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Illuminating GPCR Signaling Mechanisms by NMR Spectroscopy with Stable-Isotope Labeled Receptors

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Abstract

G protein-coupled receptors (GPCRs) exhibit remarkable structural plasticity, which underlies their capacity to recognize a wide range of extracellular molecules and interact with intracellular partner proteins. Nuclear magnetic resonance (NMR) spectroscopy is uniquely well-suited to investigate GPCR structural plasticity, enabled by stable-isotope "probes" incorporated into receptors that inform on structure and dynamics. Progress with stable-isotope labeling methods in Eukaryotic expression systems has enabled production of native or nearly-native human receptors with varied and complementary distributions of NMR probes. These advances have opened up new avenues for investigating the roles of conformational dynamics in signaling processes, including by mapping allosteric communication networks, understanding the specificity of GPCR interactions with partner proteins and exploring the impact of membrane environments on GPCR function.

Introduction

G protein-coupled receptors (GPCRs) are sensory integral membrane proteins that recognize an enormous range of extracellular stimuli and interact with numerous intracellular partner proteins to initiate cellular signaling events. It is widely appreciated that the functions of GPCRs are enabled by their inherent structural plasticity, i.e., conformational dynamics, and a complete view of GPCR function must also include knowledge of their dynamic behavior [1,2]. While crystallography and cryo-EM have made tremendous progress determining GPCR structures, concurrently, great advances investigating conformational dynamics of GPCRs have been made by spectroscopic methods, especially nuclear magnetic resonance (NMR) spectroscopy [3]. Indeed, current understanding of GPCR molecular recognition mechanisms are highly informed from NMR studies [3].

NMR spectroscopy provides several significant advantages for studying GPCR conformational dynamics, including that experiments can be carried out at physiological

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temperatures, do not require bulky tags, and frequently utilize proteins with native or nearly-native amino acid sequences. Importantly, NMR data provide information on GPCR structures and dynamics at the level of individual nuclei. This unique capability is enabled by stable-isotopes, which act as “probes” that sense changes in local structure, dynamics, and environments. By distributing NMR probes throughout the receptor, one can obtain a global view of GPCR conformational dynamics at atomic resolution. With advances in stable-isotope labeling approaches, inroads have been made into NMR studies with more challenging proteins, including GPCRs.

This review surveys stable-isotope labeling approaches for NMR studies of GPCRs, emphasizing methods that utilize NMR-observable nuclei other than ^{19}F, i.e., ^{13}C, ^{15}N, ^{2}H, and ^{1}H. ^{19}F-NMR complements experiments with these nuclei, as reviewed elsewhere [4,5]. Examples from the literature are presented that illustrate a range of expression systems for producing GPCRs and various methods for incorporating NMR probes, including stable-isotope labeling via chemical modification and via biosynthetic approaches. We discuss how advances in stable-isotope labeling and production of GPCRs have led to a more complete view of their functions by providing insights from NMR into GPCR-drug interactions, interactions with partner proteins, and impacts of the cellular environment on GPCR structure and conformational dynamics.

Overview of stable-isotope labeling approaches for NMR

Table 1 presents a survey from the literature of GPCRs expressed for NMR studies, employed expression systems, stable-isotope labeling schemes and employed membrane mimetics. GPCR NMR studies have used two general approaches for incorporating stable-isotope labels: post-translational chemical modification, especially reductive methylation of lysines, or incorporation via biosynthesis during protein expression. The majority of GPCR NMR studies have employed Eukaryotic expression systems, with insect cells (Sf9) being the most widely used organism. Most studies incorporated stable-isotopes via biosynthesis rather than chemical modification. To date, studies in solution have used mostly detergent micelles as membrane mimetics and have exclusively focused on class A GPCRs.

Studies of GPCR complexes with small molecules

A central question in GPCR signaling is how information from ligand binding at the orthosteric pocket is transmitted ~30 Å to the intracellular surface of the receptor. NMR studies covering a growing number of class A GPCRs have provided insight into allosteric transmission processes.

The adenosine A_{2A} receptor, A_{2A}AR, a class A GPCR that regulates dopamine release and myocardial blood flow, has been the focus of multiple NMR studies. Expression of A_{2A}AR in *Pichia pastoris* enabled uniform incorporation of stable-isotopes and extensive deuteration. This allowed highly resolved NMR spectra to be recorded that provided a global view of A_{2A}AR structural plasticity. 2D [^{15}N,^{1}H]-transverse relaxation-optimized spectroscopy (TROSY) [40] spectra of A_{2A}AR revealed the impact of drugs and mutations to receptor hot spots on signal transduction (Figure 1, a and b) [7].

The same methodology was employed to study $A_{2A}AR$ complexes with partial agonists [9], leading to the observation of conformations for highly conserved residues $Trp^{6.48}$ and $Phe^{6.44}$ unique from those observed in full agonist complexes (superscripts denote Ballesteros-Weinstein nomenclature). Extrinsic tryptophans were introduced using the same expression methodology to provide novel, well-dispersed $^{15}N–^{1}H$ indole signals, which showed different responses at helices V, VI, and VII correlating with changes in the efficacy of bound drugs and a ternary complex with bound agonist and polypeptide derived from the carboxy terminus of $G\alpha_S$ [8]. 2D [$^{13}C,^{1}H$]-HMQC spectra of uniformly deuterated $A_{2A}AR$ with $^{1}H/^{13}C$-labels at isoleucine δ1 methyl groups enabled experiments correlating fast side chain motions with the efficacy of bound drugs and sodium concentration [6] (Figure 1, c and d).

Adrenergic receptors, targets of catecholamine neurotransmitters, are one of the most studied class A GPCR subfamilies. NMR studies of β_2AR have so far exclusively produced the receptor in insect cells and have utilized both chemical modification and biosynthesis stable-isotope labeling approaches. Early studies of the β_2AR labeled with $\varepsilon-^{13}CH_3$-methionine observed functionally important conformational states not represented among available crystal or cryo-EM structures [20] and demonstrated how drug efficacy influenced the equilibrium of different conformational states [21]. Improvements in signal-to-noise of NMR experiments with β_2AR in lipid nanodiscs were obtained by substituting a selected set of amino acids with ^{2}H-labeled amino acids in protein expression media also containing $\varepsilon-^{13}CH_3$-methionine [22] or $\beta-^{13}CH_3$-Alanine [23]. Paramagnetic relaxation enhancement (PRE) experiments with $^{15}N,^{2}H$-leucine labeled β_2AR yielded a structural model of the agonist-bound receptor that significantly differed from available crystal structures [25] (Figure 1, e and f). NMR studies of the related β_1AR incorporated $^{15}N,^{2}H$-valines throughout the protein, which enabled visualization of how drug binding altered allosteric networks [12] and characterization of distinct conformers and quantitative measurement of their rates of exchange [13].

NMR studies have identified significant differences among the energy landscapes of class A receptors and propensities for activating specific signaling pathways. Utilizing $\varepsilon-^{13}CH_3$-methionine labeling, NMR studies of an $\alpha_{1A}A$ receptor engineered for *E. coli* expression correlated chemical shifts with ligand efficacies and conformations of receptor microswitches [11]. Microswitches are conserved clusters of amino acids though to play important roles in allosteric transmission of drug binding, as reviewed elsewhere [41]. In contrast, NMR observations of M_2R containing $\varepsilon-^{13}CH_3$-methionine observed no clear correlations between chemical shifts and the efficacy of bound drugs, suggesting a more complex energy landscape comprising multiple distinct receptor conformations [30]. Stable isotope labeling with $\varepsilon-^{13}CH_3$-methionine in combination with reductive methylation of lysine residues [35] and $\varepsilon-^{13}CH_3$- methionine in a deuterated background [34] were employed to investigate the effects of ligand pharmacology on μ-OR signaling bias. The intrinsically biased receptor ACKR3 was studied using $\varepsilon-^{13}CH_3$-methionine labeling, correlating conformational changes in the extracellular ligand-binding pocket with changes in the intracellular β-arrestin–coupling region [28]. NMR studies of a growing number of class A receptors have provided additional insights (see Table 1).

Investigations of GPCR ternary complexes

NMR experiments have expanded on work with GPCR binary complexes with ligands to studies of ternary complexes with partner signaling proteins. Observations from NMR experiments have provided insights into mechanisms of partner protein recognition and allosteric modulation of partner protein complex formation on orthosteric ligand binding

Single domain antibodies, termed nanobodies, have been used as mimetics of G proteins to investigate GPCR complex formation with partner proteins by structural and biophysical techniques, including NMR spectroscopy [12,15,17,19,20,28,30,33,35,42]. [^{1}H,^{15}N]-TROSY spectra of ^{15}N-valine labeled β_1AR in complex with nanobody Nb80 revealed allosteric communication pathways from the receptor's intracellular surface to the orthosteric binding pocket [12]. β_1AR labeled with $\varepsilon-^{13}CH_3$-methionine showed rigid receptor dynamics in a ternary complex with agonist and nanobdy Nb6b9 compared to intermediate timescale motion for complexes with agonists alone [15]. A comparison of $\varepsilon-^{13}CH_3$-methionine labeled β_1AR in complex with Nb80 and the engineered G_S protein, 'mini-Gs", showed highly similar responses of the receptor in both complexes (Figure 2, a and b) [17].

The structural basis for GPCR-G protein selectivity is not well understood. This problem was explored by NMR with reductively ^{13}C-methylated β_2AR to investigate the structural determinants as to why β_2AR preferentially forms complexes with G_S over G_I [19]. Significant chemical shift differences between complexes with G_S and G_i were observed for methylated lysine residues located on the intracellular loop 2 (ICL2) of β_2AR (Figure 2, c and d) [19]. Interactions between β_2AR ICL2 and G proteins were found to be important determinants for selectivity of G_S over G_i in signaling complexes [19].

Mechanisms of arrestin-receptor complex formation have also been investigated by NMR spectroscopy. Early studies of [u-^{15}N,^{2}H]-arrestin-1 interaction with rhodopsin observed global structural changes of arrestin-1 upon complex formation and indicated arrestin adopted a dynamic conformational ensemble [43]. A critical step preceding arrestin recruitment is phosphorylation of the disordered receptor C-terminus. The impact of phosphorylation on the conformation of the β_2AR C-terminus was studied using a segmentally [^{13}C,^{15}N]-labelled C-terminus covalently attached to the unlabeled receptor TM region using intein chemistry (Figure 2, e and f) [24]. Phosphorylation of the β_2AR C-terminus was found to bring the C-terminus proximate to the membrane surface in samples reconstituted in lipid nanodiscs, placing residues in the C-terminus closer to the TM core to facilitate arrestin binding simultaneously to both receptor regions [24].

GPCR-lipid interactions explored by NMR

Increasing evidence from experimental and computational studies highlight the critical impact of lipids on GPCR function both through specific receptor-lipid interactions and by changing the bulk physical properties of the membrane bilayer [44]. NMR studies are investigating these dual roles, utilizing membrane mimetics including lipid nanodiscs and vesicles.

Cholesterol has been thought to modulate GPCR activity both directly as an orthosteric ligand, as in the case with the class F receptor Smoothened [45], and as a potential allosteric modulator [46]. Earlier saturation-transfer NMR experiments showed β_2AR associated preferentially with cholesterol over ergosterol [47]. The role of cholesteryl hemisuccinate (CHS), a more soluble analog of cholesterol, has been investigated as a potential negative allosteric modulator of the β_1AR (Figure 3, a–c) [14]. Pressure-dependent ^{1}H–^{15}N TROSY spectra of the G protein binding-competent ^{15}N-valine-labelled β_1AR complex in the presence and absence of CHS were collected. Combining high-pressure NMR with crystallography, the location of a cavity in the receptor structure was found to correlate with a cholesterol-binding pocket. The presence of CHS was thus shown to prevent this pocket from collapsing and to block conformational changes of activation microswitches [14].

Observations correlating higher abundance of specific lipids with higher expression of certain GPCRs in some cell types led to the hypothesis that organ-specific GPCR functions may be driven by lipid-receptor interactions. For example, docosahexaenoic acid (DHA) and arachidonic acid (ARA) make up ~14% of the total lipid content in the mammalian brain striatum where A_{2A}AR is also extensively expressed [48]. 2D HMQC spectra of [[α,β,β−^{2}H,methyl-^{13}C] Met,u-^{2}H] A_{2A}AR showed distinct changes for A_{2A}AR in nanodiscs with and without DHA, especially near the intracellular surface in TM3 and TM6 (Figure 3 d–f) [10]. These changes correlated with a significant increase in GTP uptake by G proteins in complex with A_{2A}AR in the same lipid compositions [10].

Conclusions and Outlook

NMR has provided insights into GPCR structural plasticity so far predominantly for class A receptors (Table 1). Future experiments will expand on these initial studies to include more class A subfamilies and additional classes, facilitating comparison of function-related dynamics among more receptors. Exploration of GPCR complex formation with partner proteins by NMR is at the early stages, but initial literature data hint at the promise of NMR to provide improved understanding of the roles of post-translational modifications and membranes in signaling complex formation. Potentially transient complexes difficult to capture by structural techniques, such as GPCR interactions with kinases, may be more amenable to investigation by NMR. Flexible regions involved in the formation of signaling complexes, including the receptor C-terminus, are accessible to NMR and can be independently expressed, stable-isotope labeled and covalently attached to receptor cores via chemical ligation methods [24,49].

An emerging area of research where NMR will likely play a key role are investigations of the impact of the cellular environment, especially lipid membranes, on GPCR structure-function relationships. A seemingly limitless range of membrane and membrane-mimicking environments is accessible to NMR, including micelles, bicelles, nanodiscs for experiments in aqueous solutions, and vesicles for experiments in solids. Integrating data from NMR with cryo-EM structures of receptors in membrane mimetics will likely be a powerful combination to address questions on receptor-lipid interactions, including the affinities of lipids for different structural regions. Ultimately, membrane mimetics may not even

be needed. The advent of technologies for enhancing the sensitivity of NMR, including dynamic nuclear polarization [50,51], promises to provide opportunities to study GPCRs directly *in situ* in their native cellular environments.

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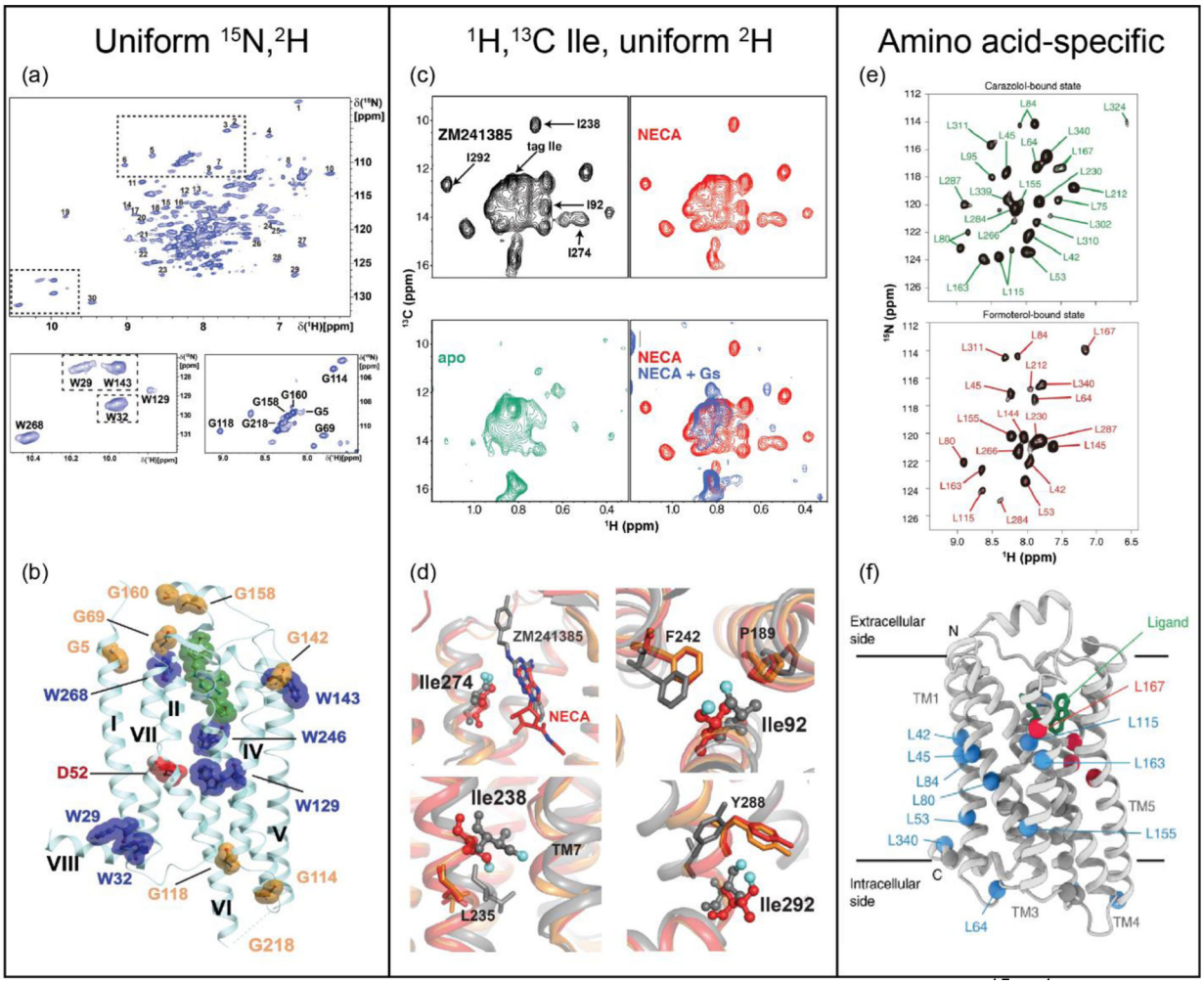

Figure 1.

Insights from NMR into ligand-stimulated GPCR activation. **(a)** 2D [^{15}N,^{1}H]-TROSY spectrum of [u-^{15}N, ~70% ^{2}H]-A_{2A}AR in complex with the antagonist ZM241385. Regions containing Trp indole ^{15}N–^{1}H and Gly backbone signals are expanded. **(b)** Assigned signals mapped onto an A_{2A}AR crystal structure (PDB 6AQF) with the antagonist ZM241385 shown in green and conserved residue Asp52 in red. **(c)** [^{13}C,^{1}H]-HMQC spectra of several complexes of [^{1}H,^{13}C-Ile δ1, u-^{2}H]-A_{2A}AR with assigned signals annotated. **(d)** Superimposed crystal structures of A_{2A}AR in complex with the antagonist ZM241385 (gray, PDB 4EIY), agonist NECA (red, PDB 2YDV), and agonist UK432097 (orange, PDB 3QAK), highlighting regions where significant chemical shift changes were observed. **(e)** 2D [^{15}N,^{1}H]-TROSY correlation spectra of [2,3,3-^{2}H,^{15}N Leu]-β_2AR in complex with the antagonist carazalol and agonist formoterol. **(f)** Chemical shift differences observed between antagonist- and agonist-bound β_2AR mapped onto the crystal structure of β_2AR in complex with carazolol (PDB 2RH1); red spheres and blue spheres denote amide signals of leucine residues with chemical shift differences >0.4 ppm and <0.4ppm, respectively. Panels **a** and **b** adapted from reference 7, panels **c** and **d** adapted from reference 6, and panels **e** and **f** adapted from reference 24, with permission.

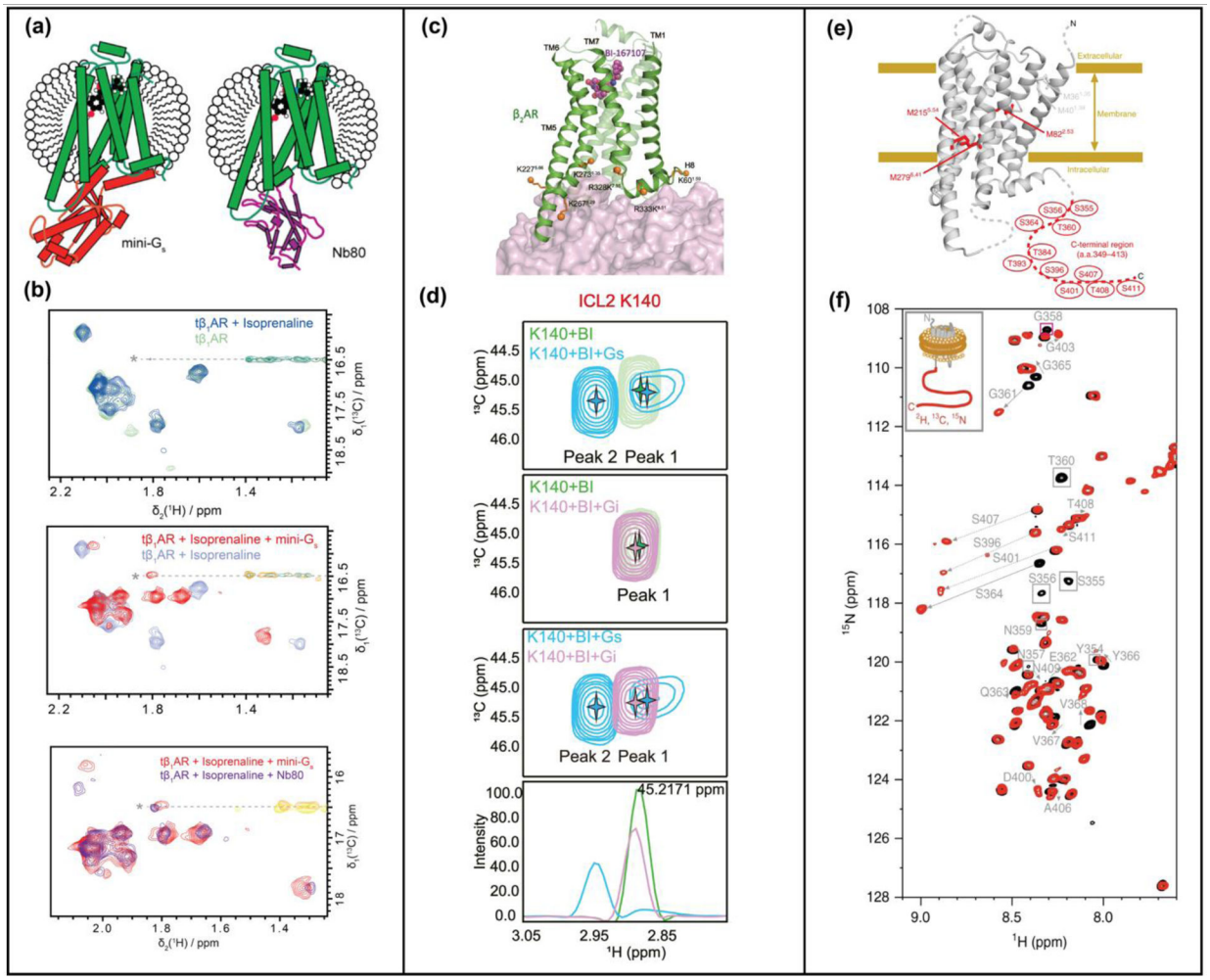

Figure 2.

NMR studies of GPCR ternary complexes. **(a)** Complexes of tβ_1AR with an engineered Gs protein (mini-Gs) and G protein-mimicking nanobody, Nb80, compared by NMR. Schematics show β_1AR ternary complexes with an agonist and mini-Gs or nanobody NB80. **(b)** Superimposed [^{13}C,^{1}H]-HMQC spectra of β_1AR in complex with an agonist and in ternary complexes with either mini-G_S or Nb80. **(c)** β_2AR-G protein interactions studied by reductive methylation of lysines. Lysines used as NMR probes are shown as orange solid spheres on the structure of the β_2AR-G_S complex (PDB 3SN6). **(d)** Expanded panels from [^{13}C,^{1}H]-HSQC spectra of β_2AR highlighting chemical shift differences of K140between complexes with G_S and G_i, indicating involvement of ICL2 in differentiating G_S and G_i. **(e)** Phosphorylated β_2AR and β_2AR in complex with arrestin studied with ε−$^{13}CH_3$-methionine labeling and segmental labeling. **(f)** Superimposed [^{15}N,^{1}H]-HSQC spectra of β_2AR (black) and phosphorylated β_2AR (red) with segmentally-labeled C-terminus. Significant chemical shift changes are indicated with arrows. Panels **a** and **b** adapted from reference 17, **c** and **d** adapted from reference 19, and **e** and **f** adapted from reference 23, with permission.

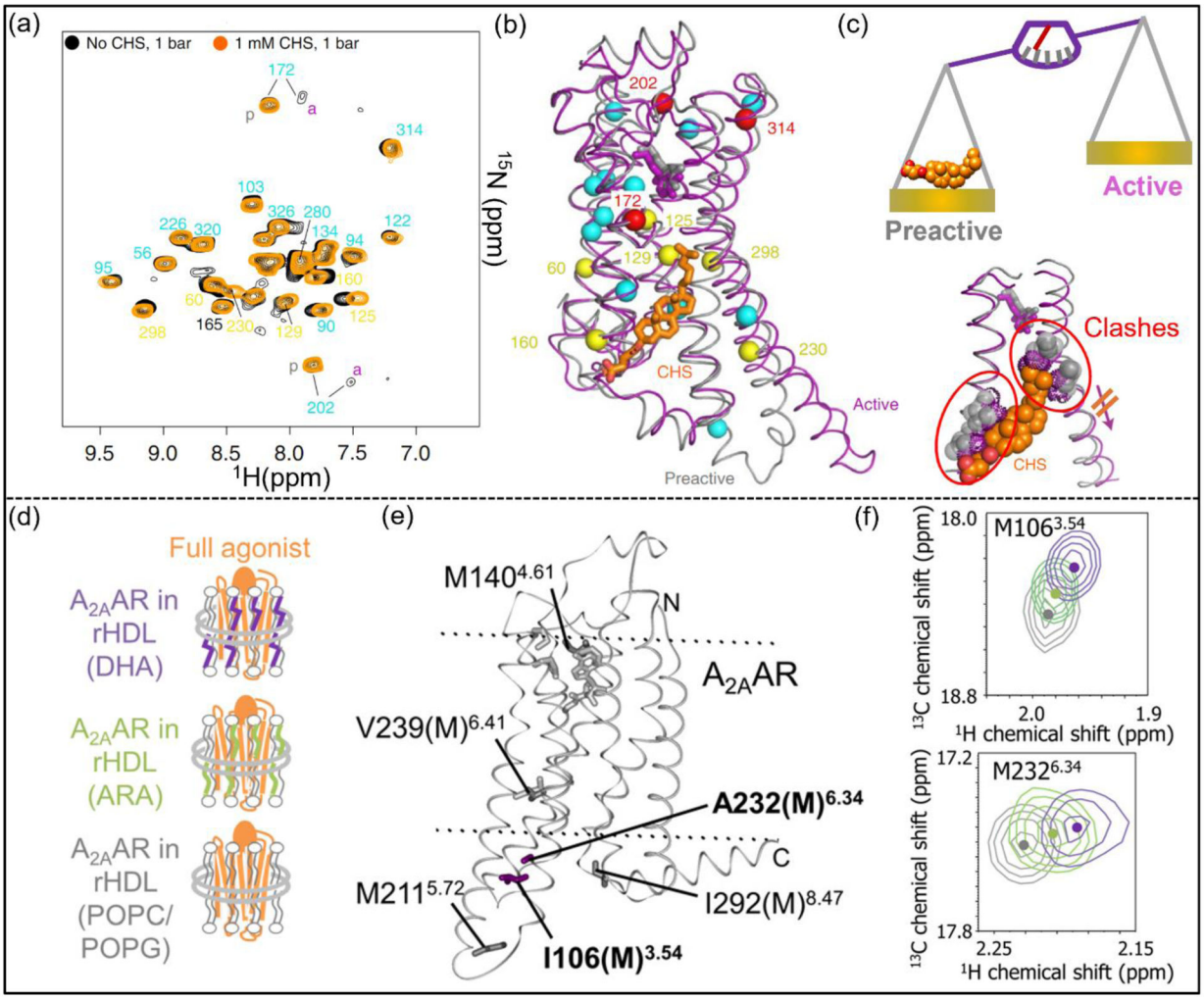

Figure 3.

NMR investigations of membrane composition on GPCR function-related dynamics. **(a)** Superposition of [^{15}N,^{1}H]-TROSY spectra of ^{15}N-valine β_1AR in the absence (black) or presence (orange) of CHS. **(b)** Responses to CHS (orange sticks) mapped onto the crystal structures of pre-active (PDB 2Y03, grey) and active (PDB 6H7J, magenta) β_1AR. Valine ^{15}N–^{1}H amides in **(a)** are shown as spheres colored by their response to CHS (red, increasing pre-active conformation population; yellow, moderate chemical shift change; cyan, small chemical shift change). **(c)** NMR data and crystal structures provided a view of CHS as a negative allosteric modulator of activation. **(d)** Schematics of A_{2A}AR reconstituted in nanodiscs containing lipids and DHA or ARA. **(e)** ε−$^{13}CH_3$-methionine used to monitor the receptor's response to DHA and ARA. I106(M) and A232(M) showed larger chemical shift differences and are shown as purple sticks. NECA and the less affected methionines are shown as grey sticks. **(f)** Signals for M106 and M232 in superimposed ^{1}H-^{13}C HMQC spectra. Peak colors correspond to the colored text in **(d)**. Panels **a-c** adapted from reference 14, and panels **d-f** adapted from reference 10, with permission.

Table 1

GPCR	Expression system	Isotope labeling	Membrane Mimetic	Ref.
$A_{2A}AR$	Yeast (*P. pastoris*)	ε−$^{13}CH_3$-Ile, ^2H	Detergent micelles	[6]
		u-^{15}N, ~70%^2H	Detergent micelles	[7], [8], [9]
		ε−$^{13}CH_3$-Met	Lipid nanodiscs	[10]
$\alpha_{1A}AR$	*E.coli*	ε−$^{13}CH_3$-Met	Detergent micelles	[11]
β_1AR	Insect cells (*Sf9*)	^{15}N-Valine	Detergent micelles	[12] , [13], [14]
		ε−$^{13}CH_3$-Met	Detergent micelles	[15]
	Insect cells (*Sf9*)	u-^{15}N, >60%^2H	Detergent micelles	[16]
	Mammalian cells	ε−$^{13}CH_3$-Met	Detergent micelles	[17]
β_2AR	Insect cells (*Sf9*)	($^{13}CH_3$)-Lys reductive dimethylation	Detergent micelles	[18], [19]
		ε−$^{13}CH_3$-Met	Detergent micelles	[20,21]
		ε−$^{13}CH_3$-Met, ^2H	Lipid nanodiscs	[22]
		β−$^{13}CH_3$-Ala, ^2H	Detergents micelles Lipid nanodiscs	[23]
	Insect cells (*Sf9*) and *E.coli*	ε−$^{13}CH_3$-Met, ^2H, c-term-^2H, ^{13}C, ^{15}N	Lipid nanodiscs	[24]
		[2,3,3-^2H, ^{15}N]-leucine	Detergent micelles	[25]
BLT_2	*E.coli*	ε−$^{13}CH_3$-Met and ε−$^{13}CH_3$-Ile	Lipid nanodiscs	[26]
CB2	Yeast (*P. pastoris*)	ε−$^{13}CH_3$-Ile	Detergent micelles	[27]
ACKR3	Insect cells	ε−$^{13}CH_3$-Met	Detergent micelles	[28]
H_1R	Yeast (*P. pastoris*)	u-^{15}N, ~70%^2H	Detergent micelles	[29]
M_2R	Insect cells (*Sf9*)	ε−$^{13}CH_3$-Met	Detergent micelles	[30]
NTR1	*E.coli*	^{13}C-MMTS	Detergent micelles	[31]
		ε−$^{13}CH_3$-Met	Detergent micelles	[32]
μOR	Insect cells	ε−$^{13}CH_3$-Met, ^2H	Detergent micelles	[33]
		($^{13}CH_3$)-Lys reductive dimethylation	Detergent micelles	[34], [35]
OX_2R	Yeast (*P. pastoris*)	ε−$^{13}CH_3$-Ile	Detergent micelles	[36]
CB1	Yeast *(P. pastoris)*	ε−$^{13}CH_3$-Ile	Detergent micelles	[36]
Rhodopsin	Mammalian cells	α,ε−^{15}N-Trp	Detergent micelles	[37],[38]
		^{13}Cβ-Ser, ^{13}Cβ-Cys, ^{13}Cα-Gly		[39]

Abbreviations: $A_{2A}AR$, adenosine A_{2A} receptor; $\alpha_{1A}AR$, α_1A-adrenergic receptor; β_1AR, β_1-Adrenergic receptor; β_2AR, β_2-Adrenergic receptor; BLT_2, leukotriene B_4 receptor 2; CB2, Cannabinoid receptor type 2; ACKR3, atypical chemokine receptor 3; H_1R, histamine H_1 receptor; M_2R, muscarinic acetylcholine receptor M2; NTR1, neurotensin receptor type 1; μOR, μ-opioid receptor; OX_2R, orexin receptor type 2; CB1, cannabinoid receptor type 1.