Advanced Review

Structure and function of G protein-coupled receptor oligomers: implications for drug discovery

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G protein-coupled receptor (GPCR) oligomers are promising targets for the design of new highly selective therapeutics. GPCRs have historically been attractive drug targets for their role in nearly all cellular processes, and their localization at the cell surface makes them easily accessible to most small molecule therapeutics. However, GPCRs have traditionally been considered a monomeric entity, a notion that greatly oversimplifies their function. As evidence accumulates that GPCRs tune function through oligomer formation and protein–protein interactions, we see a greater demand for structural information about these oligomers to facilitate oligomer-specific drug design. These efforts are slowed by difficulties inherent to studying membrane proteins, such as low expression yield, *in vitro* stability and activity. Such obstacles are amplified for the study of specific oligomers, as there are limited tools to directly isolate and characterize these receptor complexes. Thus, there is a need to develop new interdisciplinary approaches, combining biochemical and biophysical techniques, to address these challenges and elucidate structural details about the oligomer and ligand binding interfaces. In this review, we provide an overview of mechanistic models that have been proposed to underlie the function of GPCR oligomers, and perspectives on emerging techniques to characterize GPCR oligomers for structure-based drug design.

How to cite this article:
WIREs Nanomed Nanobiotechnol 2014. doi: 10.1002/wnan.1319

INTRODUCTION

G protein-coupled receptors (GPCRs) are an important superfamily of membrane proteins that share a seven α-helical transmembrane (7TM) structural motif. They are responsible for triggering many diverse cell responses, and play a key role in the central nervous system (CNS),[1] sensory functions,[2] and cancer.[3] By binding to an extracellular ligand, receptors undergo a conformational change that activates a signaling cascade by coupling to intracellular G-proteins. These trimeric G-protein complexes contain an α, β, and γ subunit, and are linked to several different signaling cascades that have been most studied for Class A GPCRs. There are 16 different Gα subunits separated into four groups that play a regulatory role in cAMP production through interactions with adenylate cyclase and calcium and potassium transport via phospholipase C (PLC) pathway, among other functions.[4] Five Gβ and 12 Gγ subunits form multiple heterodimers that together mediate a multitude of signaling cascades, including PLC mediation of calcium signaling, and are also the only known regulators of G-protein coupled inwardly-rectifying potassium (GIRK) channels.[5] Historically, GPCRs have been a target of nearly 40% of all commercially available pharmaceuticals due to their localization at the cell surface, making them easily accessible to interact with small molecule

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Conflict of interest: The authors have declared no conflicts of interest for this article.

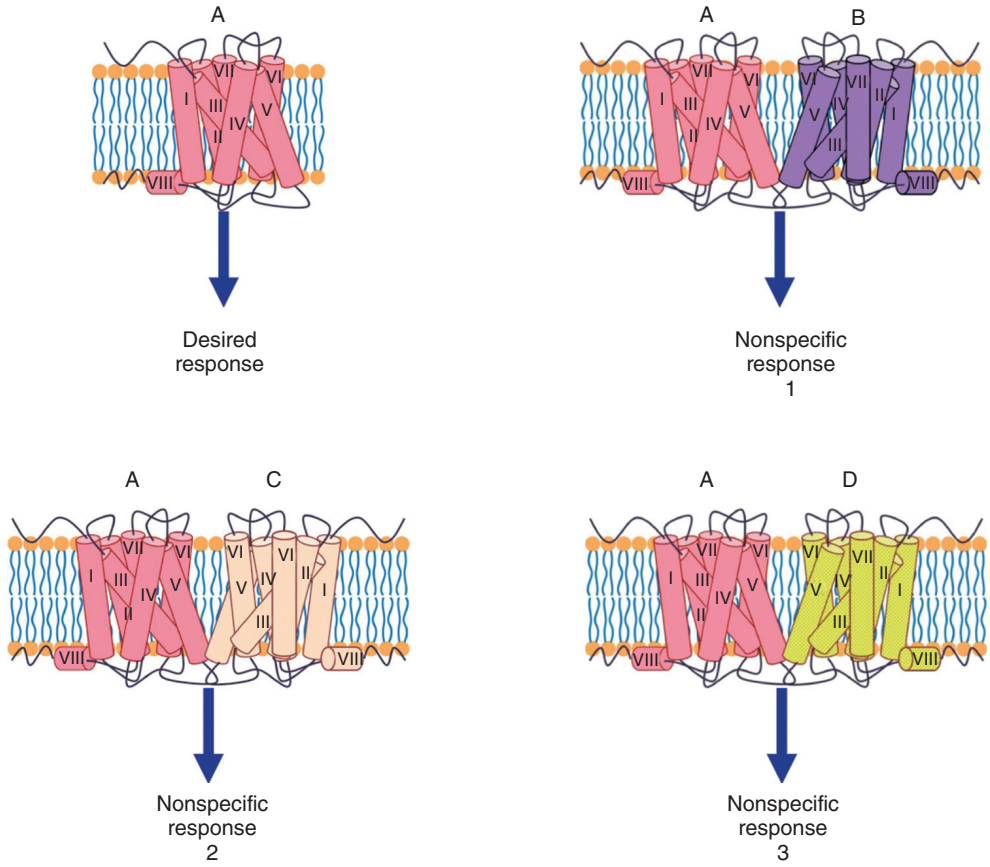

FIGURE 1 | Schematic illustrating nonspecific drug–receptor interactions with hetero-GPCR oligomers. A therapeutic targets a single receptor (receptor A), but elicits multiple nonspecific responses due to receptor A's propensity to form functionally distinct hetero-oligomers with receptors B, C, or D in membranes.

drugs.[6] Approximately 20% of the all drug targets in the human genome are GPCRs, with less than half of all 'druggable' GPCRs currently explored as targets.[7]

GPCRs were originally regarded as a monomeric functional entity that coupled to specific G-protein subunits and activated a single signaling cascade. However, it has become increasingly evident that GPCRs associate with each other in membranes to form oligomeric complexes (Figure 1), and in many cases the precise cellular response varies with the oligomer that is formed.[8–14] This means that GPCR oligomers broaden the range of cell signaling, as multiple signals are integrated among them to influence cellular behaviour. Despite this added complexity in GPCR signaling, the ability to develop highly specific treatments to target oligomers in diseases where a particular oligomer species dominates could improve efficacy and lessen side effects. Toward this goal, a great deal of structural and functional data is still needed to elucidate the structure–function relationship of GPCR oligomers and the mechanisms that drive their formation.

Drug development for the treatment of disease is a complicated process, which can take more than a decade and cost upwards of a billion dollars per drug. The discovery phase is generally broken down into two major steps: (1) choice of an appropriate target (protein or pathway) to activate or inhibit, and (2) development of screening assays to test large libraries of potential therapeutics (such as small molecules or antibodies) for a desired response.[15] GPCRs are the most heavily targeted class of proteins by both established drugs and those in clinical trials,[7] yet only four of the 24 new drugs that were FDA approved in 2013 are known to target GPCRs.[16] This low percentage is likely due, in part, to a multitude of side effects that often accompany treatment, which arise from both a lack of structural data for GPCRs and a generally poor understanding of functional consequences of GPCR oligomerization. For example, if a single receptor can form multiple functionally distinct hetero-oligomers (Figure 1), a drug meant for that receptor monomer may still interact with each hetero-oligomer, resulting in multiple unintended responses. It is thus important

to decipher specific signaling cascades associated with GPCR oligomers to more tightly control drug response. However, in order to successfully design and screen therapeutics that target a specific oligomer, it is vital to develop robust methods to isolate heterodimers (and homodimers) and elucidate molecular level structural details, such as the location of the oligomer interface and distances between respective ligand binding pockets on the receptors. Such information, for example, will aid progress toward the development of bivalent ligands that target specific oligomers, and allow for better prediction of off-target side effects.[17] This review focuses on the broad role of GPCRs in disease, including models of oligomer allosterism that underlie functional changes with highlights from the recent literature. Existing methods to isolate and characterize GPCR oligomers are discussed, with emphasis on the need for new approaches to relate molecular structure to macromolecular function, assembly, and its application to drug discovery.

ROLE OF GPCRS AND GPCR OLIGOMERS IN DISEASE

The human genome encodes for approximately 1000 GPCRs that vary greatly in sequence and length, and are known to respond to a wide array of endogenous ligands including most hormones and neurotransmitters.[18] They are divided into five major families based on overall sequence homology: Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin receptors. Not surprisingly, they have been linked to a broad range of diseases including diseases of the CNS,[19] human immunodeficiency virus (HIV),[20] and even genetic disorders such as albinism.[21]

Misassembly

It is well known that errors in protein folding and assembly can drive human disease[22] and are often linked to mutations. Although data on structural effects of mutations in GPCRs is limited due to difficulties in obtaining crystal structures, many GPCRs harbor specific mutations that are associated with particular diseases.[23] Two prominent examples are hereditary diseases of the retina, retinitis pigmentosa (rhodopsin mutations)[24] and ocular albinism (OA1 mutations).[21] For GPCRs, an obvious implication of protein misfolding due to mutation is an error in trafficking through the secretory pathway on path to the plasma membrane. For example, the GnRHR hormone receptor involved in reproduction is known to have genetic variants that affect receptor trafficking to the endoplasmic reticulum (ER), causing reduced fertility.[25] Although the propensity of receptors to form oligomers has not been directly linked to disease onset, there is a strong indication that proper folding and assembly of a GPCR dimer is important for trafficking to the plasma membrane. This oligomer formation can begin early in the ER, and was first observed for the GABA receptors, where heterodimer formation between $GABA_{B1}$ and $GABA_{B2}$ is required for proper trafficking and function.[26]

Therapeutic Targets

The functional consequences of receptor oligomerization and their role in disease are still poorly understood, and there is likely no obvious trend that connects oligomeric state with the presence of a disease. However, many recent studies have begun to unveil complicated networks of oligomeric receptor assemblies tuning function of the individual receptor, including ligand interactions,[27–30] cellular trafficking, and mobility.[31,32] Additionally, some receptors have been observed to form hetero-oligomers with several different receptors, each of which have distinct functional implications. For example the dopamine D_2 receptor forms heterodimers with the dopamine D_1 receptor,[13] dopamine D_3 receptor,[33] dopamine D_5[14] receptor, adenosine A_2a receptor,[9] serotonin 5HT,[34] cannabinoid CB_1 receptor,[35] hetero-trimers with A_2a and metabotropic glutamate receptor $mGluR_5$[36] as well as homooligomers with itself.[37,38] Each of these hetero-oligomers have been discussed as potential therapeutic targets for schizophrenia and/or Parkinson's disease as more specific alternatives to existing pharmaceuticals that target only the D_2 monomer. It is unclear whether these heterodimer states are affected by the presence of disease, or vice versa; however the D_2 homodimer has been observed to exist at higher levels in patients with schizophrenia,[39] emerging as a promising target for novel antipsychotics in the form of bivalent ligands.[17]

MODES OF OLIGOMER ALLOSTERISM

Several functional consequences of GPCR oligomerization have been proposed including novel signaling cascades, transactivation, and signal amplification. For a more complete review of the types of GPCR oligomer allostery, refer to reviews by George et al[40] and Ferre et al.[8]

The first crystal structure of the β_2-adrenergic receptor (β_2-AR) in complex with its heterotrimeric G-protein[41] has provided valuable molecular insights into G-protein coupling among GPCRs, and can be extended to understanding the mechanisms of

allostery among GPCR oligomers. In conjunction with the available GPCR oligomer structures (Table 2), the β_2-AR-G_s structure has enabled hypotheses about the potential for G-proteins to bind to oligomers, although we note that the regulation of GPCR function by oligomerization is also possible through different modes (e.g., ligand binding, trafficking). An illustrative example of this is found in the β_1-AR dimer structure, which resolves two different dimer interfaces, one of which (involving transmembrane helix (TM) 1, TM2, and C-terminal helix 8, H8) is common to other GPCR dimer structures (e.g., Rhodopsin and opioid receptors, Table 2), and upon overlaying the β_2-AR-G_s complex would enable the docking of a trimeric G-protein.[47,62] However, the second dimer interface observed, involving TM4, TM5 and the second intracellular loop (ICL2), which is also implicated in G-protein binding, does not appear to allow this interaction.[47] This suggests the possibility that this mode of dimerization may prevent G-protein binding or vice versa, and in fact some studies show that G_s actually diminishes the amount of oligomer for β_2-AR.[63] Alternatively, as the β_1-AR structure is a basal, ligand-free model, and as the TM4-5 region of the protein is known to experience conformational change upon activation for some GPCRs (e.g., D2,[64] changes at the transmembrane homodimer interface), it is possible that ligand binding causes rearrangement of the oligomeric interface, and thereby enables G-protein coupling.

This single case exemplifies the complex influence of oligomerization on the molecular basis for GPCR signaling. Here we classify and discuss further such scenarios with relevant examples with emphasis on the dopamine receptors, which have been linked to diseases of the CNS, such as schizophrenia and Parkinson's disease.

Novel G-Protein Coupling

One potential result of receptor oligomerization is activation of a novel pathway rather than either protomer's autonomous signal cascade, as illustrated for the dopamine D_2 receptor in Figure 2(a). The

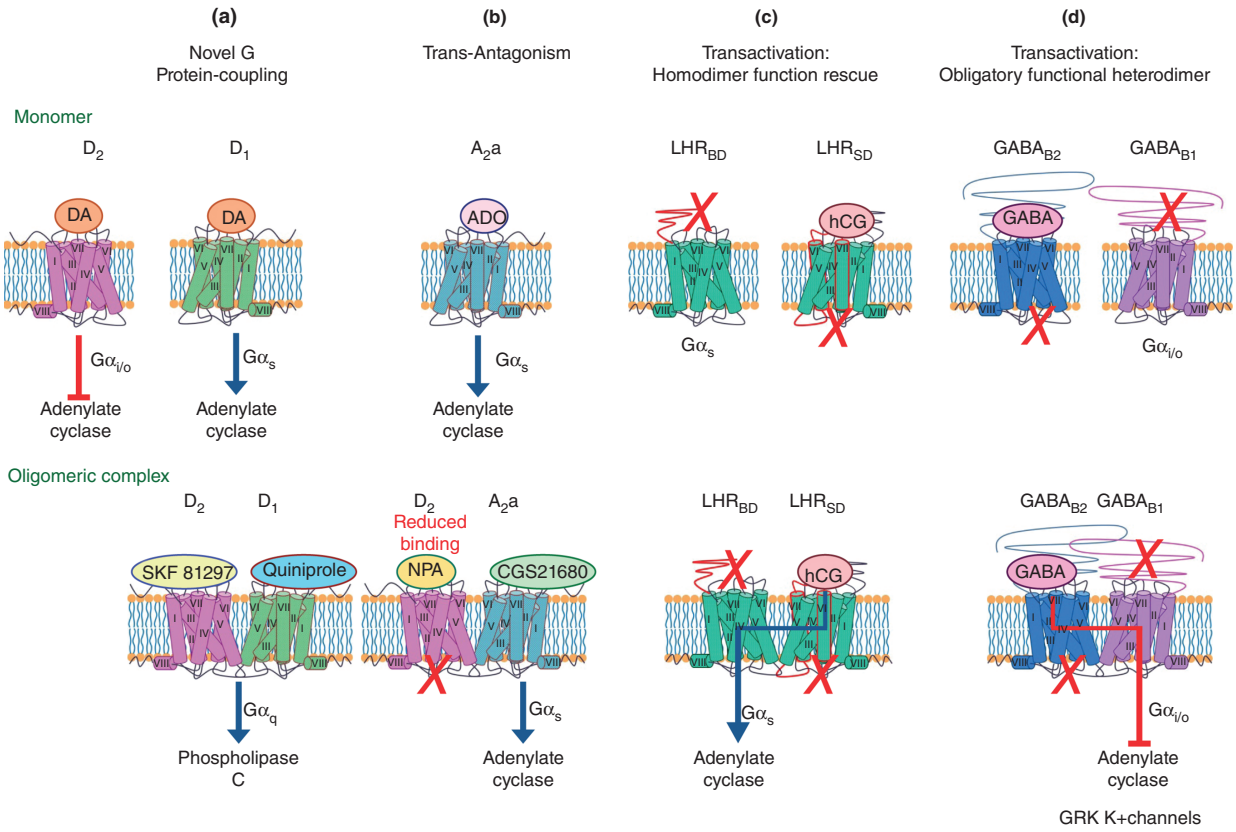

FIGURE 2 | Several modes of GPCR oligomer allostery have been experimentally observed. Novel G-protein coupling is executed through a D_2-D_1 heterodimer (a). A trans-antagonistic interaction exists between A_2a-D_2 heterodimer, in which activation of A_2a by CGS21680 agonist inhibits D_2 signaling through agonist raclopride[9] (b). Homodimeric function rescues signaling for LHR receptors, where one protomer cannot bind to ligand (hCG) and the other cannot couple to G proteins[65] (c). Finally, heterodimer transactivation occurs via endogenous ligand GABA between $GABA_{B1}$-$GABA_{B2}$ receptors[57](d). Endogenous ligands for monomeric D_1, D_2, and A_2a receptors in (a) and (b) are dopamine (DA) and adenosine (ADO), respectively.

dopamine D_2 receptor forms two heterodimer complexes that have been implicated in this type of signaling, one with the dopamine D_1 and the other with dopamine D_5. All three receptors bind to dopamine as their endogenous ligand, with the D_1 and D_5 receptors activating the adenylate cyclase pathway through coupling to a $G\alpha_s$, olf, and the D_2 receptor inhibiting the adenylate cyclase pathway by coupling to $G\alpha_{i/o}$.[66] However, in both native tissue and in cells recombinantly expressing D_1 and D_2, activation of both receptors in the dimer state results instead in a $G\alpha_q$ coupling that leads to a rapid and transient increase in intracellular Ca^{2+} concentration in the striatum.[11] In addition to $G\alpha_q$ coupling, this movement of intracellular calcium by the D_1-D_2 dimer has also been shown to be caused by $G\beta\gamma$ activation of the PLC pathway, and may not be heteromer specific.[67] For simplicity, in all examples in Figure 2, only the known $G\alpha$ signaling pathways are illustrated, as $G\beta\gamma$ signaling cascades for each oligomer are not known for all examples. One study also found that cells co-expressing D_1 and D_2 not only showed evidence of heterodimers, but also found that expression levels in HEK293 mammalian cells of each receptor were modulated (D_1 had lower cell surface expression and D_2 had higher cell surface expression compared to single-receptor expression). Upon activation of both receptors the dimers were co-internalized,[13] supporting the theory that GPCR oligomerization plays a role in trafficking. The D_2-D_5 heterodimer also modulates intracellular calcium levels through $G\alpha_q$ coupling; however unlike the D_1-D_2 dimer, the calcium response by D_2-D_5 is dependent on the influx of extracellular calcium, indicating that a different mechanism is involved.[14] Although the existence of the D_1-D_2 heterodimer has not been proven to cause schizophrenia, it has been identified as a potential therapeutic target, as patients with schizophrenia often exhibit abnormal cellular calcium regulation.[12]

Transantagonism

Alternatively, an oligomer interaction can occur in a way such that the activation of one receptor inhibits the signaling activity of the other, as depicted in Figure 2(b). First detected via co-immunoprecipitation,[68] heterodimers formed by adenosine A_2a and dopamine D_2 (A_2a-D_2) exist in the striatopallidal pathway, an important target for most antipsychotics.[10] Possible mechanisms for this interaction appear to exist at several levels: ligand binding, G-protein coupling, presynaptic, and second messenger levels.[69] Importantly for oligomer-specific pharmaceutical design, at the ligand binding level, the A_2a-D_2 heterodimer exhibits an antagonistic relationship in which the binding of an A_2a agonist lowers the binding affinity of the D_2 receptor to its agonists.[9,70] Evidence from mass spectrometry indicates that the arginine-rich epitope on the N-terminal portion of the 3rd intracellular loop (ICL3) of D_2 can interact with two regions on the A_2a C-terminus by creating a salt bridge with a phosphorylated serine (A2a S374), as well as partake in other electrostatic interactions with two consecutive aspartic acid (DD) residues.[71] ICL3 has been suggested to play an important role in G-protein coupling for many Rhodopsin family GPCRs, and as such A_2a's interaction with D_2's ICL3 may disrupt its ability to couple to $G\alpha_{i/o}$. One might infer based upon the recent crystal structure of β_2AR dimer that certain GPCR oligomer interfaces prevent G-protein binding. Alternatively, the A_2a-D_2 heterodimer could interact with the heterotrimeric G-protein complex such that A_2a couples to the $G\alpha_s$ subunit and D_2 interacts with the $G\beta\gamma$ subunits. However, neither option has been explored significantly, and much of available dimer interface data arises from homodimers rather than heterodimers. Rigid body docking simulations of the A2a-D2 heterodimer predict TM5, TM6 and ICL3 of D2 to interact with TM4 and the C-terminus of A2a.[72] Such an interface is slightly different from prevailing homodimer interfaces identified in crystal structures listed in Table 2, but is most representative of the interfaces involving TM4 and TM5 of β_2AR or TM5 and TM6 of the μ-opioid receptor. In addition to signaling consequences, extended incubation with either receptor's agonist results in elevated co-trafficking of the A_2a-D_2 dimer to the plasma membrane. Further treatment with both receptors' agonists initiates co-internalization. This example may give some insight into the functional relationship of a different hetero-oligomer involving D_1 and D_2 in spite of the different pharmacological effects observed. Since A_2a and D_1 are morphologically similar on a structural level, both having a short third intracellular loop and long C-terminus, D_1 may interact with D_2 similarly to A_2a with a phosphorylated C-terminal serine.[9]

Transactivation

Another mechanism of crosstalk between GPCR oligomers is the ability to initiate the signaling cascade of one receptor upon agonist binding to the other protomer. Experimental evidence for this transactivation has been shown for homodimers by co-expressing two receptor mutants in which one protomer of the dimer has a nonfunctional ligand binding domain, and the other protomer's ability to activate G-protein signaling is abolished, as shown in Figure 2(c).[73,65,74] This method was used to obtain the

first *in vivo* evidence of the physiological importance of GPCR homodimerization, shown for the leutenizing hormone receptor (LHR). Co-expression of ligand binding deficient LHR (signaling active) and signaling deficient LHR (able to bind ligand) in mice was able to rescue function, enabling the mice to reproduce, whereas mice expressing either mutant alone were sterile.[65]

Another example of transactivation, depicted in Figure 2(d), is an obligatory heterodimer between $GABA_{B1}$ and $GABA_{B2}$ receptors, which is required to produce prolonged inhibitory responses within the CNS.[26] Activation of this heterodimer complex has been shown to occur via agonist binding to the $GABA_{B1}$ receptor, leading to G-protein coupling via $GABA_{B2}$ receptor.[57] The $GABA_{B1}$ receptor contains an ER retention sequence that retains the $GABA_{B1}$ receptor intracellularly until a heterodimer forms with $GABA_{B2}$.[75]

MAJOR CHALLENGES IN THE STUDY OF GPCR OLIGOMERS

Expression, Purification, and Activity

The study of GPCRs suffers from difficulties inherent to working with membrane proteins, as most of them are expressed at low levels in native cellular environments and all of them are extremely hydrophobic. Combined with the presence of hundreds of other GPCRs and membrane proteins per mammalian cell, isolation of a particular protein's function is difficult.[76–78] As a result, a popular approach relies on recombinant expression of GPCRs in model microbial expression systems, which can serve as a GPCR-null system. Along with ease of use and straightforward genetics, this also allows the flexibility to add tags (e.g., fluorescent probes) to perform biophysical studies or affinity tags to facilitate purification for *in vitro* characterization for receptors of interest.

Several options for expression systems fall generally into four main categories: bacterial, yeast, insect, and mammalian. While *Escherichia coli* is undoubtedly the cheapest and least time consuming expression system, it is not suitable for the expression of many GPCRs, as it lacks eukaryotic protein secretion and post-translational processing machinery. A particularly important component of protein trafficking in eukaryotic cells is the ER, an expansive intracellular compartment that facilitates the proper folding and translocation of hydrophobic membrane proteins. *E. coli* have limited or no ability to perform many post-translational modifications such as forming multiple disulfide bonds or attaching sugar moieties, which many GPCRs require for function and stability.[79,80] Additionally, GPCRs interact dynamically with their membrane environment and for some, their stability relies on particular lipids not synthesized in *E. coli*.[81,82]

Mammalian expression hosts provide the most native-like expression environment for GPCRs, with the ability to perform complicated post-translational modifications and the most similar membrane composition to the native system. As a result, proper folding and receptor function are less of a challenge. However, mammalian cell culture is more expensive, cells grow more slowly, and overall expression yields are lower[83] compared to microbial hosts. Higher expression levels can be achieved with insect cell culture by exploiting the infectious cycle of a baculovirus to infect insect cells. Vectors typically utilize a strong promoter, such as polyhedron, to induce protein production at the end of the cell cycle making it difficult to create stable cell lines.[84] As such, most insect expression systems are transient. Despite the drawbacks of complicated transfection protocols and transient expression, 40 of all 58 GPCR crystal structures solved to date were expressed in insect cells, as can be seen in Table 1.[132]

Yeast expression systems bridge the advantages of mammalian/insect cell lines and bacterial expression. Similarly to bacterial hosts, yeast have fast doubling times (~2 h), high expression capacity and easy genetic manipulation.[133,134] However, as eukaryotes, yeast contain protein expression machinery that *E. coli* do not, namely an ER that facilitates the formation of disulfide bonds, along with glycosylation and palmitoylation. Although yeast can perform most post-translational modifications, the composition and glycan pattern are often different from mammalian systems.[78] Signal sequences encoded into vectors direct translation to the ER to facilitate proper folding and trafficking to the plasma membrane. Additionally, yeast have three native GPCRs (which can be knocked out), G-proteins, and an endogenous G-protein signaling pathway, allowing for live-cell signaling assays and oligomeric studies for some heterologously expressed GPCRs.[135,136]

Stability *in vitro* for Structural Studies

Upon successful expression of properly folded receptor, a critical task in obtaining high-resolution structural information is to purify the protein of interest, which is a major obstacle in the study of membrane proteins. With 7TM domains, GPCRs are largely hydrophobic, making them unstable in polar solvents.[137] As such, the process of removing integral membrane proteins from the membrane typically involves the use of solubilizing surfactants, which

TABLE 1 | GPCR Crystal Structures: Different Expression Systems and Crystallography Methods

Receptor	Gene Source	Expression System	Crystallography Method	Ref.	Ligand	Res. Å	PDB ID
Rhodopsin	*Bos Taurus* (Bovine)	*Bos Taurus* (native)	X-ray crystallography	85	11-cis retinal	2.80	1F88
				86		2.6	1L9H
				87		2.65	1GZM
				88		2.2	1U19
				42		4.15	2I37
				43		2.9	3CAP
				89		3.00	3PXO
				90		3.2	3DQB
		COS cells	X-ray crystallography	91	11-cis retinal	3.4	2J4Y
		HECK cells	X-ray crystallography	44	11-cis retinal	3.30	4A4M
	Todarodes pacificus (Squid)	*Todarodes pacificus* (native)	X-ray crystallography	92	11-cis retinal	2.5	2Z73
				93		3.7	2ZIY
				94		2.7	3AYN
β_1 adrenergic	*Meleagris gallopavo* (Turkey)	*Trichoplusia ni* (insect)	X-ray crystallography	95	Antag: Cyanopindolol	2.7	2VT4
				96	Par ag: dobutamine	2.65	2Y01
				97	Ag: carmoterol	2.65	2Y02
				98	Ag: isoprenaline	2.85	2Y03
				47	Par ag: salbutamol	3.05	2Y04
					Antag: carazolol	3.00	2YCW
					Antag: cyanopindolol (t148)	3.25	2YCX
					Antag: cyanopindolol (t468)	3.15	2YCY
					Antag: Iodocyanopinodolol (t756)	3.65	2YCZ
					Antag: carvedilol	2.30	4AMJ
					Antag: bucindolol	3.20	4AMI
					Basal state	3.50	4GPO
β_2 adrenergic	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	99	Inv ag: carazolol/ Fab5	3.4	2R4R
				100	Inv ag: carazolol/ Fab5[1]	3.7	2R4S
				49	Inv ag: carazolol	3.4	3KJ6
				101	Inv ag: carazolol	2.4	2RH1
				102	Inv ag: timolol	2.8	3D4S
				103	Inv ag: ICI 118,551	2.84	3NY8
				104	Inv ag: Novel molecule	2.84	3NY9
				41	Antag: alprenolol	3.16	3NYA
					Ag: BI167107/Nb80	3.50	3POG
					Ag: FAUC50	3.50	3PDS
					Ag: BI167107/Gs complex	3.20	3SN6
A_2a adenosine	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	105	Inv ag: ZM241385	2.6	3EML
				106	Ag: UK-432097	2.71	3QAK
				107	Antag: caffeine	3.60	3RFM
				108	Antag: ZM241385	3.30	3PWH

TABLE 1 | Continued

Receptor	Gene Source	Expression System	Crystallography Method	Ref.	Ligand	Res. Å	PDB ID
					Antag: XAC	3.31	3REY
					Inv ag: ZM241385	1.80	4EIY
		Trichoplusia ni (insect)	X-ray crystallography	109	Ag: adenosine	3000	2YDO
					Ag: NECA	2.60	2YDV
		Pichia pastoris (yeast)	X-ray crystallography	110	Antag: ZM241385/ Ab Fab2838	2.70	3VG9
					Antag: ZM241385	3.10	3VGA
CXCR1	*Homo sapiens*	*E. coli*	Rotationally aligned solid state NMR	111	Ag: IL-8	1.7	2LNL
CXCR4	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	45	Antag: IT1t P21	2.5	3ODU
					Antag: IT1t P1	3.1	3OE8
					Antag: IT1t P1	3.1	3OE9
					Antag: IT1I222	3.2	3OE6
					Antag: CVX15	2.9	3OE0
CCR5	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	112	Antag: Maraviroc	2.71	4MBS
Dopamine D3	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	113	Antag: R-22	2.89	3PBL
Histamine H1	*Homo sapiens*	*Pichia pastoris* (yeast)	X-ray crystallography	114	Antag: doxepin	3.1	3RZE
Sphingosine 1-phosphate	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	115	Antag: sphingolipid mimic	3.35	3V2W
			Microdiffraction			2.8	3V2Y
Muscarinic acetylcholine M2	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	116	Antag: 3-quinuclidinyl-benzilate	3.00	3UON
				117	Ag: iperoxo	3.50	4MQS
					Ag iperoxo and AM LY2119620	3.70	4MQT
Muscarinic acetylcholine M3	*Rattus norvegicus*	*S. frugiperda* (insect)	X-ray crystallography	118	Antag: tiotropium	3.40	4DAJ
κ-opioid	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	51	Antag: JDTic	2.90	4DJH
μ-opioid	*Mus musculus*	*S. frugiperda* (insect)	X-ray crystallography	53	Antag: morphinan	2.80	4DKL
δ-opioid	*Mus musculus*	*S. frugiperda* (insect)	X-ray crystallography	119	Antag: naltrindol	3.40	4EJ4
	Homo sapiens	*S. frugiperda* (insect)	X-ray crystallography	120	Antag: naltrindol	1.80	4N6H
Nociceptin/ orphanin FQ	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	121	Antag: peptide mimetic compound 24	3.01	4EAJ
Neurotensin NTS1	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	122	Ag: neurotensin	2.80	4GRV
	Rattus norvegicus	*E. coli*	X-ray crystallography	123	Ag: neurotensin	2.75	4BUO

TABLE 1 | Continued

Receptor	Gene Source	Expression System	Crystallography Method	Ref.	Ligand	Res. Å	PDB ID
						3.00	3ZEV
						3.10	4BV0
						3.57	4BWB
Protease activated PAR1	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[124]	Antag: vorapaxar	2.20	3VW7
Serotonin 5-HT_{1B}	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[125]	Ag: ergotamine	2.70	4IAR
					Ag: dihydroergotamine	2.80	4IAQ
Serotonin 5-HT_{2B}	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[126]	Ag: Ergotamine	2.70	4IB4
			Serial femtosecond crystallography	[127]		2.80	4NC3
Smoothened SMO	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[55]	Antag: LY2940680	2.45	4JKV
Purinergic $P2Y_{12}$	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[128]	Antag: AZD1283	2.62	4NTJ
				[61]	Ag: 2MeSADP	2.50	4PXZ
Class B: Corticotropin-releasing factor 1	*Homo sapiens*	*Trichoplusia ni* (insect)	X-ray crystallography	[129]	Antag: CP-376395	2.98	4D5Y
Class B: Glucagon	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[130]	Ag: glucagon	3.30	4L6R
$GABA_B$ GBR1-GBR2	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[58]	Ligand-free	2.35	4MQE
					Antag: 2-hydroxysaclofen	2.22	4MQF
					Antag: CGP54626	2.15	4MR7
					Antag: CGP35348	2.15	4MR8
					Antag: SCH50911	2.35	4MR9
					Antag: phaclofen	2.86	4MRM
					Antag: CGP46381	2.25	4MS1
					Ag: GABA	2.50	4MS3
					Ag: baclofen	1.90	4MS4
Class C: Metabotropic Glutamate mGlu1	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[59]	Negative Allosteric Modulator FITM	2.80	4OR2
GPR40	*Homo sapiens*	*S. frugiperda* (insect)	X-ray crystallography	[131]	Allosteric Ag: TAK-875	2.33	4PHU

Ag, Agonist; Antag, Antagonist; Inv ag, Inverse agonist; Par ag, partial agonist.
[1] β_2AR methylated for NMR

mimic the membrane environment. Yet in many cases, this mimetic environment on its own is not enough to maintain receptor stability and activity *in vitro*. Receptor ligand binding activity may depend on the presence of a particular lipid or surfactant alkyl chain length, which drives the size and shape of the micelle environment.[138–140] In these cases, proper folding and function of GPCRs *in vitro* often requires the addition of specific lipid molecules such as cholesterol into the surfactant environment.[140,141] Although not discussed in detail here, several studies have analyzed the effects of fatty acid chain length, chain saturation, lipid head group, and lipid additives for their effectiveness in solubilizing stable GPCRs, though these results have not yet be extended to GPCR oligomers.[76,140–142]

Aside from detergent micelles, alternative membrane mimetics such as liposomes, nanolipoprotein discs, nanodiscs and bicelles are popular membrane

mimetic environments that have been used to study membrane proteins *in vitro*.[143] Various studies have shown that lipid properties affect GPCR function profoundly, notably the photoactivation properties of Rhodopsin,[144–146] and G-protein activation of the cannabinoid receptor.[147] Therefore the membrane or surfactant environment must be carefully chosen to preserve receptor activity. Although generally more difficult to prepare than detergent micelles, if properly chosen, these systems offer a lipid environment more representative of native plasma membranes. Many studies have focused on the purification of stable and active receptors,[141,148,149] however efforts that focus on optimizing the membrane mimetic system to stabilize GPCR oligomer states are still lacking. This is due in large part to the difficulties in controlling protein to lipid or protein to surfactant concentrations, maintaining oligomeric contacts for weakly interacting oligomers, and the realization that there will likely be a mixture of homo and hetero oligomers in any given preparation.

CHARACTERIZATION OF OLIGOMERS

Existing techniques for the study of GPCR oligomers fall into two main categories: (1) low/medium resolution techniques for the detection and characterization of oligomers at the macromolecular scale, and (2) high-resolution methods to characterize structure at the molecular level. It is important to recognize that each technique provides valuable and unique information toward the goal of elucidating a comprehensive view of GPCR oligomer interactions.

Observing and Isolating GPCR Oligomers

Fluorescence to Detect and Characterize GPCR Oligomers in Membranes

Fluorescence resonance techniques have exploded in their application to study GPCR dimers and higher order oligomers in live cells and in model lipid bilayers and vesicles.[150,151] Förster Resonance Energy Transfer (FRET) exploits electric dipole–dipole interactions that result in a nonradiative energy transfer from an excited donor fluorophore such as a cyan fluorescent protein to an acceptor fluorophore such as a yellow fluorescent protein.[152] Many studies have utilized FRET to detect GPCR dimers in live cells,[150] analyze relative dimer stability[153] and diffusion of monomer or oligomer species.[154] The FRET signal received is highly dependent on inter-fluorophore distances, with FRET efficiency or quantum yield decreasing as the sixth power of increasing distance, requiring donor and acceptor fluorophores be within 100 Å for measurable signal.[151] Some limitations of FRET arise from the need for initial excitation at the excitation wavelength of the donor fluorophore, which can lead to crosstalk in the form of direct excitation of the acceptor due to overlap in the excitation spectra for the donor and acceptor. Samples can also experience photobleaching of the donor fluorophore. To circumvent these limitations, bioluminescence resonance energy transfer (BRET) utilizes a bioluminescent protein as the donor. However BRET signal tends to be lower intensity than FRET. A more comprehensive review of FRET and BRET techniques can be found by Lohse et al.[151]

Co-Immunoprecipitation

Co-immunoprecipitation (CoIP) has become a useful tool to identify and isolate GPCR oligomers.[155] Based upon antibody affinity to either particular proteins or epitope tags, this method purifies one protein, and any additional proteins that form highly specific protein–protein interactions are co-purified.[156] This approach has identified novel oligomeric interactions of pharmaceutical interest, including the discovery of the A2a-D2 heterodimer as a potential target for schizophrenia treatment.[68] CoIP can also be performed to isolate hetero-oligomeric complexes for biophysical study, though due to the high cost of antibodies and low protein yields it is not scalable.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a biochemical technique used to separate proteins by size, and it is a robust way to separate oligomeric complexes of membrane proteins within surfactant micelles. Quantification of molecular weight via SEC is more complicated for membrane proteins compared to globular proteins due to the contribution of surfactant to the total molecular weight, leading to the need for other techniques such as static light scattering and refractive index measurements to determine exact oligomeric state of elution peaks.[157–159] While SEC can be used to separate oligomer species, this process dilutes the protein sample, which may already be at low concentration. In order to facilitate most biophysical characterization methods, protein samples need to be concentrated, typically by centrifugal methods. A drawback to centrifugal protein concentrators is their tendency to also concentrate surfactant,[160] which can also force rearrangement of the protein detergent complex. This redistributes oligomer populations, making it difficult to control oligomer state and detergent concentration. Despite these complications, SEC is a powerful tool for oligomer purification if concentration issues can be overcome.

Molecular level Structure and Dynamics of GPCR Oligomers

X-Ray Crystallography

To date, 26 different GPCRs have solved crystal structures, most of which have been solved using X-ray crystallography. A summary of GPCR crystal structures is shown in Table 1. In some cases, these crystal structures consist of dimers with defined interfaces. Table 2 summarizes these oligomeric crystals, describing the main oligomeric interfaces that have been observed thus far. For example, the μ-opioid receptor was crystallized with two distinct oligomer interfaces, GABA receptor heterodimers were crystallized, and the smoothened (SMO) receptor was crystallized as a homodimer.[58,53,55] It is important to note that these oligomers described in Table 2 may or may not represent physiologically relevant oligomer interfaces due to harsh, nonphysiological crystallization procedures. While crystal structures provide high-resolution structural details of a protein, GPCR crystallization often requires significant modifications or truncations to the receptor. Often, these modifications eliminate the G-protein coupling interface, replacing it with a soluble protein, such as the insertion of T4 Lysozyme or apocytochrome c in place of the third cytoplasmic loop, as well as C-terminal truncation of the protein.[105] GPCRs have also been crystallized by thermostabilizing point mutations that enrich either the active or inactive conformations,[107,109] sometimes in conjunction with T4 Lysozyme or apocytochrome,[131] and also by binding antibody fragments or nanobodies.[99] In addition to these large modifications, crystal structures give a static picture of the protein in question, and provide no dynamic view of ligand binding or conformational shifts.

Magnetic Resonance

Magnetic resonance techniques have emerged as a multifaceted approach to gain high-resolution structure and dynamics for membrane proteins. Thus far, the only GPCR crystal structures obtained by nuclear magnetic resonance (NMR) are the first transmembrane domain of the apelin receptor[161] and the full-length CXCR1 receptor.[111] However, this method has been used to determine the structures of other membrane proteins, including structurally homologous proteorhodopsin (PR) found in marine bacteria[162] and nearly 50 other α-helical membrane proteins.[132] The CXCR1 structure solved by solid state NMR has an advantage over traditional X-ray crystallography as the experimental conditions are more similar to physiological conditions and large deletion/substitutions (such as the Receptor-T4 Lysozyme chimera) are not necessary to form crystals. Additionally, NMR based approaches allow for studies in receptor dynamics and how movements and conformational shifts relate to protein function. However, like X-ray crystallography, solution NMR requires large quantities of protein, which can be difficult to obtain. Membrane proteins also pose challenges to deciphering NMR spectra due to spectral overlap between dynamic loop regions and less exposed transmembrane domains, an obstacle which is encountered by both β-barrel and α-helical membrane proteins.[163]

At somewhat lower resolution, attachment of paramagnetic labels to specific sites on a protein via site-directed mutagenesis facilitates investigations into protein structural dynamics. Electron paramagnetic resonance (EPR) exploits the excitation of a free electron upon the application of a magnetic field to investigate side chain mobility at sites of interest (i.e., at or near an oligomer interface or ligand binding domain) and can be applied to map an oligomer interface of a protein complex[159] as well as observe precise structural conformations and conformational shifts upon activation of dynamic loop regions that are difficult to resolve with typical NMR or X-ray crystallography methods.[164] A particularly useful tool to study oligomers is double electron–electron resonance (DEER), which measures distances between two spin labels.[165] This method has been applied to measure the distance of TM6 conformational shift in rhodopsin upon activation,[166] and can also be utilized to measure distances between protomers of an oligomer species upon attaching a single spin label to each of two neighboring molecules. This approach also facilitates investigation of the orientation and any rearrangement of an oligomer species, as has been shown for the calcium dependent oligomerization of recoverin[167] and the Na+/H+ *E. coli* antiporter[168] and novel application of gadolinium spin labels to determine hexameric assembly of green PR.[169]

Often, experimental challenges combined with incomplete or unclear data give rise to the need for computational approaches to study receptor oligomerization. Molecular dynamics simulations have become a powerful tool to utilize structural data to predict oligomer interfaces,[170] analyze relative stability of GPCR oligomer interfaces for rhodopsin,[171] β_2AR, β_1AR,[62] μ-opioid and κ-opioid receptors,[172] and even analyze oligomer-G-protein interfaces.[62,173] A primary finding of the above simulations infer that homooligomer interfaces consisting of TM1, and cytoplasmic helix 8 (H8) create a more stable oligomer interface than the larger interface created by TM3/4 or TM4/5. These simulations largely rely on existing structural data and in some cases can

TABLE 2 | GPCR Crystal Structures with Oligomers

Receptor	Oligomer Interface	PDB ID	Functional Relevance
Rhodopsin	TM1, TM2, H8	2I36	Functional oligomer.[42–44]
		2I37	
		2I35	
		3CAP	
		4A4M	
CXCR4	TM5, TM6	3ODU	May be involved in signal regulation.[45,46]
β_1 adrenergic	TM1, TM2, H8	4GPO	Oligomer may facilitate trafficking to cell surface[47,48]
	TM4, TM5, ICL2		
β_2 adrenergic	TM1, H8, cholesterol and palmitic molecules	2RH1	Dimerization not required for activation, but potentially important for trafficking[49,50]
κ-opioid	TM1, TM2, H8	4DJH	Functional oligomer[51,52]
μ-opioid	TM1, TM2, H8	4DKL	Possible function tuning.[53,54]
	TM5, TM6,	4DKL	
Smoothened SMO	TM4, TM5	4JKV	Dimerization essential for activation, though unclear whether interface in crystal is the necessary contact.[55,56]
$GABA_{B!}$-$GABA_{B2}$	Novel large interface defined as regions 4,5,6.	4MQU	Dimerization essential for activation. Also necessary for trafficking of $GABA_{B1}$ to cell surface.[57,58]
		4MQF	
		4MR7	
		4MR8	
		4MR9	
		4MRM	
		4MS1	
		4MS4	
Glutamate mGlu1	Extracellular portion TM1, TM2 with cholesterol	4OR2	Functional dimer. mGluR homodimers are crosslinked by intermolecular disulfide bond.[59,60]
Purinergic P2Y	TM3, TM5 with cholesterol	4NTJ	To be explored.[61]

predict nonphysiological and thus irrelevant interactions. Nevertheless, they are a powerful tool for predicting oligomer interactions and in some cases help to direct future experiments for biophysical and biochemical characterization. Reviews that explore the utility of modeling and simulation techniques are readily available.[174,175]

PERSPECTIVES

By understanding the functional role of GPCR oligomers, and how to selectively modulate their signaling, there is an opportunity to design oligomer-specific pharmaceuticals to mitigate disease. In order to achieve this goal, we will need to greatly increase our knowledge of high-resolution oligomer structure, conformation, and dynamics, as well as gain a better understanding of the mechanisms that drive the formation of oligomers. While heterologous production and reconstitution allows biophysical characterization *in vitro*, most relevant ligand binding and G-protein signaling studies must still be carried out in native mammalian cell lines. One technique alone is not enough to construct a mechanism for GPCR oligomerization, but the combination of cellular, biochemical, and biophysical techniques will help illuminate receptor oligomer interactions and related signaling pathways. While these techniques continue to develop, it is critical to ensure that GPCR oligomers characterized *in vitro* are representative of physiologically relevant conditions.

Currently, crystallography is one of the most powerful tools at our disposal to achieve high-resolution structural details. However, this comes at the high cost of significant protein modification to stabilize the receptor in addition to harsh

crystallization conditions—we are thus observing the protein far from physiological relevance. Magnetic resonance is emerging as a promising approach to obtain structural data for membrane proteins without modification of their sequence, and in the presence of membrane mimetic lipid bilayers. Spin label magnetic resonance techniques such as EPR and DEER are being further developed to elucidate oligomer structural and dynamic details such as distance measurements between key residues, oligomer interface, ligand binding domains, and G-protein coupling interfaces. This will improve connections between high-resolution structural data and low-resolution functional studies, a concept important toward the over-arching goal of relating GPCR structure to function for structure-based drug design.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding support from the University of California Cancer Research Coordinating Committee, the American Heart Association (Award Number 14GRNT18690063), and a National Science Foundation Graduate Research Fellowship to NSS.

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