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Review

^{19}F NMR: A promising tool for dynamic conformational studies of G protein-coupled receptors

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SUMMARY

Advances in X-ray crystallography and cryoelectron microscopy enabled unprecedented insights into the activation processes of G protein-coupled receptors (GPCRs). However, these static receptor structures provide limited information about dynamics and conformational transitions that play pivotal roles in mediating signaling diversity through the multifaceted interactions between ligands, receptors, and transducers. Developing NMR approaches to probe the dynamics of conformational transitions will push the frontier of receptor science toward a more comprehensive understanding of these signaling processes. Although much progress has been made during the last decades, it remains challenging to delineate receptor conformational states and interrogate the functions of the individual states at a quantitative level. Here we cover the progress of ^{19}F NMR applications in GPCR conformational and dynamic studies during the past 20 years. Current challenges and limitations of ^{19}F NMR for studying GPCR dynamics are also discussed, along with experimental strategies that will drive this field forward.

INTRODUCTION

G protein-coupled receptors (GPCRs) form the largest human membrane protein family, which is composed of 826 family members (Lv et al., 2016). Their broad range of ligands varies from odors, hormones, and chemokines to lipids, peptides, and even proteins, which makes GPCRs to some of the most significant signaling ''messenger carriers'' across the cell membrane, with connections to almost all aspects of physiological activity (Insel et al., 2019). The typical GPCR signaling pathway starts with an agonist-binding event, which shifts the receptor conformational equilibrium to the active forms and opens the cytoplasmic cavity for G protein binding. In response to the conformational change of the receptor, the C-terminal end of the α helix from Gα protein (Cα5) of the heterotrimeric G$\alpha\beta\gamma$ complex undergoes a conformational rearrangement and starts intruding into the receptor cytoplasmic cavity. A recent study captured an intermediate state structure of the receptor, where the Cα5 half intruded into the cavity (Liu et al., 2019). Next, Cα5 binds tighter to the receptor while the Ras-like (RS) domain and the α-helical domain (AHD) of the G protein separate from each other, which facilitates nucleotide exchange, where the protein releases GDP and then binds GTP.

The first crystal structure of the ground-state bovine GPCR rhodopsin in complex with 11-*cis*-retinal was determined more than 20 years ago (Palczewski et al., 2000; Wang et al., 2022), followed by several rhodopsin/opsin structures in complex with Cα5 of the Gα subunit (Park et al., 2008) (Figure 1). The structures of the different receptor states laid the foundation for our understanding of ligand-induced GPCR activation and the subsequent engagement of their downstream signal transducers, such as heterotrimeric G proteins (G$\alpha\beta\gamma$), G protein-coupled receptor kinases (GRKs), and β-arrestins. In 2011, researchers from Stanford University broke new ground by solving the crystal structure of the human β_2 adrenergic receptor (β_{2A}R)-G$\alpha\beta\gamma$ complex (Rasmussen et al., 2011). For this breakthrough and other significant contributions toward the understanding of GPCR signaling, Brian Kobilka and Robert Lefkowitz were recognized with the 2012 Nobel Prize in Chemistry. Although the first cryoelectron microscopy (cryo-EM) structure of a membrane protein, the transient receptor potential cation channel subfamily V member 1 (TRPV1), was already solved in 2013 (Liao et al., 2013), it took until 2017 for the first successful application of cryo-EM toward the structure determination of a GPCR system, when the structure of the calcitonin receptor (CTR)-G$\alpha\beta\gamma$ complex that was stabilized by a nanobody was determined (Liang et al., 2017; Safdari et al., 2018). Nearly at the same time, the structure of the glucagon-like peptide 1 receptor (GLP-1R) bound to a G protein was published (Zhang et al., 2017). These two GPCR-G protein complex structures paved the way for cryo-EM in solving the structures of GPCRs bound to heterotrimeric G$\alpha\beta\gamma$ proteins and facilitated the understanding of GPCR signaling processes, as evident by

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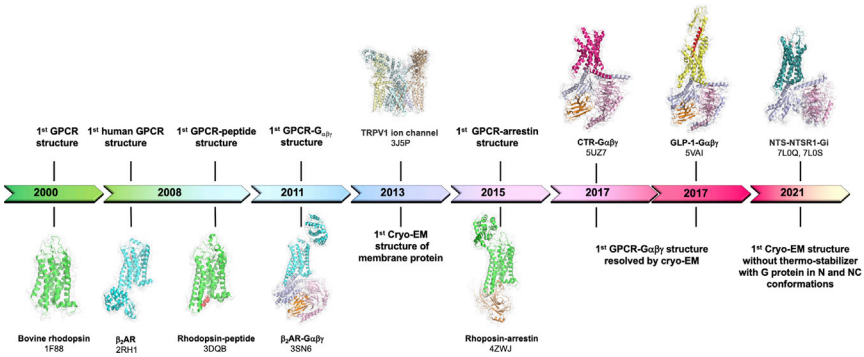

Figure 1. Timeline for GPCR structure determination using X-ray crystallography and cryo-EM

the more than 50 complex structures that have been reported in the past 5 years.

Despite breakthroughs in the structural characterization of GPCRs that originated from instrumental advances in X-ray crystallography and cryo-EM, certain limitations for the structural snapshots obtained with these techniques remain. In particular, the need for reducing the conformational flexibility by "locking" a certain conformer with thermo-stabilization—for example, through mutagenesis or by using nanobodies—and data averaging for structural model refinement might adversely affect structural elucidation. According to the prevailing ensemble view, receptor activation comprises a continuous set of conformations with accompanying transitions that are extremely challenging and likely impossible to be fully uncovered by the means of current X-ray crystallographic and cryo-EM approaches. Therefore, it is necessary to develop complementary approaches to characterize the continuous dynamic conformational transitions that occur during receptor activation. In the last decade, efforts have been made to interrogate the dynamic conformational transitions that are related to the energy landscape with the aim of connecting them with their respective pharmacological outputs. In this review, we describe the application of nuclear magnetic resonance (NMR) spectroscopy with a focus on ^{19}F NMR in profiling GPCR energy landscapes that is pushing the field of structural biology to new frontiers by expanding our understanding of GPCR signaling beyond static structural snapshots into the realm of dynamic conformational ensembles.

PROGRESS AND LIMITATIONS IN UNDERSTANDING GPCR ACTIVATION FROM CRYSTAL AND CRYO-EM STRUCTURES

X-ray crystallographic structure determination of GPCRs

More than 400 structures of over 70 GPCRs have been determined at the time of writing this review and most of them (around 90%) are crystal structures. Nearly all of these crystal structures were solved with bound ligands but without either G proteins or β-arrestins being present. Structures that contain these binding partners include the Cα5-bound rhodopsin structure (Park et al., 2008), the $G\alpha_s\beta\gamma$-bound β_{2A}R structure (Rasmussen et al., 2011), and β-arrestin-bound rhodopsin (Kang et al., 2015). Of note, over 90% of these bound ligands are inverse agonists or antagonists that capture GPCRs in their ground-state inactive conformations. However, the conformations critical for functioning are usually higher-energy states that correspond to either local minima or saddle points in the free-energy landscape, which are very challenging to capture without thermo-stabilization of the receptor. It is worth noting that there are only a few human-derived GPCR crystal structures with bound partial agonists known such as the A_{2A}R (Amelia et al., 2021) in addition to two turkey β_{1A}R structures (Warne et al., 2011), and an angiotensin II receptor type I (AT1R)-nanobody complex structure (PDB: 6DO1) that exhibited partial agonist activity (Wingler et al., 2019b). Although the available GPCR crystal structures are very informative with respect to revealing details of receptor-ligand interactions, it is difficult to form a comprehensive view of the receptor ensemble behavior based on these structures alone because they represent the inactive or active states, with the in-between region mostly inaccessible. As a result, it is still not feasible to reveal the full spectrum of functionally relevant receptor conformations that also includes the elusive intermediate states through X-ray crystallography alone, which would be needed for delineating the entire receptor activation process.

Structural investigations of GPCRs using cryo-EM

The application of cryo-EM for GPCR structure determination has led to more than 50 cryo-EM GPCR complex structures being solved, primarily with bound G proteins but also other transducer complexes since the end of 2017 (Liang et al., 2017; Safdari et al., 2018). This still-expanding list includes calcitonin gene-related peptide (CGRP) (Liang et al., 2018a), glucagon-like peptide-1 receptor (GLP-1R) (Liang et al., 2018b; Zhang et al., 2017), adenosine A_{2A} receptor (A_{2A}R) (Garcia-Nafria et al., 2018a), adenosine A_1 receptor (A_1R) (Draper-Joyce et al., 2018), neurotensin receptor type 1 (NST1R) (Kato et al., 2019), cannabinoid receptor 1 and 2 (CB1 and CB2) (Hua et al., 2020), CXC chemokine ligand 8 receptor (CXCL8) (Liu et al., 2020), human smoothened coupled receptor (Qi et al., 2019), 5-hydroxytryptamine receptor 1B (5-HT1B) (Garcia-Nafria et al., 2018b), rhodopsin (Kang et al., 2018), μ-opioid receptor (μ-OR) (Koehl et al., 2018), dopamine-1 receptor (D1R) (Zhuang et al., 2021), dopamine-2 receptor (D2R) (Yin et al., 2020; Zhuang et al., 2021), D3R (Xu et al., 2021), angiotensin receptor (Wingler et al., 2019a, 2019b), parathyroid hormone receptor type 1 (Zhao et al., 2019), and neurokinin-1 receptor (NK1R) (Harris et al., 2022) in complex with $G\alpha\beta\gamma$, and the class B CTR (Liang et al., 2017). Such astonishing progress has enriched our structural knowledge of GPCRs, providing new insights for GPCR complexes with signaling partners and shedding further light on the signal transduction process (Kang et al., 2015; Rasmussen et al., 2011).

The GPCR activation mechanism that emerged from crystal and cryo-EM structures can be described as follows (Figure 2): upon agonist binding, the receptor shifts to an active-like

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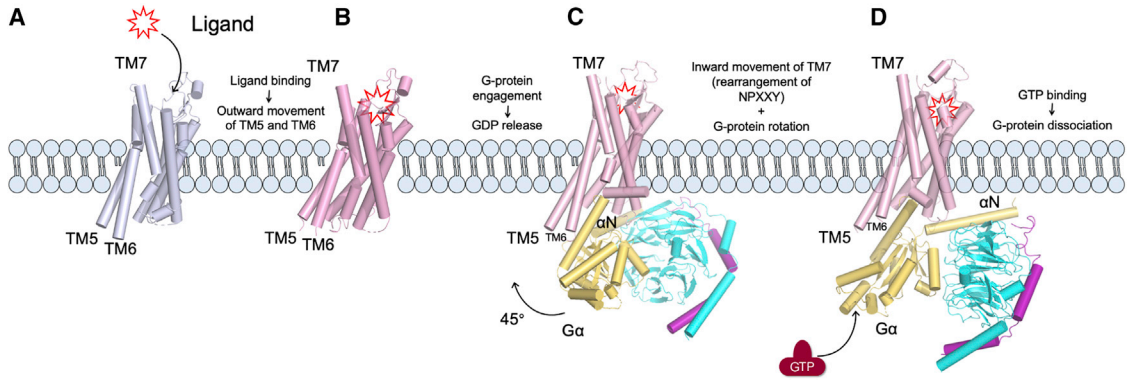

Figure 2. Model for GPCR activation

This figure is based on the activation of the hNTSR1 receptor and was adapted from Kato et al. (2019).

(A) Inactive state of the receptor.

(B) Agonist binding induces the outward movement of the TM5 and TM6 helices.

(C) GDP-bound Gαβγ engages with the receptor, which triggers the displacement of the Cα5 helix and formation of the NC state. GDP is released.

(D) Heterotrimeric Gαβγ rotates 45°, and TM7 is re-arranged, forming the C state. The C state is a more flexible conformation compared with the NC state. Nucleotide exchange is completed by binding of GTP.

conformation that allows the GDP-loaded heterotrimeric G protein to engage with the intracellular binding cavity of the GPCR. Detergent-derived cryo-EM structures have revealed two distinct Gα conformations: the C state and NC state. In the C state, the nucleotide-binding pocket adopts a more flexible conformation that might facilitate the nucleotide exchange, while the G protein in the NC state is rotated ~45° relative to the receptor and has a high propensity for GDP release (Kato et al., 2019). It was further suggested that the NC state might represent an intermediate state along the activation pathway toward the fully activated state (Kato et al., 2019). Most reports employing the recently developed circularized lipid nanodiscs (NDs) described similar interactions between the receptor and heterotrimeric G proteins with a slight rotation of 5° of the Gα protein toward the GPCR in comparison with complex structures determined in the presence of detergent (Zhang et al., 2021).

Limitations of crystal and cryo-EM structures for the elucidation of GPCR activation mechanism

It is practically impossible to determine receptor structures in complex with all candidate ligands from a large-scale drug screening campaign. The structural characterization of all possible conformational states of a receptor's ensemble is not feasible with X-ray crystallography or cryo-EM alone due to the plasticity of the receptor. NMR has a significant advantage in this respect, since all relevant conformations in principle can be probed at once using a minimally modified receptor construct or even the wild-type GPCR in a near-physiological environment (Wang et al., 2022). In contrast, all of the structurally resolved GPCR-G protein complexes were reconstituted in detergent micelles with only two exceptions at the time of writing this review: D2 dopamine receptor (DRD2) complexed with Gαβγ and the stabilizing antibody scFv16 in lipid nanodiscs (Yin et al., 2020) and the neurotensin receptor 1 bound to $G\alpha_{i1}\beta_1\gamma_1$ in a lipid bilayer (Zhang et al., 2021). Furthermore, all currently known GPCR-G protein complex structures are nucleotide free because GDP was hydrolyzed by apyrase to facilitate structure determination (Du et al., 2019). Even in the high-affinity lipid membrane system that was used for the structure determination of the neurotensin receptor 1 complex bound to $G\alpha_{i1}\beta_1\gamma_1$, both N-terminal helices of Gβ and Gγ displayed weak densities likely due to flexibility (Zhang et al., 2021). Although GPCR cryo-EM and crystal structures have radically advanced our understanding of receptor activation, questions remain as to what degree the stabilized GPCR constructs through either thermo-stabilization, extensive mutagenesis, protein fusion, or nanobody inclusion affect receptor structural ensembles and their conformational transitions, and also whether micelle incorporation can faithfully reflect receptor dynamics in native membranes. For example, allosteric sites that are often considered promising drug targets are rarely observed in crystal structures, and conformational sampling is needed to uncover the related allosteric pathway. This may be because crystal and cryo-EM structures tend to represent the global free-energy minima conformations, but we cannot rule out the influence of perturbations introduced by the measures taken to stabilize the samples. Understanding the structural and molecular mechanism of the ligand-induced effects on the receptor conformations is critical for the rational design of an allosteric drug. Despite heavy investment in allosteric drug discovery, there are only 19 approved allosteric modulators for all proteins, while 3,700 approved drugs target orthosteric sites (Abdel-Magid, 2015; Wishart et al., 2018). The AlloSteric Database (ASD) contains over 1,900 protein targets with more than 82,000 allosteric modulators (Shen et al., 2016). There is an unequivocal need to explore complementary experimental approaches to characterize allosteric pathways that are relevant for signaling processes in GPCRs.

EMERGENCE OF ^{19}F NMR FOR PROBING GPCR CONFORMATIONS AND DYNAMICS

Limitations of the currently available structure-based tools make it challenging to obtain more detailed insights into the conformational transitions, allosteric mechanisms, and dynamic properties of GPCRs. However, within the past few years NMR has emerged as a promising technique to study GPCR structures

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and conformational dynamics. Early experiments applied ^{13}C-methyl labeling to GPCRs. For example, labeled methionine residues were used to incorporate ^{13}C-methyl into the receptors β_{2A}R (Kofuku et al., 2012; Nygaard et al., 2013), β_{1A}R (Solt et al., 2017), μ-OR(Okude et al., 2015), and NTS1R (Bumbak et al., 2018) to characterize receptor dynamics and conformations. Strategies for the selective incorporation of isotopically labeled alanine and isoleucine were applied to label β_{2A}R (Kofuku et al., 2018) and A_{2A}R (Clark et al., 2017) as well. In addition, $^{13}CH_3$ groups were introduced into μOR (Sounier et al., 2015) and β_{2A}R (Bokoch et al., 2010) through di-methylated Lys residues. A clear benefit of these approaches is that the $^{13}CH_3$ group causes minimal structure-function perturbation compared with larger probes and modifications. However, the limited chemical shift span for ^{13}C and ^{1}H restrain their applications in probing and quantifying the multiple conformational states of the receptors, and these studies usually focus on global dynamics of the receptor other than the individual conformational states for that reason. Recently, a ^{1}H reporter from trimethylsilyl (TMS) was used to probe and monitor different conformational states and their changes in β_{2A}R, demonstrating that TMS could be a useful reporter for conformational changes of membrane proteins with a promise of studying individual conformational states (Hu et al., 2019).

Another promising approach was developed that uses the quantizable isotopic nucleus ^{19}F, which is much more sensitive to microenvironmental changes. For instance, the conformational states of β_2AR and A_{2A}R and their sub-populations of inactive and active-like states were quantified through ^{19}F NMR spectral deconvolution (Chung et al., 2012; Kim et al., 2013; Manglik et al., 2015; Ye et al., 2016, 2018a). The data indicated that, in the apo samples, the inactive states of β_2AR were mainly populated, whereas a large portion of active-like states were populated for A_{2A}R. These observations are consistent with pharmacological studies showing that β_2AR is a low constitutive receptor (Lerch et al., 2020; Staus et al., 2019), while A_{2A}R has high constitutive activity (Ibrisimovic et al., 2012). By probing the sub-populations of the receptor conformational states using ^{19}F NMR, recent research on β_1AR indicated that the inactive states were predominant in the apo sample (Frei et al., 2020), which is also in agreement with pharmacological data showing that this receptor has only low basal activity (Frei et al., 2020; Mani et al., 2016; Solt et al., 2017).

ADVANTAGES AND DRAWBACKS OF ^{19}F NMR IN STUDYING GPCR CONFORMATION AND DYNAMICS

Universal and selective isotopic labeling through ^{1}H, ^{13}C, and ^{15}N have provided a wealth of insights into GPCR conformational transitions and dynamics. However, the obtained information is mostly qualitative due to the insensitivity of these nuclei to micro-electrostatic changes. ^{19}F NMR, on the other hand, benefits from a broad range of chemical shift dispersion and a large gyromagnetic ratio, making it an exceptional probe to study changes resulting from conformational motions (Kitevski-LeBlanc and Prosser, 2012; Peng, 2001). Furthermore, the ^{19}F isotope is 100% naturally abundant and it is one of the most sensitive and stable NMR-active nuclei (Arntson and Pomerantz, 2016) besides ^{3}H and ^{1}H (Ye et al., 2015). The large gyromagnetic ratio of ^{19}F confers both high sensitivity and strong dipolar couplings, potentially enabling X-^{19}F NOE experiments such as ^{1}H-^{19}F, ^{19}F–^{19}F, and ^{19}F-^{31}P to measure distance restraints, analyzing topologies, and contacts with the solvent (Getmanova et al., 2004; Loewen et al., 2001). Moreover, unlike the ubiquitous H and C atoms, ^{19}F is nearly absent in native biological systems, which makes it a unique background-free bio-orthogonal nucleus for probing macromolecules (Arntson and Pomerantz, 2016). Besides a relatively broad chemical shift dispersion compared with ^{1}H, ^{19}F is additionally advantageous for studying dynamics because the broadly dispersed frequencies can be modulated to a great extent.

However, despite its many advantages, ^{19}F NMR also exhibits intrinsic limitations. For example, the rapid ^{19}F-chemical shift anisotropy (CSA)-driven relaxation with increasing magnet fields makes ongoing instrumental innovations for high-field magnets less impactful in studies of macromolecules (Boeszoermenyi et al., 2019; Hull and Sykes, 1975). CSA manifests itself in the transverse relaxation (T2) and yields undesirable linewidth broadening, which depends on the square of the rotational correlation time and the magnetic field strength. At 800 MHz, the linewidths are dominated by >50% CSA effects (Arntson and Pomerantz, 2016). For this reason, ^{19}F-labeled protein experiments should generally be carried out at field strengths of 564 MHz or even lower. In some cases, the sidechain mobility of amino acids can partially attenuate part of the CSA broadening effects, which would enable the possibility of studying large proteins (Arntson and Pomerantz, 2016). The development of pulse sequence ^{19}F-^{13}C ^{15}N-transverse relaxation optimized spectroscopy (TROSY) can also compensate for linewidth broadening; however, it requires large quantities of GPCR samples, which is a limitation and becomes another barrier for the application of this type of pulse sequences. Thus, continued innovations in sample preparation and pulse sequence design will improve the value of ^{19}F NMR and promote its future applications in dynamic conformational studies.

The development of ^{19}F probes for GPCR NMR studies

In the early days, GPCR studies using NMR mainly focused on bovine rhodopsin because it is a naturally abundant receptor. In 1999, ^{19}F NMR was used to probe rhodopsin conformations for the first time by labeling the receptor with 2,2,2-trifluoroethanethiol (TET) using a single-cysteine strategy (Klein-Seetharaman et al., 1999) (Figure 3A). TET was chemically attached to the different sites K67C, C140, K248C, and C316 through a reaction with the thiol groups of these cysteine residues. Upon illumination from the Meta II state, ^{19}F NMR up-field changes were observed for residues K67C and C140, while down-field changes were detected for residues K248C and C316, indicating a conformational transition upon receptor activation. These findings implied a rotation of K67C and C140 toward an aqueous environment, while K248C and C316 rotated toward the hydrophobic core of the G protein-binding pocket. This conformational transition agreed with electron paramagnetic resonance (EPR) spectroscopy measurements (Altenbach et al., 1996, 1999a, 1999b; Farahbakhsh et al., 1995).

In 2004, the same group reported that the receptor activation process might involve different dynamic properties of the sidechain versus backbone atoms of rhodopsin by examining ^{15}N-tryptophan in TROSY spectra (Klein-Seetharaman et al., 2004).

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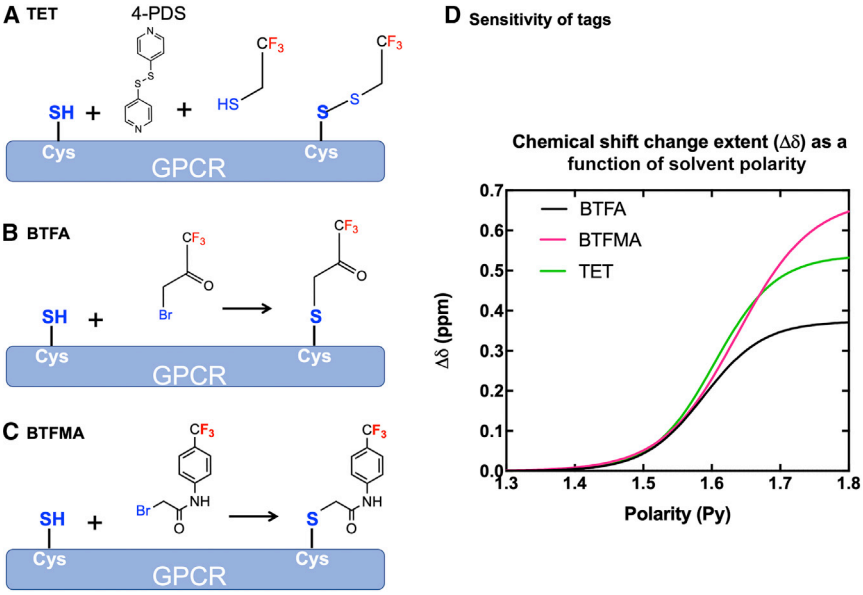

Figure 3. Structures and characteristics of TET, BTFA, and BTFMA, the three chemical ^{19}F probes that are commonly used for GPCR conformational and dynamic studies

(A–C) (A) Scheme for TET labeling to a GPCR, (B) BTFA labeling, and (C) BTFMA labeling.

(D) The sensitivities of these probes to microenvironmental changes were measured as a function of solvent polarity using a mixture of H_2O and methanol. The polarity value Py varied from 1.3 to 1.8. Figure was adapted from Ye et al. (2015) with slight modifications.

Also in 2004, the mobilities of the phosphorylated C terminus of rhodopsin in the dark and upon light activation were probed using ^{19}F-TET labeling. Solid-state rotational-echo double-resonance (REDOR) NMR was applied to characterize the internuclear distances between the ^{19}F and ^{13}C probes to study the conformational transition of the receptor upon phosphorylation (Getmanova et al., 2004).

In the following decade, two new ^{19}F reporters were developed and applied to GPCRs: 3-bromo-1,1,1-trifluoroacetate (BTFA) (Chung et al., 2012) (Figure 3B) and 2-bromo-4-trifluoromethylacetanilide (BTFMA) (Figure 3C) (Manglik et al., 2015; Ye et al., 2015). The sensitivity characteristics of these probes are summarized in Figure 3D. BTFMA is the most recently developed fluorophore, and it has the highest sensitivity to local micro-electrostatic environmental changes. BTFA and BTFMA can both be conjugated in a single chemical step, whereas TET labeling requires a two-step chemical reaction procedure that includes a 4,4′-dithiodipyridine (4-PDS) reduction step (Klein-Seetharaman et al., 1999). In this two-step reaction, the initial cysteine reduction often causes intramolecular disulfide bond breakage and off-target conjugations. Of note, many GPCRs contain multiple disulfide bonds, but not all of them are strictly required for receptor functionality. Therefore, undesirable off-target ^{19}F labeling is difficult to avoid during TET labeling even when a ligand column purification step is included. In addition, the TET labeling procedure can also yield TET-TET and thiopyridinyl-TET dimers, and these by-products remain in solution and contaminate the samples (Klein-Seetharaman et al., 1999; Susac et al., 2018). On the other hand, the smaller size of TET compared with the other two probes has the advantage of less structure-function perturbation. A shorter distance between the CF_3 group of TET and the GPCR electrostatic cloud might also help to compensate its disadvantage of having less sensitivity than BTFMA. Taken together, at this point it remains unclear which one of these probes reveals the most faithful representation of GPCR conformational sampling, and this may also depend on the specific probe location on the receptor and the nature of the receptor. Thus, the choice of probe and its labeling location on the receptor are important considerations that require further experimental validation.

Considerations for the selection of ^{19}F labeling sites

The three described ^{19}F probes have been selectively linked to cysteine-minimized receptor constructs. Therefore, radioligand binding assays or other functional assays such as surface plasmon resonance are needed to confirm the functionality of these engineered receptors. So far, four GPCRs have been investigated by ^{19}F NMR spectroscopy using cysteine-mediated chemical conjugation (Table 1). The applied labeling strategies took advantage of either native cysteines or mutated cysteine residues judiciously selected by structural topology and/or chemical shift deviations between inactive or active states using PROSHIFT (Meiler, 2003) or other chemical shift prediction programs. The labeling sites for these four receptors are shown in Figure 4. Among different approaches for selecting residues for labeling, chemical shift prediction seems a rational one considering that the underlying physical basis of NMR relies on electrostatic field strength. For example, this strategy was successfully applied to the $A_{2A}R$ receptor, and three labeling sites in the intracellular loop III (ICL3) of the receptor, R206C$^{5.67}$ in the transmembrane helix V (TM5), V229C$^{6.31}$ in TM6, and A289C$^{7.54}$ in TM7 were identified (Wang et al., 2021c; Ye et al., 2016, 2018a). Through single-site conjugation of V229C_BTFMA$^{6.31}$, the $A_{2A}R$ receptor was dissected into four conformational states, including two inactive states and two active-like states, in a commonly used detergent reconstitution system maltose neopentyl glycol-3/cholesteryl hemisuccinate (MNG-3/CHS) (Ye et al., 2016). Building on these results, the receptor was further reconstituted in a high-density lipoprotein (HDL) system. The HDL reconstituted $A_{2A}R$ data not only confirmed earlier findings (Ye et al., 2016) but also presented a few discrepancies. For instance, the fully activated conformational state was less opened compared with the partially activated conformational state indicated from ^{19}F chemical shift, while the common concept is the fully activated conformation should be more open (Huang et al., 2021). This might be due to a possible interference from the scaffolding protein MSP1D1ΔH5 when the ^{19}F probe rotated and approached the protein during the activation. However, it is important to note that incomplete structural

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Table 1. Four GPCR systems studied by chemically conjugated ^{19}F NMR spectroscopy

System	Probe	Labeling sites	Site selection	Functional purification	ICL3 maintained	Conformation number	Quantified
Rhodopsin	TET	K67C	CS	no	yes	ND	ND
		C140$^{3.55}$	NS	no	yes	ND	ND
		K245C$^{6.28}$	CS	no	yes	ND	ND
		K248C$^{6.31}$	CS	no	yes	ND	ND
		C316$^{8.53}$	NS	no	yes	ND	ND
		K311C$^{8.48}$	CS	no	yes	ND	ND
$A_{2A}R$	TET	L225C	TL	no	no	ND	ND
		A289C$^{7.54}$	TL	no	no	4	ND
		R341C$^{8.59}$	TL	no	no	ND	ND
	BTFMA	V229C$^{6.31}$	CS	yes	yes	4	yes
		A289C$^{7.54}$	CS	yes	Ye	3	yes
$\beta_{2A}R$	TET	C265$^{6.27}$	NS	no	no	2	ND
		C327$^{7.54}$	NS	no	no	2	ND
		C341$^{8.59}$	NS	no	no	ND	ND
	BTFA	C265$^{6.26}$	NS	no	yes	4	yes
	BTFMA	C265$^{6.26}$	NS	yes	yes	4	yes
$\beta_{1A}R$	TET	A282C$^{6.27}$	TL	no	no	2	ND
		C344$^{7.54}$	NS	no	no	3	ND

ND, not described." Three strategies for site selections: CS, chemical shift prediction, TL, topological location, and NS, native cysteine.

models with missing side chains, loops, or domains can cause uncertainty in theoretical chemical shift predictions and further screening, along with rigorous validation, is needed to identify suitable labeling sites for probing receptor conformations. In practical terms, there appears to be no substantial advantage of using either topological or chemical shift deviation approaches based on the currently limited data shown in Table 1. However, we hope that the discussion presented here might facilitate the further development of label-site-selection approaches.

Advances in the mechanistic understanding of GPCR activation through ^{19}F NMR delineated GPCR conformations and their quantifications

Early studies of ^{19}F NMR on rhodopsin mainly focused on qualitative measurements of the receptor to explore the behavior of an individual domain. These studies yielded a reasonable agreement between ^{19}F NMR and EPR spectroscopy regarding domain rotation and solvent accessibility of certain residues/domains. Years later, the renewed interest in using NMR to study GPCR conformation and dynamics was underscored by a study that investigated ligand-specific conformational changes of $\beta_{2A}R$ using methylated Lys (Bokoch et al., 2010). In the meantime, the two ^{19}F probes, BTFA and BTFMA, were utilized to investigate receptor conformations and dynamics of $\beta_{2A}R$ by labeling residue C265$^{6.27}$ on the TM6 helix (Kim et al., 2013; Manglik et al., 2015). Meanwhile, TET was also used to interrogate the molecular mechanism of biased signaling pathways in $\beta_{2A}R$ (Liu et al., 2012). Two conformers were observed in the TET study, whereas four conformational states, including two inactive states, an active intermediate state, and a fully activated state, were spectroscopically identified in the study with BTFMA. Of note, the two inactive states were not physically separated resonances but spectroscopically resolved through a line shape simulation. Their transition is related to the ionic lock (DRY-E) breaking between the TM3 and TM6, which triggers TM6 to rotate outward from the intracellular hydrophobic bundle. This ionic microswitch was later verified in a study of $A_{2A}R$ (Ye et al., 2016). A follow-up study, where BTFMA labeled $A_{2A}R$_V229C was incorporated into the HDL reconstitution system (Huang et al., 2021), reinforced the observations from an earlier paper using MNG-3/CHS (Ye et al., 2016). So far, four distinct conformers have been identified for $A_{2A}R$ using the single labeling site V229C$^{6.31}$. Four conformers were also identified when A289C on TM7 was labeled with the TET tag (Sušac et al., 2018). It is worthy of discussion that, if one examines the ^{19}F NMR spectra of both $A_{2A}R$ and β_2AR that were labeled with either TET or BTFMA, a very interesting finding was that for the TET labeling, the chemical shift of the probe was shifted to the high field during the receptor activation, whereas for the BTFMA labeled sample it was shifted to the down field. This phenomenon can be explained as follows: in the studies using BTFMA labeling, the cytosolic domains of the $A_{2A}R$ and β_2AR receptors were included. In contrast, in the TET study, the cytosolic domains of the receptors were replaced by T4 lysozyme, leading to a different electrostatic environment. This difference altered the electrostatic environments that surrounded the probes BTFMA and TET, causing the different observed chemical shifts.

Studies of GPCR conformational dynamics by ^{19}F NMR

Biomolecular dynamics play a major role in the context of ligand binding, receptor activation, allosteric modulation, and biased signaling, and are highly relevant to a specific physiological or pharmacological output (Latorraca et al., 2017; Wang et al., 2021b). So far, GPCR dynamics have been primarily studied computationally using molecular dynamics (MD) simulations (Mafi et al., 2022) due to the challenges of analyzing them experimentally. Previous applications of NMR for characterizing GPCR dynamics have focused on the global dynamics of a GPCR instead of a particular conformational state. A conformational

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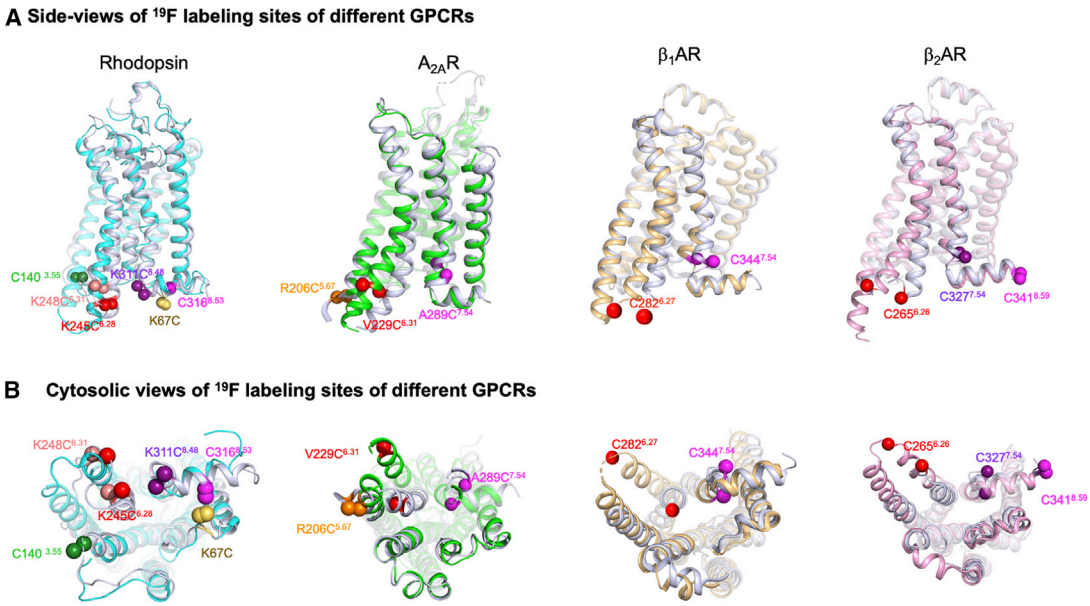

Figure 4. ^{19}F labeling sites for rhodopsin, $A_{2A}R$, $\beta_{2A}R$, and $\beta_{1A}R$
(A and B) (A) Side views and (B) cytosolic views of ^{19}F labeling sites of the respective receptors.

state can be imagined as a basin of closely related individual micro-states that are more populated; this macroscopic state has a lower free energy than its local surroundings, which gives rise to transition barriers (Latorraca et al., 2017). Understanding the dynamics of the receptor conformational states and their transitions are essential for gaining insights into the sequential activation process and the signaling matrix in response to ligand binding or microenvironmental stimuli.

Early work on β_2AR using ^{19}F NMR revealed two inactive states: an active-like state, and a fully activated state (Kim et al., 2013). Only one NMR resonance was observed for the apo sample and identified as a convoluted peak from two inactive states. The active-like state of β_2AR was only observed upon addition of a high-affinity agonist, while the accumulation of the fully activated state further needs nanobody addition (Manglik et al., 2015). The exchange rate between two inactive states was K_{ex} = 6,200 ± 830 s^{-1} when an inverse agonist was bound as measured with a CPMG relaxation experiment (Manglik et al., 2015). The evaluation of the temperature-dependent population distributions of these conformational states through ^{19}F NMR indicated that β_2AR activation is an entropically favored and enthalpically disfavored process (Kim et al., 2013). Analysis of the population equilibrium and thermodynamics of the two inactive states yielded a lifetime of 325 ± 44 μs for these two inactive states upon inverse agonist binding (Manglik et al., 2015). In the apo sample, the interconversion between these two states was reduced by approximately half with an increased lifetime to 700 ± 137 μs. Similar observations were made in a later $A_{2A}R$ study (Ye et al., 2016, 2018a) and more recent research on β_1AR (Frei et al., 2020). The lifetime and exchange rates of the receptors were proposed to be correlated with their pharmacological efficacy and should also be considered for rational drug design (Kofuku et al., 2012; Lamichhane et al., 2015; Ueda et al., 2019).

We are still at an early stage developing techniques that are capable of providing high-resolution data for the various GPCR conformational states. In addition to the ^{19}F NMR experiments described above, other ^{19}F NMR approaches have also been applied to study GPCRs and the effects of ligand binding. For instance, ligand-based 2D[^{19}F-^{19}F] exchange spectroscopy (EXSY) experiments revealed that the orthosteric pocket of the NK1 receptor undergoes a conformational transition with amplitudes of 6 to 8 Å, whereas in the crystal structure the pocket was less than 2.9 Å (Pan et al., 2022). NMR approaches that are commonly used to study the conformational dynamics of GPCRs are summarized in Figure 5.

NEW STRATEGIES OF IMPROVING ^{19}F NMR APPLICATION IN GPCR STUDIES

The high sensitivity toward surrounding microenvironmental changes, coupled with a large CSA effect, makes the ^{19}F nucleus a double-edged sword for studying the conformational dynamics of biological macromolecules. Additional challenges arise when analyzing GPCRs or other membrane proteins because they are usually reconstituted in detergents or an HDL system, and the reconstitution system itself increases the rotational correlation time and exacerbates the linewidth broadening of the ^{19}F spectrum. Coupled with the ever-present challenge of preparing GPCRs in sufficient quantities, it is not surprising that only a few GPCRs have been studied with ^{19}F NMR so far. New strategies for labeling and increasing GPCR expression as well as improvements of the reconstitution systems are required to promote the research of GPCR conformation and dynamics using ^{19}F NMR.

Reconstitution system innovations

Micelles and HDLs are the two major types of reconstitution systems that are currently used for solution-state NMR

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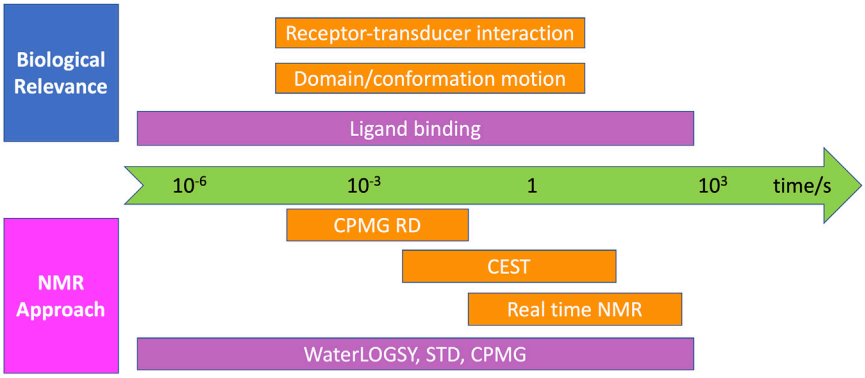

Figure 5. The most relevant NMR pulse sequences used for investigating biological activities related to receptor activation and PPI

Of note, biological activities listed on the Biological Relevance side can be investigated by the methods on the NMR Approach side labeled with corresponding time scales. For instance, ligand-binding events can be studied by either WaterLOGSY, STD, or CPMG, and domain motion can be investigated by CPMG, CEST, or real-time NMR experiments.

investigations of membrane proteins. n-Dodecyl-β-D-maltoside (DDM) micelles were first used for reconstituting rhodopsin in a ^{19}F NMR study and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was added for receptor stabilization (Klein-Seetharaman et al., 1999). Subsequent research indicated that CHS played a major role in stabilizing both the DDM micelles and incorporated receptors. This system has been frequently used in GPCR X-ray crystallography, and its success is evident from the hundreds of determined crystal structures. In 2010, a new detergent system, MNG-3, was introduced to the GPCR field. It has several advantages over the early systems, such as its exceptional stability and a low critical micelle concentration (CMC) compared with DDM (Chae et al., 2010).

Several years later, the membrane scaffold protein 1D1, delta-helix 5 deleted (MSP1D1ΔH5) was introduced for preparing nanodiscs. Nanodiscs that contain 120–160 lipid molecules wrapped by two MSPs molecules were applied in a GPCR NMR study (Hagn et al., 2013). The thickness of nanodisc ranges from 4.6 to 5.6 nm, which is largely determined by the chemical nature of the incorporated phospholipids (Denisov et al., 2004). Nanodiscs were successfully applied to analyze $A_{2A}R$ using ^{19}F NMR (Huang et al., 2021). However, some space restrictions might occur due to the rigidity of the scaffold protein. More recently, another membrane scaffold protein, named circularized NW9 (cNW9), was developed, which is a variant of MSPΔH5 (Nasr et al., 2017). Nanodiscs prepared with this scaffold protein have diameters in the range of 9-50 nm (cNW9, cNW11, cNW30, and cNW50), which allows the incorporation of GPCR complexes ranging from monomers to oligomers. cNW9 was successfully used for both cryo-EM and NMR studies. For example, neurotensin receptor 1 in cNW9 nanodiscs exhibited high stability and formed a complex with a heterotrimeric G protein without the need of a stabilizing nanobody (Zhang et al., 2021).

It is worth noting that in both $A_{2A}R$ and β_2AR studies, the HDL reconstituted receptors were much more active compared with the MNG-3/CHS reconstituted receptors (Huang et al., 2021; Staus et al., 2019), which was not fully consistent with the pharmacological observations considering β_2AR is not a high constitutive receptor. The reason for this behavior is still unclear. From this perspective, MNG-3/CHS reconsitution is a more desirable system in studying intrinstic ligand biases to avoid the effects from lipid variation in the HDL reconsititution systems while the HDL systems are more suitable for studying the effects of lipids on the receptor activation. The ability to determine GPCR-G protein complex structures without nanobodies or other stabilizers by NMR is a significant advantage. Innovations in reconstitution systems have dramatically driven the studies of GPCRs, both for determining static structures and characterizing their dynamics and conformations. However, it is important to remember that the faithfulness of the receptor structure and behavior in reconstituted systems is still under debate and needs to be further scrutinized.

Labeling strategy innovations

Integration of the ^{19}F label into the receptor can be performed either chemically or biologically. The great majority of approaches implemented so far use chemical conjugation, where a fluorophore is attached to the receptor in a solvent-exposed region. The three tri-fluorinated tags (TET, BTFA, and BTFMA) that have been discussed above are all chemically incorporated. With the current chemical conjugation approaches, a number of issues and complications can arise: (1) it is impossible to access hydrophobic regions that account for 70% of GPCR amino acid residues unless a challenging unfolding and refolding procedure is performed; (2) buried residues are inaccessible to surface-applied tags; (3) the receptor can lose function due to the bulky nature of the fluorophore and harsh environments during the chemical modification; (4) interactions can be interrupted when labels are added to interfacial regions or domains; (5) it is difficult to control the labeling efficiency because reactivity depends on the characteristics of the fluorophore and the reaction environment; (6) chemical conjugation is time consuming and increases sample-preparation times, which is critical for GPCRs considering their short lifetimes after reconstitution; (7) it can also lead to non-specific labeling; (8) site-directed mutagenesis is often needed in order to minimize non-specific labeling, e.g., by producing a cysteine-minimized construct, which might also lead to artifacts and loss of receptor functionality. These detriments emphasize the importance of developing an alternative labeling strategy that is both efficient and specific with minimal structure-function perturbations. Biosynthesis is a promising alternative to pursue; the genetic incorporation of unnatural amino acids (UAAs) (Figure 6) allows the modification of proteins without minimally affecting their functionality. For example, selenocysteine can be incorporated by encoding it through a TAG nonsense codon using a unique seryl-tRNA (Leinfelder et al., 1988). The directed evolution of orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pairs (O-tRNA/O-aaRS) from *Methanocaldococcus jannaschii* can be used for UAA incorporation in an

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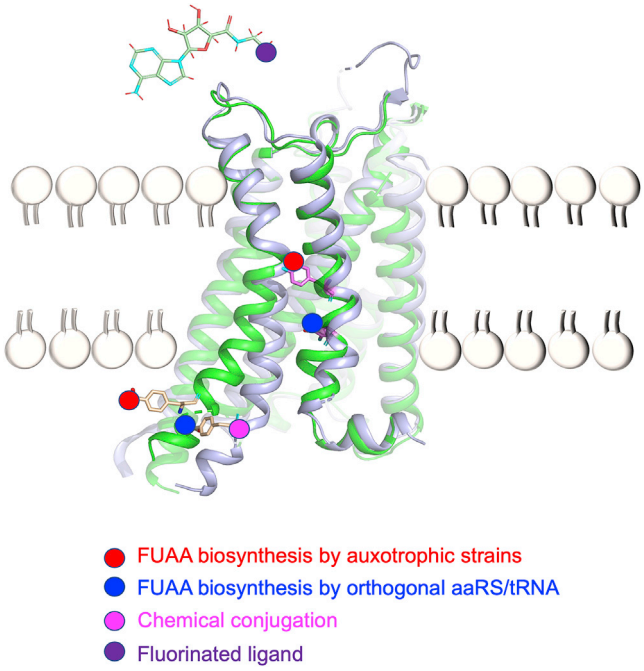

Figure 6. Summary of the different fluorinated labeling strategies using $A_{2A}R$ as a model

FUAAs can be incorporated into any places in the receptor using the biosynthesis methods, whereas chemical conjugation can only label the solvent exposure sites.

Escherichia coli expression system (Noren et al., 1989; Wang et al., 2000). To date, more than 200 UAAs have been genetically integrated into prokaryotic and eukaryotic proteins (Schmied et al., 2014; Xiao and Schultz, 2016; Young and Schultz, 2010) for various purposes, including biological process probing and industrial and pharmaceutical applications (Wang and Wang, 2008). In previous research, 4-(trifluoromethyl)-phenylalanine (tfmF) was successfully incorporated into proteins (Hammill et al., 2007), which opens another avenue for fluorophore incorporation. More recently, 3′-trifluoromethyl-phenylalanine (mtfF) was genetically incorporated into the CB1 receptor and ligand-induced receptor conformational polymorphism was studied using ^{19}F NMR (Wang et al., 2021a).

Besides genetic incorporation, fluorinated unnatural amino acids (FUAAs) biosynthesis could also be pursued by the addition of ^{19}F-labeled analogs for tyrosine, phenylalanine, and tryptophan during the culture period using auxotrophic strains. However, both approaches share the bottleneck that the incorporation of any type of fluorinated Tyr, Phe, or Trp results in a significant decrease of receptor expression levels, making it difficult to produce enough GPCRs for NMR studies, in particular for dynamic studies using CPMG and other NMR techniques. Engineering systems that significantly increase the productivity of these biosynthetic approaches is critical to facilitating the biological incorporation of fluorinated amino acids into proteins.

Advances in expression systems

Sample preparation is the key to success for biophysical studies involving GPCRs because of the intrinsic challenges in heterologous expression and receptor purification. This is even more pertinent when it comes to studying the dynamics of GPCRs by NMR, where milligram amounts of samples are often required for a single experiment. In contrast, a relatively small quantity (10–100 μg) of the receptor is sufficient for cryo-EM structure determination of GPCRs (Ye et al., 2018b; Zhao et al., 2021). Therefore, innovating expression systems to enable further NMR conformational and dynamics studies is of utmost urgency. Several established systems are currently used for GPCR expression, including yeast systems (*Saccharomyces cerevisiae*, *Pichia pastoris*), insect cell systems (sf9, sf21, Hi5 cells), and mammalian systems (HEK293, HEK293F, and COS). Each of those systems can achieve 100 μg to 1 mg of expressed protein for a few selected receptors, but none of them can be applied to a broad range of GPCRs.

Overcoming the barrier of low-yield GPCR expression may involve several steps. First, a membrane-abundant system is needed that can accommodate the expressed receptors while ensuring that the overexpressed receptors fold correctly. To address this concern, a new promising expression system was developed that uses the eyes of *Drosophila melanogaster*. The fly-eyes system consists of photoreceptor cells, which contain membrane stacks called rhabdomeres (Kumar and Ready, 1995) that offer a massive membrane space to accommodate the expressed receptor (Panneels et al., 2011). Early evidence showed its potential for expressing the glutamate receptor (DmGluRA) with a higher yield of mature receptors than conventional expression systems (Eroglu et al., 2002) and other insect cell cultures (Wiseman et al., 2020). More recently, our group established heterologous expression using *P. pastoris*, which enabled us to improve the original amount of $A_{2A}R$ receptor from 0.5 to 3 mg/L cell culture (Ye et al., 2018b) to 3 to 5 mg/L (Zhao et al., 2021). Other expression systems such as ExpiCHO cells have shown great potential for human-derived protein expression with a potential for membrane protein expression as well, but they still need to be tested in GPCR expression. Overall, considering that GPCRs must be inserted into the bilayer lipid and be folded correctly, engineering novel membrane environments that can accommodate these overexpressed receptors is a promising research direction to pursue. Additionally, chaperones that assist in GPCR folding can also be utilized to maintain the functionality of the receptors during their expression.

OUTLOOK

Structural biology has entered a new era with recent advances in the cryo-EM field as recognized with the Nobel Prize that was awarded to Jacques Dubochet, Joachim Frank, and Richard Henderson in 2018 for their contributions in advancing cryo-EM. X-ray crystallography and cryo-EM are currently the dominant methods for determining GPCR structures, with more than 50 GPCR-Gαβγ complexes being solved since 2018.

However, proteins, like anything else in life, are in motion and it is the intricacies of continuous motions from which function arises. The characterization of transitions between conformations and the connection to differential signaling outputs has therefore attracted great attention from the scientific community. Time-resolved X-ray crystallography and cryo-EM approaches

will promote further instrumental innovation. Instead of capturing the low-energy and most stable-endpoint GPCR-G protein complex structure, we assert that continued advancement in time-resolved technology will let scientists capture less-stabilized and transient intermediate complexes to bridge inactive and active endpoint conformations and fill the gaps in between. In addition to developing these mainstream tools, the community should embrace the unique advantages of NMR spectroscopy since it covers the full range of dynamics and allows measurements at (near-) physiological conditions. NMR will help us to gain a detailed understanding of the conformational transitions and dynamics of GPCRs.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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