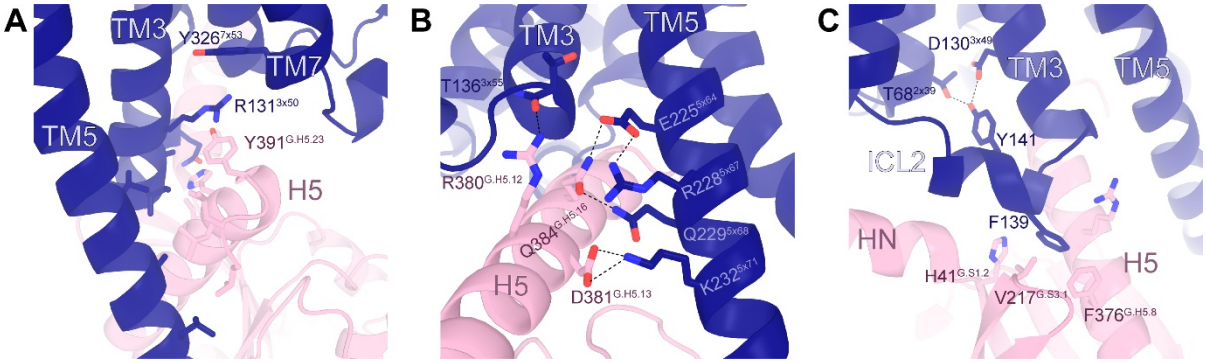


FIGURE 6. Main contacts between β_2AR and G_s protein. Shown are enlarged views of the interaction interface as observed in the structure of the active β_2AR–G_s complex (PDB ID: 3SN6). TM6 is omitted for clarity. H5 of $G\alpha_s$ (pink) is docked into a cavity on the intracellular side of the receptor (blue) arising from the relocation of TM5 and TM6. A: Main interactions of the H5 tip with the receptor core. Most interactions are non-polar. An exception is the interaction of $Y^{G.H5.23}$ in H5 with R3x50 in the conserved DRY motif of TM3. R3x50, in turn, interacts with Y7x53 within the conserved NPxxY motif of TM7. B: Interactions of the middle part of H5 with the receptor. The middle part of H5 forms a network of polar interactions with residues in TM3 and TM5. C: Interactions involving ICL2. T2x39 in TM2 and D3x49 of the DRY motif in TM3 form intramolecular interaction with Y141, which is located in a short α-helix within ICL2. These interactions position the α-helix in ICL2 so that F139 within the same domain docks into a hydrophobic pocket on the G protein surface, thereby providing a structural link between the DRY motif and the G protein.

These studies also provided important insights into the molecular rearrangements that occur in a G protein during receptor–G protein interactions and the subsequent G protein activation (344, 434). In the inactive G protein state, two universally conserved residues in H5, $Phe^{G.H5.8}$ and $Val^{G.H5.7}$, interact with residues in H1/S2/S3 and S5/S6, respectively. In the active state, these interactions are broken, allowing the tip of H5 (H5.11-H5.26) to insert deep within the receptor core, where it interacts with TM3, TM5, TM6, ICL2 and ICL3 (131, 344). The transition between the two states involves a rotation and translation of H5 away from the nucleotide binding site and towards the receptor. This results in increased flexibility and repositioning of H1, accompanied by rearrangement of the adjacent s6h5 loop

– involved in guanine-ring-binding via its TCAT motif – and the s1h1 loop (P loop) – involved in phosphate binding. Contacts between H1, GDP and the helical domain hinge region are broken, weakening GDP binding, increasing the likelihood of helical domain opening, and allowing GDP to dissociate (7, 69). $Phe^{G.H5.8}$ plays a critical role in this activation process by relaying to the P-loop the conformational changes that are triggered by receptor binding (195). Intriguingly, molecular dynamics (MD) simulations and DEER spectroscopy experiments indicate that the rearrangement of the s6h5 loop is more important for nucleotide release than the physical separation of the GTPase and helical domains, which is believed to also occur spontaneously (100).

The high affinity complex of nucleotide-free G protein and active receptor likely represent a very short-lived, transient state in the G protein activation cycle. GTP, which is present in the cytoplasm at approximately 10-times higher concentration than GDP (39), rapidly binds to the nucleotide-free G protein, triggering its dissociation form the receptor. This is also believed to initiate the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer, or, alternatively, their rearrangement, as indirectly suggested by the results of some BRET and FRET studies (45, 117). The initial events in the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer involve the stabilization of switches I, II and III in the GTPase domain by the γ-phosphate of GTP, which then propagates to other regions of the $G\alpha$ subunit (235). Importantly, the dissociation or rearrangement of the G protein subunits unmasks a shallow hydrophobic surface between switch II and H3 of the GTPase domain – which is hidden in the heterotrimeric complex – where $G\alpha$ effectors bind (64, 65, 268, 393, 421, 422).

Although most receptor–G protein interactions are mediated by the $G\alpha$ subunit, the $G\beta\gamma$ dimer seems to play an additional role. This has been better investigated in the case of rhodopsin where the results of kinetic studies suggest that the initial encounter between rhodopsin and a G protein occurs via the $G\beta\gamma$ dimer, followed by engagement of the $G\alpha$ subunit (160-162). A direct interaction between rhodopsin and the $G\beta\gamma$ dimer has been recently observed in a cryo-EM structure of rhodopsin in complex with the G_i protein, where the C-tail of rhodopsin interacts with residues in blades 6 and 7 of

the Gβ subunit (433). Further information has recently been obtained by a cryo-EM structure of the active muscarinic M1 receptor in complex with the G_{11} protein, in which 11 residues of the receptor distal to H8, which were not visible in previous structures, have been resolved. Interestingly, this C-terminal region extends into the groove formed by the Gα GTPase domain and the Gβ subunit (270). In addition, the muscarinic M3 receptor has been suggested to interact with the Gβ subunit in a ligand-independent manner via a polybasic C-terminal domain conserved among several $G_{q/11}$-coupled GPCRs (335). Furthermore, possible interactions of the Gβγ dimer with the ICL3 of M2 and M3 receptors (469) and the ICL1 of A_{2A}Rs (119) have been reported. The modality of receptor interaction with the Gβγ dimer might differ among receptors, as supported by the recently reported structures of calcitonin and GLP-1 receptors in complex with the G_s protein (254, 255, 481). These Family B GPCRs include an extended and tilted H8, which forms extensive contacts with the Gβγ dimer, a feature not seen with Family A receptors (119, 254, 255, 481). These findings suggest that Family A and Family B GPCRs might interact with the Gβγ dimer through distinct mechanisms.

D. Structural basis for G protein specificity

Although the mechanisms responsible for G protein specificity are not fully understood, important insights can be gained from the recently obtained structures of GPCRs bound to different G proteins, including G_s, $G_{i/o}$ and G_{11}.

One determinant of G protein-coupling specificity appears to reside in the degree of TM6 outward movement and the accompanying opening of the intracellular G protein binding cavity within the receptor transmembrane core (357). The relatively wider intracellular cavity found in G_s-coupled receptors might be required to accommodate the larger side chains present within H5 of $G\alpha_s$ compared to $G\alpha_i$. The only available structure of a GPCR in complex with $G\alpha_{11}$ (270) shows that the degree of TM6 outward movement is comparable to that of G_i-coupled receptors. However, the relatively bulkier side chains in H5 of $G\alpha_{11}$ are accommodated by an additional 3 Å translation of H5 towards TM1 and TM2 (270).

Another likely determinant is the primary structure of H5. While one third of H5 residues are highly conserved across species and Gα protein isoforms providing the basis for a common mode of receptor binding (110), the remaining two thirds are variable and possibly contribute to G protein specificity (313). For example, the formation of a tight interaction between $R^{G.H5.17}$ of H5 and R^{5x68} in Family A or K^{5x64} in Family B GPCRs has been suggested to contribute to the wider outward movement of TM6 associated with G_s coupling (131, 433).

Muscarinic receptors have proven particulary useful for investigating the mechanisms of G protein coupling specificity due to their preferential coupling to either $G_{i/o}$ (M2, M4) or $G_{q/11}$ (M1, M3, M4), despite a high degree of sequence homolgy. This led to the identification of a series of determinants of $G_{i/o}$ versus $G_{q/11}$ specificity in ICL3 (228, 391, 456, 457), ICL2, TM5 and TM6 (32, 33, 221, 258). In particular, the presence of a tyrosine residue at postion 5x62 appears required for efficient $G_{q/11}$ coupling (33), whereas residues present in the 6x33-6x37 region seemingly play a critical role in $G_{i/o}$ versus $G_{q/11}$ specificity (30, 258). Most of these residues do not contact the Gα subunit directly, but rather participate in intrahelical interactions within the receptor that determine the exact position and orientation of TM6, and, thus, indirectly control G protein specificity (270).

A recent structural comparison of the active M1R–G_{11} and M2R–G_{oA} complexes (270) has provided further insights into the mechanisms of G protein coupling specificity. In the M1R–G_{11} structure, H5 and the rest of the GTPase domain are rotated further away from the receptor TM5 compared to what is observed in the M2R–G_{oA} (270) and other receptor–G protein complexes. This difference may be due to the presence of more extensive interactions of the M1R ICL3 with the Gα_{11} subunit. Of note, residues in direct contact with Gα_i or Gα_{11} appear to be conserved only within the muscarinic receptor family, suggesting that, rather than sharing a common binding modality, receptors from different sub-families likely evolved different mechanisms to interact with and activate G proteins (109).

V. OPEN QUESTIONS AND EMERGING CONCEPTS IN GPCR SIGNALLING

A. Signal compartmentalization

After the discovery of the soluble second messenger cAMP (415), it was initially assumed that the effects of hormones like adrenaline or glucagon propagate rapidly within the stimulated cells to activate their intracellular effectors. However, subsequent observations in intact cells and tissues unexpectedly pointed to the presence of spatial compartmentalization in receptor signaling (21, 410). Among other observations, groundbreaking studies showed that both adrenaline and prostaglandin E1 (PGE1) induce similar increases in cAMP levels and PKA activation in heart tissue, but only adrenaline is able to significantly activate glycogen phosphorylase, increase heart contractility, and induce troponin I phosphorylation (43, 196). Later, the β-adrenergic agonist isoproterenol, but not PGE1, was found to increase the amount of cAMP and PKA activity in the particulate fraction of rabbit heart lysates, which mainly contain type-II isoforms of PKA (150). Based on these and similar observations, Buxton and Brunton proposed that β-adrenergic and PGE1 receptors might induce cAMP accumulation and PKA activation in distinct subcellular compartments, leading to different biological effects (47). However, verifying this hypothesis proved difficult, mainly due to technical limitations. The picture changed with the introduction of novel biophysical methods that allow cAMP levels and PKA signaling to be monitored in living cells with subcellular resolution (91, 300, 332, 478, 480). This approach provided direct evidence that, despite cAMP being a small, soluble second messenger, cAMP/PKA signaling downstream of GPCR activation can be highly compartmentalized in both space and time (17, 52, 59, 248, 271, 298, 301).

B. G protein signaling at intracellular sites

The classical view that GPCR signaling occurs exclusively at the plasma membrane has been challenged by work of our own and others showing that GPCRs can signal at intracellular sites, including early endosomes and the Golgi/trans-Golgi network (53, 108, 132, 176). These findings suggest that intracellular membranes serve as alternative, or, possibly, even preferred platforms for GPCR signaling. This newly appreciated modality of GPCR signaling adds yet more complexity to an already multifaceted system, raising many questions such as: How do GPCRs located at intracellular sites get

activated? Which signals dictate where inside a cell and for how long a given GPCR signals? To what extent does the location of GPCR signaling influence downstream events and the ensuing physiological responses?

The endoplasmic reticulum (ER) represents the beginning of the GPCR life cycle; here, the newly synthesized GPCRs are folded, assembled and enter the secretory pathway to be transported to the plasma membrane (418). Once at the plasma membrane, GPCRs can be activated by a wide range of physiological cues and are often internalized via arrestin-mediated CME upon prolonged stimulation as outlined above. The internalized receptors are then trafficked to endosomes where they are either dephosphorylated and recycled to the plasma membrane or directed to lysosomes for degradation (178, 226, 330, 403).

The first evidence that internalized GPCR might still be able to send signals from endosomes came from the study of non-classical, G protein-independent mechanisms (77). Soon after the identification of β-arrestins as adaptors for endocytosis, a number of signaling proteins were shown to interact with β-arrestins upon receptor stimulation. Among them are members of the Src family of tyrosine kinases (84, 266), components of the MAPK cascade (85, 267, 279), cAMP phosphodiesterases (321) and members of the diacylglycerol (DAG) kinase family (296). Probably the best characterized interaction is the one with ERK1/2, which has been suggested to promote mitogenic responses independently of G protein signaling (77). This phenomenon was first reported for the AT_1R (200, 267, 453). Furthermore, experiments using dominant-negative mutants of dynamin and β-arrestin revealed that β_2AR internalization was required to mediate ligand-dependent activation of ERK1/2 (77). Subsequent studies suggested the existence of two independent modalities or phases of ERK1/2 activation: a first, transient phase triggered by G proteins at the plasma membrane leading to ERK1/2 translocation to, and signaling, in the nucleus; and a second, sustained phase triggered by internalized receptors in the endosomal compartment, mediated by β-arrestin and characterized by ERK1/2 signaling in the cytoplasm (85, 200, 267, 427, 453). Although several lines of evidence support the notion that β-

arrestin promotes activation of ERK1/2 independently of G protein signaling, some recent studies have challenged this concept. Using genome editing and small interfering RNAs to interfere with β-arrestin 1/2, O'Hayre *et al.* provided evidence that, while β-arrestin 2 is essential for β_2AR internalization, both β-arrestin isoforms are dispensable for ERK activation (308). In agreement with these results, a subsequent study taking advantage of gene-edited cell lines lacking either Gα subunits or β-arrestins supports a model in which G proteins but not β-arrestins are required for several GPCRs to initiate ERK signaling (140).

More recently, we and other groups have revealed a previously unknown and unexpected type of classical, G protein-dependent signaling on membranes of the endocytic compartment (FIGURE 7). A first possible hint came from a study on the pheromone receptor Ste2 in the budding yeast (394). Subsequently, studies on the thyroid stimulating hormone (TSH) and parathyroid hormone (PTH) receptors independently reveled that internalization of these GPCRs together with their ligands is associated with a prolonged phase of G_s protein-dependent signaling within the endosomal compartment, which is distinct from that occurring at the plasma membrane (53, 108). Interestingly, whereas internalized PTH receptors have been shown to signal on membranes of early endosomes (108), TSH receptors appear to signal from a subdomain of the trans-Golgi network, which they reach via retromer-mediated retrograde trafficking (132). Here, they meet an endogenous pool of G_s protein, adenylyl cyclase and PKA to trigger a local cAMP/PKA signal near the Golgi, which is required to efficiently induce gene transcription in the nucleus (132). A similar requirement of endosomal signaling for efficient gene transcription has also been shown for the β_2AR (435). Furthermore, important evidence for G protein signaling induced by the β_2AR on early endosomes has been obtained using fluorescently-labeled conformation-sensitive nanobodies that selectively recognize the active receptor or G_s protein (177). This approach has also revealed the occurrence of β_1AR-mediated signaling at the Golgi complex (176). This Golgi-resident pool of β_1ARs has been shown to be rapidly activated by adrenalin and other cell-impermeable agonists that are able to cross cellular

membranes and reach the Golgi-localized receptors via transport by the organic cation transporter 3 (176). A recent study has also shown that β_1ARs located on the Golgi-complex of cardiac myocytes stimulate phosphatidylinositol-4-phosphate hydrolysis via a cAMP-mediated activation of Epac and PLCε to induce a hypertrophic response (294).

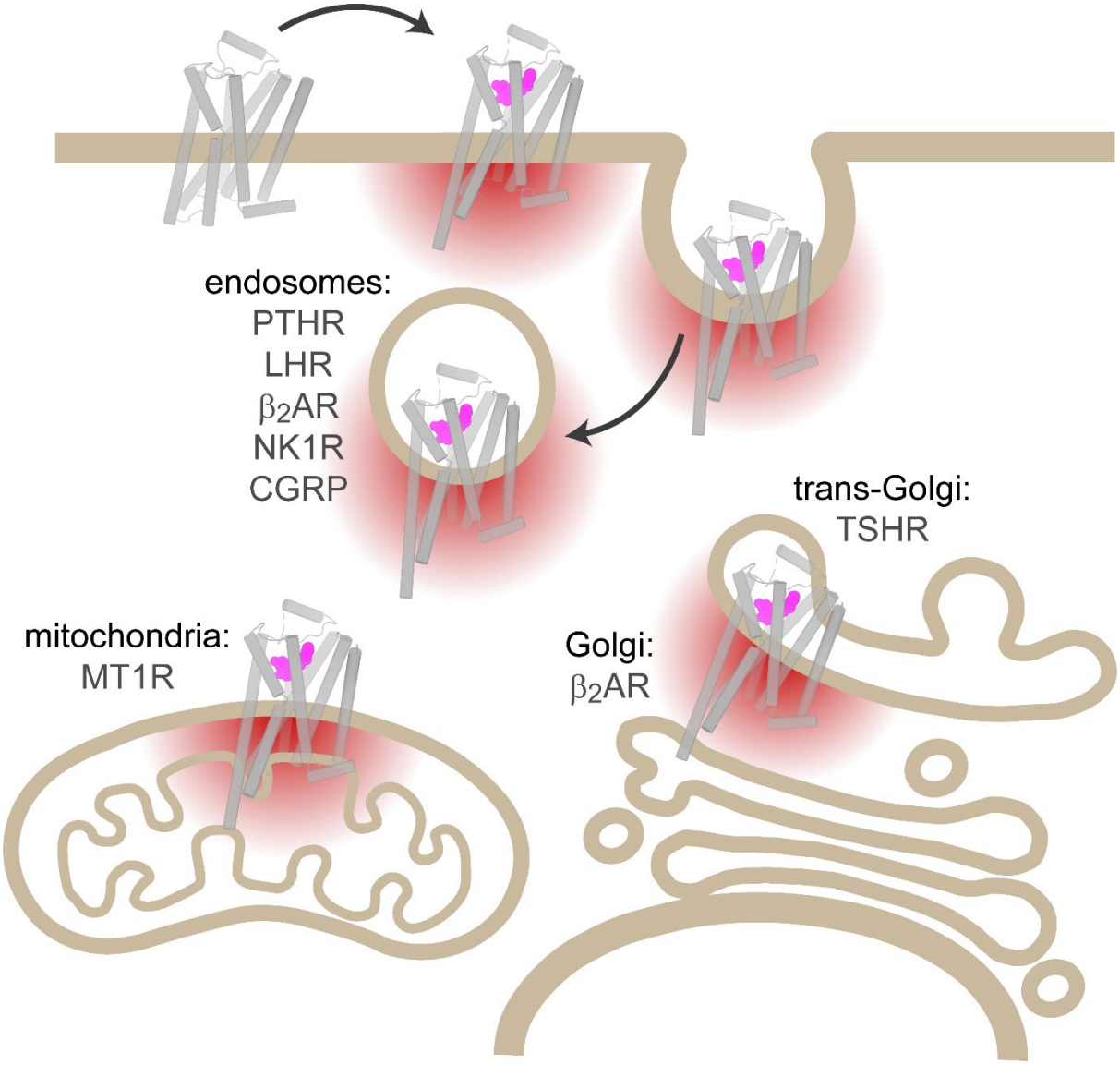


FIGURE 7. GPCR signaling at intracellular sites. Several GPCRs, including PTH, β_2AR, NKIR and CGRP receptors, have been shown to internalize to the endosomal compartment where they continue to stimulate downstream signaling (108, 177, 184, 435, 472). The TSH receptor traffics retrogradely to the trans-Golgi network where it induces a second wave of local cAMP/PKA signaling (132). Other receptors have been suggested to be already present at the Golgi (β_1AR) (176) or the outer

mitochondrial membrane (MT1R), where they are activated by either cell-membrane permeable or locally produced agonists (414).

Intriguingly, not only the location, but also the extent and duration of GPCR signaling in the endosomal compartment seem to vary substantially among receptors. For instance, TSH and PTH receptors induce marked and sustained responses, whereas those triggered by β_2-adrenerigc (177) or dopamine D1 (222) receptors appear to be smaller and more transient. Distinct factors might explain these differences, including the relative affinity of the receptors for their ligands.

It has also been proposed that prolonged endosomal G protein signaling may involve the formation of so called 'megaplexes' in which a GPCR simultaneously interacts with a G protein and an arrestin molecule (49, 426). This notion is consistent with biochemical and structural evidence that arrestin can bind to receptors via two distinct modalities: a so-called 'core' interaction, whereby arrestin occupies the receptor transmembrane core with its finger loop, sterically hindering the binding of a G protein; and a 'tail' interaction, whereby arrestin only binds to the phosphorylated receptor C tail, leaving the receptor core free. The tail interaction is believed to be the stronger of the two and allow β-arrestin to remain associated with receptors in endosomes (309, 310). This possibility is further supported by a recent cryo-EM structure of a complex containing a chimeric β_2-adrenergic-vasopressin receptor with a G protein bound to the receptor core and β-arrestin bound to its C-tail (299).

In addition to the endosomal compartment, there is evidence that GPCRs might initiate signaling from other intracellular organelles, including the ER (352), nucleus (90, 113, 187, 203, 345), mitochondria (1, 22, 23, 46, 123, 147, 414, 451), lysosomes (311, 365) and melanosomes (377). For instance, melatonin, a small lipophilic ligand, is found in high concentrations in mitochondria where it has been suggested to activate the melatonin receptor 1 (MT1R) located on the mitochondrial outer membrane (414). In addition, the mitochondrial matrix is capable of synthesizing melatonin *in situ*, from where it could reach and activate the local pool of MT1Rs (414).

With the growing number of studies investigating GPCR signaling at intracellular sites, our understanding of its physiological implications is increasing. For example, a recent study on the neurokinin 1 receptor (NK1R) provided important evidence that endosomal signaling is implicated in a pathophysiologically relevant process *in vivo*, highlighting the potential therapeutic utility of endosomally directed drugs (184). Substance P is a neuropeptide of the tachykinin family that activates NK1R in second-order spinal neurons to mediate pain transmission. Prolonged substance P stimulation, as experienced in chronic pain, has been shown to induce NK1R internalization and sustained signaling from endosomes (184). Intriguingly, the same study has shown that pharmacological inhibition or siRNA silencing of dynamin impairs substance P-evoked endocytosis of the NK1R, accompanied by inhibition of cytosolic cAMP production, PKC activation and nuclear ERK signaling (184). Importantly, blockade of endocytosis impairs sustained substance P-induced excitation of spinal cord neurons *in vitro* as well as nociception in a mouse model, suggesting that endosomal NK1R signaling is required for normal pain transmission. The same group additionally demonstrated that the calcitonin receptor-like receptor, which is co-expressed with NK1R in spinal neurons and mediates the effects of the calcitonin gene-related peptide (CGRP), also signals from endosomes (472). Inhibitors of dynamin, as well as ERK and PKC, prevents the sustained actions of CGRP in pain-transmitting neurons in the spinal cord (472). Another example is provided by a study on the LH receptor, which stimulates the final steps of oocyte maturation and triggers ovulation at mid-cycle (269). Using intact ovarian follicles isolated from mice expressing a FRET sensor for cAMP, LH receptor internalization was shown to induce a second wave of cAMP production within granulosa cells, which is required for efficient signal transmission to the oocyte to induce meiosis resumption (269).

C. Biased signaling

Early depictions of GPCR signaling envisaged linear signaling pathways. However, it soon became evident that GPCRs can receive a range of incoming signals, and in turn, initiate a multitude of outgoing

signals, albeit with potential differences in potency, magnitude and/or rate of activation (277). The concept of biased signaling, as the word implies, refers to the supposed ability of a given agonist for a given receptor and in a given cellular system to preferentially activate one (or more) of these pathways, whereas another agonist for the same receptor may preferentially activate others (281). Importantly, biased signaling has been proposed as a means to develop more selective and effective drugs (198, 272). Although a number of studies have documented effects that can be traced back to biased signaling, the concept of biased signaling remains highly debated as several unrelated factors could contribute to or even explain some of those observations without the intervention of biased signaling (395). These include system, observational and kinetic factors that often complicate data interpretation (206, 238).

Some of the first descriptions of 'bias agonists' focused on the ability of certain ligands to induce arrestin-mediated signaling while competitively antagonizing G protein signaling (446). A prime example of biased ligand is the AT_1R peptide agonist [Sar_1, Ile_4, Ile_8]-Ang (SII), which was initially shown to selectively recruit β-arrestin to the AT_1R and induce G protein-independent ERK MAPK signaling (339). Subsequently, the concept has been expanded to include G protein-biased signaling. G protein-biased compounds have been reported to not only preferentially induce G protein over arrestin signaling, but also differentiate between G protein types or even specific combinations of $G\alpha$- and $G\beta\gamma$-mediated signaling (198).

A frequently invoked mechanism to explain biased signaling is the ability of different ligands to stabilize a given receptor in distinct active conformational states, each coupled to a unique downstream signaling pathway (381). This selective stabilization of distinct conformational states could be achieved by ligands that bind to the canonical orthosteric binding site as well as by allosteric modulators or bitopic ligands that simultaneously interact with both orthosteric and allosteric sites (395). Although new important insights have recently been obtained by comparing the structures of

receptors in complex with different G proteins and arrestin, no simple relationship has been found between any given receptor conformation and its coupling to a specific transducer.

Besides the possible stabilization of receptors in different active conformations, another important factor that has been proposed to contribute to, and potentially confound, the interpretation of biased signaling is the kinetics of ligand binding and dissociation. For instance, the unique chemical structure of lysergic acid diethylamide (LSD) prolongs its residence time at both the 5-hydroxytryptamine 2A and 2B receptors compared to the ergot-derived agonist ergotamine (448). The resulting slow dissociation of LSD has been suggested to contribute to its relatively potent ability to induce β-arrestin recruitment, an effect that might be crucial for its hallucinogenic activity *in vivo* (448). Furthermore, a role of ligand binding kinetics in biased signaling is supported by a study investigating a set of agonists for the dopamine D2 receptor (206). Although it remains uncertain how different binding or dissociation kinetics translate into variable signaling responses, it has been proposed that certain ligands may dissociate slower from their receptor compared to others, which might potentially reflect the stabilization of the receptor in different conformational states that are linked to distinct cellular responses (206). At the same time, caution should be taken when interpreting the results of these and similar studies, as kinetic factors may also confound the results, especially when responses are measured at single time points (206). Since biological responses can have distinct and complex temporal profiles, single-point measurements can erroneously attribute differences in the binding or dissociation kinetics among ligands to bias effects (206).

Even assuming that different ligands induce distinct receptor conformations, a remaining question is how this translates into long-lasting, distinct cellular responses. One intriguing hypothesis is that ligands might induce unique receptor phosphorylation patterns or 'barcodes', as supported by recent studies. For example, stimulation of the β_2AR with isoproterenol or carvedilol has been shown to induce distinct patterns of β_2AR phosphorylation, mediated by GRK2 and GRK6, respectively (303). These changes correlate with different patterns of β-arrestin recruitment and MAPK phosphorylation

(349). Likewise, ligand-specific phosphorylation 'barcodes' have been reported for angiotensin (68), opioid (188, 245), serotonin (134) and chemokine (483) receptors. In the case of the C-C motif chemokine receptor 7, both of its two endogenous ligands, CCL19 and CCL21, induce G protein coupling and GRK6-dependent receptor phosphorylation. However, it has been shown that only CCL19-mediated activation results in GRK3 recruitment, leading to a more robust β-arrestin binding, receptor desensitization and internalization, possibly due to a distinct phosphorylation pattern (483). Moreover, a recent elegant study using either full length GRK2 or its C-terminal pleckstrin homology domain has revealed that κOR agonists can induce different modalities of GRK2 recruitment to the receptor (411).

The newly discovered paradigm of GPCR signaling at intracellular sites may also provide another important mechanism to explain both biased signaling and the barcode hypothesis (53, 108, 132, 176). For instance, two distinct PTH receptor agonists, PTH_{1-34} and the PTH-related peptide ($PTHrP_{1-36}$), were shown to differ in their capacity to promote receptor signaling at intracellular sites (108). Whereas $PTHrP_{1-36}$-mediated signaling is restricted to the cell surface and is rapidly reversible, PTH_{1-34} stimulation induces receptor internalization and signaling in the endosomal compartment, leading to a persistent cAMP response (108). Another example comes from a more recent study showing the existence of a signaling pool of β_1ARs on membranes the Golgi complex. Intriguingly, it has been shown that adrenaline and dobutamine can reach and activate this Golgi pool of β_1ARs via transport across membranes mediated by the organic cation transporter 3 (176). If, as it appears, different agonists for the same receptor may induce unique spatio-temporal patterns of receptor internalization, trafficking and signaling at intracellular sites, this might represent a key mechanism at the basis of biased signaling.

Following the discovery of β-arrestin-biased agonists, major efforts have been devoted to investigating their potential as novel drug candidates. The first β-arrestin-biased AT_1R agonist, SII, was shown to have beneficial effects on isolated rat cardiomyocytes, including the stimulation of cell

contractility together with a reduction of apoptosis (339). These promising results lead to the development of peptide TRV120027, a β-arrestin-biased AT_1R ligand with increased potency and efficacy towards β-arrestin-mediated signaling. TRV120027 was shown to decrease blood pressure and improve myocardial contractility in preclinical studies in rats (105, 445). However, in a phase IIb trial, TRV120027 did not produce a composite clinical benefit in acute heart failure compared with placebo (319). Further studies appear required to fully explore the pharmacological potential of β-arrestin-biased ligands.

Whereas most attention has been devoted to β-arrestin-biased ligands, G protein-biased agonists might also find therapeutic application. Perhaps the best example stems from the study of biased signaling at the μ opioid receptor (μOR). Several studies support the notion that analgesia is associated with G_i protein-mediated signaling, whereas gastrointestinal dysfunction, respiratory depression, and tolerance have been linked by some studies to β-arrestin recruitment (197, 337). These studies stemmed from the initial observations that β-arrestin 2 knock-out in mice potentiates the analgesic effect of morphine (36) while reducing tolerance (35). The results further prompted the search for G-protein biased μOR drugs. In particular, the μOR agonist oliceridine (formerly known as TRV130) has been shown to induce robust G protein activation with a potency and efficacy similar to morphine, while causing limited arrestin recruitment and μOR internalization. In a randomized, double-blind controlled trial, oliceridine displayed greater pain relief than morphine with fewer or similar side effects (399, 447). Likewise, PZM21, a subsequently developed μOR agonist with properties similar to oliceridine, exhibited improved analgesia with minimal constipation and respiratory depression (399, 447). Although oliceridine was not approved by the FDA in 2018 due to insufficient evidence of an improved benefit-risk profile (67), it was eventually approved in 2020 (437). It is also worth noting that recent efforts, including the generation of knock-in mice carrying a phosphorylation-deficient μOR, failed to replicate some of the initial observations in mice, questioning the role of β-arrestin 2 in opioid-induced tolerance, respiratory depression, and other side-effects (209, 210). Moreover, a new

potential mechanism for improved side effect profile of opioid agonists based on low intrinsic efficacy for G protein activation has been proposed (128, 129).

VI. KINETIC MODELS OF RECEPTOR–G PROTEIN INTERACTIONS: RANDOM COLLISIONS VS. PRECOUPLING/STABLE COMPLEXES

Along with the progressive elucidation of the mechanisms at the basis of GPCR signaling, several models have been proposed to describe the interactions between receptors and G proteins (156). At the time of the discovery of receptor and G proteins, the plasma membrane was considered to be a 'fluid mosaic' of proteins floating in a sea of lipids as originally proposed by Singer and Nicolson (392). In agreement with this model, receptors and effectors were initially thought to freely diffuse and interact via random collisions (317, 429). With the discovery of G proteins (361), this free collision coupling model was updated to incorporate the random collision between receptors and G proteins as well as between G proteins and effectors. An important underlying assumption of the free collision coupling model was that receptors and effectors or, as we now know, G proteins, interact only when receptors are activated by an agonist. Moreover, a general prediction of this model was that, under conditions of irreversible effector activation, receptor stimulation should eventually activate the entire pool of G proteins or effectors present on the plasma membrane. Consequently, increasing the number of receptors on the plasma membrane should result in faster and more efficient G protein/effector activation, leading to a left shift in the obtained concentration-response curve, without an increase in the maximal response. Such a behavior was observed studying adenylyl cyclase activation following βAR stimulation in turkey erythrocyte membranes, which provided evidence in support of the free collision coupling model (317, 429). However, the general validity of free collision coupling was soon challenged by a comparison with the response to A_{2A}R stimulation in the same system (428). The results revealed that maximal adenosine stimulation was able to activate 70% of the available pool of adenylyl cyclase, whereas all of it could be activated by βAR agonists (428). Moreover, the data obtained with A_{2A}R stimulation could not be satisfactorily fitted with the equations

derived for free collision coupling, but only with a model that considered a tight coupling, i.e. some degree of pre-association, between the $A_{2A}R$ and adenylyl cyclase (428). A subsequent study on the $A_{2A}R$ revealed a non-linear relationship between progressive receptor inactivation and the resulting reduction of adenylyl cyclase activity (262). In disagreement with the predictions of the free collision-coupling model, reducing the number of receptors decreased both the maximal response and the rate of adenylyl cyclase activation. In an attempt to explain these results, the authors proposed a restricted collision-coupling model based on the assumption that receptor mobility on the plasma membrane is slow compared to the speed at which ligands dissociate from the receptor. Monte Carlo simulations performed with this model could reproduce the experimental findings, suggesting that active receptors might have access only to a limited fraction of effectors located in their vicinity (139).

In the meantime, further insights into the mechanisms of receptor–G protein interactions were gained from radioligand binding experiments. Competition radioligand binding experiments revealed the existence of two inter-convertible binding sites characterized by high and low affinity for agonists, with progressive conversion of the high affinity to the low affinity site in the presence of increasing concentrations of guanine nucleotides (81). Attempts to explain these findings led to the formulation of the ternary complex model, whereby agonists were proposed to promote the formation of a high affinity complex between the agonist, the active receptor and the nucleotide-free G protein (81). In its full formulation, the ternary complex model allows the formation of receptor–G protein complexes also in the absence of agonist, consistent with several experimental observations (126, 297). Further studies on constitutively active receptor mutants led to the introduction of the extended ternary complex model (373), whereby receptors were proposed to exist in an equilibrium between an inactive and an active state, the latter stabilized by agonists. This model allows receptors to bind G proteins and initiate signaling also in the absence of agonists, explaining the basal activity measured for some receptors.

The ternary complex model has subsequently been completed to accommodate all possible states and transitions involving ligand, receptor, and G protein in what is known as the 'cubic ternary complex' model (454). This model can be visually represented as a cube, with each vertex corresponding to a state (FIGURE 8). Conveniently, transitions involving opposite faces of this cube correspond to ligand binding/dissociation, G protein coupling/uncoupling or receptor activation/deactivation (FIGURE 9). The cubic ternary complex model encompasses the collision-coupling model, the pre-coupling model, as well as the extended ternary complex model, which can be obtained by forbidding some of the transitions. The cubic ternary complex model represents a theoretical achievement as it is complete both thermodynamically and statistically (454). In spite of this, it cannot recapitulate the complex conformational dynamics that have recently been uncovered in both receptors and G proteins. Moreover, it does not take into account the complexity in the diffusion of receptors and G proteins that results from the nanoscale organization of the plasma membrane.

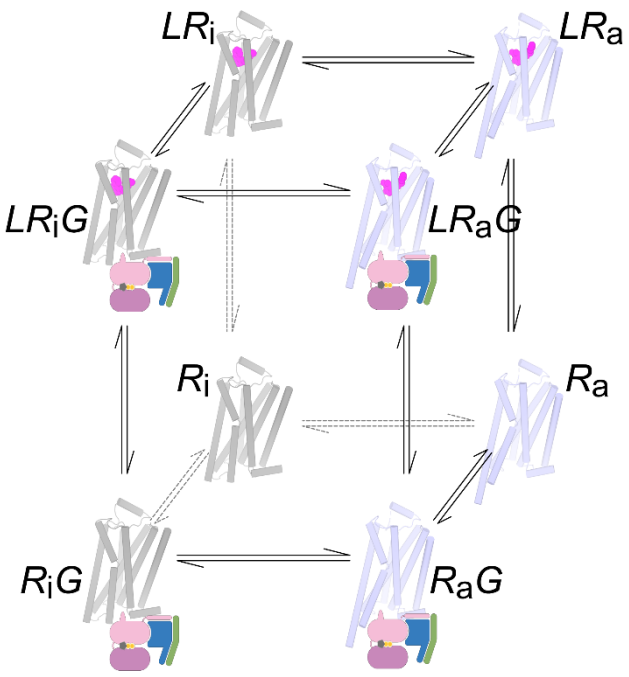


FIGURE 8. The cubic complex model. Each vertex of the cube represents a possible state, determined by the presence of a ligand, the active/inactive state of the receptor and the binding of a G protein.

Lines represent transitions between different states. *R*i, inactive receptor. *R*a, active receptor. *L*, ligand. *G*, G protein. Adapted from Weiss *et al.* 1996 (454).

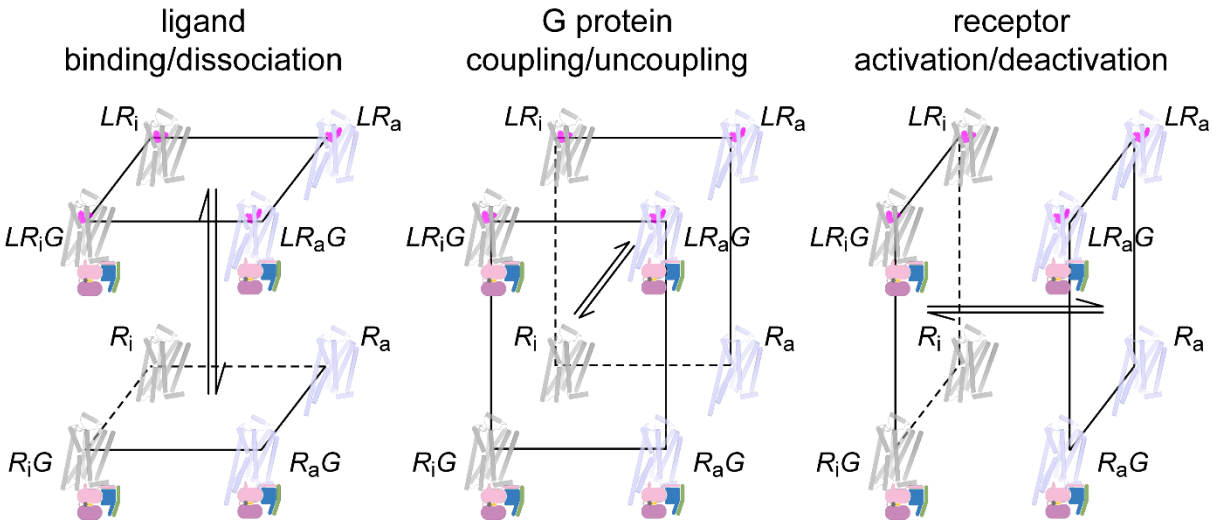


FIGURE 9. Main transitions in the cubic complex model. States are labeled as in FIGURE 8; *R*a, active receptor; *L*, ligand; *G*, G protein. Transitions between opposite faces of the cube correspond to ligand binding/dissociation, G protein coupling/uncoupling, and receptor activation/deactivation. Adapted from Weiss *et al.* (454).

VII. THE NANOSCALE ORGANIZATION OF THE PLASMA MEMBRANE

A. Lipid nanodomains

Important early evidence for the organization of membrane proteins into small domains within the plasma membrane came from biochemical studies on so-called lipid rafts. Lipid rafts were initially identified as small membrane domains, rich in sphingolipids, cholesterol, and a unique set of other molecules, including glycosylphosphatidylinositol (GPI)-anchored proteins, that are resistant to mild detergent extraction at low temperatures and can be isolated by sucrose gradient centrifugation (389). Some of these raft domains also contain caveolins, which are the building blocks of caveolae – small invaginations of the plasma membrane that had been identified by electron microscopy (390). Studies in neonatal rat cardiomyocytes revealed that β_1ARs are present in both caveolar and non-

caveolar domains, whereas β_2ARs are preferentially localized in caveolae, which they leave upon activation (367). Interestingly, it was shown that pharmacological disruption of lipid rafts/caveolae enhances and prolongs cAMP accumulation in response to β_2AR activation (470). Furthermore, although both β_1AR and β_2AR stimulate inotropic and chronotropic responses via activation of the G_s/cAMP signaling pathway, only β_1AR-mediated effects can be negatively modulated by muscarinic M2 receptors (9). The possible accumulation or exclusion of specific signaling components in lipid rafts/caveolae was advocated in an attempt to explain these findings. In particular, G_i proteins as well as certain adenylyl cyclase and PKA isoforms have been reported to be enriched in lipid rafts/caveolae (367). By increasing the local concentration of signaling molecules, lipid rafts/caveolae or similar membrane nanodomains might favor their interactions, and thus increase the speed and efficiency of specific receptor-mediated responses (174). Evidence for a high level of spatial compartmentalization in βAR signaling has also been obtained in adult cardiomyocytes, which display a highly sophisticated membrane organization, including the presence of transverse tubules (T-tubules). T-tubules are elongated invaginations of the plasma membrane that have a composition similar to caveolae and play a fundamental role in excitation-contraction coupling (468). For instance, an elegant study combining FRET and scanning ion conductance microscopy revealed that β_2ARs induce cAMP responses only in T-tubules, whereas β_1ARs do so both inside and outside T-tubules (302). Since the highly localized β_2AR responses from T-tubules are lost in failing cardiomyocytes (302) or in mice deficient of caveolin 3 (468), it has been suggested that the compartmentalization of β_2AR signaling in T-tubules plays an important role in assuring physiological responses to adrenergic stimulation in cardiomyocytes.

Although these and similar results provided important, albeit indirect, evidence for a role of lipid nanodomains in receptor signaling, the size and stability of these nanodomains has been a matter of debate (51, 104, 290, 326). Early attempts to directly visualize lipid nanodomains by immunofluorescence and electron microscopy in fixed cells produced conflicting results (278). This

controversy has been partially resolved with the introduction of single-molecule microscopy methods, which made it possible to directly observe individual GPI-anchored proteins and membrane lipids in living cells. The results revealed a much more dynamic picture than previously thought, with GPI-anchored proteins, phospholipids and gangliosides undergoing rapid diffusion on the plasma membrane (114, 220, 251). In addition, combining fluorescence correlation spectroscopy (FCS) with stimulated emission depletion (STED) microscopy researchers succeeded in precisely monitoring the diffusion of sphingolipids and GPI-anchored proteins on a nanometer scale (104, 164). The results of these studies suggest that sphingolipids and GPI-anchored proteins are transiently trapped within nanodomains of the plasma membrane of less than 20 nm in diameter, with average dwelling times in these nanodomains of approximately 10-20 ms. Cholesterol appears to play a role in the formation of these nanodomains as suggested by the results of its pharmacological depletion (104). Overall, these data are consistent with the formation of small, transient cholesterol-assisted lipid-protein complexes or nanodomains rather than stable liquid-ordered domains floating across the plasma membrane. Although lipid nanodomains might not be as stable as initially thought, lipid–protein interactions are nevertheless likely to have a major impact on the function of receptors and other membrane proteins (257).

B. Role of the cytoskeleton

The membrane skeleton, i.e. the cytoskeleton closely associated with the plasma membrane, consists of a network of actin filaments and microtubules that interact with both integral and peripheral membrane proteins (288). The membrane skeleton is involved in various cellular processes, including both endo- and exocytosis (115), and provides mechanical resistance against extracellular forces (92).

The importance of the membrane skeleton in controlling the location and movement of membrane molecules was directly demonstrated by pioneering single-particle tracking studies with gold nanoparticles (372) and optical tweezers (103, 371). These studied revealed that the membrane skeleton partitions the plasma membrane into small nanodomains (182, 231). As a consequence, it

was shown that the diffusion of both lipids and proteins in the plasma membrane is about 20-times slower than in artificial lipid bilayers – for a comprehensive review, see Kusumi *et al.* (231). As membrane proteins and lipids become transiently trapped in the nanodomains defined by the cytoskeleton, their trajectories display a characteristic 'hop' diffusion (114, 436). Hence, trajectories can be described by two distinct diffusion coefficients, depending on the considered spatio-temporal scale: a microscopic one that describes the free diffusion of molecules within the nanodomains and a macroscopic one that describes their slower effective diffusion across the plasma membrane (230). Consistent with a prominent role of the cytoskeleton in controlling the movement of membrane molecules, their diffusion was found to be sensitive to actin-depolymerizing drugs (251). In contrast, an involvement of interactions with the extracellular matrix or lipid rafts was ruled out by the lack of changes upon treatment with trypsin or cholesterol depleting agents, respectively (114). The μ opioid receptor (μOR) provided an ideal model for these experiments. By labeling μORs with small gold nanoparticles, it was possible to record the diffusion of individual receptor particles with high spatial and temporal resolution (416). At the relatively slow acquisition rate of one frame every 33 ms, μORs were seen to diffuse as expected in the case of simple Brownian motion; however, when imaged at the much higher rate of one frame every 25 μs, μORs displayed a characteristic 'hop' diffusion. Mild actin depolymerization with latrunculin B increased the apparent size of the trapping compartments, leading to an increase in the macroscopic diffusion coefficient (416). These and similar experiments led to the formulation of the so-called fence-and-picket model of the plasma membrane (232). According to this model, the actin filaments ('fences') and the integral membrane proteins associated with them ('pickets') create barriers to the diffusion of membrane proteins, leading to their transient confinement in small nanodomains of the plasma membrane. Using fluorescently labeled molecules, Murase *et al.* showed the size of these compartments to range from tens to hundreds of nanometers (291).

In addition to restricting the lateral diffusion of membrane proteins, the cytoskeleton plays a fundamental role in controlling their precise location, usually through interactions mediated by

scaffold proteins (233, 293). These mechanisms have been best investigated at chemical synapses, where a high spatio-temporal organization is required for rapid and precise synaptic transmission (385, 412). A prime example is provided by ionotropic glutamate receptors, which mediate the rapid effects of glutamate, the main excitatory neurotransmitter in the central nervous system. Single-particle tracking and super-resolution imaging in neurons have shown that the localization and lateral mobility of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, one of the two main subtypes of ionotropic glutamate receptors, is governed by interactions with the membrane skeleton (71). These interactions with the membrane skeleton have been shown to be mediated by stargazin, which is an AMPA receptor regulatory protein that interacts with the postsynaptic density protein 95 (PSD95), a synaptic scaffold protein with guanylate cyclase activity (63). Since the transient nature of these interactions allow receptors to exchange between synaptic and extra-synaptic sites, they might provide an important mechanism to control the number of receptors present within a synapse and, hence, its activity (432). Similar findings have recently been obtained for GPCRs. For instance, metabotropic $GABA_B$ receptors have been shown to be largely immobile and align along actin fibers on the plasma membrane (54). This organization results from $GABA_B$ receptor interactions – likely indirect – with the actin cytoskeleton, as supported by its disruption upon pharmacological depolymerization of actin (54). Some scaffold proteins mediating interactions between GPCRs and the membrane skeleton have also been identified. One of these is filamin A, which has been shown to interact with several GPCRs, including the somatostatin receptor type 2 (SSTR2) (322). The interactions between SSTR2, filamin A, and the actin cytoskeleton have been recently investigated by single-molecule microscopy (431). This study has shown that SSTR2 and filamin A undergo transient interactions, which occur preferentially along actin fibers and contribute to restraining SSTR2 diffusion. As supported by experiments with a filamin A dominant-negative fragment, these interactions facilitate the agonist-dependent recruitment of SSTR2 into CCPs and its subsequent internalization (431).

Several studies have also investigated the impact of the cytoskeleton on G proteins and effectors as well as downstream signaling. Insel *et al.* reported about 40 years ago that colchicine treatment, which disrupts the microtubular assembly, potentiates β-adrenergic-stimulated cAMP accumulation in S49 lymphoma cells (175). This is at least partially due to the role of the cytoskeleton in regulating the formation and spatial arrangement of caveolae/lipid domains where GPCRs, G proteins and downstream enzymes such as adenylyl cyclases are concentrated on the plasma membrane (152). Pharmacological disruption of either microtubules or actin filaments revealed that these elements restrict cAMP signaling by regulating the localization of GPCRs, G proteins, and ACs in lipid rafts/caveolae (153). Conversely, it has been suggested that G proteins can serve as regulators of microtubule assembly and dynamics. In particular, Gα has been shown to inhibit microtubule assembly (363) while Gβγ has been suggested to bind to microtubules and promote their assembly (364, 387). Furthermore, adenylyl cyclase 8 was suggested to interact directly with actin (13).

VIII. ADVANCED OPTICAL METHODS TO STUDY GPCR SIGNALING

A. Ensemble FRET and BRET

Classical biochemical and pharmacological methods require cell disruption and, therefore, have only limited temporal and, generally, no spatial resolution. These limitations have been partially overcome by the introduction of optical methods based on resonance energy transfer (RET), which allow monitoring cell signaling in living cells. RET is a physical phenomenon that consists of the non-radiative transfer of energy between a fluorescent (FRET) or bioluminescent (BRET) donor and an acceptor fluorophore. Since RET occurs only when the donor and acceptor are in close proximity, typically less than 10 nm, it can be exploited to monitor protein–protein interactions as well as conformational changes within a protein of interest, e.g. a receptor (52, 263). In order to do so, the protein(s) under investigation need to be labeled with a suitable pair of RET donor and acceptor molecules. These can be genetically encoded, allowing direct fusion to the protein(s) of interest. Blue and yellow variants of the green fluorescent protein (GFP) are often used as fluorophore pair in FRET measurements. In the

case of BRET, the donor, instead of being a fluorescent molecule, is a luciferase enzyme, which generates light through oxidation of a specific substrate (211). Since the fusion of large protein tags like GFP or luciferase can potentially alter the properties of the native protein(s), suitable functional tests should be performed to exclude this possibility.

FRET and BRET have successfully been used to monitor all key steps in GPCR signaling in living cells, including ligand binding, receptor activation, the coupling to G proteins, G protein activation, the production of soluble second messengers like cAMP, and the activation of downstream protein kinases such as PKA (5, 12, 45, 91, 300, 324, 332, 444, 478, 480). These experiments clarified a number of important questions on the mechanisms and kinetics of GPCR activation and signaling. This includes important evidence for cAMP/PKA compartmentalization (17, 52, 59, 248, 271, 298, 301). Moreover, they provided important quantitative insights into the kinetics of GPCR signaling and the mechanisms of G protein activation (45). FRET and BRET have also been widely used to investigate the formation of receptor dimers and oligomers (283).

B. Single-molecule microscopy

The advent of single-molecule microscopy (27, 287) revolutionized the study of membrane proteins. Whereas single-molecule methods were initially reserved to few, highly specialized labs, recent methodological advances, including the development of more sensitive cameras and innovative labeling strategies, have made these methods accessible to a broader scientific community. Labeling with bright organic fluorophores is often preferred in single-molecule microscopy as it allows extended imaging compared to the use of less photostable fluorescent proteins. This can be conveniently obtained via fusion of a genetically encoded self-labeling tag like the SNAP tag (199). The SNAP tag is an engineered DNA repair enzyme that reacts irreversibly with fluorescent benzylguanine substrates. A modified version of the enzyme, known as CLIP tag, reacts specifically with benzylcytosine derivatives, allowing orthogonal labeling with two different fluorophores (121).

Alternatively, receptors and other membrane proteins can be visualized using fluorescently labeled antibodies, peptides or small molecule ligands (55).

Single-molecule imaging at, or near, the plasma membrane is generally achieved via total internal reflection fluorescence (TIRF) illumination. This technique exploits the evanescent excitation field that occurs when light is reflected at the interface between two media with different refractive indices, like in the case of a cell growing on a glass coverslip. Since the intensity of the evanescent field decreases exponentially with the distance from the interface, this allows illuminating only a thin layer of approximately 100-200 nm, typically encompassing the plasma membrane in contact with the coverslip and the cytoplasm immediately above it. Since fluorophores outside this thin illumination volume are not excited, TIRF results in a high signal-to-noise ratio and low background, which facilitates single-molecule visualization. Because of the diffraction of light, individual, fluorescently labeled molecules appear in TIRF images as much larger intensity spots, with a size of approximately 200-300 nm. However, so long as the fluorophores are well separated, their position can be determined with high accuracy by fitting pixel intensity values with a 2-dimensional Gaussian distribution (FIGURE 10A). The precision of this fitting is mostly dictated by the number of the collected photons and is typically in the range of 10-30 nm. This allows breaking the resolution limit of 200-300 nm that is intrinsically associated with far field microscopy. Tracking algorithms are then used to link the particles detected at each frame of an image sequence and, thus, reconstruct their trajectories (FIGURE 10B).

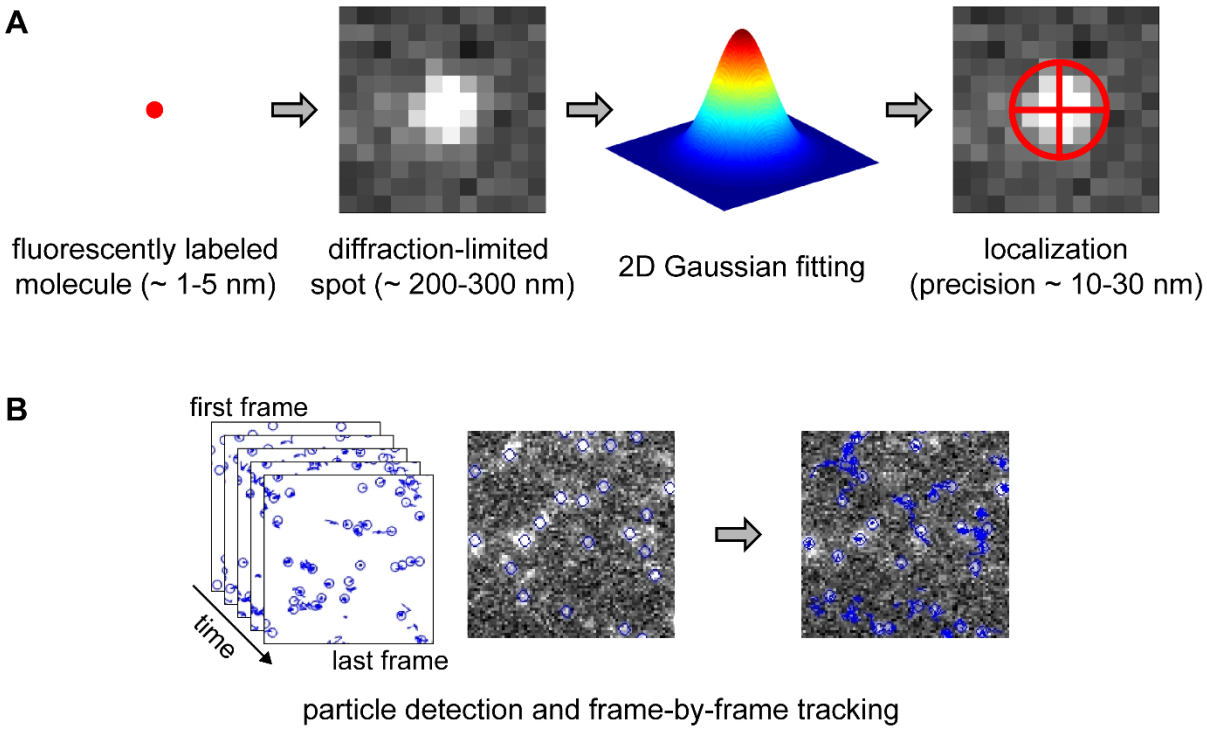


FIGURE 10. Single-molecule microscopy. A: Principle of single-molecule localization. A typical fluorescently labeled molecule appears as a diffraction-limited spot with a size (FWHM) of approximately 200-300 nm. However, the position of the molecule can be determined with high precision by fitting the pixel intensities to a 2D Gaussian distribution. The localization precision is largely dependent on the number of collected photons and is typically in the range of 10-30 nm. B: Single-particle tracking. An automated single-particle tracking algorithm is applied to the localizations in each frame of an image sequence to obtain trajectories (blue) describing the movements of the fluorescently labeled molecules over time.

TIRF microscopy has successfully been employed to study the diffusion of membrane proteins and lipids within the plasma membrane (54, 192, 193, 416). Moreover, it has found an important application in the study of the supramolecular organization and spatial arrangement of GPCRs and other membrane receptors. One of the first studies used a fluorescent agonist to investigate the muscarinic M1 acetylcholine receptor, which was observed to form transient dimers on the plasma membrane (158). Similar results were obtained using a fluorescent agonist of the N-formyl peptide

receptor (193). A further study compared β_1ARs, β_2ARs and $GABA_B$ receptors labeled with a bright organic fluorophore via the SNAP tag. While β_1ARs and β_2ARs were observed to rapidly diffuse, $GABA_B$ receptors were largely organized into arrays on the plasma membrane resulting from dynamic interactions with the actin cytoskeleton (54). Furthermore, the three receptors showed different tendencies to interact, with β_1ARs and β_2ARs predominantly existing in a monomer/dimer equilibrium, and $GABA_B$ receptors forming larger supramolecular complexes (54). Single-molecule microscopy has also been used to investigate the effects of agonists and antagonists on receptor dimerization, with variable results obtained in different receptor systems (54, 124, 191). Fast two-color TIRF microscopy using fluorescently labeled ligands has also recently been used to investigate opioid receptors, revealing short lived dimers between μORs (125), but not between κ opioid receptors (κOR) (97).

Finally, single-molecule FRET (smFRET) can be used to probe the conformational dynamics of individual proteins (141). This can be achieved by labeling a protein of interest with a suitable pair of organic fluorophores and measure FRET between them. smFRET measurements can be done on proteins in solution or in intact cells, but are mostly performed with purified proteins immobilized on a glass surface (450).

C. Single-molecule localization super-resolution microscopy

Another emerging application of single-molecule fluorescence microscopy is so-called super-resolution imaging (165, 376). The underlying principle is the same that allows single-molecule localization to bypass the resolution limit of conventional fluorescence microscopy. However, instead of working with very low densities of fluorophores to separate them in space, individual fluorophores present at high density are imaged by virtue of temporal separation. This results in only a small fraction of the fluorophores to be visible at any given time, allowing their precise localization. Through reiterating this process thousands of times, a high-resolution image is eventually reconstructed from the individual localizations. Since the entire process typically takes several minutes, single-molecule localization super-resolution microscopy has a limited temporal resolution and is most frequently

applied to fixed samples. Among popular super-resolution methods are direct stochastical optical reconstruction microscopy (*d*STORM) and photoactivated localization microscopy (PALM).

*d*STORM relies on the spontaneous switching of certain fluorophores between a dark and a fluorescent state (208, 374, 460, 466), a process that can be tuned by varying the experimental conditions. Recent studies have used *d*STORM to investigate GPCRs. In one study, *d*STORM was used to image the corticotropin-releasing hormone receptor 1 in a simple cell system (417). Moreover, *d*STORM has been applied to investigate the nanoscale organization of endogenous GPCRs in the brain (101, 385).

PALM (28) instead exploits the properties of reversibly photoswitchable fluorophores as well as irreversibly photoactivatable or photoconvertible probes. Upon irradiation with a suitable wavelength, typically near-UV, these probes can either be brought from a dark to a fluorescent state or made to emit at a different wavelength. In a typical PALM experiment, a low-power activating laser turns on only a small fraction of the fluorophores at a time, which are imaged and localized with high precision. The fluorophores are then rapidly brought to a dark state, usually via photobleaching. Similar to *d*STORM, this process is reiterated thousands of times until a super-resolved image is reconstructed. Also, PALM has been employed to investigate the nanoscale organization of GPCRs. For instance, Scarselli *et al.* employed PALM to visualize β_2AR clusters on the surface of a rat cardiomyocyte cell line (375).

D. Fluorescence correlation spectroscopy

FCS is a highly sensitive, quantitative method that is complementary to single-molecule microscopy (41). FCS is based on the detection of intensity fluctuations as fluorescent molecules diffuse through a small illumination volume, which can be generated by confocal microscopy or other methods. By performing an autocorrelation analysis of the intensity fluctuations over time, quantitative information about the average number and diffusion speed of the fluorescent molecules can be gained. One limitation of FCS is that it does not detect immobile or very slow molecules. An extension

of FCS is fluorescence cross-correlation spectroscopy (FCCS), which measures the cross correlation between two or more emission channels. FCCS finds an important application in the study of protein–protein interactions. These methods have been employed to investigate various aspects of GPCR signaling, including ligand−receptor interactions (41). Several GPCRs including β_2AR, adenosine A_1 and A_3 receptors and the histamine H_1 receptor have been investigated by FCS (42, 75, 76, 155, 282). Overall, these studies have revealed a high heterogeneity in the diffusion of receptors on the plasma membrane. Moreover, photon counting histogram analysis can be utilized to determine the size of receptor complexes. This has been employed to quantify adrenergic, muscarinic, and dopamine receptor dimers and oligomers (159, 170).

E. Advantages and disdvantages of single-molecule methods compared to ensemble methods

Single-molecule microscopy offers a number of important advantages compared to ensemble methods (TABLE 2). A first limitation of ensemble methods is that they often require overexpression of the investigated molecules, which might alter their signaling kinetics or specificity (263). Second, ensemble methods measure the average behavior of thousands or millions of fluorescently labeled molecules simultaneously (190). Since the investigated molecules are generally non-synchronized, this precludes the direct estimation of kinetic rates (55). In contrast, single-molecule methods can directly visualize and investigate individual fluorescent molecules present at low concentrations with high spatio-temporal resolution. Importantly, this allows the direct estimation of kinetic parameters, such as the rates of protein–protein association and dissociation at the plasma membrane (413). Since individual molecules are analyzed separately, single-molecule methods can also investigate complex mixtures or identify rare events that are typically hidden in ensemble measurements. Moreover, they can reach a spatial resolution of tens of nanometers, which enables researchers to directly study the nanoscale organization of the plasma membrane or the formation of receptor signaling nanodomains. An important drawback of single-molecule methods is the complexity and duration of data analysis, which is usually done on a large number of image sequences and trajectories in order to reach

sufficient statistical power. Moreover, they are not well suited in situations where the fluorescently labeled molecules reach high local concentrations, as is the case at specialized membrane structures such as neuronal synapses.

IX. LESSONS ABOUT RECEPTOR AND G PROTEIN SIGNALING FROM ENSEMBLE FRET/BRET

A. Kinetics of receptor–G protein interactions

The introduction of FRET and BRET methods proved particularly useful in investigating receptor–G protein interactions. Initially, studies focused on the apparent kinetics of G protein activation in response to agonist stimulation (16, 45, 111, 183, 474). This was achieved by measuring FRET/BRET between genetically encoded fluorophores attached to the Gα and either the Gβ or Gγ subunit. Subsequently, the association and dissociation between receptors and G proteins were also investigated by both FRET (108, 127, 156, 157, 185, 304) and BRET (14, 15, 116, 117, 229). Overall, these studies agree that receptor–G protein coupling can be very rapid (TABLE 3). In particular, FRET experiments performed with a fast superfusion system have shown that G protein coupling can occur with a time constant in the range of 50 ms, which is indistinguishable from the kinetics of receptor activation measured by FRET under the same conditions (156, 157). The rapid increase in receptor–G protein coupling observed in response to receptor activation indirectly suggested that receptors and G proteins might be in close proximity or even pre-assembled before receptor activation (116, 117). G protein activation was found to be slower, with time constants of approximately 500 ms, identifying it as the time-limiting step in this process (2, 45, 117, 157).

These studies also provided unexpected insights into the mechanisms of G protein activation. The results of classical biochemical studies indicate that Gα and Gβγ subunits dissociate upon GTP binding (40, 130). Moreover, there is evidence that the released Gα subunit might subsequently translocate from the plasma membrane to the cytosol (341, 366). Indeed, several FRET studies have reported a reduction in FRET or BRET between Gα and Gβγ following receptor activation (16, 45, 116, 157, 183, 229, 474). Although the observed reduction in FRET or BRET was often only partial, these results were

consistent with the classical model of G protein dissociation during activation. However, paradoxical increases in both FRET and BRET between Gα and Gβγ have been reported in some studies (45, 117). These responses appear to depend on the positions chosen to label the G protein subunits. These findings challenged the classical model, suggesting that instead of dissociating into Gα and Gβγ subunits, G proteins might undergo a conformational rearrangement that leads to the exposure of the intersubunit interface, which is required for their interaction with effectors. This hypothesis is further supported by the observation that non-dissociable G protein constructs are signaling competent (207, 253, 347).

Additional fundamental questions that have been investigated by FRET and BRET with discordant results regard receptor pre-coupling and the possible occurrence of preformed receptor–G protein complexes. Studies on several GPCRs and their G proteins have measured basal BRET or FRET in the absence of agonists, indirectly supporting the occurrence of pre-coupling. These include studies on the α_{2A}-adrenergic, β_2-adrenergic, vasopressin V2, thromboxane A2, calcitonin gene related peptide, bradykinin B2, protease activated 1, δ opioid, dopamine D2, muscarinic M4, adenosine A_1 and prostacyclin receptors (12, 14, 116, 117, 216, 304, 323). Based on the occurrence of BRET between a β_2AR mutant that is retained in the ER and the G_s protein, it was also suggested that receptor–G protein complexes might form intracellularly and co-traffic to the plasma membrane (102). However, no basal FRET or BRET was detected in other studies on the α_{2A}-adrenergic, β_2-adrenergic as well as muscarinic M2 and M3 receptors (16, 156, 229), providing evidence against pre-coupling. The occurrence of transient interactions between receptors and G proteins is further supported by the observation that the apparent speed of receptor–G protein association measured by FRET following agonist stimulation correlates with the expression level of both receptors and G proteins. Furthermore, a study examining muscarinic M2 and M3 as well as serotonin receptors showed that these receptors share the same pool of fully accessible G proteins, favoring a model of free collision coupling (16). These findings are also in agreement with the well-documented phenomenon of catalytic G protein activation, which is at the basis of signal amplification (388). In the special case of

rhodopsin and its G protein transducin, which have evolved to achieve maximal rapidity and sensitivity, it has been estimated that one rhodopsin molecule can activate as many as 1,000 G proteins per second in rod outer segment disk membranes (154). Intriguingly, fluorescence recovery after photobleaching (FRAP) experiments have provided evidence against the formation of stable α_2AR–G protein complexes (336). However, the same approach has revealed the possible occurrence of inactive-state complexes between muscarinic M3 receptors and the G_q protein in intact cells (335). Several factors might contribute to the apparent discrepancies among these studies, including differences among individual receptors and G proteins as well as a variable sensitivity of the employed methods. At the same time, it is worth mentioning that whereas FRET, BRET and, to some extent, FRAP are useful in monitoring changes in dynamic equilibria over time, they cannot directly probe the stability of protein–protein complexes or readily distinguish between conformational rearrangements and protein association/dissociation. This explains why, despite considerable efforts, the stability of receptor–G protein interactions has long remained elusive.

B. Evidence for GPCR and G protein association with effectors

Besides suggesting the possible occurrence of receptor–G protein pre-coupling, FRET and BRET have provided evidence for the formation of complexes of both receptors and G proteins with effectors. A well-studied case is that of GIRK/Kir3 channels, which mediate the effects of several $G_{i/o}$-coupled receptors in the brain, heart and other tissues. TIRF combined with FRET in intact cells provided evidence for the existence of trimeric G protein–GIRK/Kir3 complexes (354). Similarly, BRET and co-immunoprecipitation suggested the occurrence of stable interactions between heterotrimeric G proteins and GIRK/Kir3 channel subunits, which persist after receptor activation (346). Furthermore, it was shown that G protein–GIRK/Kir3 interactions start in the ER before the proteins reach the plasma membrane (355). In parallel, important insights came from the determination of X-ray crystallography structures of the G$\beta\gamma$–GIRK/Kir3 complex in different functional states (461). A subsequent study suggested that GIRK/Kir3 constitutively interacts with the δOR and G$\beta\gamma$ subunits,

with receptor activation leading to conformational changes at the Gβγ/Kir3 interface (353). Based on these findings, it has been proposed that receptors, G proteins and GIRK/Kir3 channels might form a preassembled multimeric complex that changes its conformation during receptor activation, allowing the Gβγ dimer to interact with and activate the GIRK/Kir3 channel while remaining bound to the receptor (292). Since the GIRK/Kir3 channel is a tetramer of four subunits, each interacting with a Gβγ dimer, four receptors might simultaneously interact with one channel, possibly occupying the grooves between two adjacent GIRK/Kir3 subunits (292).

Similar to GIRK/Kir3 channels, adenylyl cyclases have been suggested to form complexes with both receptors and G protein subunits. Constitutive interactions between G protein subunits and adenylyl cyclase 2 have been detected by BRET and co-immunoprecipitation (346). Moreover, a study on the relaxin family peptide receptor 1 (RXFP1) provided evidence for the formation of a constitutive complex containing the receptor, heterotrimeric G protein and adenylyl cyclase 2, capable of producing cAMP responses to sub-picomolar relaxin concentrations (144).

The association of receptors in macromolecular complexes with G proteins and effectors might serve as a mechanism to ensure fast, efficient and specific signaling. This might be particularly important in neuronal synaptic transmission. In hippocampal neurons, β_2ARs have been suggested to form signaling complexes containing G protein subunits, adenylyl cyclase, PKA and the voltage-dependent calcium channel $Ca_v1.2$ (80). Moreover, super-resolution imaging (*d*STORM) has recently revealed that the metabotropic glutamate receptor type 4 (mGluR4) is organized in small nanodomains with $Ca_v2.1$ channels and Munc-18-1 at cerebellar active zones (385). The short distances measured within these nanodomains are compatible with the occurrence of physical interactions between mGluR4, $Ca_v2.1$ and Munc-18-1. Such nanodomains or macromolecular complexes might contain more than one receptor, as suggested by findings on the mGluR4 (385) as well as by work on the A_{2A}R and D_2R (295).

X. NEW INSIGHTS INTO RECEPTOR–G PROTEIN SIGNALING FROM SINGLE-MOLECULE MICROSCOPY

A. GPCR activation and G protein coupling with purified proteins

Both single-molecule microscopy and FCS have been employed to investigate the conformational dynamics of purified GPCRs *in vitro*. In one of the first studies, Peleg *et al.* (320) monitored the fluorescence intensity bursts generated by the diffusion of micellae containing individual, fluorescently labeled β_2ARs through the illumination volume of a confocal microscope. β_2ARs were site specifically labeled with a small organic fluorophore at the cytoplasmic end of TM6. An analysis of the distribution of the burst intensities revealed more than one peak. Based on the results, the authors concluded that the β_2AR was likely present in at least two conformational states under basal conditions and three states after activation with the full agonist isoproterenol. Ten years later, Bockenhauer *et al*. (34) used anti-Brownian electrokinetic (ABEL) trapping to immobilize single, solubilized and fluorescently labeled β_2ARs for a time sufficient to monitor potential transitions among conformational states. The results were consistent with the β_2AR being in a dynamic equilibrium among multiple, discrete states, characterized by distinct fluorescent intensities and lifetimes. The conformational dynamics of β_2ARs was further investigated using a similar approach in lipid nanodiscs (237). Lipid nanodiscs provide a more native-like environment where receptors are surrounded by phospholipids. The results confirmed that the β_2AR can spontaneously transition between two discrete conformational states. Application of a full agonist shifted the conformational equilibrium towards the state characterized by higher fluorescence. In contrast, an inverse agonist shifted the equilibrium towards the state with lower fluorescence. An analysis of the dwell-times in the two states revealed a complex transition kinetics, with a faster and a slower component with rate constants of approximately 2-4 and 0.5 s^{-1}, respectively (237).

Overall, the above-mentioned studies indicated that agonist stimulation was only partially stabilizing the β_2AR in an active conformation, allowing the receptor to spontaneously transition to its inactive state. A likely explanation for this incomplete stabilization was the fact that the experiments had been conducted in the absence of G proteins. This hypothesis has recently been tested in an elegant study by Gregorio *et al*. (138). In this study, single-molecule FRET between two fluorophores attached to the

cytoplasmic ends of TM4 and TM6 of the receptor was used to monitor β_2AR conformational changes induced by ligands with different efficacies, in the presence or absence of G_s protein. This study showed that G protein binding is required to stabilize the β_2AR in an active conformation, which is characterized by low FRET. Moreover, it revealed a correlation between agonist efficacy and the proportion as well as time spent by individual β_2ARs in the active, low FRET state. Intriguingly, rapid addition of GDP or GTP to nucleotide-depleted β_2AR–G_s complexes gave rise to an intermediate FRET state, suggesting that the receptor–G protein complex might explore conformations that are distinct from the nucleotide-free state that has been observed crystallographically (344).

More recently, smFRET has been used in combination with X-ray crystallography to study the conformational dynamics of the platelet-activating factor receptor (PAFR) (57). A comparison of the crystal structures of the PAFR in complex with either the inverse agonist ABT-491 or the antagonist SR27417, revealed an unusual conformation in the presence of SR27417, characterized by an outward shift of TM2 and TM4 by 13 Å and 4 Å, respectively. smFRET between fluorophores inserted at the cytosolic ends of TM2 and TM4 were then used to further investigate these findings. The results confirmed the occurrence of ligand-specific conformational changes.

Single-molecule microscopy and FCS have also been used to investigate the conformational dynamics of metabotropic glutamate receptors (mGluRs), which play important physiological roles in the modulation of neurotransmitter release, neuronal excitability and synaptic plasticity (74, 348). Like other Family C GPCRs, mGluRs are dimeric receptors characterized by the presence of large extracellular ligand binding domains (LBDs) (74). A first study monitored rapid conformational changes within mGluR2 LBD dimers in solution by FCCS and smFRET with pulsed interleaved excitation (316). The ECD dimers were labeled with a pair of FRET donor and acceptor fluorophores via SNAP tags fused to their N-termini. The results of this study suggested that mGluR2 LBDs oscillate on a sub-millisecond scale between an open (inactive) and a closed (active) conformation, stabilized by the full agonist glutamate. Moreover, partial agonists were found to shift the equilibrium towards the same active

state, albeit to a lesser extent, rather than inducing distinct conformations (316). A subsequent study investigated full-length mGluR2 and mGluR3 immobilized on glass and labeled with a pair of fluorophores via SNAP and CLIP tags inserted at their N-termini (439). By monitoring smFRET between the two fluorophores, the authors concluded that the LBDs of mGluR2/3 are in equilibrium between a resting conformation, an active one, and a short-lived intermediate state, with some kinetic differences between mGluR2 and mGluR3 (439). Estimates of the receptor residence times in the resting and active conformations gave longer values than those observed for LBD dimers in solution (316), suggesting that the TMDs have a stabilizing effect on the LBDs (439). Interestingly, a subsequent study by the same group revealed that mGluR dimerization is primarily mediated by hydrophobic interactions between the LBDs rather than the formation of a covalent intersubunit disulfide bridge, as previously thought (252). Moreover, using photo-switchable agonists conjugated to one or both subunits of covalently linked mGluR dimers, this study showed that these intersubunit interactions prevent spontaneous LBD closure and mediate cooperativity in receptor activation (252).

B. Receptor–G protein interactions in living cells

Our group has recently succeeded in visualizing individual receptors and G proteins as they diffuse and interact on the surface of living cells by fast multi-color TIRF microscopy (FIGURE 11) (413). In this study, prototypical α_{2A}ARs and β_2ARs and their main interacting G proteins, G_i and G_s, were imaged simultaneously with a resolution of approximately 20 nm in space, and 30 ms in time. The receptors and G proteins were labeled with a pair of bright organic fluorophores via SNAP and CLIP tags (121, 199). This allowed tracking individual molecules for a time sufficient to analyze their diffusion and mutual interactions. Experiments were performed in both a simple cell system and in human primary endothelial cells, where α_{2A}ARs and β_2ARs play an important role on the control of vascular tone.

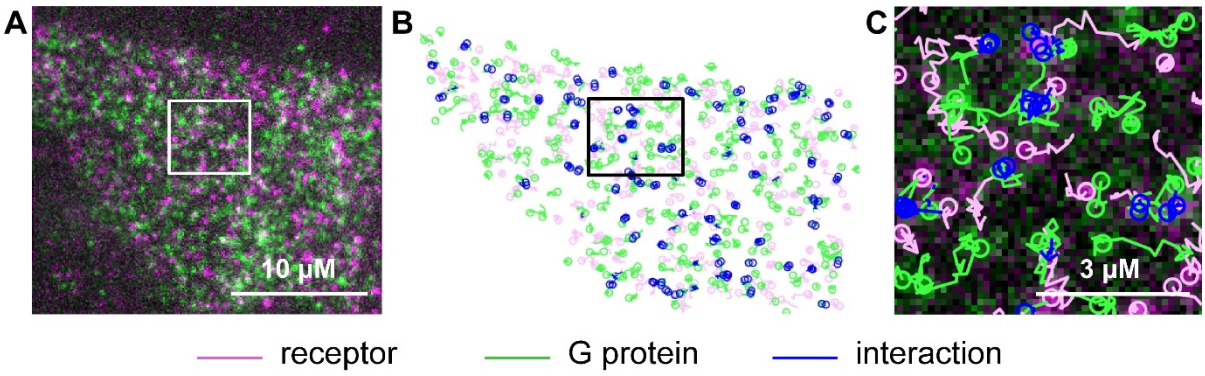


FIGURE 11. Single-molecule imaging of individual receptors and G proteins as they diffuse and interact on the surface of a living cell. Receptors (green) and G proteins (magenta) were labeled with two different fluorophores via CLIP/SNAP tags and imaged by fast two-color TIRF microscopy. An automated single particle tracking algorithm was applied to follow the movement of receptors and G proteins and detect their interactions. A: representative frame from a TIRF image sequence. B: Receptor and G protein trajectories obtained from the same image sequence. Blue denotes possible interactions. C: Enlarged view of the region corresponding to the box in A and B, with the trajectories overlaid on the image. See Sungkaworn *et al*. for further information (413).

This study revealed a high heterogeneity and complexity in the diffusion of receptors and G proteins on the plasma membrane, with both receptors and G proteins frequently switching among phases of rapid and slow diffusion (413). The phases of slow diffusion were found to result from transient trapping of receptors and G proteins in small areas of the plasma membrane. Labelling of the actin filaments and microtubules underneath the plasma membrane revealed that the trapping of receptors and G proteins was at least partially due to confinement in small nanodomains defined by the membrane skeleton, in agreement with the 'fence-and-picket' model of the plasma membrane (233). Importantly, this leads to the formation of 'hot spots' on the plasma membrane, where receptors and G proteins accumulate and preferentially interact with each other (FIGURE 12). Using a nanobody-based biosensor (Nb37) (177) that selectively binds the nucleotide-free, active G_s protein, it was also possible to show that G protein activation preferentially occurs at these hot spots, rather than homogenously across the plasma membrane (413).

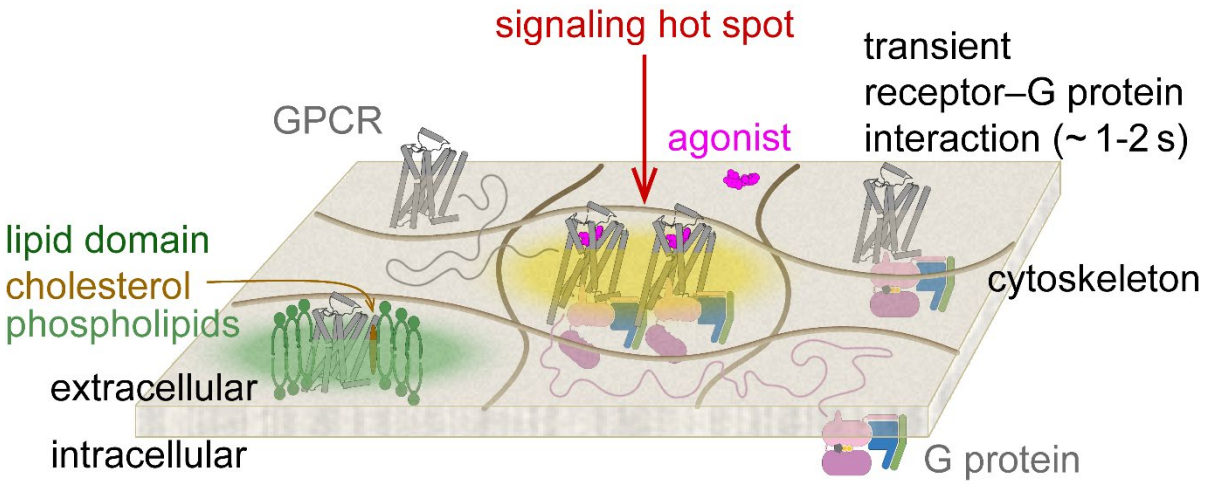


FIGURE 12. Hot spots for receptor–G protein interactions at the plasma membrane. The complexity of the plasma membrane, with important contribution of barriers provided by the cytoskeleton and its associated proteins, leads to the preferential accumulation of receptors and G proteins in small nanodomains of the plasma membrane (hot spots) where they preferentially interact and signal.

Using a novel mathematical approach based on deconvolution of the apparent colocalization times, this study also provided estimates of the microscopic rates of receptor–G protein association (k_{on}) and dissociation (k_{off}) in living cells (TABLE 3). In the absence of agonists, a low level of basal interactions between receptors and G proteins was observed ($k_{on} \approx 0.015$ $\mu m^2 molecule^{-1} s^{-1}$ for α_{2A}AR–G_i interactions), leading to transient interactions lasting approximately 1-2 s ($k_{off} \approx 0.5\text{-}1$ s^{-1}). Interestingly, agonists were shown to modulate receptor–G protein interactions mainly via increasing the association rate by approximately 10-fold. Intriguingly, this rate was approximately 10-times higher for α_{2A}AR–G_i protein than for β_2AR–G_s protein interactions, consistent with the view that receptor–G protein coupling might be more efficient in the case of G_i- than G_s-coupled receptors. The higher association rates oberved with the G_i compared to G_s protein might be the result of the relatively narrower opening of the intracellular binding cavity seen in the available structures of receptor–G protein complexes (58, 98, 119, 194, 214, 224, 344) (FIGURE 13). As further supported by a recent study (430), this kinetic difference between G_i- and G_s-coupled receptors might also explain

why, although both types of receptors can trigger Gβγ dissociation (79), only G_i-coupled receptors are capable of efficiently activating GIRK channels via Gβγ.

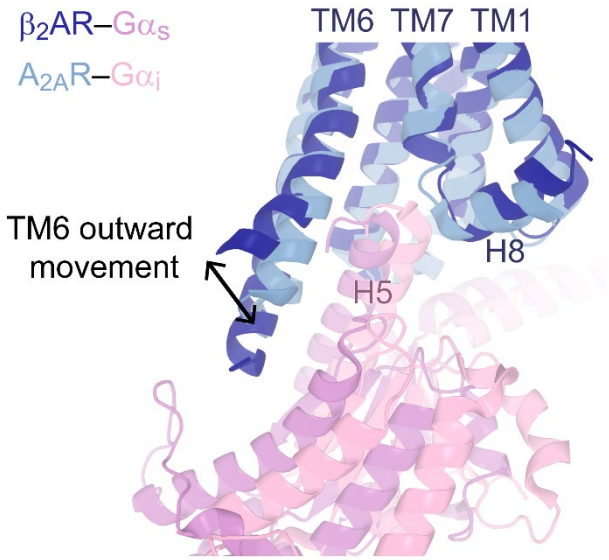


FIGURE 13. Structural comparison of the regions involved in G protein binding in the β_2AR–G_s and A_{2A}R–G_i complexes. Note the larger outward movement of the intracellular end of TM6 in the β_2AR–G_s (PDB ID: 3SN6) than in the A_{2A}R–G_i complex (PDB ID: 6D9H).

Experiments comparing a panel of agonists for the α_{2A}AR also revealed a positive correlation between agonist efficacy and the rate of receptor–G protein association, with only minor effects on the duration of the interactions (413). This is similar to what has been observed by Gregorio *et al*. on purified β_2AR by single-molecule FRET (138). Altogether, these results suggest that agonist efficacy operates at least partially at the level of receptor–G protein interactions.

Furthermore, our study shed new light on the long-debated question of whether receptors and G proteins are pre-coupled in the absence of agonists. Although a low frequency of transient interactions between G proteins and both receptors were detected, no preformed stable complexes were observed (413). These transient interactions were suppressed by treatment with an inverse agonist as well as by inactivation of the G_i protein with pertussis toxin, indicating that they result from the constitutive activity of the receptor and require a functional $G\alpha_i$ subunit (413). Importantly, these

transient interactions likely contribute to maintaining receptors and G proteins in close proximity, allowing for a rapid and efficient response to an agonist-induced receptor activation. At the same time, the transient nature of these interactions allows a single receptor to catalytically activate more than one G proteins, leading to signal amplification.

C. Towards a microscopic interpretation of protein–protein interaction kinetics

Recent single-molecule studies have provided quantitative information about the microscopic kinetics of receptor–G protein interactions. These results can be better interpreted in the light of microscopic models of protein–protein interactions (379). Although a complete analytical description of protein–protein interactions is presently unattainable, simplified models that can recapitulate the overall microscopic kinetics of protein interactions haven been developed (379).

In order for two proteins *A* and *B* to bind to each other, their contact surfaces need to be in a favorable orientation. Additionally, some proteins require large conformational changes to bind to their partners. To take these important aspects into account, it is convenient to consider protein–protein interactions to occur via the formation of an intermediate, nanosecond-lived state, generally known as the encounter complex (here referred to as *A*B*). The encounter complex can then undergo a series of conformational changes that ultimately lead to a fully assembled complex, called the productive complex (here referred to as *AB*). These reactions can be summarized as:

$$A + B \underset{k_{-\mathrm{D}}}{\overset{k_{\mathrm{D}}}{\rightleftharpoons}} A^{*}B \underset{k_{-\mathrm{c}}}{\overset{k_{\mathrm{c}}}{\rightleftharpoons}} AB, \qquad (1)$$

where k_D is the diffusion-controlled rate of formation of the encounter complex, k_{-D} is the diffusion-controlled rate of dissociation of the encounter complex, k_c is the rate of conversion of the encounter complex to the productive complex and k_{-c} is the rate of conversion of the productive complex to the encounter complex. The diffusion-controlled association rate k_D has units $mol^{-1}s^{-1}$ in solution, while the other rates have units s^{-1}.

On a relatively large scale (i.e., considering an entire cell), the time evolution of the concentrations of both encounter and productive complexes can be mathematically described by the following system of two first-order differential equations (86):

$$\begin{cases} \dfrac{\mathrm{d}[A^*B]}{\mathrm{dt}} = k_{\mathrm{D}}[A][B] - (k_{-\mathrm{D}} + k_{\mathrm{c}})[A^*B] + k_{-\mathrm{c}}[AB], \\ \dfrac{\mathrm{d}[\mathrm{AB}]}{\mathrm{dt}} = k_{\mathrm{c}}[A^*B] - k_{-\mathrm{c}}[AB]. \end{cases} \quad (2)$$

The three terms on the right hand side of the first equation, which describes the evolution of the concentration of the encounter complex, correspond to the formation of the encounter complex from *A* and *B*, its disappearance via dissociation or conversion to the productive complex, and its reverse conversion from the productive complex, respectively. The two terms on the right hand side of the second equation, which describes the evolution of the concentration of the productive complex, correspond to the formation of the productive complex from the encounter complex and its reverse conversion to the encounter complex. At equilibrium, the left hand side terms of both equations vanish. Under these conditions, one can express the overall rates for the formation (k_{on}) and dissociation (k_{off}) of the productive complex from *A* and *B* as:

$$\begin{cases} k_{\mathrm{on}} = \dfrac{k_{\mathrm{c}}k_{\mathrm{D}}}{k_{-\mathrm{D}} + k_{\mathrm{c}}}, \\ k_{\mathrm{off}} = \dfrac{k_{-\mathrm{c}}k_{-\mathrm{D}}}{k_{-\mathrm{D}} + k_{\mathrm{c}}}. \end{cases} \quad (3)$$

These equations predict two main scenarios. On the one hand, when the conformational rearrangement is fast compared to the dissociation of the encounter complex ($k_{\mathrm{c}} \gg k_{\text{-D}}$), the reaction is diffusion-limited and the protein–protein interaction is governed by the rate of formation of the encounter complex, i.e. $k_{\mathrm{on}} \approx k_{\mathrm{D}}$. At the same time, the overall dissociation rate is relatively low and can be approximated as $k_{\mathrm{off}} \approx k_{\text{-c}}k_{\text{-D}}/k_{\mathrm{c}}$. In this limit, *A* and *B* associate nearly instantaneously upon their encounter, so that most collisions are productive. On the other hand, when the conformational changes leading to the productive complex are much slower than diffusion ($k_{\text{-D}} \gg k_{\mathrm{c}}$), the conformational changes become rate limiting and the overall association constant $k_{\mathrm{on}} \approx k_{\mathrm{c}}k_{\mathrm{D}}/k_{\text{-D}}$ is

small. Under these conditions, most encounter complexes dissociate before leading to the formation of a productive complex. While the microscopic kinetics of protein–protein interactions have been mostly studied for purified proteins in solution (i.e. in 3 dimensions), limited experimental evidence exists for membrane-anchored proteins (i.e. in 2 dimensions), especially in their native context (122, 333, 379).

Extensive theoretical work has been devoted to modeling and interpreting the diffusion-controlled association rate k_D. In fact, k_D is a macroscopic constant that encompasses a number of microscopic phenomena, such as the capability of the reactants to effectively explore the medium or the occurrence of electrostatic interactions, and can be thought as the flux of molecules *A* that arrive onto a target molecule *B*. The simple case of an idealized reaction in which the encounter complex is formed instantaneously upon each collision between *A* and *B* was first studied by Smoluchowski in 1916 (396). In Smoluchowski's formulation, the two reactants are spherical objects that diffuse in a homogeneous three-dimensional medium. This leads to the well-known relationship:

$$k_D = 4\pi DR, \qquad (4)$$

where *D* is the sum of the diffusion coefficients of the two reactants and *R* is the sum of their radii. Smoluchowski's work marked a historical milestone in the development of a kinetic model of chemical reactions based on the theory of diffusion. However, Smoluchowski's formulation did not take into account the complexity of protein–protein interactions and the heterogeneity of the biological media, e.g. the cytoplasm or the plasma membrane in which those reactions take place. These phenomena cause the observed k_D values to be several orders of magnitude lower that the theoretically predicted values.

An important factor causing a significant departure from Smoluchowski's prediction lies in the fact that biological media are crowded and heterogeneous. This leads to deviations of the diffusion of biological molecules from the behavior expected for normal Brownian motion, a phenomenon known as 'anomalous diffusion'. Anomalous diffusion has been documented both in the cytoplasm (455, 465,

467) and on the cell membrane (54, 275, 413). Different theoretical models have been proposed to explain anomalous diffusion. One model considers an accessible medium with a fractal geometry, as may result from the presence of obstacles such as the cytoskeleton (369). Under such conditions, molecules tend to revisit the same locations several times. As a consequence, their mean first passage time towards a target is longer compared to normal diffusion (73). Analogous results have been obtained considering a model of molecules diffusing according to a continuous time random walk process (72, 148). This process is characterized by the alternation of short diffusive jumps and long stalling periods, as could be expected in the case of relatively long and frequent interactions with immobile cellular structures. Similarly, fluctuations in the diffusivity of individual molecules over time, e.g. due to changes in their conformation or in the local environment, have been shown to increase the mean first passage time towards a target by a multiplicative factor that depends on the amplitude of the diffusivity fluctuations (241, 406). However, such diffusivity fluctuations also lead to a broadening of the distribution of first passage times, effectively reducing the time required for the fastest molecules to reach the target (241, 406). Although these and similar theoretical models (240, 280, 424) have predicted a reduction of reaction rates, they almost always assume that the reactant disappears after first contact with the target. Whereas this can be assumed to be true in the case of irreversible enzymatic reactions, it does not recapitulate the behavior of more complex reactions like those frequently observed in cell signaling. This includes receptor–G protein interactions, where G proteins can interact multiple times with the same receptor and have a complex activation cycle, characterized by phases of high and low affinity for receptors.

A second important factor lies in the fact that the interacting molecules, e.g. a receptor and a G protein, are usually far from being perfect, inert spheres. If the two molecules bear opposite charges, electrostatic effects enhance their collision probability (384). Conversely, charges of the same sign generate repulsive forces, reducing the collision probability (384). Moreover, proteins usually exist in a dynamic equilibrium among different conformations with reactive sites typically occupying only a small area of their surface (379, 400). As a consequence, proteins typically need to undergo more than

one collision and explore different relative orientations and conformations before an encounter complex can be formed (137, 379). Altogether, these effects lead to important deviations from Smoluchowski's predictions as supported by both experimental measurements and Brownian dynamics simulations (379, 482).

After two proteins have collided, they usually need to undergo a series of conformational changes for their encounter complex to be converted into a productive complex. The kinetics of this transition can be rationalized by considering that the encounter and productive complexes correspond to two metastable states separated by an energy barrier. A protein complex configuration can jump over this energy barrier thanks to random fluctuations due to thermal rearrangements of its molecular components.

Hendrik Kramers shed important light on this process by developing a quantitative theory of how thermal fluctuations can lead to energy barrier crossing (223). These thermal fluctuations have an energy in the order of $k_B T$, where k_B is the Boltzmann constant and T is the absolute temperature. As a consequence, higher temperatures imply faster and more frequent conformational changes. Kramers' original approach looks at the problem in 'reaction coordinates', whereby conformational states are assumed to lie one after another on a single linear path (FIGURE 14). Each state is associated with an energy level. In the simple case of the transition between an inactive state (A) and an active state (C) separated by an energy barrier (B), random conformational rearrangements can be described by a random walk in a double well potential. The transition rate from A to C can be shown to be exponentially dependent on the height of the energy barrier. Adding an auxiliary molecule to the complex, e.g. a ligand or an allosteric modulator, is thought to modify the height of the energy barrier and/or the relative energy level of the two pre-existing states (87, 212). Lowering the energy barrier implies faster transition rates in both directions. Decreasing or increasing the relative energy of one of the two states will respectively increase or decrease the transitions towards that state. Since its

initial formulation, several extensions of Kramers' theory have been developed. For a thorough review, see Hänggi *et al*. (145).

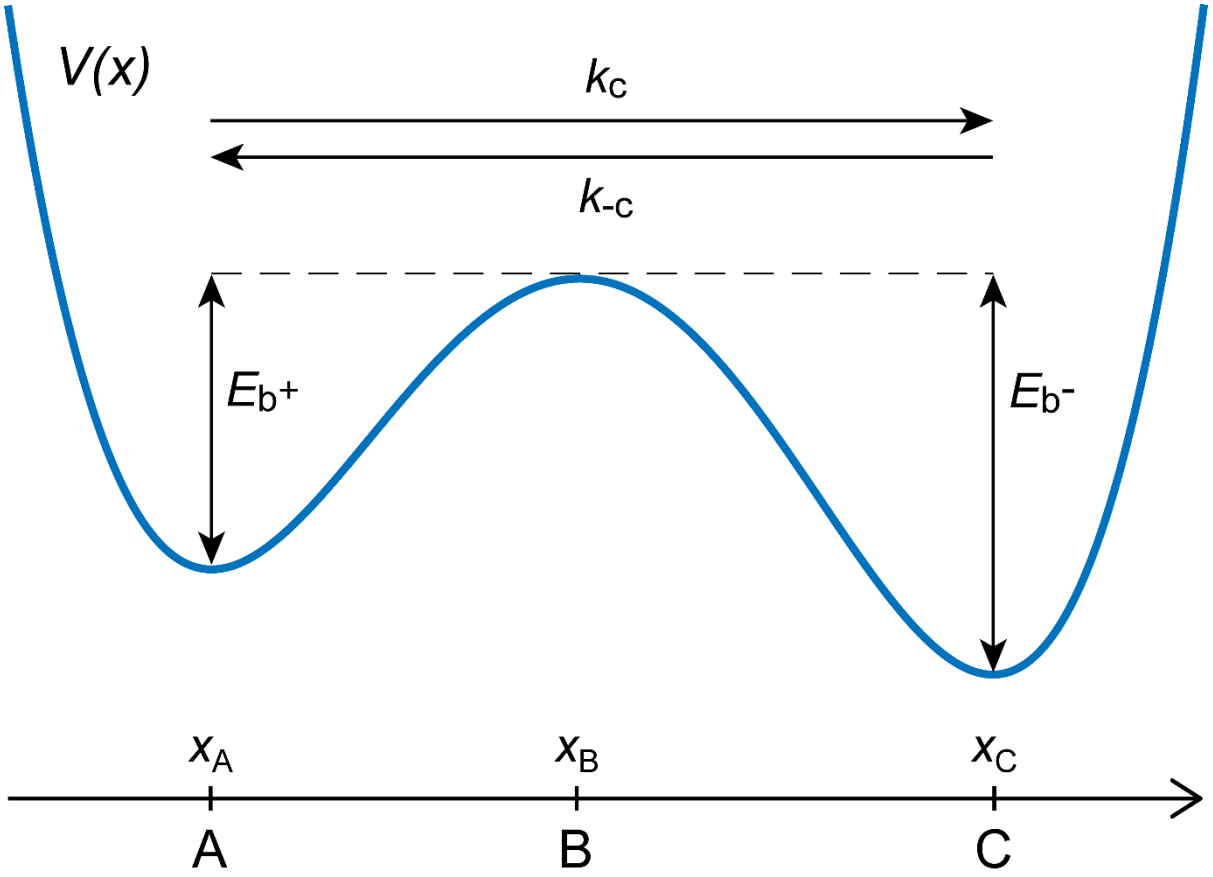


FIGURE 14. Diagram illustrating Kramers' theory applied to conformational changes. An inactive (A) and an active (C) state are separated by an energy barrier (B) of height E_b^+ in one direction and E_b^- in the opposite direction.

D. Lessons on kinetics of receptor–G protein interactions from single-molecule studies

Applying the above-described theoretical framework to the data obtained from single-molecule microscopy (138, 413) allows drawing a series of conclusions about the mechanisms that control the kinetics of receptor–G protein signaling and their implications (FIGURE 15).

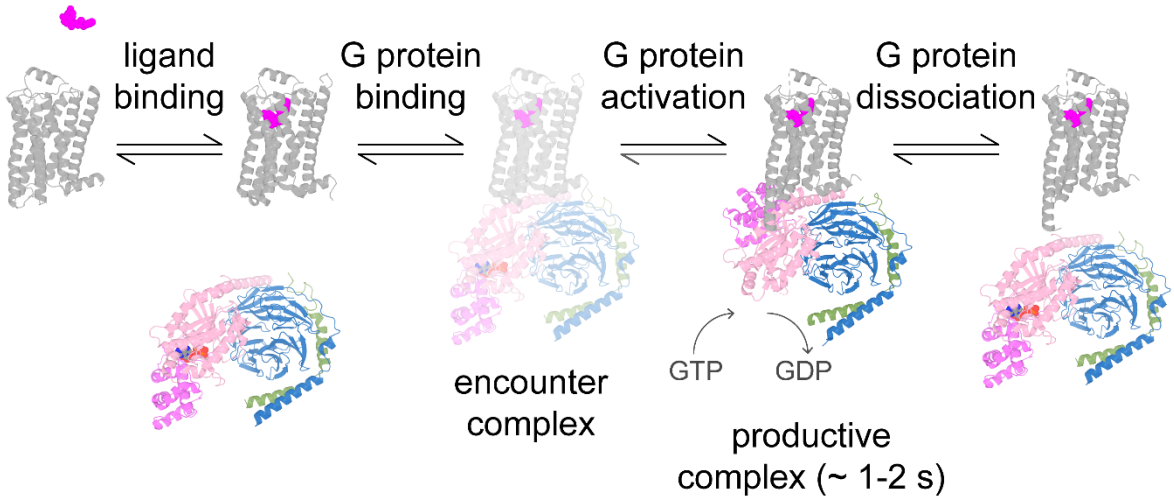


FIGURE 15. New kinetic model of receptor–G protein interactions based on recent single-molecule studies. Receptor–G protein collisions lead to the formation of a short-lived encounter complex, which converts into a productive complex lasting approximately 1-2 s. In the absence of agonists, there is a low conversion probability, leading to basal constitutive activity. Agonists increase the conversion probability, likely by favoring a receptor conformational state that is required for G protein interaction, ultimately leading to increased G protein activation.

A first aspect regards the interpretation of receptor–G protein association rates and how they are affected by agonist stimulation. Strikingly, agonist stimulation in living cells (413) or with purified receptors and G proteins (138) have shown a very similar effect, i.e. an approximately 7-10-fold increase in the overall rate of receptor–G protein association. In addition, both studies agree that receptor–G protein association rates are low in the absence of agonist (138, 413), being several orders of magnitude smaller than the diffusion limited association rates observed for macromolecules in solution (106). Moreover, they both revealed a positive correlation between agonist efficacy and the measured rates of receptor–G protein association (138, 413). Altogether, these findings suggest that receptor–G protein interactions do not operate in a diffusion-limited regime, but are rather controlled by the rate of the conformational changes required for the formation of the productive complex. This conclusion is in very good agreement with the growing evidence from structural and biophysical

studies that the formation of receptor–G protein complexes requires large conformational changes in the interacting G protein and, to a lesser extent, receptor. In the context of Kramers' transition state theory, these results can be explained by the existence of an unfavorable energy barrier for the conformational rearrangement under basal conditions, resulting in a low probability of encounter complexes being converted to productive ones, and, thus, in low G protein coupling and activation. Agonists likely lower this energy barrier and/or the energy level of the productive complex, thus favoring the transition from encounter to productive complex. This interpretation is in agreement with energy landscapes inferred from structural and biophysical studies on purified receptors (87, 212). Thus, the resulting low association rate under basal conditions and its efficacy-dependent increase upon agonist stimulation emerge as fundamental regulatory mechanisms that allow agonists to modulate GPCR signaling. Such regulatory mechanisms could not operate if receptor–G protein interactions were instead diffusion-limited. In this respect, it is tempting to speculate that nature might have evolved receptors and G proteins to undergo large conformational changes during their interactions so that their association rate can be controlled by agonists.

A second aspect regards the duration of receptor–G protein interactions. Interestingly, the average duration of 1-2 s measured by single-molecule microscopy is significantly longer than the time required to activate effectors such as GIRK channels, which can occur within 100 ms (156). These findings suggest that G proteins might stimulate GIRK/Kir3 channels, and potentially other effectors, while still bound, perhaps in a loose conformation, to the receptor. This view is supported by the results of computer simulations of GPCR signaling based on the experimentally measured kinetics parameters (413). Moreover, it is consistent with previous observations that G proteins might not dissociate into Gα and G$\beta\gamma$ subunits after activation (45, 117) and that receptors, G proteins and effectors, including both GIRK channels and adenylyl cyclases, might associate, at least transiently, in multimolecular complexes (292, 353).

A final aspect regards the formation of signaling hot spots and their consequences on the speed, efficiency and specificity of GPCR signaling. Importantly, the results of stochastic simulations comparing different diffusion scenarios indicate that the presence of hot spots approximately doubles the probability of receptor–G protein interactions (413). The reasons for this appear complex. In fact, the reduced exploration of the plasma membrane resulting from anomalous diffusion has been predicted to decrease reaction rates (148). However, the combination of basal interactions with the possibility of re-association after a previous interaction leads to the accumulation of receptors and G proteins within the hot spots. The overall outcome is an increase in the receptor–G protein association rate (413)**.** Based on these considerations, we predicted the hot spots to have a series of consequences on GPCR signaling (413). First, by increasing the local concentration of receptors and G proteins and keeping them in close proximity after a previous interaction, they likely enhance the efficiency and speed of GPCR signaling. Second, by allowing GPCRs to produce local responses, they might give an important contribution to the high specificity observed in GPCR signaling.

XI. CONCLUDING REMARKS

More than 40 years of intense research on receptor–G protein interactions have provided important insights into one of the most critical mechanism in human physiology. Thanks to recent advances in structural biology, we now possess snapshots with atomistic details of receptors and G proteins in different conformational states. Combined with biophysical investigations on purified proteins, these studied have revealed an unexpected flexibility and structural dynamics in both receptors and G proteins. In parallel, the introduction of new methods based on single-molecule microscopy is giving us a unique opportunity to investigate receptor–G protein interactions and other signaling events in living cells with unprecedented spatio-temporal resolution. These approaches are helping to clarify some of the most fundamental and still unresolved questions about GPCR signaling. The results obtained so far have revealed a complex picture, whereby dynamic interactions among receptors, G proteins and the surrounding cellular environment shape GPCR signaling in both space and time. This

complexity leads to the formation of GPCR signaling hot spots on the plasma membrane, where GPCRs and G proteins preferentially interact and signal. The accumulation of receptors and proteins in these hot spots appears to increase the speed and efficiency of GPCR signaling. Moreover, since individual receptors and G proteins might signal in different nanodomains both at the cell surface and, as we have recently learnt, intracellularly, this complexity might help cells to discriminate between the myriad of signals initiated by the hundreds of different GPCRs that are simultaneously expressed in a typical cell.

As it is often the case in science, for every question that is answered many more questions and hypotheses are generated that will have to be further investigated in the future. One important question is whether the emerging spatio-temporal organization of GPCR signaling is altered in disease and/or contributes to the pathogenesis of common conditions. An example is the above-mentioned change in the nanoscale organization of β-adrenergic receptor signaling on the surface of cardiomyocytes that has been implicated in the pathogenesis of heart failure (302).

A second question is whether the new information might be exploited to develop innovative drugs. Current drug screening strategies are based on the assumption that GPCR are simple on/off switches. However, this approach is associated with an overall poor success rate and a high economic burden, largely caused to the frequently late failure of new experimental drugs due to lack of efficacy in humans. Thus, there is an urgent need for new and more rational drug development strategies based on better models, predictive strategies and testing approaches. Single-molecule microscopy could give a major contribution to achieve this goal by providing highly quantitative data about the complex spatio-temporal dynamics and microscopic kinetics of GPCR signaling. Together with the growing richness of structural information and rapid advances in computer science, this will hopefully lead to advanced dynamic models, capable of reproducing the complexity of GPCR signaling across different scales and, eventually, predicting the responses to new experimental treatments.

One particularly intriguing question for future research is whether the new information about the nanoscale organization of GPCR signaling can be exploited to more selectively modulate GPCR signaling in space and time, for instance by activating or inhibiting receptors only in specific subcellular nanodomains at the plasma membrane or inside cells. Another intriguing possibility would be to identify new strategies capable of reverting the changes in the nanoscale organization of GPCR signaling found in disease. If successful, these approaches could pave the way to innovative drugs with improved pharmacological profiles and, hopefully, fewer side effects for the therapy of common conditions such as diabetes or heart failure.

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REFERENCES

1. Abadir PM, Walston JD and Carey RM. Subcellular characteristics of functional intracellular renin–angiotensin systems. *Peptides* 38: 437-445, 2012.
2. Adjobo-Hermans MJ, Goedhart J, van Weeren L, Nijmeijer S, Manders EM, Offermanns S, Gadella TW Jr. Real-time visualization of heterotrimeric G protein G_q activation in living cells. *BMC Biol* 9: 32, 2011.
3. Ahmadian MR, Stege P, Scheffzek K, Wittinghofer A. Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. *Nat Struct Biol* 4: 686-689, 1997.
4. Akgoz M, Kalyanaraman V and Gautam N. G protein $\beta\gamma$ complex translocation from plasma membrane to Golgi complex is influenced by receptor γ subunit interaction. *Cell signal* 18: 1758-1768, 2006.
5. Alcobia DC, Ziegler AI, Kondrashov A, Comeo E, Mistry S, Kellam B, Chang A, Woolard J, Hill SJ, Sloan EK. Visualizing ligand binding to a GPCR *in vivo* using NanoBRET. *iScience* 6: 280-288, 2018.
6. Alewijnse AE, Timmerman H, Jacobs EH, Smit MJ, Roovers E, Cootecchia S, Leurs R. The effect of mutations in the DRY Motif on the constitutive activity and structural instability of the histamine H2 receptor. *Mol Pharmacol* 57: 890-898, 2000.
7. Alexander NS, Preininger AM, Kaya AI, Stein RA, Hamm HE, Meiler J. Energetic analysis of the rhodopsin–G-protein complex links the α5 helix to GDP release. *Nat Struct Mol Biol* 21: 56-63, 2014.
8. Alvaro CG and Thorner J. Heterotrimeric G protein-coupled receptor signaling in yeast mating pheromone response. *J Biol Chem* 291: 7788-7795, 2016.
9. Aprigliano O, Rybin VO, Pak E, Robinson RB, Steinberg SF. β_1- and β_2-adrenergic receptors exhibit differing susceptibility to muscarinic accentuated antagonism. *Am J Physiol* 272: H2726-2735, 1997.

10. Assmann SM. Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *Plant Cell* 14: S355-S373, 2002.

11. Attwood TK and Findlay JB. Fingerprinting G protein-coupled receptors. *Protein Eng* 7: 195-203, 1994.

12. Audet N, Gales C, Archer-Lahlou E, Vallieres M, Schiller PW, Bouvier M, Pineyro G. Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing δ opioid receptors and heterotrimeric G proteins. *J Biol Chem* 283: 15078-15088, 2008.

13. Ayling LJ, Briddon SJ, Halls ML, Hammond GR, Vaca L, Pacheco J, Hill SJ, Cooper DM. Adenylyl cyclase AC8 directly controls its micro-environment by recruiting the actin cytoskeleton in a cholesterol-rich milieu. *J Cell Sci* 125: 869-886, 2012.

14. Ayoub MA, Maurel D, Binet V, Fink M, Prezeau L, Ansanay H, Pin JP. Real-time analysis of agonist-induced activation of protease-activated receptor 1/$G\alpha_{i1}$ protein complex measured by bioluminescence resonance energy transfer in living cells. *Mol Pharmacol* 71: 1329-1340, 2007.

15. Ayoub MA, Trinquet E, Pfleger KD, Pin JP. Differential association modes of the thrombin receptor PAR1 with $G\alpha_{i1}$, $G\alpha_{12}$, and β-arrestin 1. *FASEB J* 24: 3522-3535, 2010.

16. Azpiazu I and Gautam N. A fluorescence resonance energy transfer-based sensor indicates that receptor access to a G protein is unrestricted in a living mammalian cell. *J Biol Chem* 279: 27709-27718, 2004.

17. Bacskai B, Hochner B, Mahaut-Smith M, Adams, Kaang B, Kandel E, Tsien R. Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. *Science* 260: 222-226, 1993.

18. Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, Javitch JA. Activation of the β_2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* 276: 29171-29177, 2001.

19. Baltoumas FA, Theodoropoulou MC and Hamodrakas SJ. Interactions of the α-subunits of heterotrimeric G proteins with GPCRs, effectors and RGS proteins: a critical review and analysis of interacting surfaces, conformational shifts, structural diversity and electrostatic potentials. *J Struct Biol* 182: 209-218, 2013.

20. Baruscotti M, Bucchi A and Difrancesco D. Physiology and pharmacology of the cardiac pacemaker ("funny") current. *Pharmacol Ther* 107: 59-79, 2005.

21. Beavo JA and Brunton LL. Cyclic nucleotide research – still expanding after half a century. *Nat Rev Mol Cell Biol* 3: 710-718, 2002.

22. Belous A, Wakata A, Knox CD, Nicoud IB, Pierce J, Anderson CD, Pinson CW, Chari RS. Mitochondrial P2Y-Like receptors link cytosolic adenosine nucleotides to mitochondrial calcium uptake. *J Cell Biochem* 92: 1062-1073, 2004.

23. Bénard G, Massa F, Puente N, Lourenço J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutiérrez S, Martín-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, López-Rodríguez ML, Grandes P, Rossignol R, Marsicano G. Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nat Neurosci* 15: 558-564, 2012.

24. Berman DM, Kozasa T and Gilman AG. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J Biol Chem* 271: 27209-27212, 1996.

25. Berman DM, Wilkie TM and Gilman AG. GAIP and RGS4 are GTPase-activating proteins for the G_i subfamily of G protein α subunits. *Cell* 86: 445-452, 1996.

26. Bers DM. Cardiac excitation-contraction coupling. *Nature* 415: 198-205, 2002.

27. Betzig E. Single molecules, cells, and super-resolution optics (Nobel Lecture). *Angew Chem Int Ed Engl* 54: 8034-8053, 2015.

28. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313: 1642-1645, 2006.

29. Bigay J, Faurobert E, Franco M, Chabre M. Roles of lipid modifications of transducin subunits in their GDP-dependent association and membrane binding. *Biochemistry* 33: 14081-14090, 1994.

30. Blin N, Yun J and Wess J. Mapping of single amino acid residues required for selective activation of $G_{q/11}$ by the M3 muscarinic acetylcholine receptor. *J Biol Chem* 270: 17741-17748, 1995.

31. Blumer JB and Lanier SM. Activators of G protein signaling exhibit broad functionality and define a distinct core signaling triad. *Mol Pharmacol* 85: 388-396, 2014.

32. Blüml K, Mutschler E and Wess J. Functional role of a cytoplasmic aromatic amino acid in muscarinic receptor-mediated activation of phospholipase C. *J Biol Chem* 269: 11537-11541, 1994.

33. Blüml K, Mutschler E and Wess J. Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. *J Biol Chem* 269: 402-405, 1994.

34. Bockenhauer S, Furstenberg A, Yao XJ, Kobilka BK, Moerner WE. Conformational dynamics of single G protein-coupled receptors in solution. *J Phys Chem B* 115: 13328-13338, 2011.

35. Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. Mu-opioid receptor desensitization by β-arrestin 2 determines morphine tolerance but not dependence. *Nature* 408: 720-723, 2000.

36. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking β-arrestin 2. *Science* 286: 2495-2498, 1999.

37. Bokoch GM, Katada T, Northup JK, Ui M, Gilman AG. Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J Biol Chem* 259: 3560-3567, 1984.

38. Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi HJ, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L, Kobilka BK. Ligand-specific

regulation of the extracellular surface of a G protein-coupled receptor. *Nature* 463: 108-112, 2010.

39. Bos JL, Rehmann H and Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. *Cell* 129: 865-877, 2007.

40. Bourne HR, Sanders DA and McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348: 125-132, 1990.

41. Briddon SJ, Kilpatrick LE and Hill SJ. Studying GPCR pharmacology in membrane microdomains: Fluorescence correlation spectroscopy comes of age. *Trends Pharmacol Sci* 39: 158-174, 2018.

42. Briddon SJ, Middleton RJ, Cordeaux Y, Flavin FM, Weinstein JA, George MW, Kellam B, Hill SJ. Quantitative analysis of the formation and diffusion of A_1-adenosine receptor-antagonist complexes in single living cells. *Proc Natl Acad Sci USA* 101: 4673-4678, 2004.

43. Brunton LL, Hayes JS and Mayer SE. Hormonally specific phosphorylation of cardiac troponin I and activation of glycogen phosphorylase. *Nature* 280: 78-80, 1979.

44. Brust TF, Conley JM and Watts VJ. $G\alpha_{i/o}$-coupled receptor-mediated sensitization of adenylyl cyclase: 40 years later. *Eur J Pharmacol* 763: 223-232, 2015.

45. Bünemann M, Frank M and Lohse MJ. G_i protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci USA* 100: 16077-16082, 2003.

46. Busquets-Garcia A, Bains J and Marsicano G. CB_1 receptor signaling in the brain: Extracting specificity from ubiquity. *Neuropsychopharmacology* 43: 4-20, 2018.

47. Buxton IL and Brunton LL. Compartments of cyclic AMP and protein kinase in mammalian cardiomyocytes. *J Biol Chem* 258: 10233-10239, 1983.

48. Cabrera JL, de Freitas F, Satpaev DK, Slepak VZ. Identification of the Gβ5-RGS7 protein complex in the retina. *Biochem Biophys Res Commun* 249: 898-902, 1998.

49. Cahill TJ 3rd, Thomsen AR, Tarrasch JT, Plouffe B, Nguyen AH, Yang F, Huang LY, Kahsai AW, Bassoni DL, Gavino BJ, Lamerdin JE, Triest S, Shukla AK, Berger B, Little Jt, Antar A, Blanc A, Qu CX, Chen X, Kawakami K, Inoue A, Aoki J, Steyaert J, Sun JP, Bouvier M, Skiniotis G, Lefkowitz

RJ. Distinct conformations of GPCR–β-arrestin complexes mediate desensitization, signaling, and endocytosis. *Proc Natl Acad Sci USA* 114: 2562-2567, 2017.

50. Calebiro D and Godbole A. Internalization of G protein-coupled receptors: Implication in receptor function, physiology and diseases. *Best Pract Res Clin Endocrinol Metab* 32: 83-91, 2018.

51. Calebiro D and Koszegi Z. The subcellular dynamics of GPCR signaling. *Mol Cell Endocrinol* 483: 24-30, 2019.

52. Calebiro D and Maiellaro I. cAMP signaling microdomains and their observation by optical methods. *Front Cell Neurosci* 8: 350, 2014.

53. Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, Persani L, Lohse MJ. Persistent cAMP-signals triggered by internalized G protein-coupled receptors. *PLoS Biol* 7: e1000172, 2009.

54. Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A, Cocucci E, Zürn A, Lohse MJ. Single-molecule analysis of fluorescently labeled G protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc Natl Acad Sci USA* 110: 743-748, 2013.

55. Calebiro D and Sungkaworn T. Single-molecule imaging of GPCR interactions. *Trends Pharmacol Sci* 39: 109-122, 2017.

56. Camps M, Hou C, Sidiropoulos D, Stock JB, Jakobs KH, Gierschik P. Stimulation of phospholipase C by guanine-nucleotide-binding protein βγ subunits. *Eur J Biochem* 206: 821-831, 1992.

57. Cao C, Tan Q, Xu C, He L, Yang L, Zhou Y, Zhou Y, Qiao A, Lu M, Yi C, Han GW, Wang X, Li X, Yang H, Rao Z, Jiang H, Zhao Y, Liu J, Stevens RC, Zhao Q, Zhang XC, Wu B. Structural basis for signal recognition and transduction by platelet-activating-factor receptor. *Nat Struct Mol Biol* 25: 488-495, 2018.

58. Carpenter B, Nehme R, Warne T, Leslie AG, Tate CG. Structure of the adenosine A_{2A} receptor bound to an engineered G protein. *Nature* 536: 104-107, 2016.

59. Castro LR, Gervasi N, Guiot E, Cavellini L, Nikolaev VO, Paupardin-Tritsch D, Vincent P. Type 4 phosphodiesterase plays different integrating roles in different cellular domains in pyramidal cortical neurons. *J Neurosci* 30: 6143-6151, 2010.

60. Chan RK and Otte CA. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by a factor and α factor pheromones. *Mol Cell Biol* 2: 11-20, 1982.

61. Chan RK and Otte CA. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by a factor and α factor pheromones. *Mol Cell Biol* 2: 21-29, 1982.

62. Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, He W, Wensel TG, Simon MI. Instability of GGL domain-containing RGS proteins in mice lacking the G protein β-subunit Gβ5. *Proc Natl Acad Sci USA* 100: 6604-6609, 2003.

63. Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408: 936-943, 2000.

64. Chen S, Lin F and Hamm HE. RACK1 binds to a signal transfer region of G$\beta\gamma$ and inhibits phospholipase C-β2 activation. *J Biol Chem* 280: 33445-33452, 2005.

65. Chen Z, Singer WD, Danesh SM, Sternweis PC, Sprang SR. Recognition of the activated states of Gα_{13} by the rgRGS domain of PDZRhoGEF. *Structure* 16: 1532-1543, 2008.

66. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC. High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science* 318: 1258-1265, 2007.

67. Choi MHV, Zacharoff K. (October 11, 2018). Final summary minutes of the anesthetic and analgesic drug products advisory committee meeting [Online]. Food and Drug Administration, Center for Drug Evaluation and Research. https://www.fda.gov/media/119281/download [13 May 2020].

68. Christensen GL, Kelstrup CD, Lyngso C, Sarwar U, Bogebo R, Sheikh SP, Gammeltoft S, Olsen JV, Hansen JL. Quantitative phosphoproteomics dissection of seven-transmembrane receptor signaling using full and biased agonists. *Mol Cell Proteomics* 9: 1540-1553, 2010.

69. Chung KY, Rasmussen SG, Liu T, Li S, DeVree BT, Chae PS, Calinski D, Kobilka BK, Woods VL Jr, Sunahara RK. Conformational changes in the G protein G_s induced by the β_2-adrenergic receptor. *Nature* 477: 611-615, 2011.

70. Coleman DE, Berghuis AM, Lee E, Linder ME, Gilman AG, Sprang SR. Structures of active conformations of $G\alpha_{i1}$ and the mechanism of GTP hydrolysis. *Science* 265: 1405-1412, 1994.

71. Compans B, Choquet D and Hosy E. Review on the role of AMPA receptor nano-organization and dynamic in the properties of synaptic transmission. *Neurophotonics* 3: 041811, 2016.

72. Condamin S, Bénichou O and Klafter J. First-passage time distributions for subdiffusion in confined geometry. *Phys Rev Lett* 98: 250602, 2007.

73. Condamin S, Bénichou O, Tejedor V, Voituriez R, Klafter J. First-passage times in complex scale-invariant media. *Nature* 450: 77-80, 2007.

74. Conn PJ and Pin JP. Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol* 37: 205-237, 1997.

75. Cordeaux Y, Briddon SJ, Alexander SP, Kellam B, Hill SJ. Agonist-occupied A_3 adenosine receptors exist within heterogeneous complexes in membrane microdomains of individual living cells. *FASEB J* 22: 850-860, 2008.

76. Corriden R, Kilpatrick LE, Kellam B, Briddon SJ, Hill SJ. Kinetic analysis of antagonist-occupied adenosine A_3 receptors within membrane microdomains of individual cells provides evidence of receptor dimerization and allosterism. *FASEB J* 28: 4211-4222, 2014.

77. Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, Lefkowitz RJ. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* 273: 685-688, 1998.

78. dal Maso E, Glukhova A, Zhu Y, Garcia-Nafria J, Tate CG, Atanasio S, Reynolds CA, Ramírez-Aportela E, Carazo J-M, Hick CA, Furness SGB, Hay DL, Liang Y-L, Miller LJ, Christopoulos A, Wang M-W, Wootten D, Sexton PM. The molecular control of calcitonin receptor signaling. *ACS Pharmacol Transl Sci* 2: 31-51, 2019.

79. Dascal N and Kahanovitch U. The roles of Gβγ and Gα in gating and regulation of GIRK channels. *Int Rev Neurobiol* 123: 27-85, 2015.

80. Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW. A β_2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel Cav1.2. *Science* 293: 98-101, 2001.

81. De Lean A, Stadel JM and Lefkowitz RJ. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* 255: 7108-7117, 1980.

82. de Mendoza A, Sebe-Pedros A and Ruiz-Trillo I. The evolution of the GPCR signaling system in eukaryotes: modularity, conservation, and the transition to metazoan multicellularity. *Genome Biol Evol* 6: 606-619, 2014.

83. De Waard M, Liu H, Walker D, Scott VE, Gurnett CA, Campbell KP. Direct binding of G protein βγ complex to voltage-dependent calcium channels. *Nature* 385: 446-450, 1997.

84. DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, Bunnett NW. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex. *Proc Natl Acad Sci USA* 97: 11086-11091, 2000.

85. DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. β-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148: 1267-1281, 2000.

86. DeLisi C. The biophysics of ligand–receptor interactions. *Q Rev Biophys* 13: 201-230, 2009.

87. Deupi X and Kobilka BK. Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. *Physiology (Bethesda)* 25: 293-303, 2010.

88. Devost D, Sleno R, Petrin D, Zhang A, Shinjo Y, Okde R, Aoki J, Inoue A, Hébert TE. Conformational profiling of the AT_1 angiotensin II receptor reflects biased agonism, G protein coupling, and cellular context. *J Biol Chem* 292: 5443-5456, 2017.

89. DeVree BT, Mahoney JP, Velez-Ruiz GA, Rasmussen SG, Kuszak AJ, Edwald E, Fung JJ, Manglik A, Masureel M, Du Y, Matt RA, Pardon E, Steyaert J, Kobilka BK, Sunahara RK. Allosteric coupling from G protein to the agonist-binding pocket in GPCRs. *Nature* 535: 182-186, 2016.

90. Di Benedetto A, Sun L, Zambonin CG, Tamma R, Nico B, Calvano CD, Colaianni G, Ji Y, Mori G, Grano M, Lu P, Colucci S, Yuen T, New MI, Zallone A, Zaidi M. Osteoblast regulation via ligand-activated nuclear trafficking of the oxytocin receptor. *Proc Natl Acad Sci USA* 111: 16502-16507, 2014.

91. DiPilato LM, Cheng X and Zhang J. Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci USA* 101: 16513-16518, 2004.

92. Discher DE, Winardi R, Schischmanoff PO, Parra M, Conboy JG, Mohandas N. Mechanochemistry of protein 4.1's spectrin-actin-binding domain: ternary complex interactions, membrane binding, network integration, structural strengthening. *J Cell Biol* 130: 897-907, 1995.

93. Dohlman HG, Apaniesk D, Chen Y, Song J, Nusskern D. Inhibition of G-protein signaling by dominant gain-of-function mutations in Sst2p, a pheromone desensitization factor in *Saccharomyces cerevisiae*. *Mol Cell Biol* 15: 3635-3643, 1995.

94. Dohlman HG, Song J, Ma D, Courchesne WE, Thorner J. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein α subunit). *Mol Cell Biol* 16: 5194-5209, 1996.

95. Dore AS, Robertson N, Errey JC, Ng I, Hollenstein K, Tehan B, Hurrell E, Bennett K, Congreve M, Magnani F, Tate CG, Weir M, Marshall FH. Structure of the adenosine A_{2A} receptor in complex with ZM241385 and the xanthines XAC and caffeine. *Structure* 19: 1283-1293, 2011.

96. Downes GB and Gautam N. The G protein subunit gene families. *Genomics* 62: 544-552, 1999.

97. Drakopoulos A, Koszegi Z, Lanoiselee Y, Hübner H, Gmeiner P, Calebiro D, Decker M. Investigation of inactive state κ opioid receptor homodimerization via single molecule microscopy using new antagonistic fluorescent probes. *J Med Chem* 63:3596-3609, 2020.

98. Draper-Joyce CJ, Khoshouei M, Thal DM, Liang YL, Nguyen ATN, Furness SGB, Venugopal H, Baltos JA, Plitzko JM, Danev R, Baumeister W, May LT, Wootten D, Sexton PM, Glukhova A, Christopoulos A. Structure of the adenosine-bound human adenosine A1 receptor–G_i complex. *Nature* 558: 559-563, 2018.

99. Dror RO, Arlow DH, Maragakis P, Mildorf TJ, Pan AC, Xu H, Borhani DW, Shaw DE. Activation mechanism of the β_2-adrenergic receptor. *Proc Natl Acad Sci USA* 108: 18684-18689, 2011.

100. Dror RO, Mildorf TJ, Hilger D, Manglik A, Borhani DW, Arlow DH, Philippsen A, Villanueva N, Yang Z, Lerch MT, Hubbell WL, Kobilka BK, Sunahara RK, Shaw DE. Structural basis for nucleotide exchange in heterotrimeric G proteins. *Science* 348: 1361-1365, 2015.

101. Dudok B, Barna L, Ledri M, Szabo SI, Szabadits E, Pinter B, Woodhams SG, Henstridge CM, Balla GY, Nyilas R, Varga C, Lee SH, Matolcsi M, Cervenak J, Kacskovics I, Watanabe M, Sagheddu C, Melis M, Pistis M, Soltesz I, Katona I. Cell-specific STORM super-resolution imaging reveals nanoscale organization of cannabinoid signaling. *Nat Neurosci* 18: 75-86, 2015.

102. Dupré DJ, Robitaille M, Ethier N, Villeneuve LR, Mamarbachi AM, Hébert TE. Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking. *J Biol Chem* 281: 34561-34573, 2006.

103. Edidin M, Kuo S and Sheetz M. Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. *Science* 254: 1379-1382, 1991.

104. Eggeling C, Ringemann C, Medda R, Schwarzmann G, Sandhoff K, Polyakova S, Belov VN, Hein B, von Middendorff C, Schonle A, Hell SW. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* 457: 1159-1162, 2009.

105. Felker GM, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, Filippatos G, Levy PD, Metra M, Ponikowski P, Soergel DG, Teerlink JR, Violin JD, Voors AA, Pang PS. Heart failure therapeutics on the basis of a biased ligand of the angiotensin-2 type 1 receptor. Rationale and design of the BLAST-AHF study (Biased Ligand of the Angiotensin Receptor Study in Acute Heart Failure). *JACC Heart Fail* 3: 193-201, 2015.

106. Ferguson A, Wang L, Altman RB, Terry DS, Juette MF, Burnett BJ, Alejo JL, Dass RA, Parks MM, Vincent CT, Blanchard SC. Functional dynamics within the human ribosome regulate the rate of active protein synthesis. *Mol Cell* 60: 475-486, 2015.

107. Ferguson SS, Barak LS, Zhang J, Caron MG. G protein-coupled receptor regulation: role of G protein-coupled receptor kinases and arrestins. *Can J Physiol Pharm* 74: 1095-1110, 1996.

108. Ferrandon S, Feinstein TN, Castro M, Wang B, Bouley R, Potts JT, Gardella TJ, Vilardaga JP. Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat Chem Biol* 5: 734-742, 2009.

109. Flock T, Hauser AS, Lund N, Gloriam DE, Balaji S, Babu MM. Selectivity determinants of GPCR–G-protein binding. *Nature* 545: 317-322, 2017.

110. Flock T, Ravarani CNJ, Sun D, Venkatakrishnan AJ, Kayikci M, Tate CG, Veprintsev DB, Babu MM. Universal allosteric mechanism for Gα activation by GPCRs. *Nature* 524: 173-179, 2015.

111. Frank M, Thumer L, Lohse MJ, Bünemann M. G protein activation without subunit dissociation depends on a $G\alpha_i$-specific region. *J Biol Chem* 280: 24584-24590, 2005.

112. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB. The G protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63: 1256-1272, 2003.

113. Fu Q and Xiang YK. Trafficking of β-adrenergic receptors: Implications in intracellular receptor signaling. *Prog Mol Biol Transl* 132: 151-188, 2015.

114. Fujiwara T, Ritchie K, Murakoshi H, Jacobson K, Kusumi A. Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol* 157: 1071-1081, 2002.

115. Gaidarov I, Santini F, Warren RA, Keen JH. Spatial control of coated-pit dynamics in living cells. *Nat Cell Biol* 1: 1-7, 1999.

116. Gales C, Rebois RV, Hogue M, Trieu P, Breit A, Hébert TE, Bouvier M. Real-time monitoring of receptor and G protein interactions in living cells. *Nat Methods* 2: 177-184, 2005.

117. Gales C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M. Probing the activation-promoted structural rearrangements in preassembled receptor–G protein complexes. *Nat Struct Mol Biol* 13: 778-786, 2006.

118. Garcia-Marcos M, Ghosh P and Farquhar MGJ. GIV/Girdin transmits signals from multiple receptors by triggering trimeric G protein activation. *J Biol Chem* 290: 6697-6704, 2015.

119. Garcia-Nafria J, Lee Y, Bai X, Carpenter B, Tate CG. Cryo-EM structure of the adenosine A_{2A} receptor coupled to an engineered heterotrimeric G protein. *Elife* 7: e35946, 2018.

120. Garcia-Nafria J, Nehme R, Edwards PC, Tate CG. Cryo-EM structure of the serotonin 5-HT_{1B} receptor coupled to heterotrimeric G_o. *Nature* 558: 620-623, 2018.

121. Gautier A, Juillerat A, Heinis C, Correa IR, Jr., Kindermann M, Beaufils F, Johnsson K. An engineered protein tag for multiprotein labeling in living cells. *Chem Biol* 15: 128-136, 2008.

122. Gavutis M, Jaks E, Lamken P, Piehler J. Determination of the two-dimensional interaction rate constants of a cytokine receptor complex. *Biophys J* 90: 3345-3355, 2006.

123. Gbahou F, Cecon E, Viault G, Gerbier R, Jean-Alphonse F, Karamitri A, Guillaumet G, Delagrange P, Friedlander RM, Vilardaga JP, Suzenet F, Jockers R. Design and validation of the first cell-impermeant melatonin receptor agonist. *Br J Pharmacol* 174: 2409-2421, 2017.

124. Ge B, Lao J, Li J, Chen Y, Song Y, Huang F. Single-molecule imaging reveals dimerization/oligomerization of CXCR4 on plasma membrane closely related to its function. *Sci Rep* 7: 16873, 2017.

125. Gentzsch C, Seier K, Drakopoulos A, Jobin ML, Lanoiselee Y, Koszegi Z, Maurel D, Sounier R, Hubner H, Gmeiner P, Granier S, Calebiro D, Decker M. Selective and wash-resistant fluorescent dihydrocodeinone derivatives allow single-molecule imaging of μ opioid receptor dimerization. *Angew Chem Int Ed Engl* 59: 5958-5964, 2019.

126. Gether U and Kobilka BK. G protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* 273: 17979-17982, 1998.

127. Gibson SK and Gilman AG. $G\alpha_i$ and Gβ subunits both define selectivity of G protein activation by α_2-adrenergic receptors. *Proc Natl Acad Sci USA* 103: 212-217, 2006.

128. Gillis A, Gondin AB, Kliewer A, Sanchez J, Lim HD, Alamein C, Manandhar P, Santiago M, Fritzwanker S, Schmiedel F, Katte TA, Reekie T, Grimsey NL, Kassiou M, Kellam B, Krasel C, Halls ML, Connor M, Lane JR, Schulz S, Christie MJ, Canals M. Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Sci Signal* 13: eaaz3140, 2020.

129. Gillis A, Sreenivasan V and Christie MJ. Intrinsic efficacy of opioid ligands and its importance for apparent bias, operational analysis, and therapeutic window. *Mol Pharmacol* 98: 410-424, 2020.

130. Gilman AG. G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* 56: 615-649, 1987.

131. Glukhova A, Draper-Joyce CJ, Sunahara RK, Christopoulos A, Wootten D, Sexton PM. Rules of engagement: GPCRs and G proteins. *ACS Pharmacol Transl Sci* 1: 73-83, 2018.

132. Godbole A, Lyga S, Lohse MJ, Calebiro D. Internalized TSH receptors en route to the TGN induce local G_s-protein signaling and gene transcription. *Nat Commun* 8: 443, 2017.

133. Goddard AD and Watts A. Regulation of G protein-coupled receptors by palmitoylation and cholesterol. *BMC Biol* 10: 27, 2012.

134. Gonzalez-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, Lira A, Bradley-Moore M, Ge Y, Zhou Q, Sealfon SC, Gingrich JA. Hallucinogens recruit specific cortical 5-HT_{2A} receptor-mediated signaling pathways to affect behavior. *Neuron* 53: 439-452, 2007.

135. Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. β-arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. *Nature* 383: 447-450, 1996.

136. Granier S, Kim S, Shafer AM, Ratnala VR, Fung JJ, Zare RN, Kobilka B. Structure and conformational changes in the C-terminal domain of the β_2-adrenoceptor: insights from fluorescence resonance energy transfer studies. *J Biol Chem* 282: 13895-13905, 2007.

137. Grebenkov DS. Imperfect diffusion-controlled reactions. In: *Chemical kinetics: Beyond the textbook*, edited by Lindenberg K, Metzler R and Oshanin G. World Scientific, 2019.

138. Gregorio GG, Masureel M, Hilger D, Terry DS, Juette M, Zhao H, Zhou Z, Perez-Aguilar JM, Hauge M, Mathiasen S, Javitch JA, Weinstein H, Kobilka BK, Blanchard SC. Single-molecule analysis of ligand efficacy in β_2AR–G-protein activation. *Nature* 547: 68-73, 2017.

139. Gross W and Lohse MJ. Mechanism of activation of A2 adenosine receptors. II. A restricted collision-coupling model of receptor–effector interaction. *Mol Pharmacol* 39: 524-530, 1991.

140. Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, Ruttiger N, Ziegler N, Benkel T, Schmitt NK, Ishida S, Müller I, Reher R, Kawakami K, Inoue A, Rick U, Kühl T, Imhof D, Aoki J, König GM, Hoffmann C, Gomeza J, Wess J, Kostenis E. Lack of β-arrestin signaling in the absence of active G proteins. *Nat Commun* 9: 341, 2018.

141. Ha T, Enderle T, Ogletree DF, Chemla DS, Selvin PR, Weiss S. Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc Natl Acad Sci USA* 93: 6264-6268, 1996.

142. Haga K and Haga T. Activation by G protein βγ subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. *J Biol Chem* 267: 2222-2227, 1992.

143. Haga K, Kruse AC, Asada H, Yurugi-Kobayashi T, Shiroishi M, Zhang C, Weis WI, Okada T, Kobilka BK, Haga T, Kobayashi T. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* 482: 547-551, 2012.

144. Halls ML and Cooper DM. Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, β-arrestin 2, PDE4D3 complex. *EMBO J* 29: 2772-2787, 2010.

145. Hänggi P, Talkner P and Borkovec M. Reaction-rate theory: fifty years after Kramers. *Rev Mod Phys* 62: 251-341, 1990.

146. Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY, Velasquez J, Kuhn P, Stevens RC. A specific cholesterol binding site is established by the 2.8 Å structure of the human β_2-adrenergic receptor. *Structure* 16: 897-905, 2008.

147. Harkany T and Horvath TL. (S)Pot on mitochondria: Cannabinoids disrupt cellular respiration to limit neuronal activity. *Cell Metab* 25: 8-10, 2017.

148. Haugh JM. Analysis of reaction-diffusion systems with anomalous subdiffusion. *Biophys J* 97: 435-442, 2009.

149. Hauser AS, Attwood MM, Rask-Andersen M, Schiöth HB, Gloriam DE. Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* 16: 829, 2017.

150. Hayes JS, Brunton LL and Mayer SE. Selective activation of particulate cAMP-dependent protein kinase by isoproterenol and prostaglandin E1. *J Biol Chem* 255: 5113-5119, 1980.

151. He W, Danilova V, Zou S, Hellekant G, Max M, Margolskee RF, Damak S. Partial rescue of taste responses of α-gustducin null mice by transgenic expression of α-transducin. *Chem Senses* 27: 719-727, 2002.

152. Head BP, Patel HH and Insel PA. Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. *Biochim Biophys Acta* 1838: 532-545, 2014.

153. Head BP, Patel HH, Roth DM, Murray F, Swaney JS, Niesman IR, Farquhar MG, Insel PA. Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. *J Biol Chem* 281: 26391-26399, 2006.

154. Heck M and Hofmann KP. Maximal rate and nucleotide dependence of rhodopsin-catalyzed transducin activation: initial rate analysis based on a double displacement mechanism. *J Biol Chem* 276: 10000-10009, 2001.

155. Hegener O, Prenner L, Runkel F, Baader SL, Kappler J, Haberlein H. Dynamics of β_2-adrenergic receptor−ligand complexes on living cells. *Biochemistry* 43: 6190-6199, 2004.

156. Hein P, Frank M, Hoffmann C, Lohse MJ, Bünemann M. Dynamics of receptor/G protein coupling in living cells. *EMBO J* 24: 4106-4114, 2005.

157. Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, Engelhardt S, Berlot CH, Lohse MJ, Bünemann M. G_s activation is time-limiting in initiating receptor-mediated signaling. *J Biol Chem* 281: 33345-33351, 2006.

158. Hern JA, Baig AH, Mashanov GI, Birdsall B, Corrie JE, Lazareno S, Molloy JE, Birdsall NJ. Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc Natl Acad Sci USA* 107: 2693-2698, 2010.

159. Herrick-Davis K, Grinde E, Cowan A, Mazurkiewicz JE. Fluorescence correlation spectroscopy analysis of serotonin, adrenergic, muscarinic, and dopamine receptor dimerization: the oligomer number puzzle. *Mol Pharmacol* 84: 630-642, 2013.

160. Herrmann R, Heck M, Henklein P, Henklein P, Kleuss C, Hofmann KP, Ernst OP. Sequence of interactions in receptor−G protein coupling. *J Biol Chem* 279: 24283-24290, 2004.

161. Herrmann R, Heck M, Henklein P, Hofmann KP, Ernst OP. Signal transfer from GPCRs to G proteins: role of the Gα N-terminal region in rhodopsin–transducin coupling. *J Biol Chem* 281: 30234-30241, 2006.

162. Herrmann R, Heck M, Henklein P, Kleuss C, Wray V, Hofmann KP, Ernst OP. Rhodopsin–transducin coupling: role of the Gα C-terminus in nucleotide exchange catalysis. *Vision Res* 46: 4582-4593, 2006.

163. Holst B, Nygaard R, Valentin-Hansen L, Bach A, Engelstoft MS, Petersen PS, Frimurer TM, Schwartz TW. A conserved aromatic lock for the tryptophan rotameric switch in TM-VI of seven-transmembrane receptors. *J Biol Chem* 285: 3973-3985, 2010.

164. Honigmann A, Mueller V, Ta H, Schoenle A, Sezgin E, Hell SW, Eggeling C. Scanning STED-FCS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells. *Nat Commun* 5: 5412, 2014.

165. Huang B, Bates M and Zhuang X. Super-resolution fluorescence microscopy. *Annu Rev Biochem* 78: 993-1016, 2009.

166. Huang CL, Slesinger PA, Casey PJ, Jan YN, Jan LY. Evidence that direct binding of Gβγ to the GIRK1 G protein-gated inwardly rectifying K^+ channel is important for channel activation. *Neuron* 15: 1133-1143, 1995.

167. Huang W, Masureel M, Qianhui Q, Janetzko J, Inoue A, Kato HE, Robertson MJ, Nguyen KC, Glenn JS, Skiniotis G, Kobilka BK. Structure of the neurotensin receptor 1 in complex with β-arrestin 1. *Nature* 579: 303-308, 2020.

168. Iiri T, Backlund PS Jr, Jones TL, Wedegaertner PB, Bourne HR. Reciprocal regulation of $G\alpha_s$ by palmitate and the βγ subunit. *Proc Natl Acad Sci USA* 93: 14592-14597, 1996.

169. Ikeda S. Modulation of Ca^{2+} channels by G protein βγ subunits. *Nature* 380: 258-262, 1996.

170. Ilien B, Glasser N, Clamme JP, Didier P, Piemont E, Chinnappan R, Daval SB, Galzi JL, Mely Y. Pirenzepine promotes the dimerization of muscarinic M1 receptors through a three-step binding process. *J Biol Chem* 284: 19533-19543, 2009.

171. Illenberger D, Walliser C, Nurnberg B, Diaz Lorente M, Gierschik P. Specificity and structural requirements of phospholipase C-β stimulation by Rho GTPases versus G protein βγ dimers. *J Biol Chem* 278: 3006-3014, 2003.

172. Inanobe A, Morishige KI, Takahashi N, Ito H, Yamada M, Takumi T, Nishina H, Takahashi K, Kanaho Y, Katada T, Kurachi Y. Gβγ directly binds to the carboxyl terminus of the G protein-gated muscarinic K^+ channel, GIRK1. *Biochem Biophys Res Commun* 212: 1022-1028, 1995.

173. Iñiguez-Lluhi JA, Simon MI, Robishaw JD, Gilman AG. G protein βγ subunits synthesized in Sf9 cells. Functional characterization and the significance of prenylation of γ. *J Biol Chem* 267: 23409-23417, 1992.

174. Insel PA, Head BP, Patel HH, Roth DM, Bundey RA, Swaney JS. Compartmentation of G protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem Soc Trans* 33: 1131-1134, 2005.

175. Insel PA and Kennedy MS. Colchicine potentiates β-adrenoreceptor-stimulated cyclic AMP in lymphoma cells by an action distal to the receptor. *Nature* 273: 471-473, 1978.

176. Irannejad R, Pessino V, Mika D, Huang B, Wedegaertner PB, Conti M, von Zastrow M. Functional selectivity of GPCR-directed drug action through location bias. *Nat Chem Biol* 13: 799-806, 2017.

177. Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, von Zastrow M. Conformational biosensors reveal GPCR signalling from endosomes. *Nature* 495: 534-538, 2013.

178. Irannejad R, Tsvetanova NG, Lobingier BT, von Zastrow M. Effects of endocytosis on receptor-mediated signaling. *Curr Opin Cell Biol* 35: 137-143, 2015.

179. Isberg V, de Graaf C, Bortolato A, Cherezov V, Katritch V, Marshall FH, Mordalski S, Pin JP, Stevens RC, Vriend G, Gloriam DE. Generic GPCR residue numbers – aligning topology maps while minding the gaps. *Trends Pharmacol Sci* 36: 22-31, 2015.

180. Isogai S, Deupi X, Opitz C, Heydenreich FM, Tsai CJ, Brueckner F, Schertler GF, Veprintsev DB, Grzesiek S. Backbone NMR reveals allosteric signal transduction networks in the β1-adrenergic receptor. *Nature* 530: 237-241, 2016.

181. Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP, Stevens RC. The 2.6 Å crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. *Science* 322: 1211-1217, 2008.

182. Jacobson K, Sheets ED and Simson R. Revisiting the fluid mosaic model of membranes. *Science* 268: 1441-1442, 1995.

183. Janetopoulos C, Jin T and Devreotes P. Receptor-mediated activation of heterotrimeric G proteins in living cells. *Science* 291: 2408-2411, 2001.

184. Jensen DD, Lieu T, Halls ML, Veldhuis NA, Imlach WL, Mai QN, Poole DP, Quach T, Aurelio L, Conner J, Herenbrink CK, Barlow N, Simpson JS, Scanlon MJ, Graham B, McCluskey A, Robinson PJ, Escriou V, Nassini R, Materazzi S, Geppetti P, Hicks GA, Christie MJ, Porter CJH, Canals M, Bunnett NW. Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci Transl Med* 9: eaal3447, 2017.

185. Jensen JB, Lyssand JS, Hague C, Hille B. Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K+ channels. *J Gen Physiol* 133: 347-359, 2009.

186. Ji TH, Grossmann M and Ji I. G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* 273: 17299-17302, 1998.

187. Joyal JS, Nim S, Zhu T, Sitaras N, Rivera JC, Shao Z, Sapieha P, Hamel D, Sanchez M, Zaniolo K, St-Louis M, Ouellette J, Montoya-Zavala M, Zabeida A, Picard E, Hardy P, Bhosle V, Varma DR, Gobeil F Jr, Beausejour C, Boileau C, Klein W, Hollenberg M, Ribeiro-da-Silva A, Andelfinger G, Chemtob S. Subcellular localization of coagulation factor II receptor-like 1 in neurons governs angiogenesis. *Nat Med* 20: 1165-1173, 2014.

188. Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E, Mann A, Doll C, Trinidad JC, Burlingame AL, von Zastrow M, Schulz S. Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol Pharmacol* 83: 633-639, 2013.

189. Kahsai AW, Xiao K, Rajagopal S, Ahn S, Shukla AK, Sun J, Oas TG, Lefkowitz RJ. Multiple ligand-specific conformations of the β_2-adrenergic receptor. *Nat Chem Biol* 7: 692-700, 2011.

190. Kankanamge D, Ratnayake K, Senarath K, Tennakoon M, Harmon E, Karunarathne A. Optical approaches for single-cell and subcellular analysis of GPCR–G protein signaling. *Anal Bioanal Chem* 411: 4481-4508, 2019.

191. Kasai RS, Ito SV, Awane RM, Fujiwara TK, Kusumi A. The class-A GPCR dopamine D2 receptor forms transient dimers stabilized by agonists: Detection by single-molecule tracking. *Cell Biochem Biophys* 76: 29-37, 2018.

192. Kasai RS and Kusumi A. Single-molecule imaging revealed dynamic GPCR dimerization. *Curr Opin Cell Biol* 27: 78-86, 2014.

193. Kasai RS, Suzuki KG, Prossnitz ER, Koyama-Honda I, Nakada C, Fujiwara TK, Kusumi A. Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J Cell Biol* 192: 463-480, 2011.

194. Kato HE, Zhang Y, Hu H, Suomivuori CM, Kadji FMN, Aoki J, Krishna Kumar K, Fonseca R, Hilger D, Huang W, Latorraca NR, Inoue A, Dror RO, Kobilka BK, Skiniotis G. Conformational transitions of a neurotensin receptor 1–G_{i1} complex. *Nature* 572: 80-85, 2019.

195. Kaya AI, Lokits AD, Gilbert JA, Iverson TM, Meiler J, Hamm HE. A conserved phenylalanine as a relay between the α5 helix and the GDP binding region of heterotrimeric G_i protein α subunit. *J Biol Chem* 289: 24475-24487, 2014.

196. Keely SL. Prostaglandin E1 activation of heart cAMP-dependent protein kinase: apparent dissociation of protein kinase activation from increases in phosphorylase activity and contractile force. *Mol Pharmacol* 15: 235-245, 1979.

197. Kelly E. Efficacy and ligand bias at the μ opioid receptor. *Br J Pharmacol* 169: 1430-1446, 2013.

198. Kenakin T. Biased receptor signaling in drug discovery. *Pharmacol Rev* 71: 267-315, 2019.

199. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. *Nat biotechnol* 21: 86-89, 2003.

200. Kim J, Ahn S, Rajagopal K, Lefkowitz RJ. Independent β-arrestin2 and G_q/protein kinase Cζ pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem* 284: 11953-11962, 2009.

201. Kim TH, Chung KY, Manglik A, Hansen AL, Dror RO, Mildorf TJ, Shaw DE, Kobilka BK, Prosser RS. The role of ligands on the equilibria between functional states of a G protein-coupled receptor. *J Am Chem Soc* 135: 9465-9474, 2013.

202. Kimple AJ, Bosch DE, Giguere PM, Siderovski DP. Regulators of G-protein signaling and their Gα substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev* 63: 728-749, 2011.

203. Kinsey CG, Bussolati G, Bosco M, Kimura T, Pizzorno MC, Chernin MI, Cassoni P, Novak JF. Constitutive and ligand-induced nuclear localization of oxytocin receptor. *J Cell Mol Med* 11: 96-110, 2007.

204. Kjeldgaard M, Nyborg J and Clark BF. The GTP binding motif: variations on a theme. *FASEB J* 10: 1347-1368, 1996.

205. Klayman LM and Wedegaertner PB. Inducible inhibition of Gβγ reveals localization-dependent functions at the plasma membrane and Golgi. *J Biol Chem* 292: 1773-1784, 2017.

206. Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, Javitch JA, Christopoulos A, Lane JR. The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun* 7: 10842, 2016.

207. Klein S, Reuveni H and Levitzki A. Signal transduction by a nondissociable heterotrimeric yeast G protein. *Proc Natl Acad Sci USA* 97: 3219-3223, 2000.

208. Klein T, Löschberger A, Proppert S, Wolter S, van de Linde S, Sauer M. Live-cell *d*STORM with SNAP-tag fusion proteins. *Nat Methods* 8: 7-9, 2011.

209. Kliewer A, Gillis A, Hill R, Schmiedel F, Bailey C, Kelly E, Henderson G, Christie MJ, Schulz S. Morphine-induced respiratory depression is independent of β-arrestin 2 signalling. *Br J Pharmacol* 177: 2923-2931, 2020.

210. Kliewer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, Levitt ES, Williams JT, Christie MJ, Schulz S. Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* 10: 367, 2019.

211. Kobayashi H, Picard LP, Schonegge AM, Bouvier M. Bioluminescence resonance energy transfer-based imaging of protein–protein interactions in living cells. *Nat Protoc* 14: 1084-1107, 2019.

212. Kobilka BK and Deupi X. Conformational complexity of G protein-coupled receptors. *Trends Pharmacol Sci* 28: 397-406, 2007.

213. Koch WJ, Inglese J, Stone WC, Lefkowitz RJ. The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase. *J Biol Chem* 268: 8256-8260, 1993.

214. Koehl A, Hu H, Maeda S, Zhang Y, Qu Q, Paggi JM, Latorraca NR, Hilger D, Dawson R, Matile H, Schertler GFX, Granier S, Weis WI, Dror RO, Manglik A, Skiniotis G, Kobilka BK. Structure of the μ opioid receptor–G_i protein complex. *Nature* 558: 547-552, 2018.

215. Koelle MR and Horvitz HR. EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell* 84: 115-125, 1996.

216. Koestler M, Heithier H, Baumann M, Dees C, Hekman M, Vaz W. Association-dissociation of purified subunits of GTP-binding proteins measured by fluorescence energy transfer. *J Protein Chem* 8: 406, 1989.

217. Kofuku Y, Ueda T, Okude J, Shiraishi Y, Kondo K, Maeda M, Tsujishita H, Shimada I. Efficacy of the β_2-adrenergic receptor is determined by conformational equilibrium in the transmembrane region. *Nat Commun* 3: 1045, 2012.

218. Kofuku Y, Ueda T, Okude J, Shiraishi Y, Kondo K, Mizumura T, Suzuki S, Shimada I. Functional dynamics of deuterated β_2-adrenergic receptor in lipid bilayers revealed by NMR spectroscopy. *Angew Chem Int Ed Engl* 53: 13376-13379, 2014.

219. Kolakowski LF Jr. GCRDb: A G-protein-coupled receptor database. *Receptors Channels* 2: 1-7, 1994.

220. Komura N, Suzuki KG, Ando H, Konishi M, Koikeda M, Imamura A, Chadda R, Fujiwara TK, Tsuboi H, Sheng R, Cho W, Furukawa K, Furukawa K, Yamauchi Y, Ishida H, Kusumi A, Kiso M. Raft-based interactions of gangliosides with a GPI-anchored receptor. *Nat Chem Biol* 12: 402-410, 2016.

221. Kostenis E, Gomeza J, Lerche C, Wess J. Genetic analysis of receptor–Gα_q coupling selectivity. *J Biol Chem* 272: 23675-23681, 1997.

222. Kotowski SJ, Hopf FW, Seif T, Bonci A, von Zastrow M. Endocytosis promotes rapid dopaminergic signaling. *Neuron* 71: 278-290, 2011.

223. Kramers HA. Brownian motion in a field of force and the diffusion model of chemical reactions. *Physica* 7: 284-304, 1940.

224. Krishna Kumar K, Shalev-Benami M, Robertson MJ, Hu H, Banister SD, Hollingsworth SA, Latorraca NR, Kato HE, Hilger D, Maeda S, Weis WI, Farrens DL, Dror RO, Malhotra SV, Kobilka BK, Skiniotis G. Structure of a signaling cannabinoid receptor 1–G protein complex. *Cell* 176: 448-458 e412, 2019.

225. Krishnan A and Schioth HB. The role of G protein-coupled receptors in the early evolution of neurotransmission and the nervous system. *J Exp Biol* 218: 562-571, 2015.

226. Krupnick JG and Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annual Rev Pharmacol* 38: 289-319, 1998.

227. Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM, Christopoulos A, Felder CC, Gmeiner P, Steyaert J, Weis WI, Garcia KC, Wess J, Kobilka BK. Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* 504: 101-106, 2013.

228. Kubo T, Bujo H, Akiba I, Nakai J, Mishina M, Numa S. Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Lett* 241: 119-125, 1988.

229. Kuravi S, Lan TH, Barik A, Lambert NA. Third-party bioluminescence resonance energy transfer indicates constitutive association of membrane proteins: application to class A G protein-coupled receptors and G proteins. *Biophys J* 98: 2391-2399, 2010.

230. Kusumi A, Fujiwara TK, Chadda R, Xie M, Tsunoyama TA, Kalay Z, Kasai RS, Suzuki KG. Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annu Rev Cell Dev Bi* 28: 215-250, 2012.

231. Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, Murakoshi H, Kasai RS, Kondo J, Fujiwara T. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct* 34: 351-378, 2005.

232. Kusumi A and Sako Y. Cell surface organization by the membrane skeleton. *Curr Opin Cell Biol* 8: 566-574, 1996.

233. Kusumi A, Suzuki KG, Kasai RS, Ritchie K, Fujiwara TK. Hierarchical mesoscale domain organization of the plasma membrane. *Trends Biochem Sci* 36: 604-615, 2011.

234. Lagerström MC and Schiöth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* 7: 339-357, 2008.

235. Lambright DG, Noel JP, Hamm HE, Sigler PB. Structural determinants for activation of the α-subunit of a heterotrimeric G protein. *Nature* 369: 621-628, 1994.

236. Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB. The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature* 379: 311-319, 1996.

237. Lamichhane R, Liu JJ, Pljevaljcic G, White KL, van der Schans E, Katritch V, Stevens RC, Wüthrich K, Millar DP. Single-molecule view of basal activity and activation mechanisms of the G protein-coupled receptor β_2AR. *Proc Natl Acad Sci USA* 112: 14254-14259, 2015.

238. Lane JR, May LT, Parton RG, Sexton PM, Christopoulos A. A kinetic view of GPCR allostery and biased agonism. *Nat Chem Biol* 13: 929-937, 2017.

239. Lania AG, Mantovani G and Spada A. Mechanisms of disease: Mutations of G proteins and G protein-coupled receptors in endocrine diseases. *Nat Clin Pract Endoc* 2: 681-693, 2006.

240. Lanoiselée Y and Grebenkov DS. Non-Gaussian diffusion of mixed origins. *J Phys A* 52: 304001, 2019.

241. Lanoiselée Y, Moutal N and Grebenkov DS. Diffusion-limited reactions in dynamic heterogeneous media. *Nat Commun* 9: 4398, 2018.

242. Laporte SA, Oakley RH, Holt JA, Barak LS, Caron MG. The interaction of β-arrestin with the AP-2 adaptor is required for the clustering of β_2-adrenergic receptor into clathrin-coated pits. *J Biol Chem* 275: 23120-23126, 2000.

243. Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS. The β_2-adrenergic receptor/β-arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* 96: 3712-3717, 1999.

244. Laschet C, Dupuis N and Hanson J. The G protein-coupled receptors deorphanization landscape. *Biochem Pharmacol* 153: 62-74, 2018.

245. Lau EK, Trester-Zedlitz M, Trinidad JC, Kotowski SJ, Krutchinsky AN, Burlingame AL, von Zastrow M. Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. *Sci Signal* 4: ra52, 2011.

246. Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG, Tate CG. Agonist-bound adenosine A_{2A} receptor structures reveal common features of GPCR activation. *Nature* 474: 521-525, 2011.

247. Lee Y, Warne T, Nehmé R, Pandey S, Dwivedi-Agnihotri H, Edwards PC, García-Nafría J, Leslie AGW, Shukla AK, Tate CG. Molecular determinants of β-arrestin coupling to formoterol-bound β_1-adrenoceptor. *BioRxiv* 2020. doi: 10.1101/2020.03.27.011585.

248. Lefkimmiatis K and Zaccolo M. cAMP signaling in subcellular compartments. *Pharmacol Ther* 143: 295-304, 2014.

249. Lefkowitz RJ. Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol Sci* 25: 413-422, 2004.

250. Lefkowitz RJ, Rajagopal K and Whalen EJ. New roles for β-arrestins in cell signaling: not just for seven-transmembrane receptors. *Mol Cell* 24: 643-652, 2006.

251. Lenne PF, Wawrezinieck L, Conchonaud F, Wurtz O, Boned A, Guo XJ, Rigneault H, He HT, Marguet D. Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J* 25: 3245-3256, 2006.

252. Levitz J, Habrian C, Bharill S, Fu Z, Vafabakhsh R, Isacoff EY. Mechanism of Assembly and Cooperativity of Homomeric and Heteromeric Metabotropic Glutamate Receptors. *Neuron* 92: 143-159, 2016.

253. Levitzki A and Klein S. G protein subunit dissociation is not an integral part of G protein action. *Chembiochem* 3: 815-818, 2002.

254. Liang YL, Khoshouei M, Glukhova A, Furness SGB, Zhao P, Clydesdale L, Koole C, Truong TT, Thal DM, Lei S, Radjainia M, Danev R, Baumeister W, Wang MW, Miller LJ, Christopoulos A, Sexton PM, Wootten D. Phase-plate cryo-EM structure of a biased agonist-bound human GLP-1 receptor–Gs complex. *Nature* 555: 121-125, 2018.

255. Liang YL, Khoshouei M, Radjainia M, Zhang Y, Glukhova A, Tarrasch J, Thal DM, Furness SGB, Christopoulos G, Coudrat T, Danev R, Baumeister W, Miller LJ, Christopoulos A, Kobilka BK,

Wootten D, Skiniotis G, Sexton PM. Phase-plate cryo-EM structure of a class B GPCR–G-protein complex. *Nature* 546: 118-123, 2017.

256. Linder ME, Pang I-H, Duronio RJ, Gordon JI, Sternweis PC, Gilman AG. Lipid modifications of G protein subunits. Myristoylation of $G\alpha_o$ increases its affinity for $\beta\gamma$. *J Biol Chem* 266: 4654-4659, 1991.

257. Lingwood D and Simons K. Lipid rafts as a membrane-organizing principle. *Science* 327: 46-50, 2010.

258. Liu J, Conklin BR, Blin N, Yun J, Wess J. Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. *Proc Natl Acad Sci USA* 92: 11642-11646, 1995.

259. Liu JJ, Horst R, Katritch V, Stevens RC, Wuthrich K. Biased signaling pathways in β_2-adrenergic receptor characterized by ^{19}F-NMR. *Science* 335: 1106-1110, 2012.

260. Liu W, Clark WA, Sharma P, Northup JK. Mechanism of allosteric regulation of the rod cGMP phosphodiesterase activity by the helical domain of transducin α subunit. *J Biol Chem* 273: 34284-34292, 1998.

261. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature* 325: 321-326, 1987.

262. Lohse MJ, Klotz KN and Schwabe U. Mechanism of A_2 adenosine receptor activation. I. Blockade of A2 adenosine receptors by photoaffinity labeling. *Mol Pharmacol* 39: 517-523, 1991.

263. Lohse MJ, Nuber S and Hoffmann C. Fluorescence/bioluminescence resonance energy transfer techniques to study G protein-coupled receptor activation and signaling. *Pharmacol Rev* 64: 299-336, 2012.

264. Loomis WF. Cell signaling during development of Dictyostelium. *Dev Biol* 391: 1-16, 2014.

265. Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ. Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J Biol Chem* 272: 4637-4644, 1997.

266. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. β-arrestin-dependent formation of β_2 adrenergic receptor–Src protein kinase complexes. *Science* 283: 655-661, 1999.

267. Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ. Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc Natl Acad Sci USA* 98: 2449-2454, 2001.

268. Lutz S, Shankaranarayanan A, Coco C, Ridilla M, Nance MR, Vettel C, Baltus D, Evelyn CR, Neubig RR, Wieland T, Tesmer JJ. Structure of $G\alpha_q$–p63RhoGEF–RhoA complex reveals a pathway for the activation of RhoA by GPCRs. *Science* 318: 1923-1927, 2007.

269. Lyga S, Volpe S, Werthmann RC, Gotz K, Sungkaworn T, Lohse MJ, Calebiro D. Persistent cAMP Signaling by internalized LH receptors in ovarian follicles. *Endocrinology* 157: 1613-1621, 2016.

270. Maeda S, Qu Q, Robertson MJ, Skiniotis G, Kobilka BK. Structures of the M1 and M2 muscarinic acetylcholine receptor/G protein complexes. *Science* 364: 552-557, 2019.

271. Maiellaro I, Lohse MJ, Kittel RJ, Calebiro D. cAMP signals in Drosophila motor neurons are confined to single synaptic boutons. *Cell Rep* 17: 1238-1246, 2016.

272. Mallipeddi S, Janero DR, Zvonok N, Makriyannis A. Functional selectivity at G protein coupled receptors: Advancing cannabinoid receptors as drug targets. *Biochem Pharmacol* 128: 1-11, 2017.

273. Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, Prosser RS, Kobilka BK. Structural insights into the dynamic process of β_2-adrenergic receptor signaling. *Cell* 161: 1101-1111, 2015.

274. Manglik A and Kobilka BK. The role of protein dynamics in GPCR function: insights from the β_2AR and rhodopsin. *Curr Opin Cell Biol* 27: 136-143, 2014.

275. Manzo C, Torreno-Pina JA, Massignan P, Lapeyre GJ, Lewenstein M, Garcia Parajo MF. Weak ergodicity breaking of receptor motion in living cells stemming from random diffusivity. *Phys Rev X* 5: 011021, 2015.

276. Markby DW, Onrust R and Bourne HR. Separate GTP binding and GTPase activating domains of a Gα subunit. *Science* 262: 1895-1901, 1993.

277. Masuho I, Ostrovskaya O, Kramer GM, Jones CD, Xie K, Martemyanov KA. Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. *Sci Signal* 8: ra123, 2015.

278. Mayor S and Maxfield FR. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. *Mol Biol Cell* 6: 929-944, 1995.

279. McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ. β-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* 290: 1574-1577, 2000.

280. Meroz Y, Sokolov IM and Klafter J. Subdiffusion of mixed origins: when ergodicity and nonergodicity coexist. *Phys Rev E Stat Nonlin Soft Matter Phys* 81: 010101, 2010.

281. Michel MC and Charlton SJ. Biased Agonism in Drug Discovery – Is It Too Soon to Choose a Path? *Mol Pharmacol* 93: 259-265, 2018.

282. Middleton RJ, Briddon SJ, Cordeaux Y, Yates AS, Dale CL, George MW, Baker JG, Hill SJ, Kellam B. New fluorescent adenosine A_1 receptor agonists that allow quantification of ligand-receptor interactions in microdomains of single living cells. *J Med Chem* 50: 782-793, 2007.

283. Milligan G and Bouvier M. Methods to monitor the quaternary structure of G protein-coupled receptors. *FEBS J* 272: 2914-2925, 2005.

284. Milligan G, Ward RJ and Marsango S. GPCR homo-oligomerization. *Curr Opin Cell Biol* 57: 40-47, 2019.

285. Mixon MB, Lee E, Coleman DE, Berghuis AM, Gilman AG, Sprang SR. Tertiary and quaternary structural changes in $G\alpha_{i1}$ induced by GTP hydrolysis. *Science* 270: 954-960, 1995.

286. Mizuno N and Itoh H. Functions and regulatory mechanisms of G_q-signaling pathways. *Neurosignals* 17: 42-54, 2009.

287. Moerner WE. Single-molecule spectroscopy, imaging, and photocontrol: Foundations for super-resolution microscopy (Nobel Lecture). *Angew Chem Int Ed Engl* 54: 8067-8093, 2015.

288. Morone N, Fujiwara T, Murase K, Kasai RS, Ike H, Yuasa S, Usukura J, Kusumi A. Three-dimensional reconstruction of the membrane skeleton at the plasma membrane interface by electron tomography. *J Cell Biol* 174: 851-862, 2006.

289. Munk C, Mutt E, Isberg V, Nikolajsen LF, Bibbe JM, Flock T, Hanson MA, Stevens RC, Deupi X, Gloriam DE. An online resource for GPCR structure determination and analysis. *Nat Methods* 16: 151-162, 2019.

290. Munro S. Lipid rafts: elusive or illusive? *Cell* 115: 377-388, 2003.

291. Murase K, Fujiwara T, Umemura Y, Suzuki K, Iino R, Yamashita H, Saito M, Murakoshi H, Ritchie K, Kusumi A. Ultrafine membrane compartments for molecular diffusion as revealed by single molecule techniques. *Biophys J* 86: 4075-4093, 2004.

292. Nagi K and Pineyro G. Kir3 channel signaling complexes: focus on opioid receptor signaling. *Front Cell Neurosci* 8: 186, 2014.

293. Nakada C, Ritchie K, Oba Y, Nakamura M, Hotta Y, Iino R, Kasai RS, Yamaguchi K, Fujiwara T, Kusumi A. Accumulation of anchored proteins forms membrane diffusion barriers during neuronal polarization. *Nat Cell Biol* 5: 626-632, 2003.

294. Nash CA, Wei W, Irannejad R, Smrcka AV. Golgi localized β_1-adrenergic receptors stimulate Golgi PI4P hydrolysis by PLCε to regulate cardiac hypertrophy. *Elife* 8: e48167 2019.

295. Navarro G, Cordomi A, Casado-Anguera V, Moreno E, Cai NS, Cortes A, Canela EI, Dessauer CW, Casado V, Pardo L, Lluis C, Ferre S. Evidence for functional pre-coupled complexes of receptor heteromers and adenylyl cyclase. *Nat Commun* 9: 1242, 2018.

296. Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, Lefkowitz RJ. Targeting of diacylglycerol degradation to M1 muscarinic receptors by β-arrestins. *Science* 315: 663-666, 2007.

297. Neubig RR, Gantzos RD and Thomsen WJ. Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* 27: 2374-2384, 1988.

298. Neves SR, Tsokas P, Sarkar A, Grace EA, Rangamani P, Taubenfeld SM, Alberini CM, Schaff JC, Blitzer RD, Moraru, II, Iyengar R. Cell shape and negative links in regulatory motifs together control spatial information flow in signaling networks. *Cell* 133: 666-680, 2008.

299. Nguyen AH, Thomsen ARB, Cahill TJ 3rd, Huang R, Huang LY, Marcink T, Clarke OB, Heissel S, Masoudi A, Ben-Hail D, Samaan F, Dandey VP, Tan YZ, Hong C, Mahoney JP, Triest S, Little Jt, Chen X, Sunahara R, Steyaert J, Molina H, Yu Z, des Georges A, Lefkowitz RJ. Structure of an endosomal signaling GPCR–G protein–β-arrestin megacomplex. *Nat Struct Mol Biol* 26: 1123-1131, 2019.

300. Nikolaev VO, Bünemann M, Hein L, Hannawacker A, Lohse MJ. Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem* 279: 37215-37218, 2004.

301. Nikolaev VO, Bünemann M, Schmitteckert E, Lohse MJ, Engelhardt S. Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching β_1-adrenergic but locally confined β_2-adrenergic receptor-mediated signaling. *Circ Res* 99: 1084-1091, 2006.

302. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. β_2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science* 327: 1653-1657, 2010.

303. Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ. Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci Signal* 4: ra51, 2011.

304. Nobles M, Benians A and Tinker A. Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci USA* 102: 18706-18711, 2005.

305. Noel JP, Hamm HE and Sigler PB. The 2.2 Å crystal structure of transducin-α complexed with GTPγS. *Nature* 366: 654-663, 1993.

306. Nygaard R, Frimurer TM, Holst B, Rosenkilde MM, Schwartz TW. Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci* 30: 249-259, 2009.

307. Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS, Kobilka BK. The dynamic process of β_2-adrenergic receptor activation. *Cell* 152: 532-542, 2013.

308. O'Hayre M, Eichel K, Avino S, Zhao X, Steffen DJ, Feng X, Kawakami K, Aoki J, Messer K, Sunahara R, Inoue A, von Zastrow M, Gutkind JS. Genetic evidence that β-arrestins are dispensable for the initiation of β_2-adrenergic receptor signaling to ERK. *Sci Signal* 10: eaal3395, 2017.

309. Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* 274: 32248-32257, 1999.

310. Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, β-arrestin1, and β-arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* 275: 17201-17210, 2000.

311. Oksche A, Boese G, Horstmeyer A, Furkert J, Beyermann M, Bienert M, Rosenthal W. Late endosomal/lysosomal targeting and lack of recycling of the ligand-occupied endothelin B receptor. *Mol Pharmacol* 57: 1104-1113, 2000.

312. Okude J, Ueda T, Kofuku Y, Sato M, Nobuyama N, Kondo K, Shiraishi Y, Mizumura T, Onishi K, Natsume M, Maeda M, Tsujishita H, Kuranaga T, Inoue M, Shimada I. Identification of a conformational equilibrium that determines the efficacy and functional selectivity of the μ opioid receptor. *Angew Chem Int Ed Engl* 54: 15771-15776, 2015.

313. Oldham WM and Hamm HE. Heterotrimeric G protein activation by G protein-coupled receptors. *Nat Rev Mol Cell Biol* 9: 60-71, 2008.

314. Oldham WM, Van Eps N, Preininger AM, Hubbell WL, Hamm HE. Mechanism of the receptor-catalyzed activation of heterotrimeric G proteins. *Nat Struct Mol Biol* 13: 772-777, 2006.

315. Olivella M, Caltabiano G and Cordomi A. The role of Cysteine 6.47 in class A GPCRs. *BMC Struct Biol* 13: 3, 2013.

316. Olofsson L, Felekyan S, Doumazane E, Scholler P, Fabre L, Zwier JM, Rondard P, Seidel CA, Pin JP, Margeat E. Fine tuning of sub-millisecond conformational dynamics controls metabotropic glutamate receptors agonist efficacy. *Nat Commun* 5: 5206, 2014.

317. Orly J and Schramm M. Coupling of catecholamine receptor from one cell with adenylate cyclase from another cell by cell fusion. *Proc Natl Acad Sci USA* 73: 4410-4414, 1976.

318. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739-745, 2000.

319. Pang PS, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, Filippatos G, Levy PD, Metra M, Ponikowski P, Teerlink JR, Voors AA, Bharucha D, Goin K, Soergel DG, Felker GM. Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF). *Eur Heart J* 38: 2364-2373, 2017.

320. Peleg G, Ghanouni P, Kobilka BK, Zare RN. Single-molecule spectroscopy of the β_2 adrenergic receptor: observation of conformational substates in a membrane protein. *Proc Natl Acad Sci USA* 98: 8469-8474, 2001.

321. Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ. Targeting of cyclic AMP degradation to β_2-adrenergic receptors by β-arrestins. Science 298: 834-836, 2002.

322. Peverelli E, Giardino E, Treppiedi D, Vitali E, Cambiaghi V, Locatelli M, Lasio GB, Spada A, Lania AG, Mantovani G. Filamin A (FLNA) plays an essential role in somatostatin receptor 2 (SST2) signaling and stabilization after agonist stimulation in human and rat somatotroph tumor cells. *Endocrinology* 155: 2932-2941, 2014.

323. Philip F, Sengupta P and Scarlata S. Signaling through a G protein-coupled receptor and its corresponding G protein follows a stoichiometrically limited model. *J Biol Chem* 282: 19203-19216, 2007.

324. Picard LP, Schonegge AM, Lohse MJ, Bouvier M. Bioluminescence resonance energy transfer-based biosensors allow monitoring of ligand- and transducer-mediated GPCR conformational changes. *Commun Biol* 1: 106, 2018.

325. Pierce KL, Premont RT and Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3: 639-650, 2002.

326. Pike LJ. Lipid rafts: bringing order to chaos. *J Lipid Res* 44: 655-667, 2003.

327. Pin JP, Kniazeff J, Prezeau L, Liu JF, Rondard P. GPCR interaction as a possible way for allosteric control between receptors. *Mol Cell Endocrinol* 486: 89-95, 2019.

328. Pin JP, Neubig R, Bouvier M, Devi L, Filizola M, Javitch JA, Lohse MJ, Milligan G, Palczewski K, Parmentier M, Spedding M. International Union of Basic and Clinical Pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol Rev* 59: 5-13, 2007.

329. Pitcher J, Lohse MJ, Codina J, Caron MG, Lefkowitz RJ. Desensitization of the isolated β_2-adrenergic receptor by β-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry* 31: 3193-3197, 1992.

330. Pitcher JA, Freedman NJ and Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem* 67: 653-692, 1998.

331. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ. Role of βγ subunits of G proteins in targeting the β-adrenergic receptor kinase to membrane-bound receptors. *Science* 257: 1264-1267, 1992.

332. Ponsioen B, Zhao J, Riedl J, Zwartkruis F, van der Krogt G, Zaccolo M, Moolenaar WH, Bos JL, Jalink K. Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Rep* 5: 1176-1180, 2004.

333. Prakash MK. Insights on the role of (dis)order from protein−protein interaction linear free-energy relationships. *J Am Chem Soc* 133: 9976-9979, 2011.

334. Qi X, Liu H, Thompson B, McDonald J, Zhang C, Li X. Cryo-EM structure of oxysterol-bound human Smoothened coupled to a heterotrimeric G_i. *Nature* 571: 279-283, 2019.

335. Qin K, Dong C, Wu G, Lambert NA. Inactive-state preassembly of G_q-coupled receptors and G_q heterotrimers. *Nat Chem Biol* 7: 740-747, 2011.

336. Qin K, Sethi PR and Lambert NA. Abundance and stability of complexes containing inactive G protein-coupled receptors and G proteins. *FASEB J* 22: 2920-2927, 2008.

337. Raehal KM, Schmid CL, Groer CE, Bohn LM. Functional selectivity at the μ opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev* 63: 1001-1019, 2011.

338. Rahmeh R, Damian M, Cottet M, Orcel H, Mendre C, Durroux T, Sharma KS, Durand G, Pucci B, Trinquet E, Zwier JM, Deupi X, Bron P, Baneres JL, Mouillac B, Granier S. Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. *Proc Natl Acad Sci USA* 109: 6733-6738, 2012.

339. Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, Coffman TM, Rockman HA, Lefkowitz RJ. β-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci USA* 103: 16284-16289, 2006.

340. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, Lefkowitz RJ. β-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci USA* 107: 628-632, 2010.

341. Ransnas LA, Svoboda P, Jasper JR, Insel PA. Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G protein between cytosol and membranes. *Proc Natl Acad Sci USA* 86: 7900-7903, 1989.

342. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK. Structure of a nanobody-stabilized active state of the β_2 adrenoceptor. *Nature* 469: 175-180, 2011.

343. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK. Crystal structure of the human β_2 adrenergic G protein-coupled receptor. *Nature* 450: 383-387, 2007.

344. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK. Crystal structure of the β_2 adrenergic receptor–G_s protein complex. *Nature* 477: 549-555, 2011.

345. Re M, Pampillo M, Savard M, Dubuc C, McArdle CA, Millar RP, Conn PM, Gobeil F Jr, Bhattacharya M, Babwah AV. The human gonadotropin releasing hormone type I receptor is a functional intracellular GPCR expressed on the nuclear membrane. *PloS One* 5: e11489, 2010.

346. Rebois RV, Robitaille M, Gales C, Dupré DJ, Baragli A, Trieu P, Ethier N, Bouvier M, Hébert TE. Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. *J Cell Sci* 119: 2807-2818, 2006.

347. Rebois RV, Warner DR and Basi NS. Does subunit dissociation necessarily accompany the activation of all heterotrimeric G proteins? *Cell Signal* 9: 141-151, 1997.

348. Reiner A and Levitz J. Glutamatergic signaling in the central nervous system: Ionotropic and metabotropic receptors in concert. *Neuron* 98: 1080-1098, 2018.

349. Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G protein-coupled receptor kinases govern G protein and β-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci USA* 102: 1448-1453, 2005.

350. Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *BBA – Mol Cell Res* 1451: 1-16, 1999.

351. Reuveny E, Slesinger PA, Inglese J, Morales JM, Iniguez-Lluhi JA, Lefkowitz RJ, Bourne HR, Jan YN, Jan LY. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature* 370: 143-146, 1994.

352. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307: 1625-1630, 2005.

353. Richard-Lalonde M, Nagi K, Audet N, Sleno R, Amraei M, Hogue M, Balboni G, Schiller PW, Bouvier M, Hébert TE, Pineyro G. Conformational dynamics of Kir3.1/Kir3.2 channel activation via δ opioid receptors. *Mol Pharmacol* 83: 416-428, 2013.

354. Riven I, Iwanir S and Reuveny E. GIRK channel activation involves a local rearrangement of a preformed G protein channel complex. *Neuron* 51: 561-573, 2006.

355. Robitaille M, Ramakrishnan N, Baragli A, Hébert TE. Intracellular trafficking and assembly of specific Kir3 channel/G protein complexes. *Cell Signal* 21: 488-501, 2009.

356. Rodbell M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* 284: 17-22, 1980.

357. Rose AS, Elgeti M, Zachariae U, Grubmüller H, Hofmann KP, Scheerer P, Hildebrand PW. Position of transmembrane helix 6 determines receptor G protein coupling specificity. *J Am Chem Soc* 136: 11244-11247, 2014.

358. Rose MD. Nuclear fusion in the yeast Saccharomyces cerevisiae. *Annu Rev Cell Dev Bi* 12: 663-695, 1996.

359. Ross EM. Coordinating speed and amplitude in G-protein signaling. *Curr Biol* 18: R777-R783, 2008.

360. Ross EM and Gilman AG. Resolution of some components of adenylate cyclase necessary for catalytic activity. *J Biol Chem* 252: 6966-6969, 1977.

361. Ross EM, Howlett AC, Ferguson KM, Gilman AG. Reconstitution of hormone-sensitive adenylate-cyclase activity with resolved components of enzyme. *J Biol Chem* 253: 6401-6412, 1978.

362. Ross EM and Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* 69: 795-827, 2000.

363. Roychowdhury S, Panda D, Wilson L, Rasenick MM. G protein α subunits activate tubulin GTPase and modulate microtubule polymerization dynamics. *J Biol Chem* 274: 13485-13490, 1999.

364. Roychowdhury S and Rasenick MM. G protein $\beta_1\gamma_2$ subunits promote microtubule assembly. *J Biol Chem* 272: 31576-31581, 1997.

365. Rozenfeld R and Devi LA. Regulation of CB_1 cannabinoid receptor trafficking by the adaptor protein AP-3. *FASEB J* 22: 2311-2322, 2008.

366. Rudolph U, Koesling D, Hinsch KD, Seifert R, Bigalke M, Schultz G, Rosenthal W. G-protein α-subunits in cytosolic and membranous fractions of human neutrophils. *Mol Cell Endocrinol* 63: 143-153, 1989.

367. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* 275: 41447-41457, 2000.

368. Sadana R and Dessauer CW. Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. *Neurosignals* 17: 5-22, 2009.

369. Sadegh S, Higgins JL, Mannion PC, Tamkun MM, Krapf D. Plasma membrane is compartmentalized by a self-similar cortical actin meshwork. *Phys Rev X* 7: 011031, 2017.

370. Saini DK, Kalyanaraman V, Chisari M, Gautam N. A family of G protein βγ subunits translocate reversibly from the plasma membrane to endomembranes on receptor activation. *J Biol Chem* 282: 24099-24108, 2007.

371. Sako Y and Kusumi A. Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether. *J Cell Biol* 129: 1559-1574, 1995.

372. Sako Y and Kusumi A. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. *J Cell Biol* 125: 1251-1264, 1994.

373. Samama P, Cotecchia S, Costa T, Lefkowitz RJ. A mutation-induced activated state of the β_2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* 268: 4625-4636, 1993.

374. Sauer M and Heilemann M. Single-molecule localization microscopy in eukaryotes. Chem Rev 117: 7478-7509, 2017.

375. Scarselli M, Annibale P and Radenovic A. Cell type-specific β_2-adrenergic receptor clusters identified using photoactivated localization microscopy are not lipid raft related, but depend on actin cytoskeleton integrity. *J Biol Chem* 287: 16768-16780, 2012.

376. Schermelleh L, Ferrand A, Huser T, Eggeling C, Sauer M, Biehlmaier O, Drummen GPC. Super-resolution microscopy demystified. *Nat Cell Biol* 21: 72-84, 2019.

377. Schiaffino MV, d'Addio M, Alloni A, Baschirotto C, Valetti C, Cortese K, Puri C, Bassi MT, Colla C, De Luca M, Tacchetti C, Ballabio A. Ocular albinism: evidence for a defect in an intracellular signal transduction system. *Nat Genet* 23: 108-112, 1999.

378. Schmidt C, Thomas T, Levine M, Neer EJ. Specificity of G protein β and γ subunit interactions. *J Biol Chem* 267: 13807-13810, 1992.

379. Schreiber G, Haran G and Zhou HX. Fundamental aspects of protein–protein association kinetics. *Chem Rev* 109: 839-860, 2009.

380. Scott JK, Huang SF, Gangadhar BP, Samoriski GM, Clapp P, Gross RA, Taussig R, Smrcka AV. Evidence that a protein–protein interaction 'hot spot' on heterotrimeric G protein βγ subunits is used for recognition of a subclass of effectors. *EMBO J* 20: 767-776, 2001.

381. Seyedabadi M, Ghahremani MH and Albert PR. Biased signaling of G protein coupled receptors (GPCRs): Molecular determinants of GPCR/transducer selectivity and therapeutic potential. *Pharmacol Ther* 200: 148-178, 2019.

382. Shi L and Javitch JA. The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol* 42: 437-467, 2002.

383. Shnerb T, Lin N and Shurki A. What is the role of the helical domain of $G\alpha_s$ in the GTPase reaction? *Biochemistry* 46: 10875-10885, 2007.

384. Shoup D and Szabo A. Role of diffusion in ligand binding to macromolecules and cell-bound receptors. *Biophys J*l 40: 33-39, 1982.

385. Siddig S, Aufmkolk S, Doose S, Jobin M-L, Werner C, Sauer M, Calebiro D. Super-resolution imaging reveals the nanoscale organization of metabotropic glutamate receptors at presynaptic active zones. *Sci Adv* 6: eaay7193, 2020.

386. Siehler S. Regulation of RhoGEF proteins by $G_{12/13}$-coupled receptors. *Br J Pharmacol* 158: 41-49, 2009.

387. Sierra-Fonseca JA, Bracamontes C, Saldecke J, Das S, Roychowdhury S. Activation of β- and α_2-adrenergic receptors stimulate tubulin polymerization and promote the association of Gβγ with microtubules in cultured NIH3T3 cells. *Biochem Biophys Res Commun* 503: 102-108, 2018.

388. Simon MI, Strathmann MP and Gautam N. Diversity of G proteins in signal transduction. *Science* 252: 802-808, 1991.

389. Simons K and Ikonen E. Functional rafts in cell membranes. *Nature* 387: 569, 1997.

390. Simons K and Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1: 31-39, 2000.

391. Singer-Lahat D, Liu J, Wess J, Felder CC. The third intracellular domain of the M3 muscarinic receptor determines coupling to calcium influx in transfected Chinese hamster ovary cells. *FEBS Lett* 386: 51-54, 1996.

392. Singer SJ and Nicolson GL. The fluid mosaic model of the structure of cell membranes. *Science* 175: 720-731, 1972.

393. Slep KC, Kercher MA, He W, Cowan CW, Wensel TG, Sigler PB. Structural determinants for regulation of phosphodiesterase by a G protein at 2.0 Å. *Nature* 409: 1071-1077, 2001.

394. Slessareva JE, Routt SM, Temple B, Bankaitis VA, Dohlman HG. Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein α subunit at the endosome. *Cell* 126: 191-203, 2006.

395. Smith JS, Lefkowitz RJ and Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* 17: 243-260, 2018.

396. Smoluchowski M. Drei Vorträge über Diffusion, Brownsche Molekularbewegung und Koagulation von Kolloidteilchen. *Physikalische Zeitschbrift* 17: 557-571, 1916.

397. Smrcka A. G protein $\beta\gamma$ subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci* 65: 2191-2214, 2008.

398. Smrcka AV and Fisher I. G-protein $\beta\gamma$ subunits as multi-functional scaffolds and transducers in G-protein-coupled receptor signaling. *Cell Mol Life Sci* 76: 4447-4459, 2019.

399. Soergel DG, Subach RA, Burnham N, Lark MW, James IE, Sadler BM, Skobieranda F, Violin JD, Webster LR. Biased agonism of the mu-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: A randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* 155: 1829-1835, 2014.

400. Šolc K and Stockmayer WH. Kinetics of diffusion-controlled reaction between chemically asymmetric molecules. II. Approximate steady-state solution. *Int J Chem Kinet* 5: 733-752, 1973.

401. Solt AS, Bostock MJ, Shrestha B, Kumar P, Warne T, Tate CG, Nietlispach D. Insight into partial agonism by observing multiple equilibria for ligand-bound and G_s-mimetic nanobody-bound β_1-adrenergic receptor. *Nat Commun* 8: 1795, 2017.

402. Sondek J, Bohm A, Lambright DG, Hamm HE, Sigler PB. Crystal structure of a G-protein $\beta\gamma$ dimer at 2.1Å resolution. *Nature* 379: 369-374, 1996.

403. Sorkin A and von Zastrow M. Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol* 10: 609-622, 2009.

404. Soundararajan M, Willard FS, Kimple AJ, Turnbull AP, Ball LJ, Schoch GA, Gileadi C, Fedorov OY, Dowler EF, Higman VA, Hutsell SQ, Sundström M, Doyle DA, Siderovski DP. Structural diversity in the RGS domain and its interaction with heterotrimeric G protein α-subunits. *Proc Natl Acad Sci USA* 105: 6457-6462, 2008.

405. Sounier R, Mas C, Steyaert J, Laeremans T, Manglik A, Huang W, Kobilka BK, Demene H, Granier S. Propagation of conformational changes during μ opioid receptor activation. *Nature* 524: 375-378, 2015.

406. Sposini V, Chechkin A and Metzler R. First passage statistics for diffusing diffusivity. *J Phys A* 52: 04LT01, 2019.

407. Squires KE, Montañez-Miranda C, Pandya RR, Torres MP, Hepler JR. Genetic analysis of rare human variants of regulators of G protein signaling proteins and their role in human physiology and disease. *Pharmacol Rev* 70: 446-474, 2018.

408. Stadel JM, Nambi P, Shorr RG, Sawyer DF, Caron MG, Lefkowitz RJ. Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *Proc Natl Acad Sci USA* 80: 3173-3177, 1983.

409. Staus DP, Hu H, Robertson MJ, Kleinhenz ALW, Wingler LM, Capel WD, Latorraca NR, Lefkowitz RJ, Skiniotis G. Structure of the M2 muscarinic receptor–β-arrestin complex in a lipid nanodisc. *Nature* 579: 297-302, 2020.

410. Steinberg SF and Brunton LL. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu Rev Pharmacol* 41: 751-773, 2001.

411. Stoeber M, Jullié D, Li J, Chakraborty S, Majumdar S, Lambert NA, Manglik A, von Zastrow M. Agonist-selective recruitment of engineered protein probes and of GRK2 by opioid receptors in living cells. *eLife* 9: e54208, 2020.

412. Sudhof TC. The presynaptic active zone. *Neuron* 75: 11-25, 2012.

413. Sungkaworn T, Jobin ML, Burnecki K, Weron A, Lohse MJ, Calebiro D. Single-molecule imaging reveals receptor–G protein interactions at cell surface hot spots. *Nature* 550: 543-547, 2017.

414. Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, Leronni D, Mihalik AC, He Y, Cecon E, Wehbi VL, Kim J, Heath BE, Baranova OV, Wang X, Gable MJ, Kretz ES, Di Benedetto G, Lezon TR, Ferrando LM, Larkin TM, Sullivan M, Yablonska S, Wang J, Minnigh MB, Guillaumet G, Suzenet F, Richardson RM, Poloyac SM, Stolz DB, Jockers R, Witt-Enderby PA, Carlisle DL, Vilardaga JP, Friedlander RM. Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proc Natl Acad Sci USA* 114: E7997-E8006, 2017.

415. Sutherland EW and Rall TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J Biol Chem* 232: 1077-1091, 1958.

416. Suzuki K, Ritchie K, Kajikawa E, Fujiwara T, Kusumi A. Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques. *Biophys J* 88: 3659-3680, 2005.

417. Szalai AM, Armando NG, Barabas FM, Stefani FD, Giordano L, Bari SE, Cavasotto CN, Silberstein S, Aramendía PF. A fluorescence nanoscopy marker for corticotropin-releasing hormone type 1 receptor: computer design, synthesis, signaling effects, super-resolved fluorescence imaging, and *in situ* affinity constant in cells. *Phys Chem Chem Phys* 20: 29212-29220, 2018.

418. Tao YX and Conn PM. Chaperoning G protein-coupled receptors: from cell biology to therapeutics. *Endocr Rev* 35: 602-647, 2014.

419. Tehan BG, Bortolato A, Blaney FE, Weir MP, Mason JS. Unifying family A GPCR theories of activation. *Pharmacol Ther* 143: 51-60, 2014.

420. Tesmer JJ, Berman DM, Gilman AG, Sprang SR. Structure of RGS4 bound to $AlF4^-$-activated $G\alpha_{i1}$: stabilization of the transition state for GTP hydrolysis. *Cell* 89: 251-261, 1997.

421. Tesmer JJ, Sunahara RK, Gilman AG, Sprang SR. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G\alpha_s$-GTPγS. *Science* 278: 1907-1916, 1997.

422. Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T, Tesmer JJ. Snapshot of activated G proteins at the membrane: the $G\alpha_q$–GRK2–G$\beta\gamma$ complex. *Science* 310: 1686-1690, 2005.

423. Thal DM, Vuckovic Z, Draper-Joyce CJ, Liang YL, Glukhova A, Christopoulos A, Sexton PM. Recent advances in the determination of G protein-coupled receptor structures. *Curr Opin Struct Biol* 51: 28-34, 2018.

424. Thiel F and Sokolov IM. Weak ergodicity breaking in an anomalous diffusion process of mixed origins. *Phys Rev E Stat Nonlin Soft Matter Phys* 89: 012136, 2014.

425. Thomas JH and Robertson HM. The *Caenorhabditis chemoreceptor* gene families. *BMC Biol* 6: 42, 2008.

426. Thomsen ARB, Plouffe B, Cahill TJ, 3rd, Shukla AK, Tarrasch JT, Dosey AM, Kahsai AW, Strachan RT, Pani B, Mahoney JP, Huang L, Breton B, Heydenreich FM, Sunahara RK, Skiniotis G, Bouvier M, Lefkowitz RJ. GPCR–G protein–β-arrestin super-complex mediates sustained G protein signaling. *Cell* 166: 907-919, 2016.

427. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. β-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT_{1a} receptor stimulation. *J Biol Chem* 277: 9429-9436, 2002.

428. Tolkovsky AM and Levitzki A. Coupling of a single adenylate cyclase to two receptors: adenosine and catecholamine. *Biochemistry* 17: 3811-3817, 1978.

429. Tolkovsky AM and Levitzki A. Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocytes. *Biochemistry* 17: 3795, 1978.

430. Touhara KK and MacKinnon R. Molecular basis of signaling specificity between GIRK channels and GPCRs. *Elife* 7: e42908, 2018.

431. Treppiedi D, Jobin ML, Peverelli E, Giardino E, Sungkaworn T, Zabel U, Arosio M, Spada A, Mantovani G, Calebiro D. Single-molecule microscopy reveals dynamic FLNA interactions governing SSTR2 clustering and internalization. *Endocrinology* 159:2953-2965, 2018.

432. Triller A and Choquet D. Synaptic structure and diffusion dynamics of synaptic receptors. *Biol Cell* 95: 465-476, 2003.

433. Tsai CJ, Marino J, Adaixo R, Pamula F, Muehle J, Maeda S, Flock T, Taylor NM, Mohammed I, Matile H, Dawson RJ, Deupi X, Stahlberg H, Schertler GFX. Cryo-EM structure of the rhodopsin–$G\alpha_i\beta\gamma$ complex reveals binding of the rhodopsin C-terminal tail to the $G\beta$ subunit. *Elife* 8: e46041, 2019.

434. Tsai CJ, Pamula F, Nehme R, Muhle J, Weinert T, Flock T, Nogly P, Edwards PC, Carpenter B, Gruhl T, Ma P, Deupi X, Standfuss J, Tate CG, Schertler GFX. Crystal structure of rhodopsin in complex with a mini-G_o sheds light on the principles of G protein selectivity. *Sci Adv* 4: eaat7052, 2018.

435. Tsvetanova NG and von Zastrow M. Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nat Chem Biol* 10: 1061-1065, 2014.

436. Umemura YM, Vrljic M, Nishimura SY, Fujiwara TK, Suzuki KG, Kusumi A. Both MHC class II and its GPI-anchored form undergo hop diffusion as observed by single-molecule tracking. *Biophys J* 95: 435-450, 2008.

437. US Food & Drug Adminisptration. FDA approves new oppioid for intravenous use in hospitals, other controlled clinical settings [media release]. 7 Aug 2020. https://www.fda.gov/. Accessed 21 Nov 2020.

438. Vadas O, Dbouk HA, Shymanets A, Perisic O, Burke JE, Abi Saab WF, Khalil BD, Harteneck C, Bresnick AR, Nurnberg B, Backer JM, Williams RL. Molecular determinants of PI3Kγ-mediated

activation downstream of G-protein-coupled receptors (GPCRs). *Proc Natl Acad Sci USA* 110: 18862-18867, 2013.

439. Vafabakhsh R, Levitz J and Isacoff EY. Conformational dynamics of a class C G protein-coupled receptor. *Nature* 524: 497-501, 2015.

440. Van Eps N, Preininger AM, Alexander N, Kaya AI, Meier S, Meiler J, Hamm HE, Hubbell WL. Interaction of a G protein with an activated receptor opens the interdomain interface in the α subunit. *Proc Natl Acad Sci USA* 108: 9420-9424, 2011.

441. Varki A. Biological roles of glycans. *Glycobiology* 27: 3-49, 2017.

442. Venkatakrishnan AJ, Deupi X, Lebon G, Heydenreich FM, Flock T, Miljus T, Balaji S, Bouvier M, Veprintsev DB, Tate CG, Schertler GF, Babu MM. Diverse activation pathways in class A GPCRs converge near the G protein-coupling region. *Nature* 536: 484-487, 2016.

443. Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Molecular signatures of G protein-coupled receptors. *Nature* 494: 185-194, 2013.

444. Vilardaga JP, Bünemann M, Krasel C, Castro M, Lohse MJ. Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat Biotechnol* 21: 807-812, 2003.

445. Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M, Lark MW. Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* 335: 572-579, 2010.

446. Violin JD and Lefkowitz RJ. β-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* 28: 416-422, 2007.

447. Viscusi ER, Webster L, Kuss M, Daniels S, Bolognese JA, Zuckerman S, Soergel DG, Subach RA, Cook E, Skobieranda F. A randomized, phase 2 study investigating TRV130, a biased ligand of the μ opioid receptor, for the intravenous treatment of acute pain. *Pain* 157: 264-272, 2016.

448. Wacker D, Wang S, McCorvy JD, Betz RM, Venkatakrishnan AJ, Levit A, Lansu K, Schools ZL, Che T, Nichols DE, Shoichet BK, Dror RO, Roth BL. Crystal structure of an LSD-bound human serotonin receptor. *Cell* 168: 377-389, 2017.

449. Wall MA, Coleman DE, Lee E, Iñiguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR. The structure of the G protein heterotrimer $G\alpha_{i1}\beta_1\gamma_2$. *Cell* 83: 1047-1058, 1995.

450. Walter NG, Huang C-Y, Manzo AJ, Sobhy MA. Do-it-yourself guide: how to use the modern single-molecule toolkit. *Nat Methods* 5: 475-489, 2008.

451. Wang Q, Zhang H, Xu H, Guo D, Shi H, Li Y, Zhang W, Gu Y. 5-HTR3 and 5-HTR4 located on the mitochondrial membrane and functionally regulated mitochondrial functions. *Sci Rep* 6: 37336, 2016.

452. Warne T, Edwards PC, Doré AS, Leslie AGW, Tate CG. Molecular basis for high-affinity agonist binding in GPCRs. *Science* 364: 775-778, 2019.

453. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ. Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* 100: 10782-10787, 2003.

454. Weiss JM, Morgan PH, Lutz MW, Kenakin TP. The cubic ternary complex receptor-occupancy model I. Model description. *J Theor Biol* 178: 151-167, 1996.

455. Weiss M, Elsner M, Kartberg F, Nilsson T. Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells. *Biophys J* 87: 3518-3524, 2004.

456. Wess J, Bonner TI, Dörje F, Brann MR. Delineation of muscarinic receptor domains conferring selectivity of coupling to guanine nucleotide-binding proteins and second messengers. *Mol Pharmacol* 38: 517-523, 1990.

457. Wess J, Brann MR and Bonner TI. Identification of a small intracellular region of the muscarinic M3 receptor as a determinant of selective coupling to PI turnover. *FEBS Lett* 258: 133-136, 1989.

458. Westfield GH, Rasmussen SG, Su M, Dutta S, DeVree BT, Chung KY, Calinski D, Velez-Ruiz G, Oleskie AN, Pardon E, Chae PS, Liu T, Li S, Woods VL, Jr., Steyaert J, Kobilka BK, Sunahara RK, Skiniotis G. Structural flexibility of the $G\alpha_s$ α-helical domain in the β_2-adrenoceptor–G_s complex. *Proc Natl Acad Sci USA* 108: 16086-16091, 2011.

459. Wettschureck N and Offermanns S. Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85: 1159-1204, 2005.

460. Whelan DR, Holm T, Sauer M, Bell TDM. Focus on super-resolution imaging with direct stochastic optical reconstruction microscopy (*d*STORM). *Aust J Chem* 67: 179-183, 2014.

461. Whorton MR and MacKinnon R. Crystal structure of the mammalian GIRK2 K^+ channel and gating regulation by G proteins, PIP2, and sodium. *Cell* 147: 199-208, 2011.

462. Wickman KD, Iniguez-Lluhl JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE. Recombinant G-protein $\beta\gamma$-subunits activate the muscarinic-gated atrial potassium channel. *Nature* 368: 255-257, 1994.

463. Wingler LM, Elgeti M, Hilger D, Latorraca NR, Lerch MT, Staus DP, Dror RO, Kobilka BK, Hubbell WL, Lefkowitz RJ. Angiotensin analogs with divergent bias stabilize distinct receptor conformations. *Cell* 176: 468-478, 2019.

464. Witherow DS, Slepak VZ. A novel kind of G protein heterodimer: The $G\beta_5$–RGS complex. *Recept Channels* 9: 205-212, 2003.

465. Witzel P, Gotz M, Lanoiselée Y, Franosch T, Grebenkov DS, Heinrich D. Heterogeneities shape passive intracellular transport. *Biophys J* 117: 203-213, 2019.

466. Wolter S, Loschberger A, Holm T, Aufmkolk S, Dabauvalle MC, van de Linde S, Sauer M. rapidSTORM: accurate, fast open-source software for localization microscopy. *Nat Methods* 9: 1040-1041, 2012.

467. Wong IY, Gardel ML, Reichman DR, Weeks ER, Valentine MT, Bausch AR, Weitz DA. Anomalous diffusion probes microstructure dynamics of entangled F-actin networks. *Phys Rev Lett* 92: 178101, 2004.

468. Wright PT, Nikolaev VO, O'Hara T, Diakonov I, Bhargava A, Tokar S, Schobesberger S, Shevchuk AI, Sikkel MB, Wilkinson R, Trayanova NA, Lyon AR, Harding SE, Gorelik J. Caveolin-3 regulates compartmentation of cardiomyocyte β_2-adrenergic receptor-mediated cAMP signaling. *J Mol Cell Cardiol* 67: 38-48, 2014.

469. Wu G, Benovic JL, Hildebrandt JD, Lanier SM. Receptor docking sites for G-protein $\beta\gamma$ subunits: Implications for signal regulation. *J Biol Chem* 273: 7197-7200, 1998.

470. Xiang Y, Rybin VO, Steinberg SF, Kobilka BK. Caveolar localization dictates physiologic signaling of β_2-adrenoceptors in neonatal cardiac myocytes. *J Biol Chem* 277: 34280-34286, 2002.

471. Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC. Structure of an agonist-bound human A_{2A} adenosine receptor. *Science* 332: 322-327, 2011.

472. Yarwood RE, Imlach WL, Lieu T, Veldhuis NA, Jensen DD, Klein Herenbrink C, Aurelio L, Cai Z, Christie MJ, Poole DP, Porter CJH, McLean P, Hicks GA, Geppetti P, Halls ML, Canals M, Bunnett NW. Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc Natl Acad Sci USA* 114: 12309-12314, 2017.

473. Ye L, Van Eps N, Zimmer M, Ernst OP, Prosser RS. Activation of the A_{2A} adenosine G protein-coupled receptor by conformational selection. *Nature* 533: 265-268, 2016.

474. Yi TM, Kitano H and Simon MI. A quantitative characterization of the yeast heterotrimeric G protein cycle. *Proc Natl Acad Sci USA* 100: 10764-10769, 2003.

475. Yin J, Chen KM, Clark MJ, Hijazi M, Kumari P, Bai XC, Sunahara RK, Barth P, Rosenbaum DM. Structure of a D2 dopamine receptor–G-protein complex in a lipid membrane. *Nature* 584: 125-129, 2020.

476. Yin W, Li Z, Jin M, Yin Y-L, de Waal PW, Pal K, Yin Y, Gao X, He Y, Gao J, Wang X, Zhang Y, Zhou H, Melcher K, Jiang Y, Cong Y, Zhou XE, Yu X, Xu HE. A complex structure of arrestin-2 bound to a G protein-coupled receptor. *Cell Res* 29: 971-983, 2019.

477. Yuan S, Filipek S, Palczewski K, Vogel H. Activation of G protein-coupled receptors correlates with the formation of a continuous internal water pathway. *Nat Commun* 5: 4733, 2014.

478. Zaccolo M, De Giorgi F, Cho CY, Feng L, Knapp T, Negulescu PA, Taylor SS, Tsien RY, Pozzan T. A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Nat Cell Biol* 2: 25-29, 2000.

479. Zhang FL and Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem* 65: 241-269, 1996.

480. Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci USA* 98: 14997-15002, 2001.

481. Zhang Y, Sun B, Feng D, Hu H, Chu M, Qu Q, Tarrasch JT, Li S, Sun Kobilka T, Kobilka BK, Skiniotis G. Cryo-EM structure of the activated GLP-1 receptor in complex with a G protein. *Nature* 546: 248-253, 2017.

482. Zhou H-X. Disparate ionic-strength dependencies of on and off rates in protein-protein association. *Biopolymers* 59: 427-433, 2001.

483. Zidar DA, Violin JD, Whalen EJ, Lefkowitz RJ. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci USA* 106: 9649-9654, 2009.

TABLE 1. Gα subunits and their functions.

α-subunit	α-subunit name	gene	exemplary downstream effector	reference
G protein subunits α, group s				
α_s	GNAS complex locus	*GNAS*	activates AC1-9	(368) (459)
α_{olf}	G protein subunit alpha L	*GNAL*	activates AC (predominately isoforms 2, 3, 5)	
G protein subunits α, group q				
α_q	G protein subunit alpha q	*GNAQ*	activate PLC-β1/3/4	(286)
α_{11}	G protein subunit alpha 11	*GNA11*		
α_{14}	G protein subunit alpha 14	*GNA14*		
α_{15}	G protein subunit alpha 15	*GNA15*	activates PLC- β1/2/s3	
G protein subunits α, group i				
α_{i1}	G protein subunit alpha i1	*GNAI1*	inhibit AC1/5/6	(151) (368) (459)
α_{i2}	G protein subunit alpha i2	*GNAI2*		
α_{i3}	G protein subunit alpha i3	*GNAI3*		
α_o	G protein subunit alpha o1	*GNAO1*	inhibits AC1	
$\alpha_{t\text{-}rod}$	G protein subunit alpha transducin 1	*GNAT1*	activate phosphodiesterase 6	
$\alpha_{t\text{-}cone}$	G protein subunit alpha transducin 2	*GNAT2*		
α_{gust}	G protein subunit alpha gustducin 3	*GNAT3*		
α_z	G protein subunit alpha z	*GNAZ*	inhibits AC1/5/6	
G protein subunits α, group 12/13				
α_{12}	G protein subunit alpha 12	*GNA12*	activate RhoGEFs	(386)
α_{13}	G protein subunit alpha 13	*GNA13*		

TABLE 2. Comparison of ensemble and single-molecule techniques.

	single-molecule	**ensemble**
expression level of target molecules	low physiological level is achievable	overexpression of target molecules
signal accumulation (e.g. at specialized membranes)	unsuitable due to high local concentrations of signal (precise localization of molecules is impossible)	suitable for analysis of concentrated fluorescent signal
measurement of target molecule's behavior	directly visualize and measure the behavior of individual molecules	measure the behavior of thousands of molecules
kinetics measurements	direct estimation of protein–protein association and dissociation rates	limited or based on thousands of molecules' averaged behavior
analysis of complex behavior/ identification of rare events	possible due to the visualization of individual molecules	impossible due to averaging of thousands of molecules
spatial resolution	ca. 20 nm	ca. 200 nm (limited by the basic principles of light microscopy)
analysis	time and computational power-consuming	relatively easy

TABLE 3. Estimates of GPCR–G protein interaction kinetic parameters from ensemble and single-molecule studies.

EMSEMBLE STUDIES					
live cells					
receptor	**G protein**	**stimulation**	**τ_{on}***	**τ_{off}***	**reference**
α_{2A}AR	G_i	noradrenaline	50-90 ms	13 s	(156)
A_{2A}R	G_s	adenosine	50 ms	15 s	(157)
β_1AR	G_s	noradrenaline	60 ms	8 s	
M1R	G_q	oxo-M	200 ms	3.7 s	(185)
PTH1R	G_s	PTH_{1-34}	1 s	-	(108)
PTH1R	G_s	$PTHrP_{1-36}$	1.6 s	50 s	
SINGLE-MOLECULE STUDIES					
purified receptor in micellae					
receptor	**G protein**	**stimulation**	**k_{on}**	**k_{off}**	**reference**
β_2AR	G_s	adrenaline	0.005 $\mu M^{-1}s^{-1}$	-	(138)
live cells					
receptor	**G protein**	**stimulation**	**k_{on}**	**k_{off}**	**reference**
β_2AR	G_s	no	0.005 $\mu m^2 molecule^{-1}s^{-1}$	0.4 s^{-1}	(413)
β_2AR	G_s	isoproterenol	0.015 $\mu m^2 molecule^{-1}s^{-1}$	0.6 s^{-1}	
α_{2A}AR	G_i	no	0.015 $\mu m^2 molecule^{-1}s^{-1}$	0.5-1 s^{-1}	
α_{2A}AR	G_i	UK-14,304	0.1 $\mu m^2 molecule^{-1}s^{-1}$	0.9 s^{-1}	