

Chapter 3

Structural Basis of Dopamine Receptor Activation

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Abstract G protein-coupled receptors (GPCRs) are seven transmembrane (TM) proteins representing the largest and most universally expressed cell surface receptors and are present in almost all species and in a wide variety of cells. Here we will focus our attention on the catecholamine-binding GPCRs and in particular on the dopamine receptors. The catecholamine-binding GPCRs form a group of rhodopsin-like GPCRs composed of adrenoceptors, which are endogenously activated by epinephrine and norepinephrine, and dopamine receptors. We review the different “molecular switches” involved in GPCR activation and we emphasize the importance of extracellular loop 2 (ECL2) in ligand binding. A better understanding of the functional role of ECL2 can be achieved after the release of the crystal structures of B2AR and rhodopsin, which are consistent with dopamine D2 receptor substituted cysteine accessibility method (SCAM) experimental data. Even though reconstituted GPCR monomers appear sufficient to activate a G protein, in the native setting their dimerization/oligomerization may modulate activation through changes at the dimerization interface or a larger-scale reorientation of the protomers. Therefore, the structural aspects of oligomerization and their importance for receptor activation and signaling are also addressed.

Keywords Catecholamine-binding GPCRs · Dopamine receptors · Binding site · ECL2 · GPCR oligomerization · GPCR–G Protein interaction · Activation · Structural rearrangements

3.1 Introduction

G protein-coupled receptors (GPCRs) are seven transmembrane (TM) proteins representing the largest and most universally expressed cell surface receptors. GPCRs

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are present in almost all species and in a wide variety of cells [1–8]. They play important roles in a broad array of cellular functions and in disease and represent the targets for a large fraction of existing drugs [9–12]. GPCRs are classified into three major classes based on the size of the N termini, on sequence homology, the identity of conserved residues within the seven TM domains that participate in ligand binding, mode of action, and pharmacology [13, 14]. The largest family is Class A (more than 90%), which comprises rhodopsin as well as receptors for biogenic amines, peptides, and odorants. Class B receptors are a much smaller group and include receptors for large peptides such as secretin, cytokines, thrombin, and glucagon. Class C receptors (comprised of approximately 12 members) include the γ -aminobutyric acid B receptor (GABA_B), eight metabotropic glutamate receptors, the Ca²⁺ sensing receptor, as well as some pheromone and taste receptors [15]. GPCRs, upon ligand binding, induce dissociation of G proteins into their G_α and G_{βγ} components and ultimately modulate the activity of enzyme or ion channel effectors [5, 16–19].

Structurally, GPCRs are made up of seven TM segments connected by three intracellular and three extracellular loops (ICL, ECL), and Class A receptors share important functionally conserved sites identified as structural motifs that act as functional microdomains, such as the D(E)RY motif in TM3 and NPXXY in TM7 [8, 20–28]. The first GPCR structure, bovine rhodopsin, was solved in 2000 [29], and there was much anticipation that many other GPCR structures would be rapidly forthcoming. Although a number of different rhodopsin structures were solved, 7 years passed without any other GPCR structures, in support of the unique biochemical properties of rhodopsin, including its high abundance and its unusual stability, retaining function under conditions that denature other GPCRs, due to the covalently bound 11-*cis*-retinal, which maintains the receptor in an inactive conformation [30–32].

At the end of 2007, two new crystal structures of the human β 2 adrenergic receptor (B2AR) were solved, including the wild-type receptor bound to an antibody fragment and an engineered receptor with T4 lysozyme inserted into the third intracellular loop [31–33]. Although the B2AR crystallographic structures are quite similar to rhodopsin with a root mean square deviation of 1.6 Å, there are some interesting differences, which impact on considerations of the structure of the dopamine receptor family, for which a structure is not yet available. Very recently two new structures of the β 1 adrenergic receptor [34] and the adenosine A2 receptor [35] have been solved, and while there are interesting differences, the overall structures are again quite similar.

Here we will focus our attention on the catecholamine-binding GPCRs and in particular on the dopamine receptors. The catecholamine-binding GPCRs form a group of rhodopsin-like GPCRs composed of adrenoceptors, which are endogenously activated by epinephrine and norepinephrine, and dopamine receptors [36]. For the adrenoceptors, there are three main classes based on their pharmacological properties, amino acid sequences, and signaling mechanisms. These adrenoceptor classes were subsequently divided in humans into three subtypes each: α_1 (α_{1A} , α_{1B} , α_{1D}), α_2 (α_{2A} , α_{2B} , α_{2C}), and β (β_1 , β_2 , β_3). These receptors respond to the

neurotransmitters/hormones, norepinephrine and epinephrine, which play key roles in regulation of cardiovascular function, energy metabolism, and blood pressure [15]. In contrast to adrenoceptors, dopamine receptors in human are divided into two classes: D₁-like receptors (D_{1A} or D₁ and D_{1B} or D₅) and D₂-like receptors (D₂, D₃, and D₄). While sharing some common properties, each receptor displays unique properties including affinity for dopamine, specificity for G protein coupling and signaling, and specific neuronal distributions [37]. Furthermore, in the case of the D₂ receptor (D2R) subfamily, there are two isoforms: the long isoform D_{2L} and the short isoform D_{2S}, generated by alternative splicing of an 87-bp exon. This splicing event leads to an additional 29 amino acids in the ICL3 of the isoform D_{2L} [38].

3.2 Transmembrane Segments and Activation

TM segment interactions are a key determinant in the assembly and stability of the native structure of membrane proteins [39–41]. As the sequence conservation within the membrane-spanning regions is high, it is thought that class A GPCRs share a similar architecture [16, 17, 41, 42], which has been supported to date by the four different receptors for which we have crystal structures. The catecholamine-binding GPCRs share within their TM regions 20–26% sequence identity with rhodopsin [36]. For example, the sequence identity between the TM domains of rhodopsin and B2AR is 21%, between rhodopsin and D2R is 25%, and between D2R and B2AR is 38% [36].

Some of the most important features of the TM domains are the kinks and bends generated by prolines and glycines, respectively [23, 43–46]. Serines, threonines, and cysteines can also bend the α -helices that constitute the TM domains [24, 47]. In rhodopsin, TM1 possesses a proline-induced kink that bends it inward, toward the helix bundle. It was proposed that other GPCRs, which do not have this proline in TM1, including the D2R, might be packed somewhat differently with TM1 more distant from the bundle [8, 24, 48]. Consistent with such an orientation of TM1, the extracellular segment of TM1 of D2R did not seem to contribute to the binding site based on substituted cysteine accessibility method (SCAM) studies [8, 49]. The B2AR 3D structure validated this hypothesis because its TM1 is comparatively straight [32]. Moreover, although the TM segments in rhodopsin and B2AR have similar orientations, there are some differences: the angles between TM1, TM3, and TM6 and the membrane are different from their counterparts in rhodopsin, TM4 is translated away from the center of the receptor, and TM5 is translated closer to the center of the receptor [32].

It is hypothesized that GPCRs exist as an ensemble of various conformational states that are in a dynamic equilibrium, and that agonist binding and subsequent activation occur through a series of conformational intermediates [50, 51]. Ligands have the ability to stabilize or possibly induce specific conformations [52]. Mutations that disrupt stabilizing non-covalent interactions favor more active receptor conformations by increasing the movement of the TM segments relative to

each other [52]. Although there are different ligand-binding modes in the different GPCR classes, activation processes are thought to result from similar conformational changes involving the TM domain [53–59]. In particular, rotation and outward movement of TM6 likely open a crevice allowing for interaction with the C terminus of the G protein α -subunit and triggering GPCR activation [59–61].

Many GPCRs show a considerable amount of basal, agonist-independent activity, reflecting GPCR structural flexibility and the existence of conformational ensembles [52]. The study of constitutively active GPCRs has contributed to our understanding of the activation mechanism [57, 62–65]. Mutation of certain residues in GPCRs significantly increases their constitutive activation [66, 67] by breaking crucial intramolecular interactions between amino acid residues that normally constrain the receptor to its inactive state [21, 24, 62]. Many residues that produce constitutive activation when mutated are linked through packing interactions with residues that are essential for receptor activation by side chain rearrangement on adjacent TMs and/or by larger-scale TM movements [62, 68–70]. Some of the most well-known constitutively active mutants (CAMs) are those that disrupt the highly conserved (D/E)R(Y/W) amino acid sequence present in 72% of class A GPCRs. By contrast, an “ionic lock” is crucially involved in maintaining the inactive state of the receptor [21, 24, 71–73]. This is exemplified in a network of hydrogen bonding and charge interactions between Glu134^{3,49} and Arg135^{3,50} at the cytoplasmic end of TM3 and Glu247^{6,30} and Thr251^{6,34} at the cytoplasmic end of TM6 of rhodopsin (Ballesteros general number in the superscript [7]) [23, 24, 74]. In the B2AR structures, the “ionic lock” is broken, which may account for the residual basal activity of the B2AR bound to the inverse agonist carazolol [31, 33, 51]. Recent computational studies suggest that the ionic lock dynamically forms and unforms in association with conformational change in ICL2 [75].

Besides the “ionic lock,” there are other “molecular switches” involving non-covalent intramolecular interactions that must be altered to achieve an active state. The “rotamer toggle switch” involves Phe290^{6,52}, which is accessible in the binding site crevice and serves as a “sensor,” a change in the bend of TM6 at the highly conserved residue Pro288^{6,50}, and a change in the rotamer of Trp265^{6,48} upon activation of rhodopsin and related family members [46]. Although carazolol does not directly interact with the “toggle switch” in the B2AR TM6, it seems to modulate the rotameric state of Trp286 indirectly by interacting with Phe289^{6,51} and Phe290^{6,52} [31–33, 76, 77]. These kinds of molecular switches can be studied experimentally and computationally [6, 25, 71, 72].

3.3 The Binding Site

SCAM studies, experimental approaches such as studies of chimeric receptors and point mutants, as well as molecular modeling allowed for the identification of amino acids that line the putative binding pocket of the D2R [48, 78–96] (Fig. 3.1). The findings for D2R are in agreement with results for other catecholamine-binding receptors [36]. The binding crevice has two polar regions common to all these

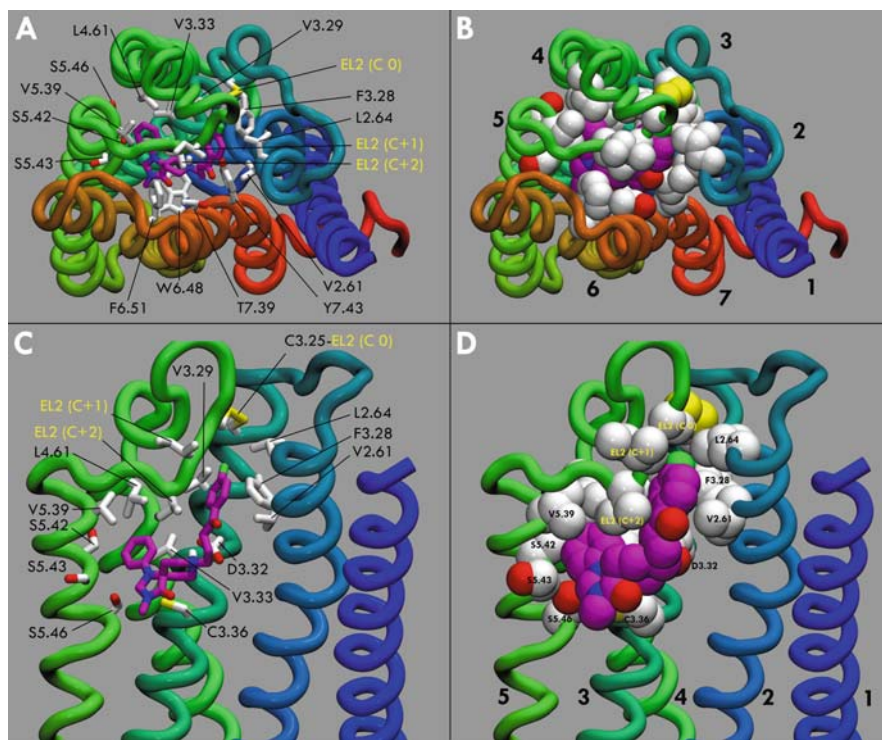


Fig. 3.1 *N*-Methylspiperone (MSP) docked into the binding site crevice of the dopamine D₂ receptor (D2R). Panel (A): From an extracellular perspective, TMs 1–7 are colored from *blue* to *red*. MSP (carbon *magenta*, stick) is nestled in the binding site and capped by EL2. D2R side chains within 4 Å of MSP are shown (carbon *white*, stick) and labeled according to the Ballesteros and Weinstein indexing system. Residues in EL2, Ile183^{EL2(C+1)}, and especially Ile184^{EL2(C+2)} (labeled in *yellow*), provide substantial hydrophobic contacts to the ligand. Other residues within 4 Å of MSP are Val91^{2.61}, Leu94^{2.64}, Phe110^{3.28}, Val111^{3.29}, Asp114^{3.32}, Val115^{3.33}, Cys118^{3.36}, Leu171^{4.61}, Cys182^{EL2(C 0)}, Val190^{5.39}, Ser193^{5.42}, Ser194^{5.43}, Ser197^{5.46}, Trp357^{6.48}, Phe360^{6.51}, Thr383^{7.39}, and Tyr387^{7.43} [D2R (*short*) UNIPROT sequence P14416-2]. Panel (B): To emphasize the hydrophobic packing of the EL2 and crevice residues with bound MSP, the contact side chains from A are rendered as van der Waals spheres. TMs are numbered. Panel (C): Peering into the crevice from a side view (TMs 6–7 are removed) reveals some key interactions between MSP and the D2R binding site crevice and EL2. Key interactions include a hydrogen bond-reinforced ionic interaction between Asp114^{3.32} and the ligand’s amine moiety, deep occupancy of the MSP’s phenyl-imidazolidinone group in the primary binding cleft centered between TMs 3 and 5–6, and contacts between the fluorophenyl group with residues in EL2 and TMs 2,3, and 7 (not shown). Missing from this view are the aromatic contacts from TM6 to the ligand’s piperazine and phenyl-imidazolidinone moieties and potential hydrogen bonding between Thr383^{7.39} and Tyr387^{7.43} and the butyrophenonyl keto group. Panel (D): Same view as Panel C with side chains rendered as van der Waals spheres. EL2 contact residues are labeled in *yellow*. TMs are numbered

receptors: Asp^{3.32}, which forms ionic interactions with the protonated amine of biogenic amines, and Ser^{5.42}, Ser^{5.46}, and Ser^{5.43} of TM5, which interact by hydrogen bonding with the *meta*-OH and *para*-OH of the catecholamine. The β -hydroxyl group of (nor)epinephrine, which is not found in dopamine, interacts with Asn^{6.55}. Phe^{5.47}, Trp^{6.48}, Phe^{6.51}, and Phe^{6.52} are also expected to interact with the aromatic ring of the ligands [36]. In an exhaustive computational study, Xhaard et al. [36] demonstrated that the docked ligand tends to be in an extended conformation because Asp^{3.32} and TM5 residues are distant from each other, at the opposite ends of the binding pocket.

Prior to the determination of the crystal structure of rhodopsin, Simpson et al. [91] used data from SCAM studies to guide an exploration of the structural basis of the pharmacological specificity of D2R and D4R. Combined substitution of four to six of the residues that faced the binding site crevice in the D2R but were not conserved in the D4R switched the affinity of the receptors for several chemically distinct D4-selective antagonists by three orders of magnitude in both directions (D2- to D4-like and D4- to D2-like). The mutated residues were in TM2, TM3, and TM7 and were predicted to form a divergent cluster that differentiated D2R and D4R binding, which has been supported as well by subsequent studies [94, 95, 97]. Ortore et al. [98] have docked different ligands to both D2R and D4R and have proposed that another difference between the two receptors seems to be due to the extracellular loop 2 (ECL2) region (see below), which is differentially situated in the receptor models, although it should be noted that loop modeling is a complex and developing science [99, 100]. Ligand binding to many members of the GPCR family is regulated allosterically by cations. For example, Na⁺ is important for the D2R, and Zn²⁺ was shown to interact with D1, D2, and D4 receptors [101].

3.4 Extracellular Loop 2

It is widely accepted that the extracellular loops (especially ECL2) are of great importance for accommodating high molecular weight GPCR ligands (peptides and proteins). In the rhodopsin structure ECL2 forms a lid-like structure over retinal, but the precise role of ECL2 in binding other lower molecular weight, drug-like ligands is less clear [102]. In more than 800 GPCRs encoded in the human genome, the average size of ECL2 is 27 residues, with a deviation of 13 residues [103]. For nearly all rhodopsin-like GPCRs, the disulfide bond between Cys^{3.25} (Cys-107 in D2R) and the conserved Cys in E2 (Cys_e2, Cys-182 in D2R) connects ECL2 with the extracellular end of TM3, and this disulfide bond (SS-E2) is crucial to the structural integrity and function of many GPCRs. This disulfide bridge is found in more than 90% of GPCRs [14, 103]. The removal of SS-E2 by mutagenesis severely disrupts ligand binding to muscarinic acetylcholine receptors [104, 105] and destabilizes the high-affinity state of the B2AR [106]. Moreover, antagonist protected the B2AR from the effects of reduction by dithiothreitol [107]. Thus, SS-E2 is protected by a conformational change or steric block within the binding site.

In rhodopsin, ECL2 forms a twisted, buried β -hairpin structure that folds deeply into the TM domain with one strand contacting retinal and forming interactions with other extracellular loops [102]. It forms a lid-like structure that shields the retinal in a hydrophobic pocket [51]. The orientation of ECL2 is maintained by the SS-E2 described above [29, 108].

Several reports have implicated ECL2 in ligand specificity in aminergic and other small molecule ligand GPCRs. Zhao et al. [109] found that substitution of three consecutive residues in ECL2 interconverted the ligand specificity for particular antagonists between that of α_{1B} AR and α_{1A} AR. Substitution of a single residue in ECL2 interconverted the pharmacological specificities of canine 5-HT_{1D} and human 5-HT_{1D} receptor [110]. Similarly, substitution of ECL2 and TM5 changed the subtype specificity of the 5-HT_{1D} receptor to that of the 5-HT_{1B} receptor and vice versa [111]. Thus, although it has been argued that the presence of ECL2 within the TMD may be a feature unique to rhodopsin [112, 113], it has also been proposed that ECL2 contributes directly to forming the binding site of aminergic and certain other small molecule ligand GPCRs [114]. To address this issue, SCAM studies were carried out in the short ECL2 of D2R [115]. The reaction of five of these mutants with sulfhydryl reagents inhibited antagonist binding, and bound antagonist protected two, I184C and N186C, the second and fourth residues after the highly conserved Cys_e2 (C+2, C+4). The pattern of accessibility in ECL2 was consistent with a structure similar to that of bovine rhodopsin, in which E2b, the part of ECL2 C-terminal to the conserved disulfide bond, is deeper in the binding site crevice than is E2a, the N-terminal part of ECL2, and E2b was inferred to contribute directly to the binding site in the D2R and probably in other aminergic GPCRs as well (Fig. 3.1).

More recently, the effects of ECL2 mutations on agonist and antagonist binding have been studied in the V1a vasopressin receptor (Class A) ECL2 by a systematic alanine-scanning mutagenesis technique that identified four aromatic amino acids, located in the middle of the ECL2 near the conserved disulfide bond and conserved throughout this subfamily of peptide GPCRs, as important for agonist binding and receptor activation [116]. Trp206^(C+1) and Phe209^(C+4) were hypothesized to be important for ligand binding and Tyr218^(C+13) and Phe189^(C-16) appear to be important for orientation/stability of ECL2 over the binding pocket [116]. Furthermore, Klco et al. showed that disruption of ECL2 of the complement C5a receptor (C5aR) by random mutagenesis generated many receptors able to activate G proteins even in the absence of ligands [14]. The authors postulated that ECL2 acts as a negative regulator of C5aR activation possibly by making multiple contacts with the TM domain to stabilize the inactive state.

Through their studies of the serotonin 5-HT_{4(a)} receptor Baneres et al. have suggested the existence of different arrangements of ECL2 depending whether the bound ligand was an agonist (partial or full) or an inverse agonist [117]. In contrast, antagonist binding was inferred not to induce any structural changes of ECL2. Therefore, as in the case of D2R, ECL2 appears to participate in the binding site and rearranges upon activation. Despite the constraint provided by the conserved disulfide bond between ECL2 and the top of TM3, Avlani et al. showed that the

flexibility in ECL2 of the muscarinic acetylcholine M₂ receptor (M₂ mAChR) and its capacity to achieve an open conformation is necessary for the binding of both allosteric and orthosteric ligands [4]. They postulated ECL2 as a gatekeeper with respect to entrance into the orthosteric binding site crevice.

Other studies stress ECL2's importance for ligand binding as in the M₃ muscarinic acetylcholine receptor (M₃R) and the thyroid-stimulating hormone receptor (TSHR). ECL2 of the M₃R was subjected to random mutagenesis. In contrast to the model proposed by Klco et al. [14], the results of this study suggested that specific ECL2 residues stabilize the active state of the M₃R, and are required for efficient agonist-induced M₃R activation [108]. The authors also proposed a mechanism in which conformational flexibility in the ECL2 loop is required for efficient receptor activation [108]. Kleinau et al. suggested an activation mechanism in which TM6 glides along ECL2 according to the diverse receptor activation states. Disruption of this critical interface by introduction of mutations in the TSHR alters its basal activity [118].

The crystal structure of B2AR provided the first non-rhodopsin ECL2 structure with which to address these hypothesized functional roles. The conformation of B2AR ECL2 and its orientation to the TMD are significantly different from that in rhodopsin, rendering the binding site crevice of B2AR directly exposed to the water phase [31–33]. Strikingly, however, the ligand-binding residue positions in E2b of rhodopsin and B2AR are remarkably consistent, if counted from CysE2, namely the C+2 and C+4 positions, even though rhodopsin has eight extra residues between CysE2 and the start of TM5 at position 5.36. This is in remarkable agreement with the SCAM studies in D2R [115], in which protection by antagonist suggests that the same two positions, Ile184 and Asn186, face the binding site crevice. In this study several residues in D2R were also found to be accessible to MTS reagent but not protected by ligand, and these were proposed to line the ligand entry pathway. This is most obvious in the C+1 position, because E2b can easily be aligned between D2R and B2AR, whereas E2a varies significantly. In the B2AR structure, C+1 is in the vestibule through which the extracellular milieu gains access to the bound carazolol [31–33]. Given the consistency between the D2R SCAM experimental data for ECL2 with both the rhodopsin and B2AR structures, it is likely that C+2 and C+4 play an important role in ligand binding in the other catecholamine receptors as well, and play an important role in ligand specificity (see Fig. 3.1).

3.5 GPCR Oligomerization

Class C GPCRs, including the metabotropic glutamate receptors and γ -aminobutyric acid type B (GABA_B) receptors, have been shown to form homo- and heterodimers in the plasma membrane, with important consequences for trafficking of receptors to the cell surface and for ligand-induced activation and G protein coupling [119]. Class C GPCRs have unique characteristics with dimerization potential: an N-terminal Venus flytrap (VTF) module with structural and functional homology to bacterial periplasmic proteins [120], and cysteine-rich domains (CRDs) [121].

Although the formation of dimers for Class C GPCRs is clear, there is still some controversy regarding the existence of dimers in Class A receptors [122, 123]. Nonetheless, there is increasing agreement that Class A GPCRs can interact to form homo- or heterodimers/oligomers [40, 124–149]. Evidence for dopamine receptor homo- and heteromerization is reviewed extensively in Chapter 10; here we focus on structural aspects of oligomerization and the relationship of oligomerization to receptor activation and signaling.

For rhodopsin, dimers and higher-order oligomers have been visualized in disc membranes by atomic force microscopy [150], and an oligomeric arrangement has been inferred in its native environment [12, 151]. Oligomerization also has been inferred from ligand-binding studies [132, 152–155]. Guo et al. recently demonstrated using biophysical and biochemical approaches that the D2R forms higher-order oligomers in living cells at physiological levels of expression [147].

3.5.1 GPCR Oligomerization and Signaling

What is physiologically most relevant is understanding the role of the dimeric or oligomeric organization of GPCRs in signaling [156, 157]. Indeed, one of the great challenges in GPCR biology today is strengthening the weak mechanistic link between the physical interactions of receptors in the membrane and signaling cross talk of presumed heterodimers or hetero-oligomers. There is a great deal of evidence from many laboratories that many GPCRs interact as heterodimers (reviewed in [158, 159]). As indicated above, a number of findings support the existence of higher-order homo-oligomers as well [150, 155, 160, 161]. This raises the possibility that GPCR heteromers may interact not as heterodimers per se but rather as higher-order hetero-oligomers composed of homodimer subunits.

A large number of studies have demonstrated signaling cross talk between coexpressed GPCRs [162]. In almost all cases, however, the mechanistic link between heteromerization and signaling is tenuous. Although activation of two coexpressed receptors may be essential, signaling cross talk could nonetheless take place downstream of parallel homomeric receptor-mediated G protein activation and in such a case would not be a direct result of heteromeric signaling. Such a downstream cross talk mechanism, while often ignored, is very difficult to rule out. One example of this complexity is a recent fascinating study of a putative D1–D2R heterodimer that has been carried out both in heterologous cells [163] and in the brain [164]. These receptors appear to be coexpressed in some neurons in vivo [164]. In heterologous cells they have been inferred to physically interact based on fluorescence resonance energy transfer (FRET) [165, 166] as well as co-internalization [167, 168] and co-retention of mutants [169]. Activating both D1 and D2Rs leads to Gq-mediated signaling [163, 164], whereas D1 signaling is normally Gs/olf mediated and D2 signaling is normally Go/i mediated. These findings are intriguing and open exciting avenues of drug design targeted selectively to specific heteromers [170]. However, the plot appears thicker, as D1R-mediated Gq signaling has been observed in the brain [171, 172] where in some studies it has been shown to be insensitive to D2R

blockade [173], suggesting a role for other cellular factors in the coupling of D1R to the Gq pathway. Evidence for a priming effect for D1R-mediated Gq signaling is an example of such a potential mechanism [174, 175].

D2R has also been reported to interact with the dopamine D₃ receptor (D3R), and coexpression of D₂ and D₃ receptors has been reported to modulate the function of both receptors [176, 177]. More recently the D2R has been shown to modulate and to physically associate with the dopamine transporter as well [178, 179].

In addition to its reported interactions with receptors from the dopamine subfamily, there is a substantial literature on heteromerization of D2R with multiple other Class A receptors. There is evidence for direct physical interaction between D2R and the SST5 somatostatin receptor [180], D2R and adenosine A2A receptor [181, 182], and D2R and CB1 cannabinoid receptor [183]. In each of these cases, changes in signaling were observed upon receptor coexpression, with either altered D2R pharmacology by the partner protomer and/or an alteration in the properties of the partner in response to drugs acting at the D2R. In the case of the D2R–CB1 heteromer, dual-agonist mediated activation of G_s was reported, although neither receptor alone is able to activate this G α subunit [183]. These results are intriguing and suggest the possibility of an untapped level of pharmacological diversity for new compound development, as well as a host of potential roles for *in vivo* signaling specificity for these putative heteromers. However, in none of these studies is it possible to rule out downstream signaling cross talk and thus to establish incontrovertibly that direct signaling by the D2R heteromer is responsible for the cross talk.

Such a mechanistic interrogation of heteromeric signaling in Class A GPCRs has been difficult. Our mechanistic understanding of the functional role of GPCR dimerization is more advanced in the Class C receptors, due in part to the availability of a clever adaptation of the endoplasmic reticulum (ER) retention signal from the GABA_B receptor to enable controlled cell surface expression and signaling by defined metabotropic glutamate receptor (mGluR) heterodimers [184]. These studies have shown evidence for asymmetric activation of the heterodimer [185, 186]. Furthermore, one agonist can activate the dimer, but two agonists are required for full activation [187]. In addition, within the same Class C, T1R3 taste receptors are known to form functional heterodimers with either T1R1 or T1R2 in order to respond to a large panel of ligands and to trigger umami and sweet taste sensations, respectively (reviewed in [188]).

Unfortunately, related approaches with ER retention signals have been unsuccessful in Class A receptors, and it has not been possible to differentiate clearly the role of each subunit in homomeric and heteromeric signaling with coexpressed receptors. However, multiple lines of study do suggest interaction between Class A receptors in a heteromeric functional unit. Thus, for example, ligand-binding dissociation kinetics have recently been linked to the GPCR dimerization process (reviewed in [189]). In chemokine receptor heteromers, a CCR2-selective drug accelerates the dissociation of a CCR5- or CXCR4-selective drug when the receptors are coexpressed in heterologous cells and in native lymphocytes [190–192]. Moreover, although it remains to be proven conclusively, it seems reasonable to

infer that bivalent drugs engaging two different receptors, i.e., heteromer-selective compounds, might act simultaneously on two protomers in a heteromer and thereby directly activate downstream heteromer-specific signaling machinery [193–195] raising the possibility of their selective therapeutic potential [196]. Although there is evidence of G protein signaling by coexpressed nonfunctional receptor chimeras, this was proposed to occur by transmembrane domain swapping [197], which is unlikely to be universal [198]. Curiously, coexpression of two loss of function glycoprotein hormone receptors (receptors with either agonist binding or the ability to activate G proteins compromised) [199–201] led to function, but among Class A receptors such rescue seems to be limited to glycoprotein hormone receptors, which have very large extracellular N-terminal binding sites. This is similar to the trans-activation seen in the Class C GABA_B receptor, in which agonist binding to one protomer signals to G protein through the second protomer [184].

Another major question facing the field is the relationship between findings in heterologous cells and in *ex vivo* or *in vivo* cell systems. Most studies have focused on heterologous cells, but new approaches are being developed, including heteromer-specific antibodies (L. Devi, personal communication) as well as transgenic approaches with modified receptors.

Recent studies of purified B2AR and rhodopsin reconstituted into nanodiscs [202, 203] or in detergent solution [204] have demonstrated clearly that these receptors as monomers *can* activate G proteins. If, however, these receptors are indeed organized as dimers (or higher-order units) in native membranes, these elegant biophysical studies beg the physiologically relevant question. That is, if the receptors are capable of functioning as monomers but are closely associated as dimers or oligomers in the membrane, then what functional role does the second protomer play in drug binding and G protein activation? For example, in the GABA_B receptor the GB2 subunit is necessary for high-affinity binding of agonist to GB1 [205]. Studies in the D2R indicate that conformational change at the TM4 dimer interface is part of the receptor activation mechanism [145], although we cannot as yet establish whether this is achieved by changes in one or both protomers. Similarly, in the LBT4 Baneres and colleagues have shown evidence receptor for conformational changes in protomer B upon agonist binding to protomer A [185], again consistent with a role for the dimer interface in activation.

3.5.2 GPCR Oligomers – Structural Considerations

Dimer interface has been the subject of various studies over the years because of its crucial value in elucidating the structural mechanism(s) for cross talk between receptors within an oligomeric arrangement [145]. Guo et al. have shown that in the D2R TM4 forms a symmetrical dimer interface and that a conformational change at this interface is part of the receptor activation mechanism [145, 206]. Based on atomic force microscopy (AFM) maps of rhodopsin, Liang et al. proposed an oligomeric model in which TM4, TM5, and ECL2 form a dimeric interface, whereas contacts between TM1, TM2, and the cytoplasmic loop connecting TM5 and TM6

facilitate the formation of oligomers [151, 160, 207, 208]. TM1 and TM4 were postulated to be the most common interfaces of oligomerization by a correlated mutation analysis-based method [143, 144, 209].

It is unclear if other dimer orientations are also permissible [119]. For example, besides TM4 and TM5 of rhodopsin [160], other TMs have been implicated in dimer interfaces [210]: TM6 of the β_2 -adrenergic, cholecystokinin, and leukotriene B₄ receptors [211, 212], TM5 and TM6 of the adrenergic–muscarinic chimera [213–216], TM1 and TM4 of the D2R [145, 147, 206], TM1 and TM7 of the α -adrenergic receptor [217], TM1 and TM4 of the chemokine receptor [218], TM4, TM1, and TM5/6 in the β_1 -adrenoceptor [130], TM1, TM2, and TM4 in the complement C5a [219], and TM5 in the adenosine A2A receptor [220].

Bouvier et al. showed that a peptide derived from the TM6 of the B2AR inhibits dimerization of these receptors and proposed a helix–helix interaction involving a conserved GxxxG motif on TM6 [221]. Although TM1 and TM4 can form simultaneous symmetric interfaces in an oligomeric structure [147], TM6 cannot form a symmetrical interface in this oligomer, although it might contribute to an asymmetrical interface.

3.5.3 Oligomer Rearrangements upon Activation

Even though GPCR monomers appear sufficient to activate a G protein [122, 202, 203, 222], their dimerization may modulate this activation through changes at the dimerization interface or a larger-scale reorientation of the two subunits [145, 147, 162, 223, 224]. Cross-linking in the D2R homodimer suggested a conformational rearrangement at the TM4 dimer interface upon receptor activation, passing from a conformation consistent with the 1N3M pdb file to an alternative TM4 interface [145, 147]. Consequently, the D2R inactive state is consistent with the AFM model while the active state is consistent with a squid rhodopsin electron cryomicroscopy (ECM) model [145, 147]. Consistent with this proposal, cross-links of the TM4 interface activated D2R, even in the absence of agonist [145, 147]. This idea was substantiated by recent studies. Brock et al. showed that an agonist-induced rearrangement may indeed occur in the activation of the dimeric metabotropic glutamate receptors [225]. Similarly, a possible dimeric rearrangement was also observed in the mGluR1 α receptor. Upon ligand binding, although the distance between ICL1 and ICL2 in each protomer is unchanged, the distance between the ICL1s becomes larger, whereas that between ICL2s becomes smaller [226]. Damian et al. have also shown that in the leukotriene B₄ (LTB₄) receptor, conformational changes take place in one of the protomers upon activation of the other [185].

These observations suggest that in addition to the activation-related conformational changes within a GPCR protomer after activation (mainly a conformational change in TM6 and an associated opening of a binding cleft for G protein between TM6 and TM3) [227], it seems that a rearrangement of the interface of the two protomers is also vital for activation [145, 147]. Mechanisms that might account for this conformational rearrangement include a rigid body clockwise rotation of

contacting TM4s upon activation, protomer displacement involving a large movement and reorganization, or partner change among protomer partners [145, 147], although Niv et al. found using computational methods that rigid body rotation of interacting TM4s is an unlikely mechanism [228].

3.5.4 GPCR Oligomerization and GPCR–G Protein Interactions

mGlu receptor heteromers have been inferred to activate with individual protomers in an asymmetrical relationship [186, 229]. For the BLT1 receptor, the active form of the receptor dimer also is nonsymmetric with only one subunit reaching the fully active state [230]. A single agonist per dimer appears to be sufficient for activation of heterodimeric receptors such as GABA_B [205, 231] and T1R receptors [232]. Similar findings have been reported for the mGlu receptor [187], but other findings in mGlu receptors suggest that activation by agonist binding to both protomers produces greater activation [187]. G protein-specific interactions have been proposed to account for such asymmetric behavior [185, 233, 234]. Jastrzebska et al. speculated that activation of a GPCR dimer could be achieved by a single protomer and that the combination of interactions including the regions of specific trimeric G proteins and two protomers facilitates more efficient coupling [235].

In the classic view, supported by innumerable mutagenesis studies of GPCRs, a monomeric GPCR interacts through ICL2, ICL3, and/or proximal carboxyl-terminal regions with a single heterotrimeric G protein. Structural studies of the receptor–G protein interface have led to the identification of several points of contact between the G protein and the receptor on both α - and β/γ -subunits [236]. When the first crystal structure of a heterotrimeric G protein was solved, it was argued that the surface area of a GPCR monomer was too small to account for the simultaneous interaction with both α - and β/γ -subunits of a G protein [29, 60, 236–238]. A single G protein molecule might instead interact with a GPCR dimer [19, 29, 160, 236, 239–241]. Consistent with this, Baneres and Parelo have shown that activated leukotriene B₄ (LTB₄) receptor BLT1 dimer and G $_{\alpha_{12}\beta_{1}\gamma_{2}}$ form an assembly containing one G protein heterotrimer and one receptor dimer (242). If the signaling unit is a GPCR dimer complexed with a heterotrimeric G protein, then both *cis*- and *trans*-activation between two protomers may occur [145].

In recent years, increasing attention has been placed on developing an improved understanding of the interaction between G proteins and the D2R. Senogles et al. have demonstrated that random point mutations in the ICL3 of D2R_s modify G_i protein coupling specificity. Specifically, ICL3 mutations R233G and A234T alter the predicted helical character of ICL3 and disrupt the D2R_s/G protein interface [243]. Moreover, a receptor-mimetic peptide derived from the N terminus of D2R ICL3 (D₂N) directly activates G_i/G_o proteins [244, 245]. The crystallographic structure of D₂N with G $_{\alpha_{i1}}$ has further elucidated D₂N/G protein interactions, suggesting that the $\alpha 4/\beta 6$ region of G α (residues Q304/E308 and T321) is connected to a short basic cluster of D₂N and ¹¹RRRK¹⁴ (corresponding to ²¹⁶RRRK²¹⁹ in human D2R) [246].

3.5.5 Consequences of GPCR Oligomerization

One of the most fundamental aspects of oligomerization is its importance for GPCR pharmacology. Ligand binding to GPCRs may result in changes in the binding characteristics of additional ligands targeting the same GPCR, creating a cooperative effect on the binding of another GPCR through an allosteric mechanism [247–249]. Stabilization of a particular conformation of the dimer by a bifunctional agonist might lead to an increase of specificity and efficacy of the signaling [250]. A number of different functionalities and pharmacologic characteristics have been reported and attributed to the generation of GPCR heterodimer/oligomer complexes. Nevertheless, it is crucial to keep in mind that these effects can not only be attributed to direct protein–protein interactions but also to indirect effects produced via downstream signaling and feedback control [251].

The capacity of a GPCR to alter the binding affinity of its binding partner may ultimately be applied clinically in future drug development. More than 50% of all drugs with annual worldwide sales of more than \$50 billion regulate the function and activity of many GPCRs in attempts to treat various diseases and disorders [131, 252]. As previously mentioned, GPCR dimerization is important prior to plasma membrane delivery, and incorrect folding may interfere with dimerization and can lead to alteration in cell surface delivery and function [253–255]. In designing potential drugs that may take advantage of our growing knowledge of GPCR structure and function, taking into account oligomerization and heterodimer formation may be critical. Moreover, since receptor heterodimers can generate distinct signals from their corresponding homodimers, understanding the structural basis for higher-order receptor structure may offer a means to improve tissue selectivity and improve drug therapeutic function [251].

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