



Resolving GPCR Activation and Signaling Dynamics by Solution NMR Spectroscopy

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Abstract: G protein-coupled receptors (GPCRs) are inherently dynamic transmembrane receptors that recognize various extracellular stimuli and mediate diverse signaling events. NMR spectroscopy is an important method to resolve the complex conformational energy landscapes of GPCRs and to probe the dynamic receptor-ligand/receptor-transducer interactions, which are critical in modulating functional diversity but are generally lacking in the static structures obtained by cryo-EM and X-ray crystallography studies. This review summarizes current advances in applying solution NMR to study GPCR structural dynamics during activation and signaling. We cover recent progress in sample preparation and isotopic labeling strategies, as well as critical NMR experimental methodologies that help illuminate transient interactions and functional intermediates. We then highlight representative studies that utilize the power of NMR to provide unique insights into GPCR signaling mechanisms that are difficult to access by other methods.

Keywords: G protein-coupled receptors; solution NMR spectroscopy; conformational dynamics; signal transduction

1. Introduction

G protein-coupled receptors (GPCRs) are the largest family of cell membrane receptors and one of the essential drug targets [1,2]. They exhibit profound signaling diversity and are structurally highly dynamic. Cryo-EM and X-ray crystallography provide high-resolution structural snapshots but usually offer limited direct information on conformational exchange, transient intermediates, and state populations. A key feature underlying GPCR signaling diversity is that rather than following a simplified “inactive-to-active” two-state exchange model, GPCR activation often results in an interconverting, multi-state conformational ensemble, and the relative populations and exchange kinetics among these conformational substates can together shape receptor activity, ligand efficacy, signaling bias and specificity [3,4]. Molecular insights in understanding the relationship between GPCR dynamics and functions have been gained largely through spectroscopic methods, in particular solution nuclear magnetic resonance (NMR) spectroscopy.

Solution NMR spectroscopy has several unique advantages for studying GPCR dynamics. Firstly, NMR is highly sensitive to local chemical environments, and is exceptionally suitable for providing information on local structural changes at residue/atomic-level. There are multiple state-of-the-art NMR methodologies developed to efficiently detect low-populated conformational species, to obtain spatial and temporal information regarding conformational changes, and to measure exchange kinetics between different conformational states. Secondly, NMR experiments can be performed under solution conditions that more closely approximate native-like environments, including selected lipid-mimetic systems such as nanodiscs. In addition, NMR measurements commonly utilize stable isotopes as atomic probes to report on receptor conformational states, consequently alleviating the need for extensive protein modifications and/or the use of bulky tags or domains as reporters. These features ensure that receptors can maximally retain their natural flexibility and conformational dynamics when examined by NMR. In the meantime, a major technical challenge for NMR is its intrinsic low sensitivity, whereas GPCRs are generally difficult to express and label in large quantity. Therefore, applying NMR in GPCR studies requires a lot of efforts in developing and optimizing new expression and isotopic labeling strategies.



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This review presents an overview of the recent advances in applying solution NMR methods to investigate GPCR functional dynamics, covering progress in sample preparation and labeling, as well as NMR experimental and analytical methods. We will compare different strategies, and discuss a few representative cases showing the unique power of NMR in shedding new light on GPCR signaling.

2. Sample Preparation and Isotopic Labeling Strategies

Protein NMR studies traditionally rely on uniform $^{15}\text{N}/^{13}\text{C}$ -isotope labeling, which is commonly achieved using the *Escherichia coli* bacterial expression system [5,6]. However, most GPCRs are extremely difficult to express in bacterial cells, and are generally produced using eukaryotic systems. To overcome this challenge, three major approaches have been adopted: (1) optimizing protein sequences to achieve expression in bacterial cells; (2) optimizing the eukaryotic systems to achieve ^{15}N or ^{13}C labeling (labeling during expression); and (3) introducing additional NMR probes via chemical modifications (labeling after expression). In the following sections, we summarize recent applications of these different strategies. In addition, we will also briefly discuss receptor segmental labeling that falls outside these two groups, as well as the choice of membrane mimetics for in vitro NMR studies (Figure 1).

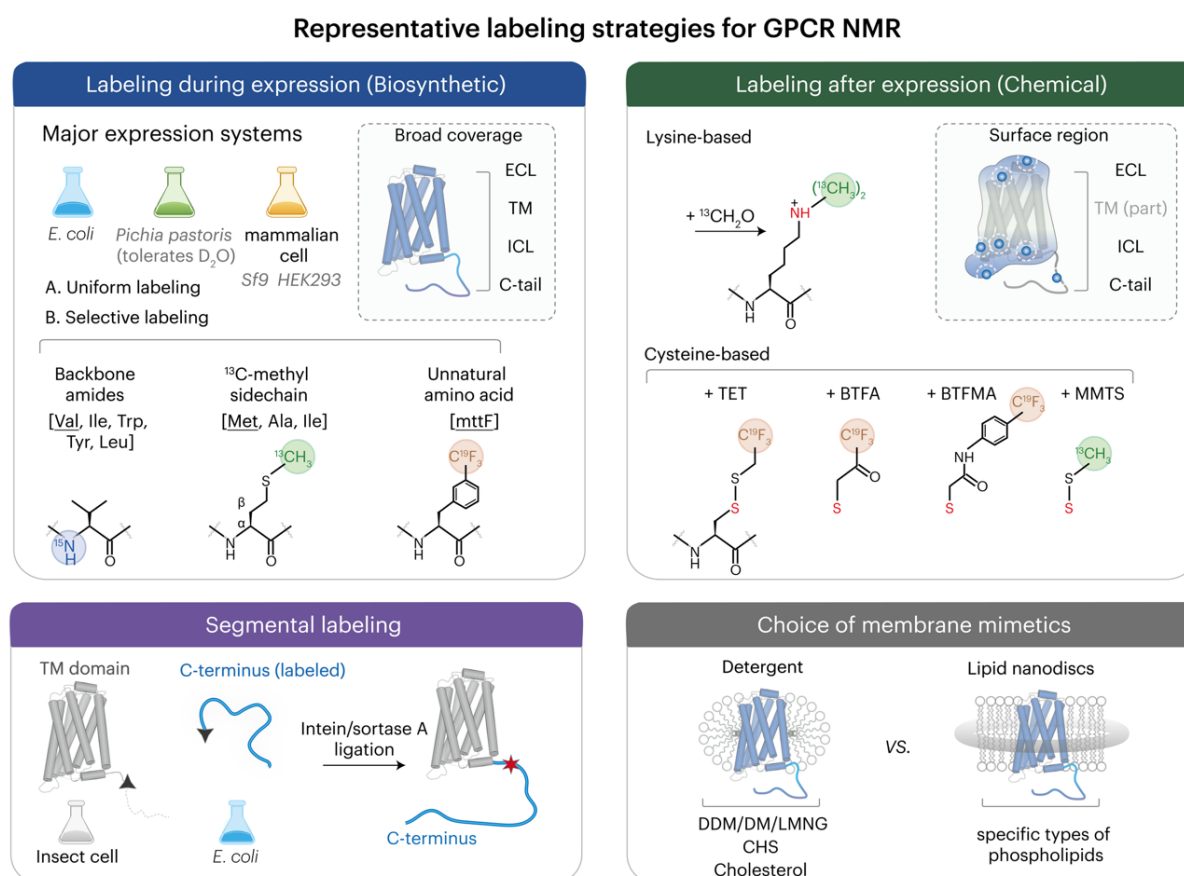


Figure 1. Representative labeling strategies for GPCR NMR. Schematics of major labeling strategies used in GPCR NMR studies, including biosynthetic labeling during expression, post-expression chemical labeling and segmental labeling. Representative membrane-mimetic systems commonly used for GPCR NMR are also illustrated.

2.1. Expression and Labeling in *E. coli* Cells

While most GPCRs fail to fold correctly in prokaryotic systems like *E. coli*, continuous efforts have been made to generate thermostabilized mutants suitable for bacterial expression. Success has been reported for a limited number of receptors, such as α_{1A} -adrenergic receptor ($\alpha_{1A}\text{AR}$), α_{1B} -adrenergic receptor ($\alpha_{1B}\text{AR}$), and human neurotensin receptor 1 (NTS₁R) [7–12], through a directed evolution strategy [13]. Through extensive protein engineering, this approach overcomes the inherent instability and low yields of GPCRs in *E. coli*, allowing for robust isotope labeling. For instance, ^{15}N -labeling with partial deuteration was applied in an $\alpha_{1B}\text{AR}$ thermostabilized mutant (containing 8 substitutions) [12]. Moreover, ϵ - $^{13}\text{CH}_3$ -methionine labeling was employed for both the $\alpha_{1A}\text{AR}$ -A4 variant (with 15 substitutions) and the enNTS₁ variant (with approximately 20

substitutions), enabling the collection of high-resolution 2D ^1H - ^{13}C HMQC spectra at tens of micromolar receptor concentrations, paving the way for in-depth mechanistic studies, particularly regarding NTS₁R [8–11]. Nevertheless, it is important to note that expression in *E. coli* remains unfeasible for the majority of GPCRs, and that extensive sequence engineering may introduce deviations from their native dynamics and functional activities.

2.2. Biosynthetic Labeling during Expression in Eukaryotic Systems

Among the commonly used eukaryotic expression systems, yeast cells (*Pichia pastoris*) most closely resemble the prokaryotic system in their capacity for large-scale protein production and uniform ^{15}N and ^{13}C isotopic enrichment, while at the same time offering eukaryotic cellular environment to assist proper folding and post-translational modifications. Moreover, *P. pastoris* tolerates D_2O -based growth media and is beneficial for global deuteration, which is favorable for studies of membrane proteins as it reduces signal line widths and improves spectral quality. Expression using *P. pastoris* has been successfully applied to human A_{2A} adenosine receptor (A_{2A}AR or A_{2A}R), allowing the production of extensively deuterated receptor samples that are either uniformly ^{15}N -labeled, or ^{13}C -labeled at specific methyl groups [14–16].

Mammalian HEK293 cells and insect cells (usually the *Sf9* cells) are both highly suitable for expressing GPCRs in their native conformations. In rhodopsin studies, stable HEK293 cell lines were employed to produce milligram quantities of receptor in defined media supplemented with isotope-labeled amino acids, thereby enabling analyses of the dynamics in the TM domain and C-terminal tail [17–19]. However, *Sf9* cells are by far the most widely used platform for non-rhodopsin GPCR studies, because they are highly efficient for expressing properly folded and functional receptors at relatively high yields compared to mammalian cells (Figure 1). Similar to mammalian cells, insect cells are auxotrophic for many amino acids and lack the capacity to synthesize them from simpler precursors. Consequently, achieving isotopic labeling requires custom-made growth media supplemented with isotope-labeled amino acids or exogenously prepared labeled ingredients, while being depleted of specific amino acids (or of all amino acids and carbohydrate sources). Given that complex metabolic pathways can lead to differential isotope scrambling in ^{15}N - or ^{13}C -labeling strategies as well as in deuteration, readers are referred to existing reviews for detailed discussions on this topic [20–22].

In principle, uniform ^{15}N labeling can provide the maximum amount of structural information. However, due to the limited sensitivity and spectral resolution, as well as the difficulty in acquiring backbone assignments via conventional triple-resonance experiments, selective ^{15}N labeling of a one or a few amino acid types is widely used in practice. For example, selective ^{15}N labeling of residues such as valine, isoleucine, tryptophan and tyrosine has been used to monitor local structural responses in the β_1 -adrenergic receptor (β_1 AR) via backbone amide detection [23–26].

Alternatively, side-chain methyl labeling is another highly attractive approach for GPCR studies, not only because methyl groups have high sensitivity and often retain favorable relaxation properties even in large complexes [27], but also because they are commonly present in the hydrophobic transmembrane core, where they can serve as sensitive reporters of local structure and dynamics. Partly because of its resistance to metabolic scrambling, and partly because of the limited number of naturally occurring methionine residues in most receptors (typically 5–15), which ensures a simplified, well-resolved spectrum, ϵ - $^{13}\text{CH}_3$ -methionine labeling in *Sf9* cells has become one of the most widely used strategies and has been successfully applied to a variety of receptors, such as β_1 AR, β_2 -adrenergic receptor (β_2 AR), M₂ muscarinic receptor (M₂R), atypical chemokine receptor 3 (ACKR3), and μ opioid receptor (μ OR), etc. [28–34]. In comparison, $^{13}\text{CH}_3$ -Ala labeling strategy has also been developed and it requires more careful control of metabolic scrambling to avoid unintended background signals [35].

In addition, deuteration is much more difficult to achieve in insect cells compared to yeast. Fractional deuteration strategies were developed by using ^{13}C or ^{15}N -labeled amino acids with deuteration at the α - and β -positions (e.g., α,β - ^2H , methyl- ^{13}C -Met, or α,β,β - ^2H , ^{15}N -Leu) to reduce strong intra-residual dipolar interactions [33,36,37]. Further deuteration level and spectral sensitivity enhancement could be obtained by supplementing the growth media with a cocktail of 8–9 different amino acids that are fully-deuterated [33,36,38,39]. Moreover, Opitz and co-workers developed a more economical method for uniform $^{15}\text{N}/^{13}\text{C}$ isotope labeling by adding labeled yeast extract into the insect cell growth media, and this method also supports over 60% deuteration if the yeast cells were grown in D_2O -based media [40]. Representative applications of biosynthetic labeling in eukaryotic cell-based NMR studies of GPCRs are summarized in Table 1.

Table 1. Representative GPCR solution NMR studies using biosynthetic labeling.

GPCR	Labeled Residue	Labeled Region	Special NMR Experiment	Membrane Mimetic	Ref.
A _{2A} AR	ϵ - ¹³ CH ₃ -Ile, ² H background	TM	triple-quantum (3Q) coherence relaxation; solvent PRE	micelles	[14]
	uniform ¹⁵ N with ~70% ² H	TM backbone, Trp/Gly	—	micelles	[15]
	² H/ ϵ - ¹³ CH ₃ -Met	TM	solvent PRE	nanodiscs	[16]
	uniform ¹⁵ N with ~70% ² H	TM backbone	—	micelles	[41]
β ₂ AR	ϵ - ¹³ CH ₃ -Met with partial ² H	TM	—	micelles	[28]
	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[29]
	β - ¹³ C-Ala with partial ² H	TM	—	micelles	[35]
	ϵ - ¹³ CH ₃ -Met with partial ² H	TM	—	nanodiscs	[36]
	¹⁵ N-Leu with partial ² H	TM backbone	PRE	micelles	[37]
	¹⁵ N-Val	TM backbone	—	micelles	[23]
β ₁ AR	¹⁵ N-Val; ¹⁵ N/ ² H backbone	TM backbone	<i>R</i> ₁ / <i>R</i> ₂ relaxation	micelles	[24]
	¹⁵ N-Val	TM	pressure perturbation NMR	micelles	[25]
	¹⁵ N-Val/Ile/Trp/Tyr	TM backbone	—	micelles	[26]
	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[30]
	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[42]
μ OR	ϵ - ¹³ CH ₃ -Met with partial ² H	TM	—	nanodiscs	[33]
	ϵ - ¹³ CH ₃ -Met with partial ² H	TM	solvent PRE	micelles	[38]
	ϵ - ¹³ CH ₃ -Met with partial ² H	TM	—	micelles	[39]
	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[34]
M ₂ R	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[31]
ACKR3	ϵ - ¹³ CH ₃ -Met	TM	—	micelles	[32]
CB1	3'-trifluoromethyl-phenylalanine (mtfF)	TM6/TM7	—	micelles	[43]

Note: Representative applications of biosynthetic isotope labeling in GPCR solution NMR studies. Entries list the receptor, labeling scheme, reporter region, special NMR experiment, membrane mimetic, and reference; the table is not intended as a comprehensive survey.

2.3. Chemical Labeling after Expression

Post-expression chemical modification of preselected sites in GPCRs after protein purification presents an appealing approach since it is much less constrained by the expression level of the target receptor, offers greater operational flexibility, and significantly reduces the labeling cost. This strategy requires the presence of solvent-exposed, chemically reactive amino acids located at the desired observing sites. Currently, the most widely used post-expression labeling methods for GPCRs are the lysine-based ¹³C-methylation and the cysteine-based introduction of ¹⁹F probes (Figure 1). Here, we summarize representative post-expression chemical labeling strategies used in GPCR solution NMR studies, with selected examples listed in Table 2.

In the lysine-based labeling scheme, the ϵ -amino group is subjected to reductive methylation using ¹³C-enriched formaldehyde to generate ¹³C-dimethyllysine [44]. This approach has been successfully applied to investigate the functional dynamics of the extracellular and intracellular loops (ECLs and ICLs) of the adrenergic receptor β ₂AR and the opioid receptor μ OR [34,45–47]. The application of this strategy can be limited by the abundance of lysine residues in the native sequence. The presence of too many lysine residues would cause severe signal overlap, particularly because they are generally solvent exposed and have similar local chemical environments. In this case, extensive mutations and necessity to confirm functional integrity for these mutants would be a great challenge.

On the other hand, cysteine-based ¹⁹F labeling is another highly attractive and promising approach, and has been reviewed extensively in multiple studies [48,49]. The core advantage of this approach is the high natural abundance (100%) and the high gyromagnetic ratio (close to ¹H) of the ¹⁹F nucleus and consequently high experimental sensitivity, which is particularly valuable for GPCR studies. Additional advantages include: (1) Proteins contain no endogenous fluorine and are therefore free of background signals; (2) ¹⁹F has a wide chemical shift range and is highly sensitive to local environmental changes, making it an ideal probe to track subtle conformational fluctuations as well as weak, transient interactions. Currently, there are multiple commercially available ¹⁹F probes, including TET, BTFA, and BTFMA, that have been applied in the structural dynamics studies

of the prototypical receptors β_2 AR, β_1 AR and A_{2A}AR [42,50–55]. Moreover, developments in new ^{19}F probes with improved sensitivity and chemical properties [56–59], together with advances in labeling strategies [60,61], would facilitate the application of ^{19}F NMR in a wider range of GPCR signaling pathways. In addition, biosynthetic incorporation of a fluorinated unnatural amino acid (UAA) into a cannabinoid receptor 1 (CB1) using an engineered *Sy9* expression system (labeling during expression) has also been reported [43]. The method is particularly beneficial for introducing ^{19}F probes into the solvent-inaccessible TM core, while circumventing the need for extensive cysteine mutations.

Table 2. Representative GPCR solution NMR studies using post-expression chemical labeling.

GPCR	Labeled Probe	Labeled Region	Special NMR Experiment	Membrane Mimetic	Ref.
μOR	^{13}C -dimethyl-Lys	intracellular surface	—	micelles	[34]
	^{13}C -dimethyl-Lys	ECL and intracellular surface	—	micelles	[47]
	^{13}C -dimethyl-Lys	ECL	—	micelles	[45]
	^{13}C -dimethyl-Lys	intracellular surface	—	micelles	[46]
$\beta_2\text{AR}$	^{19}F -TET-Cys	TM6/TM7	—	micelles	[50]
	^{19}F -BTFA-Cys	TM6	—	micelles	[51]
	^{19}F -BTFA-Cys	TM6	CPMG; saturation transfer	micelles	[52]
	^{19}F -TET-Cys	TM6	—	micelles	[62]
	^{19}F -BTfMA-Cys	TM6	Saturation transfer	micelles	[54]
	^{19}F -TET-Cys	TM6/TM7/H8	Saturation transfer; ^{19}F - ^{19}F EXSY	micelles	[55]
A _{2A} AR	^{19}F -BTfMA-Cys	TM6	—	nanodiscs	[63]
	^{19}F -BTfMA-Cys	TM6	—	nanodiscs	[64]
	^{19}F -TET-Cys	TM7	—	nanodiscs	[65]
	^{19}F -BTfMA-Cys	TM6/TM7	—	nanodiscs	[66]
$\beta_1\text{AR}$	^{19}F -TET-Cys	TM6/TM7	CEST; CPMG; saturation transfer	micelles	[53]
	^{19}F -TET/BTFA-Cys	TM7	—	micelles	[42]

Note: Representative GPCR solution NMR studies using post-expression chemical labeling, including lysine ^{13}C -dimethylation and cysteine-based ^{19}F labeling.

2.4. Segmental Labeling

While the above labeling methods were mostly applied to achieve global or site-selective labeling in the 7TM domain, segmental labeling offers an alternative strategy to specifically investigate the intrinsically disordered receptor C-terminal tail, which confers important regulatory and signaling functions. In this strategy, the isolated receptor C-terminal tail is expressed using the *E. coli* system to achieve uniform $^{15}\text{N}/^{13}\text{C}$ labeling, whereas the receptor 7TM core (with the C-terminal tail truncated) is expressed in insect cells without isotopic labeling (Figure 1). The two components are separately purified and subsequently conjugated via either an intein-mediated ligation system or a sortase-catalyzed reaction. This approach has been successfully applied in segmental ^{15}N -labeling of the β_2 AR C-terminal tail to investigate phosphorylation-related conformational changes [67], and its self-inhibitory mechanism [68].

2.5. Choice of Membrane Mimetics

Membrane environment is an important factor that needs to be considered during GPCR sample preparation, since it influences not only receptor stability and spectral quality, but also receptor conformational equilibria. Among the two major categories of membrane mimetics, detergent micelles remain most widely used in GPCR NMR studies (Figure 1). Their overall smaller particle size is important for minimizing transverse relaxation rates, and is beneficial for obtaining NMR spectra with higher resolution and sensitivity. Commonly used detergents that are favorable for preserving receptor native state include LMNG (lauryl maltose neopentyl glycol), DDM (n-dodecyl β -D-maltoside) and DM (decyl maltose) [26,31,45,47,69], whereas optimizations for specific receptors are often needed. On the other hand, lipid nanodiscs offer a more native-like membrane environment and allow tunable control of lipid composition. This makes them particularly advantageous for GPCR structural studies, especially in elucidating receptor-transducer complex formations, as comprehensively reviewed elsewhere [70]. For NMR applications, however, the relatively large size of nanodiscs leads to increased transverse relaxation. To address this, efforts have been made to generate

nanodiscs with smaller diameters to facilitate NMR experiments [71], whereas the more sensitive ^{19}F NMR is often the preferred method for delineating GPCR dynamics in nanodiscs [63–66].

Comparisons of ^1H – ^{15}N TROSY spectra obtained in micelles and lipid nanodiscs have been reported for $\alpha_{1\text{B}}\text{AR}$ and $\text{A}_{2\text{A}}\text{AR}$ [12,72]. Schuster et al. reported that a deuterated $\alpha_{1\text{B}}\text{AR}$ variant exhibits higher ^1H – ^{15}N TROSY spectral quality in the LMNG micelles compared to DMPG/DMPC nanodiscs [12]. They attributed the loss of backbone amide signals to restricted water accessibility and reduced proton back-exchange. Conversely, the ^1H – ^{13}C methyl spectrum in nanodiscs is unaffected by this effect, although subtle chemical shift differences likely reflect changes in receptor conformational equilibria. Guo et al. compared the ^1H – ^{15}N TROSY spectra of ~70% deuterated- $\text{A}_{2\text{A}}\text{AR}$ collected in LMNG/CHS micelles and POPC/POPS nanodiscs. Their data suggest that while the spectra are globally similar, tryptophan residues tend to locate at the lipid–water interface and their resonances are most sensitively affected by membrane mimetic differences [72].

More importantly, growing evidence has suggested that detergent micelles and lipid nanodiscs have different effects on receptor conformational dynamics as well as functional activities. In particular, Staus et al. showed that $\beta_2\text{AR}$ exhibits markedly enhanced constitutive activity in lipid nanodiscs compared with detergent micelles, and they linked the functional differences to a change in the equilibrium between inactive and active receptor states by using ^{19}F NMR spectroscopy [73]. Thakur et al. suggested that the cellular signaling behavior of $\text{A}_{2\text{A}}\text{AR}$ correlates with the temperature-dependent conformational equilibria measured by ^{19}F NMR in lipid nanodiscs, but not in detergent micelles [65]. Results from multiple studies indicate that not only the population distributions of inactive/active receptor conformations, but also exchange rates between different conformational species can be affected by lipid environments [36,63,65,72,74,75]. Moreover, specific types of phospholipids and cholesterol are important in modulating GPCR conformational dynamics [9,16,25,64]. These findings underscore the necessity of investigating GPCR dynamics in lipid nanodiscs to fully elucidate their activation and functional mechanisms.

3. NMR Experiments and Analysis in GPCR Studies

Corresponding to the currently applicable isotopic labeling methods, solution NMR studies of GPCRs commonly employ routine two-dimensional (2D) ^1H – ^{15}N or ^1H – ^{13}C heteronuclear correlation experiments (for ^{15}N - or ^{13}C -labeled samples), or routine one-dimensional (1D) ^{19}F experiments (for ^{19}F -labeled samples). In the following sections, we briefly summarize how these methods are applied in GPCR systems to obtain residue-specific structural dynamics information.

3.1. 2D Heteronuclear Correlation Spectra and Resonance Assignments

For ^{15}N - or ^{13}C -labeled receptors, the conventional 2D HSQC or HMQC experiments allow simultaneous detection of multiple sites and provide structural information at the residue level. TROSY-type experiments were also often employed to improve spectral quality. Changes in chemical shifts, peak intensities and line shapes under different functional conditions are important indicators to be exploited to elucidate local conformational fluctuations. However, because of the fast transverse relaxation and the difficulties in GPCR labeling, assigning the resonances in these 2D spectra cannot be achieved through conventional triple-resonance experiments. Therefore, peak assignments were generally done via extensive mutations, which could be the most labor-intensive and time-consuming step for GPCR studies.

For example, in the study of $\text{A}_{2\text{A}}\text{AR}$ using full ^{15}N -labeling, assignments of eight Gly mainchain and six Trp sidechain ^1H – ^{15}N resonances were achieved through mutation at individual sites [15]. Complete assignments of all 28 Leu backbone ^1H – ^{15}N resonances were obtained for Leu-selective ^{15}N -labeled $\beta_2\text{AR}$ using the same strategy [37]. More recently, Wu et al. proposed an innovative method for GPCR resonance assignments that combines nanobody binding with pseudocontact shift (PCS) effects, termed as the Global Positioning System by PCS (GPS-PCS) approach [76]. Briefly, lanthanide-DOTA tag is attached to a nanobody that specifically binds to receptor TM core, generating PCS effects on ^{15}N -labeled backbone atoms in the receptor $\beta_1\text{AR}$. By tagging the lanthanide center at multiple surface sites in the nanobody, the PCS data could be used like a GPS system to triangulate the atoms of the receptor and resolve the assignment problems. By using this approach, the authors were able to successfully assign 81 backbone and sidechain ^1H – ^{15}N resonances of a $\beta_1\text{AR}$ variant, and used them to trace the receptor activation dynamics [26].

In sidechain ^{13}C -methyl labeling, assignment completeness is largely affected by the number of labeled-methyl groups present in the protein sequence and their inherent spectral dispersion. For instance, $\text{A}_{2\text{A}}\text{AR}$ contains only six Met residues (excluding the N-terminus) in its native sequence, resulting in well-separated peaks and allowing complete assignments via mutagenesis [16]. Similarly, complete Met resonance assignments (eight in total) could also be obtained for the chemokine receptor ACKR3 [32]. Conversely, $\beta_2\text{AR}$ contains a total of nine

Met residues, and some of them (particularly those on the receptor exterior) generate strong, overlapping resonances. To simplify the ^1H – ^{13}C spectra, a $\beta_2\text{AR}$ - $\Delta 5\text{M}$ mutant was generated to eliminate the structurally non-essential Met residues while retaining and assigning those closely associated with activation-correlated conformational changes [29]. The same tactic is commonly adopted in cases of spectral overlap, including the studies of Met-labeled $\beta_1\text{AR}$ [30], M_2R [31], and μOR [38].

3.2. Backbone ^{15}N and Sidechain ^{13}C Relaxation Rate Measurements

Backbone ^{15}N relaxation measurements, including the longitudinal relaxation rate (R_1), transverse relaxation rate (R_2) and heteronuclear NOE (hetNOE), are conventional NMR methods used to probe structural dynamics of small proteins [77]. For GPCRs, however, the application of these methods is highly challenging because they require extensive data collection time to gain an adequate signal-to-noise ratio for extracting accurate information, whereas the sample concentration and stability of most GPCRs cannot meet the demands. Grahl and co-workers, by using thermostabilized turkey $\beta_1\text{AR}$ variants combined with valine-specific ^{15}N -labeling, reported the measurements of backbone ^{15}N relaxation transverse and longitudinal relaxation rates, providing information regarding local structural mobility and conformational exchanges [24].

For ^{13}C -methyl-labeled GPCR samples, efforts have also been made to measure the sidechain dynamics. Clark and co-workers prepared deuterated Ile $\delta 1$ - $^1\text{H}/^{13}\text{C}$ -methyl-labeled wild-type $\text{A}_{2\text{A}}\text{AR}$ sample [14], and employed a ^1H – ^{13}C HMQC-based relaxation experiment developed by Kay's group to measure the single quantum (SQ) to triple quantum (3Q) coherence transfer rate η to reflect sidechain motions on the ps-ns timescale [78]. In principle, the η value could be quantitated to calculate the sidechain order parameter S^2_{axis} . However, the limited signal-to-noise ratio for GPCR samples leads to substantial experimental errors and still poses a major bottleneck for the accurate determination of microscopic motional parameters.

3.3. Paramagnetic Relaxation Enhancement (PRE) Methods

The PRE effect, originated from the dipole-dipole interaction between an unpaired electron (the paramagnetic center) and a nucleus, is also used in GPCR studies to provide additional conformational information. On one hand, by using water-soluble paramagnetic probes (such as Gd^{3+} -DTPA and its derivatives), solvent PRE has been applied in the studies of $\text{A}_{2\text{A}}\text{AR}$ and μOR receptors to probe the solvent accessibility of ^{13}C -methyl-labeled Ile or Met residues under different ligand-binding states [14,16,38]. On the other hand, paramagnetic probes can also be attached to the protein to obtain intra-molecular distance restraints. Imai and co-workers introduced a paramagnetic probe MTSL at five distinct sites on the cytoplasmic surface of leucine-specific ^{15}N -labeled $\beta_2\text{AR}$ via cysteine-based ligation. The resulting 86 experimental Γ_2 values, corresponding to the PRE-induced increase in transverse relaxation rates, were used to derive long-range distance restraints to build a model of an agonist-bound conformation [37]. Heng and co-workers introduced a paramagnetic probe TEMPOL to the cytoplasmic tip of TM6 in a C-tail segmentally ^{15}N -labeled $\beta_2\text{AR}$ sample, and observed changes in receptor C-tail conformations associated with its autoinhibitory role [68].

3.4. 1D ^{19}F NMR Experiments

Compared to the ^{15}N - or ^{13}C -based NMR experiments, ^{19}F NMR measurements of GPCR structural dynamics are much simpler as it only requires the conventional 1D ^{19}F spectra. Because ^{19}F probes are usually attached at a single site, spectral analysis becomes straightforward. ^{19}F chemical shifts are highly sensitive to the local environment and can report on subtle conformational changes. Furthermore, spectral parameters, such as peak intensity, linewidth, and the number of observable peaks, are critical for providing a comprehensive description of receptor conformational equilibria.

More importantly, the high sensitivity of the ^{19}F nucleus, combined with the rapid data acquisition of 1D experiments, not only allows this approach to be applied to challenging receptors with limited yields or stability, but also opens up possibilities for measuring dynamics data with higher precision. For example, ^{19}F relaxation rates R_1 and R_2 could be measured with high accuracy, enabling estimation of internal correlation time τ_c and squared order parameter S^2 [69]. In addition, CPMG relaxation dispersion (RD) experiments are particularly useful for quantitative characterization of chemical/conformational exchange processes on the microsecond-to-millisecond timescale [79]. ^{19}F -based CPMG RD experiments have been conducted for the adrenergic receptors $\beta_1\text{AR}$ and $\beta_2\text{AR}$, with ^{19}F probes attached to the cytoplasmic ends of TM6 and TM7 to extract structural dynamics parameters, such as the populations of different substates and their exchange rates, when binding to different ligands [52,53].

Saturation transfer mediated by chemical/conformational exchange is another powerful NMR method to quantitatively measure protein dynamics occurring on the millisecond-to-second timescale [80]. By selectively

Earlier NMR studies of prototypical GPCRs have already demonstrated extensive dynamics associated with receptor activation. For example, ϵ - $^{13}\text{CH}_3$ -methionine spectra of $\beta_2\text{AR}$ suggested that the binding of agonist alone results in substantial receptor conformational heterogeneity rather than stabilizing a single active conformation [29]. The conformational landscape of agonist-bound $\beta_2\text{AR}$ was further delineated by using leucine-selective amide ^{15}N -labeled samples combined with PRE data, which revealed a profile consistent with a multistate equilibrium [37]. More detailed insights came from a series of ^{19}F NMR studies focusing on TM6 dynamics (Figure 2A). For both $\text{A}_{2\text{A}}\text{AR}$ and $\beta_2\text{AR}$, agonists primarily populate pre-active or partially-active states, whereas G proteins or G protein-mimetic nanobodies are required to shift the equilibrium towards the fully active conformations [15,30,52,54,63,66]. Relaxation-based kinetic analyses further distinguished rapid exchange (hundreds of μs to ms timescale) within the inactive ensembles from the slower inter-conversion (sometimes reaching the sub-second to second regime) between inactive and active states. These findings suggest that agonists function by redistributing receptor pre-existing conformations, whereas downstream binding partners stabilize the most signaling-competent states.

A follow-up question is how ligand binding in the extracellular pocket allosterically couples to the rearrangement of intracellular conformational ensembles. Based on assignments of six tryptophan indole and eight glycine backbone resonances of ^{15}N -labeled $\text{A}_{2\text{A}}\text{AR}$, Eddy and co-workers mapped signaling-related dynamics and identified D52^{2.50} as a critical allosteric switch connecting two semi-independent subdomains to drive GPCR signaling [15]. A recent study provided a more comprehensive analysis by tracking a total of 81 ^1H - ^{15}N resonances from valine, isoleucine, tryptophan and tyrosine residues in $\beta_1\text{AR}$ during activation (Figure 2A) [26]. The authors resolved four distinct conformational states—inactive, preactive, active, and transducer-bound active states—and mapped the propagation of the activation signal from the extracellular ligand-binding pocket to the intracellular side. The work proposes a hierarchical activation model by dissecting the receptor into two distinct structural modules: an early-stabilized, more rigidly-coupled structural module that undergoes substantial rearrangements across a high energy barrier during the preactive-active transition, and a more loosely-coupled module at the intracellular side that retains the plasticity to accommodate various transducers.

4.2. Understanding Ligand Efficacy and Signaling Bias

Ligand efficacy defines the capacity of a ligand to elicit receptor activation, whereas ligand bias describes the preferential activation of one signaling pathway over another. Elucidating the mechanisms underlying these two phenomena is of key importance in GPCR pharmacology. However, many receptor structures determined by cryo-EM and X-ray crystallography often exhibit high similarity when bound to different ligands, likely because these techniques preferentially capture thermodynamically stable, low-energy conformational states stabilized by downstream binding partners. To bridge this gap, extensive NMR studies have been conducted to probe the relationships between receptor dynamics and ligand efficacy or signaling bias.

A series of NMR studies on $\beta_2\text{AR}$ provided some of the earliest insights for ligand efficacy. By using ^{13}C -methyl-labeled lysines and methionines, ^{15}N -labeled backbone amides, and ^{19}F probes distributed across different receptor regions, these studies revealed ligand-specific spectral changes at distinct sites, reflecting coordinated changes in receptor conformational dynamics [28,29,37,45,50–52,62]. By monitoring the spectral signatures of a methionine probe M82^{2.53} located within the TM core, Kofuku and co-workers observed chemical shift changes that correlate with efficacy-dependent shifts in conformational equilibrium among multiple states (Figure 2B) [28]. Similar patterns were observed at additional sites proximal to the PIF motif, including L212^{5.51}, L284^{6.46}, and L287^{6.49} [37]. Collectively, these observations imply that ligand efficacy is governed by the redistribution of active-like substates within the TM core. These findings are consistent with the earlier ^{19}F NMR study focusing on the cytoplasmic TM6 and TM7 regions, where the population of active-like states was positively associated with signaling output [50].

Similar ligand-dependent spectral changes have also been observed in other GPCRs, including $\text{A}_{2\text{A}}\text{AR}$ and $\beta_1\text{AR}$ [15,24,41,53]. For instance, in the case of $\beta_1\text{AR}$, both ^{19}F labeling at the cytoplasmic side and the ^{15}N labeling of certain valine amides in the TM core have revealed an approximately linear correlation between chemical shifts and ligand efficacies. However, $^{13}\text{CH}_3$ - ϵ -methionine NMR studies of M2R suggest a more complex conformational landscape, where ligands with different efficacy profiles stabilize distinct receptor conformations, particularly those exhibiting supra-physiological efficacies [31]. This divergence may be due to the non-canonical PVF motif at the allosteric core of M2R, which likely enhances conformational heterogeneity and leads to a distinct mechanism for encoding ligand efficacies [82].

NMR studies also provided essential insights into mechanisms underlying signaling bias. An earlier ^{19}F NMR study of $\beta_2\text{AR}$ by Liu et al. suggested that signaling bias towards the β -arrestin pathway is predominantly associated with conformational changes in helix TM7 (Figure 2B) [50]. Cong et al. adopted a dual-labeling strategy to monitor spectral changes of both methionine and lysine residues in the opioid receptor μOR [34]. By integrating

these NMR data with molecular dynamics (MD) simulations, they demonstrated that the intracellular loop 1 (ICL1) and helix 8 (H8) regions are key determinants in modulating signaling bias. A parallel finding was reported in M2R, where the L54M probe introduced in ICL1 was shown to adopt a distinct conformation when bound to an arrestin-biased ligand compared to a G protein-biased one [31]. Taken together, these results highlight the ICL1/TM7-H8 structural region as a key hotspot underlying biased signaling.

4.3. Visualizing the Intrinsically Disordered Regions in GPCR

Intrinsically disordered regions (IDRs) are prevalent in GPCRs but often unresolved in static structures due to their inherent flexibility. The C-terminal tail and intracellular loop 3 (ICL3), in particular, are functionally important for phosphorylation, β -arrestin recruitment and modulation of receptor activity and selectivity. To understand these mechanisms, segmental ^{15}N -labeling can serve as a crucial tool to monitor IDR dynamics at atomic resolution.

Shiraishi and colleagues examined phosphorylation-induced conformational changes in β_2 AR via ^{13}C -Methyl-labeling in the TM core, in combination with segmental ^{15}N - or Thr γ 2/Ile δ 1-specific ^{13}C -methyl-labeling of the C-terminal tail (Figure 2C) [67]. Their results suggested that the phosphorylated tail associates with either the TM core or the lipid bilayer, and that the C-terminal phosphorylation allosterically modulates the TM5 and TM6 conformations. Another study by Heng et al. characterized the interaction between the segmentally ^{15}N -labeled β_2 AR C-terminal tail and the TM core (Figure 2C). Combined with single-molecule FRET data and MD simulations, the findings support an autoinhibitory role of the receptor tail in G protein signaling [68].

4.4. Monitoring Transducer Proteins in GPCR Signaling

The precise signaling selectivity of GPCRs is governed not only by receptor conformations, but also by the dynamic interactions with downstream transducer proteins, including G proteins and β -arrestins. In particular, transiently formed receptor-transducer intermediate complexes have been suggested to play critical roles in determining signaling specificity. Capturing these transient events necessitates the use of solution NMR spectroscopy to monitor the structural dynamics of transducer proteins (Figure 3A).

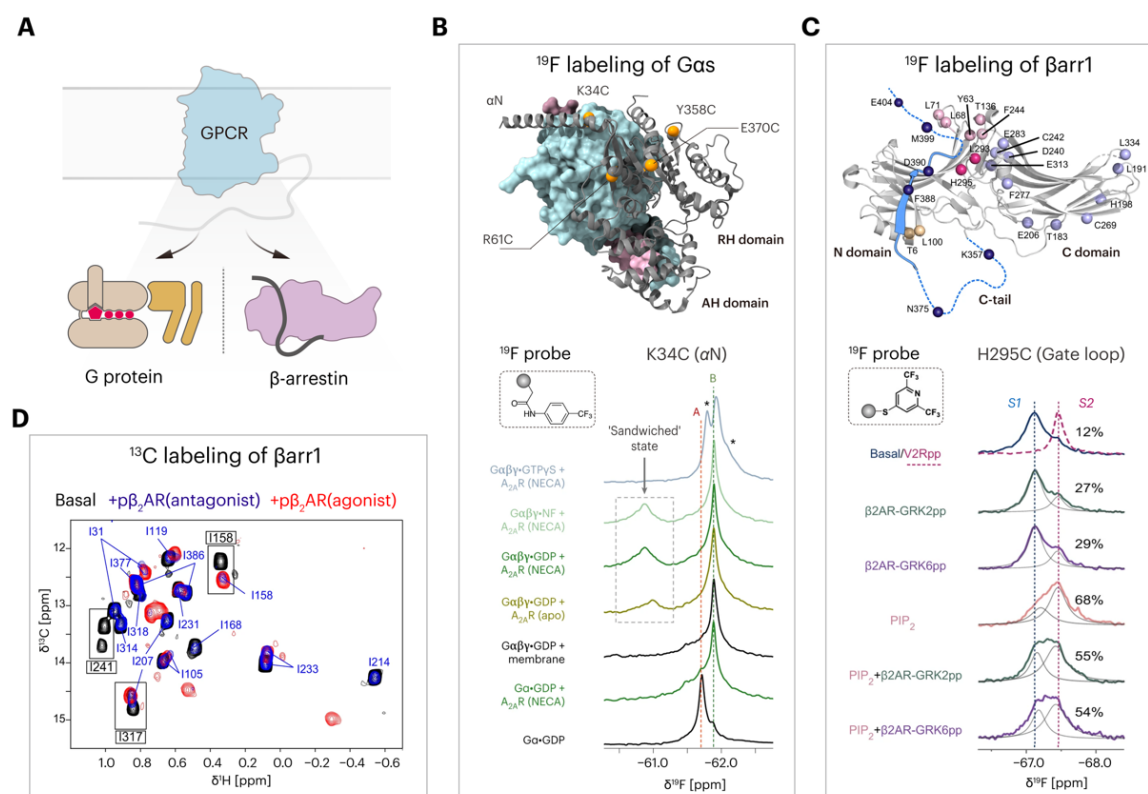


Figure 3. NMR studies of GPCR signaling partners. **(A)** Schematics of GPCR signaling partners, including G proteins and β -arrestin. **(B)** ^{19}F NMR spectra of BTFMA-labeled G α s as isolated G α , within the G $\alpha\beta\gamma$ heterotrimer, in the membrane-associated state, and in the A $_{2A}$ AR-coupled complex. Image adapted with permission from ref. [83]. **(C)** ^{19}F NMR spectra of wPSP-6F-labeled β arr1 under receptor phosphopeptide- and PIP $_2$ -bound conditions. Image adapted with permission from ref. [84]. **(D)** Ile-specific ^{13}C -methyl NMR spectra and analyses of β arr1 under receptor tail- and core-engaged conditions. Image adapted with permission from ref. [85].

A representative study by Huang et al. utilized ^{19}F NMR to dissect the conformational landscape of the Gs protein (Figure 3B) [83]. Fluorine probes introduced into specific regions of the $\text{G}\alpha$ subunit allowed for monitoring the conformational equilibria in the $\text{G}\alpha$ subunit alone, within the intact $\text{G}\alpha\beta\gamma$ heterotrimer, and in complex with A_2AAR . The results uncovered a dynamic equilibrium between two global states, which was governed by a complex interplay of nucleotide binding and interactions with the $\beta\gamma$ subunit, the membrane, and the receptor.

On the other hand, multiple NMR strategies have been utilized to investigate the conformational dynamics and interactions of β -arrestins. For example, UAA-based site-specific ^{19}F or trimethylsilyl- ^1H probes have been introduced into β -arrestin-1 (βarr1) to track its conformational changes upon interaction with different phosphopeptides or with receptors to understand its functional diversity [86–89]. A more recent cysteine-based ^{19}F NMR study introduced fluorine probes at 26 distinct sites in βarr1 , providing a more comprehensive characterization of βarr1 activation induced not only by phosphopeptides, but also by the membrane phosphoinositide PIP_2 via a distinct mechanism (Figure 3C) [84]. A methyl-TROSY study of [$u\text{-}^2\text{H}$, Ile δ 1- $^{13}\text{CH}_3$]-labeled βarr1 suggested that the receptor phospho-tail alone induces only partial activation, whereas combined engagement of the phospho-tail and TM core is required to shift the βarr1 conformational equilibrium towards the fully activated state, highlighting a biphasic activation mechanism (Figure 3D) [85].

5. Summary, Limitations and Future Directions

To summarize, the past decade has seen significant advances in solution NMR applications in GPCR research, providing atomic-resolution insights into conformational dynamics that complement the rapidly expanding repertoire of cryo-EM structures. The rich NMR toolbox offers various probes and methods for detecting dynamic processes across diverse structural regions of GPCRs, thereby addressing specific mechanistic questions regarding signal transduction. In particular, NMR-based characterization of the conformational equilibria among multiple intermediate states has elucidated the mechanistic foundations of GPCR activation and signaling, shedding light on the allosteric networks mediating transmembrane signal propagation and the dynamic determinants of signaling diversity and selectivity.

Nevertheless, NMR applications in this field still face many challenges, most notably the limited sensitivity and therefore the scarcity of experimental observables for such large, membrane-embedded and inherently unstable complexes. These constraints often hinder the building of high-resolution, comprehensive models of receptor structural dynamics, and limit the application of NMR in a wider range of pharmacologically important receptors. Future advances in NMR methodology, isotope-labeling strategies and newly developed probes may help improve sensitivity, expand the number of observable sites and enable more quantitative characterization of GPCR conformational ensembles.

From the application perspective, an equally important challenge is how to integrate current NMR data with complementary methods for characterizing GPCR structure, dynamics and function, thereby providing a deeper understanding of GPCR signal transduction. Notably, recent years have witnessed rapid advances in cryo-EM and AI-driven structural biology. Looking forward, integrative approaches combining solution NMR with complementary methods, such as single-molecule FRET spectroscopy, cryo-EM, X-ray crystallography, and computational methods, may improve mechanistic understanding of GPCR signaling and support structure- and dynamics-informed ligand design.

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