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Abstract

Over recent years, nuclear magnetic resonance (NMR) spectroscopy has developed into a powerful mechanistic tool for the investigation of G protein-coupled receptors (GPCRs). NMR provides insights which underpin the dynamic nature of these important receptors and reveals experimental evidence for a complex conformational energy landscape that is explored during receptor activation resulting in signalling. NMR studies have highlighted both the dynamic properties of different receptor states as well as the exchange pathways and intermediates formed during activation, extending the static view of GPCRs obtained from other techniques. NMR studies can be undertaken in realistic membrane-like phospholipid environments and an ever-increasing choice of labelling strategies provides comprehensive, receptor-wide information. Combined with other structural methods, NMR is contributing to our understanding of allosteric signal propagation and the interaction of GPCRs with intracellular binding partners (IBP), crucial to explaining cellular signalling.

Keywords	GPCR; NMR; dynamics; methionine; methyl; 19F

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The role of NMR spectroscopy in mapping the conformational landscape of GPCRs

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Abstract

Over recent years, nuclear magnetic resonance (NMR) spectroscopy has developed into a powerful mechanistic tool for the investigation of G protein-coupled receptors (GPCRs). NMR provides insights which underpin the dynamic nature of these important receptors and reveals experimental evidence for a complex conformational energy landscape that is explored during receptor activation resulting in signalling. NMR studies have highlighted both the dynamic properties of different receptor states as well as the exchange pathways and intermediates formed during activation, extending the static view of GPCRs obtained from other techniques. NMR studies can be undertaken in realistic membrane-like phospholipid environments and an ever-increasing choice of labelling strategies provides comprehensive, receptor-wide information. Combined with other structural methods, NMR is contributing to our understanding of allosteric signal propagation and the interaction of GPCRs with intracellular binding partners (IBP), crucial to explaining cellular signalling.

Introduction

The first crystal structures of the GPCRs rhodopsin and the β_2 adrenergic receptor (β_2AR) were released in 2000 and 2007 respectively [1,2]. Since then a surge of new crystal and more recently cryo-electron microscopy (cryo-EM) structures [3–5] of these proteins, which are central to cellular signalling pathways and are targeted by around 35% of drugs currently approved by the US Food and Drugs Administration (FDA) [6,7], has occurred. As understanding of GPCR structural biology has increased, the range of available crystal structures, spectroscopic and MD simulation studies have all pointed to the central role of GPCR dynamics and allosteric modulation in signalling [8]. NMR spectroscopists have combined established methodologies with new techniques to study the conformational energy landscape of these complex signalling proteins. These investigations have revealed the highly dynamic nature of GPCRs and the existence of functionally relevant low populated receptor states [9]. Together these studies are providing new insights into the role of conformational sampling for function and on the pathways of receptor activation, as well as contributing to the understanding of partial and biased agonism and allosteric modulation. Receptor specific differences in energy landscapes and dynamics are starting to become apparent. In parallel, many studies of GPCR interactions with ligands, essential to drug discovery, have been carried out for which NMR is a central technique [7,9]; however, in this current contribution we focus on recent protein-based studies. The combined information available from different structural techniques is providing a comprehensive molecular picture of these receptors and their signalling mechanisms, furthering our understanding of cellular processes and helping to guide the design of novel drugs that show improved properties.

Labelling

Traditional NMR isotope labelling approaches typically involve uniform ^{15}N and ^{13}C labelling with deuteration for large proteins and this is often suitable for GPCR interaction partners and their mimetics. In contrast, spectroscopic challenges related to fast spin relaxation together with difficulties in obtaining ^{13}C, ^{15}N uniformly labelled and perdeuterated receptors have so far hindered the success of comprehensive sequential assignment strategies for GPCRs. Instead, selective labelling approaches have been used with great success to investigate a subset of amino acid positions, with spectral assignments obtained via point mutations. For example, backbone amide positions in the β_1-adrenergic receptor (β_1AR) were ^{15}N labelled at valines using insect cell expression [10]. For receptors that can be expressed in *Pichia pastoris* (*P. pastoris*), a uniform labelling strategy on a deuterated background is possible. For the A_{2A} adenosine receptor (A_{2A}AR), Gly backbone and Trp indole signals have been studied using uniformly ^{15}N labelled samples on a deuterated background

[11,12]. However, *P. pastoris* is not a widely used expression host for GPCRs and most current studies rely on insect cell expression. *Escherichia coli* (*E.coli)* expression of a ^{15}N labelled thermo-stabilized variant of rat neurotensin receptor 1 (NTR1) has also been shown [13].

The favourable spectroscopic properties of methyl groups have encouraged several studies to use side chain ^{13}C methyl labelling of a range of amino acids, including methionine in the case of β_1AR [14], β_2-adrenergic receptor (β_2AR) [15,16], μ-opioid receptor (μOR) [17], neurotensin receptor 1 (NTS_1) [18], alanine for β_2AR [19], isoleucine for A_{2A}AR [20] and ^{13}C-dimethylated lysine via reductive methylation for μOR [21] and β_2AR [22]. To decrease spectral overlap the number of labelled amino acids can be reduced via mutagenesis. While selective labelling results in reduced coverage of the protein compared to uniform labelling, further NMR active reporters may be introduced at key locations through mutagenesis, provided these do not interfere with receptor function.

Deuteration is a key technique to improve NMR studies of GPCRs, by reducing the major contributions to relaxation [23]. In order to lower the proton density in the vicinity of reporter groups, carefully selected deuterated amino acid types along with algal amino acid mixtures may be used in insect cell expression [17,24]. The use of deuterated yeast extract has been shown for ^{2}H,^{15}N labelling of chemokine receptor type 5 (CCR5) and β_1AR [25,26]. Further gains through optimisation of such methods can be expected in the future. D_2O-based expression is readily available in *E. coli* as shown for the leukotriene B4 receptor (BLT2) [27] and *P. pastoris* as shown for A_{2A}AR [11,12,20].

It is also possible to use cell-free expression for GPCRs, however, so far this has not been widely used for NMR isotope labelling due to the challenges of producing folded, functional protein at good yields [28,29].

^{19}F NMR studies rely on site-specific tagging of a reactive cysteine side chain thiol with a fluorine-containing reporter group [30,31]. A variety of tagging reagents are available that introduce groups that differ in their chemical and spectroscopic properties. Here the benefits of larger spectral dispersion need to be offset against the increased steric demands of bulkier tags. Larger tags may interfere with receptor function. Amongst the most successful reporter tags used on GPCRs are 2,2,2-trifluoroethanethiol (TET) [32–34], 3-bromo-1,1,1-trifluoroacetone (BTFA) [35–37] and 2-bromo-4-trifluoromethylacetanilide (BTFMA) [38,39] (Figure 1). It may be necessary to reduce the number of cysteines via mutagenesis in order to avoid tagging at multiple sites or alternatively the chemical tagging reaction can be conducted with the GPCR still embedded in the membrane, which results in very high selectivity towards solvent-exposed cysteines [40].

In many NMR studies of unstable receptors, thermostabilised versions of GPCRs have been used as for X-ray crystallography. Thermostabilised receptors are often essential to obtain sufficient yields of stable protein for NMR studies and to enable investigations at elevated

temperatures. Thermostabilisation shifts the receptor towards a particular state influenced by the properties of the orthosteric ligand in the presence of which stabilisation was conducted, frequently an inactive state. Furthermore, it increases the activation energy reducing the overall dynamics, observed in crystal structures and MD simulations, and demonstrated by NMR for the M90A mutant of β_1AR [14,41]. Crystal structures of thermostabilised β_1AR show near identical conformations regardless of agonist efficacy [42] while mutation of the conserved tyrosines Y227$^{5.58}$ and Y343$^{7.53}$ in β_1AR (superscripts refer to Ballesteros–Weinstein numbering scheme [43]) was shown to prevent G protein activation [10]. However, the amount of thermostabilisation may be reduced for NMR studies and dynamic regions, such as loops reintroduced allowing NMR to provide information on closer to wild type constructs.

Ligand binding in an efficacy dependent equilibrium

GPCRs are understood to function via conformational changes. The extended ternary complex model explains receptor function via a series of equilibria between an inactive state and an active, signalling form of the receptor bound to ligand and IBP, via an activated state that is competent to couple to G proteins and other IBPs [44,45].

In support of this multi-equilibrium model, NMR studies of ligand-bound receptors have confirmed the existence of multiple conformations. Recent work demonstrated that binding of orthosteric ligands of increasing efficacy to the β_1AR and β_2AR resulted in changes in the chemical shifts of key reporter residues (Figure 2A) in 2D correlation spectra, in a response that correlated with ligand efficacy [10,14,15]. This response was observed for β_1AR methionine reporters M223$^{5.54}$ and M296$^{6.41}$ on transmembrane helix 5 (TM5) and TM6 respectively using side chain methionine methyl group signals, and for V226$^{5.57}$ on TM5, using the backbone amide signals of valine residues. Similar observations were made earlier for M82$^{2.53}$ in β_2AR, located on TM2 adjacent to the ligand binding pocket [15] (Figure 2B-D). These results indicate the presence of fast exchange equilibria on the NMR timescale between the signals of the apo (β_1AR) or inverse agonist bound inactive form (β_2AR) of the receptor and the conformational state when bound to full-agonist. Measurements in reconstituted high density lipoprotein (rHDL), also known as lipid nanodiscs, and detergent micelles, suggested exchange on the millisecond (ms) timescale [24]. This implies that partial agonism relates to a conformational equilibrium in the transmembrane region of the receptor, with higher efficacy agonists increasing the population of a receptor form that is competent to bind G protein or other intracellular signalling partners that interact with the cytoplasmic side of the receptor.

Studies by ^{19}F NMR using cysteine-attached CF_3 probes revealed that next to the dynamic processes affecting the membrane core region, exchange was also present at the cytoplasmic side of the receptors, as evidenced by changes taking place at the tip of TM6

and TM7 in β_2AR. A population shift between two exchanging conformational states, described as inactive and active, was seen in the presence of agonists of varying efficacy [34] (Figure 2E). In contrast to the faster conformational processes in the vicinity of the orthosteric binding pocket, indicative of relatively low free energy differences, this exchange was slow on the NMR timescale, likely due to the larger resulting chemical shift changes as well as slower kinetics, in agreement with the substantial cytoplasmic conformational rearrangements taking place. Overall this suggests a relatively loose, inefficient allosteric coupling of different regions of the receptor, which modulates the propagation of the signal towards the cytoplasmic side of the GPCR [34]. Similar observations relating to equilibria were also made for A_{2A}AR ^{19}F labelled at the tip of TM6, with partial agonist stabilising an active state at the expense of the inactive ensemble [39].

Low populated/intermediate states

While the above studies convincingly emphasize the dynamic nature of GPCRs they also indicate a more complex situation than suggested by the extended ternary complex model, with receptors adopting several additional conformational states that change their population in a ligand-dependent manner. These states are often invisible to other structure determination techniques either due to their low population, unfavourable exchange regime or inherent flexibility, or a bias in static structures driven by the requirement to form molecular contacts, thus trapping structures in a local energy minimum. Thus, the available static structures should be viewed as snapshots on a pathway between inactive (inverse agonist bound) and fully active state (bound to full agonist and G protein or an IBP mimetic). The additional low populated states are likely to play a crucial part in the versatility of GPCR signalling, allowing related receptors to achieve diverse functions. ^{13}C NMR studies have highlighted that the 'inactive' state of GPCRs in fact involves several states, for example via the two signals observed for M82 in β_2AR in the inverse agonist bound state [15] (Figure 2B). Two inactive conformations exchanging on the ms timescale are seen for the cytoplasmic part of TM6 in A_{2A}AR using ^{19}F NMR [39]. These are argued to represent states with the ionic lock salt bridge between TM3 (R131$^{3.50}$) and TM6 (E268$^{6.30}$) broken and formed respectively, consistent with crystal structures with (PDB accession code: 3PWH, [46]) and without an intact salt bridge (PDB accession code: 3EML, [47]; 3QAK [48]). Saturation transfer experiments indicate that the state with the open ionic lock is in equilibrium with an active state in both apo and partial agonist bound forms, with partial agonist causing a shift towards the active state. A more recent ^{19}F NMR study on the A_{2A}AR [49] using TET labelled A289C$^{7.54}$ on TM7 shows two states in the apo and antagonist bound forms shifting to three states with full agonists. Based on the changes in the positions of proximal aromatic residues in antagonist and full-agonist bound static structures relative to the ^{19}F reporter, the authors succeeded in assigning antagonist and full-agonist bound NMR states by comparing differences between expected and observed ring current shifts. The remaining

unassigned signals in the antagonist and agonist-bound states represent minor conformational states, shown to be mainly in slow exchange with the major states observed by crystallography [49]. In contrast, these minor states are not detected by crystallography. Studies on β_2AR with a BTFA tag observed only an equilibrium between inactive states, suggesting a very low population of the corresponding active state [37], while studies using the TET tag observed an inactive-active equilibrium in the apo form [34]. Although there are discrepancies between the two studies, likely related to the constructs, ^{19}F reporters and membrane mimetics employed, the unifying conclusion is the presence of multiple states that are in exchange, with different ligands shifting the equilibria between these states.

Pre-active states

Many studies on GPCRs conducted with a range of techniques agree with the observation that the full-agonist bound receptor state in the absence of an IBP does not reveal the fully active state [42,45,50]. Instead NMR studies highlight the existence of a pre-active or activation-intermediate state, which is in exchange with the inactive state. The extensive dynamics of this state indicate increased flexibility on the µs-ms timescale. Accordingly, for β_1AR bound to the full-agonist isoprenaline, the signal from M90 situated below the ligand binding pocket was lost and signals from M223$^{5.54}$ and M296$^{6.41}$, located in the extracellular halves of TM5 and TM6 respectively, showed temperature-dependent peak broadening [14] (Figure 2C). Results for the β_2AR also suggest transitions between several states taking place on an intermediate timescale in the agonist-bound form, with weaker signals observed at the corresponding positions M215$^{5.54}$ and M279$^{6.41}$ and with MD simulations showing substantial mobility at the cytoplasmic ends of TM5 and TM6 [16]. Temperature dependence for ^{19}F signals on TM6 and TM7 (Cys265$^{6.27}$ and Cys327$^{7.54}$) in TET-tagged β_2AR indicates slow exchange between active and inactive states with the active state showing greater flexibility amongst a range of conformations [34]. Temperature dependence of methionine ^{13}C methyl-labelled µOR was also observed for M245$^{5.49}$ indicating fast-exchange between multiple conformations in the full-agonist bound form, with biased full-agonists shifting the equilibrium towards the G protein, or β-arrestin activating forms [17]. Based on ^{19}F NMR data, the exchange between the inactive and active states involving TM6 and TM7 of β_2AR revealed an enthalpy value of ΔH ~40 kJmol^{-1}, indicating extensive molecular rearrangements, with an exchange rate constant of < 10 s^{-1} (280 K) [32]. The exchange was shown to be enthalpically disfavoured and entropically favoured [32,36]. Faster side chain dynamics on the ps-ns timescale were observed for A_{2A}AR in the agonist-bound state in comparison to the inverse agonist bound form when using a triple quantum relaxation experiment in combination with isoleucine δ1-methyl ^{13}C labelled receptor [20]. A quantitative assessment of S^2 order parameters for β_2AR was obtained for different ligand-bound states, indicating the greatest flexibility for the pre-active state. [36].

Overall the various NMR studies corroborate a situation where the full-agonist bound form represents an on-pathway intermediate to the ternary, fully active, G protein bound state seen in crystal structures. This pre-active/activation-intermediate state remains in exchange with one or more inactive states with agonists of increasing efficacy shifting the population towards the more flexible pre-active state, while inverse agonists bias towards the inactive state(s).The pre-active state is conformationally dynamic, showing both increased fast timescale motions (ps-ns dynamics) and sampling a range of conformations on a µs-ms timescale, that enable a receptor to couple to different intracellular binding partners e.g. G proteins or β-arrestin (Figure 3).

Fully active/ternary complexes

GPCRs are known to bind to a range of intracellular signalling proteins such as G proteins and arrestins, which enables signalling via multiple downstream pathways. Different receptors show selectivity for, and different ligands bias receptors towards the different pathways. NMR studies have typically used nanobodies e.g. Nb80 [10,16,51], Nb6B9 [14,52] and Nb33 [21] or the G_α C-terminal peptide [39] as mimics for G_α proteins to stabilize the fully active state of a receptor. While overall structures of receptors in ternary complexes with nanobodies or G protein are very similar, small conformational differences between the ternary complexes have been described e.g. in the case of β_2AR and µOR [51,53–56]. Substantial chemical shift changes in β_1AR are observed on binding of a G protein mimic in helices TM3-TM6, consistent with large scale conformational changes in the receptor and indicating the G protein bound form is a state distinct from that of the pre-active/activation-intermediate [10,14,16]. Changes in β_1AR extend from the ligand-binding pocket to the extracellular and cytoplasmic side of the receptor indicative of long-distance communication across the membrane [10,14]. Similarly in the case of the lysine ^{13}C-dimethylated µOR, binding of Nb33 and full-agonist is needed to stabilise the fully active state with substantial changes observed in TM5 and TM6 and a two-way connection between the extracellular ligand-binding and intracellular G protein coupling domains detected [21]. In this study large changes in ICL1 and H8 are observed in the presence of full-agonist but without Nb33, suggesting that this region may be the site of initial interaction between G protein and GPCR [21].

^{19}F NMR studies of β_2AR bound to Nb80 and full-agonist confirm that TM6 adopts a new conformation, which is conformationally less mobile as shown by CPMG measurements [37]. The latter is consistent with the methyl-group signal intensity analysis of β_1AR indicating rigidification on the µs-ms timescale of the receptor in the IBP bound form [14] (Figure 2G). This data suggests further that although motionally more restricted, the ternary complexes retain an inherent amount of mobility rather than becoming completely rigid.

For β_1AR in the ternary complex with Nb6B9, residues in TM4-6 and ICL2 revealed an equilibrium between two conformations in fast-exchange, with the equilibrium position correlating with ligand-efficacy (Figure 2F). Thus, the two conformations were postulated as a less active coupled ternary form, likely representative of basal receptor activity, and a fully-active ternary form with full-agonist and a G protein mimic nanobody bound [14] (Figure 3). Accordingly, higher efficacy agonists not only increase the propensity to bind IBP via increasing the population of a pre-active state of the receptor, but also affect the conformation of the resulting active ternary signalling complex, likely influencing the coupling efficiency to G protein and hence the level of signalling potentially through formation of a tighter or looser coupled complex [14,57]. Thus, ligand efficacy is reflected both in the inactive/pre-active state equilibrium of the receptor as well as in the equilibrium involving less/more active ternary complexes, which can be hypothesised translates into a ligand influencing both the initial interaction with an IBP (propensity to bind, and IBP selectivity) as well as the subsequent signalling activity through modulation of the GPCR-IBP interaction (Figure 3).

The allosteric coupling interplay of different receptor regions remains a prominent feature of GPCR-IBP interactions revealed by NMR and links into the important question of biased signalling. A key NMR study investigated phosphorylation of the β_2AR C-terminal region by GPCR kinases (GRKs), via a segmental labelling approach, indicating that the phosphorylated C-terminus adheres to the membrane potentially forming part of the β-arrestin binding interface. Conformational changes at M215$^{5.54}$ and exchange-broadening of M279$^{6.41}$ in the full-agonist bound, phosphorylated state were indicative of the receptor sampling multiple conformations, suggesting that tail-phosphorylation causes the β_2AR to adopt a β-arrestin binding conformation, leading to selectivity for the β-arrestin signalling pathway and may represent an on-pathway activation intermediate [58].

Lipid environment

Most structural studies of GPCRs so far have been carried out using detergent micelles, with *n*-β-dodecylmaltoside (DDM) and lauryl-maltose neopentyl glycol (LMNG) frequently used for NMR studies, in some cases doped with cholesterol hemisuccinate (CHS). Differences in receptor behaviour that relate to changes in the environment are clearly observed, with for example three distinct states in ^{19}F NMR observed for β_2AR in LMNG, compared to only two in DDM, an effect attributed to the different off-rates of the two detergents [35]. NMR studies have enabled comparisons of GPCR structures and dynamics in different media with membrane scaffold protein (MSP) nanodiscs (rHDL nanoparticles) a popular choice to enable studies of membrane proteins in a lipid-like, close-to-native environment [24,59]. A detailed comparison of the β_2AR in DDM vs POPC:POPG nanodiscs indicated slower exchange rates between the different conformations of M82 in the lipid environment, along with a shift in the equilibrium position towards the active conformation in response to different lipids [24].

Due to the large size of the nanodisc particles and hence the slower rotational diffusion, such investigations strongly benefit from receptor deuteration. Saposin-derived phospholipid nanoparticles have also been proposed as a suitable medium to study GPCRs, providing a membrane-like environment with the benefits of a high protein incorporation efficiency [60]. Although the number of studies in any of the lipid-based mimetics is still small, the available evidence suggests that phospholipids exert a significant effect on the energetics and kinetics of GPCR activation and also affect receptor interaction with intracellular binding partners [61,62]. Studies involving binding partners that are unstable in detergents are aided through the use of lipid membrane mimetics, for example the binding of heterotrimeric G protein to thermostabilized NTR1 embedded in circularized nanodiscs [13].

Relating solution NMR to static structure information

In assessing the structural implications of NMR investigations, it can be helpful to relate the data to existing X-ray and cryo-EM structures. NMR studies based on selective labelling approaches primarily use chemical shift changes to detect variations in protein conformations, which may relate to states observed in crystal structures, or to other unseen or dynamic states, as discussed above. Changes in chemical shifts result from variations in the electronic environment. Thus, residues in the immediate proximity of an NMR reporter have the greatest influence. Of particular value are ring current shift contributions from aromatic amino acids. For short distances between an aromatic ring and the NMR probe these have been shown to correlate with observed ^{1}H and ^{19}F chemical shift changes for both methyl and CF_3 groups [14,15,63]. The measured ring current shifts can be compared with the calculated expectations [64,65] based on static structures. Conversely, the absence of chemical shift changes at a reporter site may reflect a lack of aromatic residues in its vicinity. Such considerations are relevant for choosing the location of potential reporter sites, and for interpreting NMR data.

Outlook

Solution NMR has provided considerable insight into the mechanistic understanding of GPCRs through the characterization of conformational dynamics and their timescales, the description of conformational equilibria, by providing evidence of the existence of low populated states, and by delineating allosteric networks involved in the ligand-stimulated signal transfer. This trend looks set to continue as more receptors are investigated. Future studies are likely to focus even more on the dynamic nature of GPCRs and will provide a more quantitative description of the phenomena described above. This will help to explain some of the inherent similarities as well as disparities in the behaviour of different GPCRs,

including closely related receptor sub-types. Several studies have already extracted dynamic parameters and exchange kinetics using ^{19}F CPMG experiments [37,38] or saturation transfer [49], as well as limited side chain dynamics [20]. However, to fully realise the potential of relaxation data for backbone and side chain residues, and thus provide a protein-wide description of receptor dynamics enabling correlation with MD simulations, protocols for deuteration of receptors will need to be further developed to provide adequate signal-to-noise. High levels of deuteration are currently only available in *E. coli* or *P. pastoris*.

As discussed earlier, like X-ray crystallography, some NMR studies conducted on inherently unstable GPCRs rely on thermostabilised mutants to ensure sufficient receptor stability throughout NMR measurements. In order to accurately describe receptor dynamics, thermostabilisation will need to be reduced, which has been demonstrated, for example with the β_1AR [10,14]. Further development of expression techniques and use of lipid-based membrane mimetics will likely allow thermostabilisation to be reduced further. Crucially, NMR studies are able to investigate flexible regions of receptors e.g. loops, which are likely to be essential for receptor function and we expect future studies will further investigate these regions including with wild-type sequences.

The relevance of lipids is only beginning to be understood and the introduction of powerful membrane mimetics in the form of nanodiscs and nanoparticles, possibly in combination with receptor deuteration, will enable investigation of stoichiometric interactions between lipids and receptors and their role as allosteric modulators as well as bulk effects related to the lipid bilayer and its composition. In addition, solution NMR studies to date have only explored a small subset of class A GPCRs (Table 1), so studies on other receptors and receptor classes will reveal how far current observations are general principles.

Using alternative nuclei may also prove interesting. Examples include ^{31}P to investigate detergent/lipid components, as well as metal ions such as ^{23}Na NMR used in a recent study to investigate the binding dynamics of Na^+ ions in the A_{2A} receptor via CPMG experiments [38]. This highlighted a connection between metal ion dynamics and the activation process. Using such alternative reporters will provide new insights into the versatility of GPCR receptor activation in the future.

Conclusion

Solution NMR studies have explored several class A GPCRs (Table 1) using a variety of backbone and side chain reporters as well as reporter tags. This has enabled NMR to investigate the underlying receptor dynamics and exchange kinetics of class A receptors. Based on the limited number of studies available, investigations have revealed that receptors sample multiple states in equilibrium, with agonists of different efficacies altering

the populations of the different receptor states. The full agonist bound state is observed to show increased conformational exchange dynamics (µs-ms timescale), potentially priming the receptor to interact with different intracellular binding proteins. Biased agonists are observed to influence the population of the various pre-active states thus activating different pathways to varying amounts. On interaction with an intracellular binding partner and full agonist, the receptor adopts a less dynamic, fully active state. Thus, GPCRs are seen to occupy a complex energy landscape, with some general principles shared across the receptors investigated to date, and also receptor specific differences noticed. The resulting receptor conformational and exchange dynamics are likely to be critical in specifying the activation pathway and level of activation for a particular GPCR. Solution NMR has proved central to the general understanding of GPCRs and future studies will deepen our knowledge of this essential class of proteins, of the receptor specific differences, as well as exploring the variations among non-class A receptors.

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** of outstanding interest

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Competing interests

There are no competing interests.

Table 1 Solution NMR studies of GPCRs

Receptor	Expression system	Solubilisation system	Labelling	NMR experiments	Reference
$A_{2A}AR$	*P. pastoris*	LMNG	^{19}F-BTFMA	^{19}F; 1D, STD	[39]
$A_{2A}AR$	*P. pastoris*	LMNG	^{19}F-BTFMA; metal ions	^{19}F, $^{23}Na^+$, $^{25}Mg^+$; 1D, CPMG	[38]
$A_{2A}AR$	*P. pastoris*	LMNG/CHS	U-^{15}N, ~70% ^{2}H	^{1}H,^{15}N TROSY	[12]
$A_{2A}AR$	*P. pastoris*	LMNG/CHS	U-^{15}N, ~70% ^{2}H	^{1}H,^{15}N TROSY	[11]
$A_{2A}AR$	*P. pastoris*	DDM	$^{13}CH_3$ Ile δ1 / ^{2}H	^{1}H,^{13}C HMQC, 3Q-relaxation	[20]
β_1AR	Sf9 or Sf21	LMNG	$^{13}CH_3$-Met	^{1}H,^{13}C-HMQC	[14]
β_1AR	High five	DM	^{15}N-Val	^{1}H,^{15}N HSQC	[10]
β_1AR	Sf9	DDM	*u*-^{2}H,^{15}N	^{1}H,^{15}N TROSY	[25]
β_2AR	β_2AR: expressSF+ C-terminal tail: *Escherichia coli*	POPC/POPG nanodiscs	β_2AR [^{2}H-9AA, αβγ^{2}H-,$^{13}CH_3$-Met] C-tail: U-[^{2}H, ^{13}C, ^{15}N] or $^{13}CH_3$ Thr γ2 & Ile δ1	^{1}H,^{13}C-HMQC; ^{1}H,^{15}N HSQC; cross-saturation	[58]
β_2AR	Sf9	DDM	$^{13}CH_3$-Met	^{1}H,^{13}C HSQC	[16]
β_2AR	Sf9	DDM/CHS	^{19}F-TET	^{19}F; 1D	[34]
β_2AR	expressSF+	DDM	$^{13}CH_3$-Met or α,β,β-$^{2}H_3$-,$^{13}CH_3$-Met	^{1}H,^{13}C-HMQC	[15]
β_2AR	expressSF+	POPC/POPG nanodiscs	^{2}H-9AA, αβγ^{2}H-, $^{13}CH_3$-Met	^{1}H,^{13}C-HMQC	[24]
β_2AR	Sf9	LMNG	^{19}F-BTFA	^{19}F; 1D, T_1, T_2	[36]
β_2AR	Sf9	DDM/CHS	^{19}F-TET	^{19}F; 1D, 2D EXSY	[32]
β_2AR	Sf9	DDM/CHS or LMNG	^{19}F-3-bromo-1,1,1-trifluoroacetone	^{19}F; 1D, T_1, T_2	[35]
β_2AR	Sf9	DDM	$^{13}CH_3$-Lys (reductive methylation)	STD-filtered ^{1}H,^{13}C HMQC; ^{1}H,^{13}C HSQC	[22]
β_2AR	Sf9	LMNG	^{19}F-BTFA	^{19}F; 1D, CPMG, STD	[37]
BLT2	*E. coli*	DMPC/CHS nanodiscs	u-^{2}H, ^{12}C Ile-[δ1-$^{13}CH_3$], ^{12}C Met-[ε-$^{13}CH_3$]	^{1}H,^{13}C-HMQC	[27]
CCR5	Sf9	DDM	*u*-^{2}H,^{15}N	^{1}H,^{15}N TROSY	[26]
NTS_1	*E. coli*	DDM	$^{13}CH_3$-Met	^{1}H,^{13}C-HMQC	[18]
NTR1	*E. coli*	DMPC/DMPC nanodiscs	*u*-^{15}N	^{1}H,^{15}N TROSY	[13]

μOR	Sf9	LMNG/CHS	$^{13}CH_3$ -Lys (reductive methylation)	$^{1}H,^{13}C$-HMQC	[21]
μOR	Sf9	LMNG/ CHS	^{2}H-8AA, αβ-^{2}H-$^{13}CH_3$-Met	$^{1}H,^{13}C$-HMQC	[17]

$A_{2A}AR$, A_{2A} adenosine receptor; β_1AR, β_1-adrenergic receptor; β_2AR, β_2-adrenergic receptor; BLT2, leukotriene B4 receptor; BTFA, 3-bromo-1,1,1-trifluoroacetone; BTFMA, 2-bromo-4-trifluoromethylacetanilide; CCR5, C-C chemokine receptor type 5; CHS, cholesterol hemisuccinate; DDM, dodecyl maltoside; EXSY, exchange spectroscopy; HMQC, heteronuclear multiple-quantum correlation; HSQC, heteronuclear single-quantum correlation; LMNG, lauryl maltose neopentyl glycol; μOR, μ-opioid receptor; NTS_1, NTR1, neurotensin receptor 1; STD, saturation-transfer difference; TET, 2,2,2-trifluoroethanethiol; TROSY, transverse relaxation optimized spectroscopy; 3Q-, triple quantum.

Figure captions

Figure 1 | Functional studies of GPCRs by NMR: NMR studies of GPCRs require incorporation of an NMR active isotope. Amino acid type selective or uniform ^{15}N labeling [10–12], or ^{13}C labelling of methyl groups has been used [14–20], in a range of expression hosts, with most studies using insect cell expression. NMR active groups can also be incorporated post-translationally via attachment of ^{19}F-containing tags to reactive cysteine thiol groups [30–40], or via reductive ^{13}C methylation of lysine groups [21,22]. High levels of receptor deuteration that improve the performance of NMR experiments can be obtained in *E. coli* [27] and *P. Pastoris* [11,12,20], with comparable methods being less established in insect cells [17,24,25]. NMR samples can be prepared with the GPCR solubilized in detergent micelles or embedded in a phospholipid-bilayer as part of an MSP nanodisc [24,59] or saposin nanoparticle [60]. Depending on the isotope labelling scheme a range of 1D and 2D NMR spectra (^{1}H,^{15}N TROSY; ^{1}H,^{13}C HMQC) are typically recorded to monitor the conformational response of the receptor to a range of activating (agonists)/inactivating ligands (inverse agonists), lipids, IBPs etc. Residue specific assignments frequently require the introduction of point mutations. Conformational interchange and dynamics taking place on a range of timescales can be investigated for every residue available and quantitated via CPMG, STD, 3Q-relaxation, T_1, T_2 and EXSY experiments, for example. Integrated with data from other studies, the resulting NMR information can be used to propose a model that informs on the conformational plasticity of the receptor, providing insight into the dynamic nature of the GPCR in the context of ligand-based activation, allosteric activation, G protein signaling, β arrestin signalling, partial agonism, biased agonism etc. (Figure 3). The studies on different receptors can be compared, revealing receptor specific differences due to variations in their energy landscape.

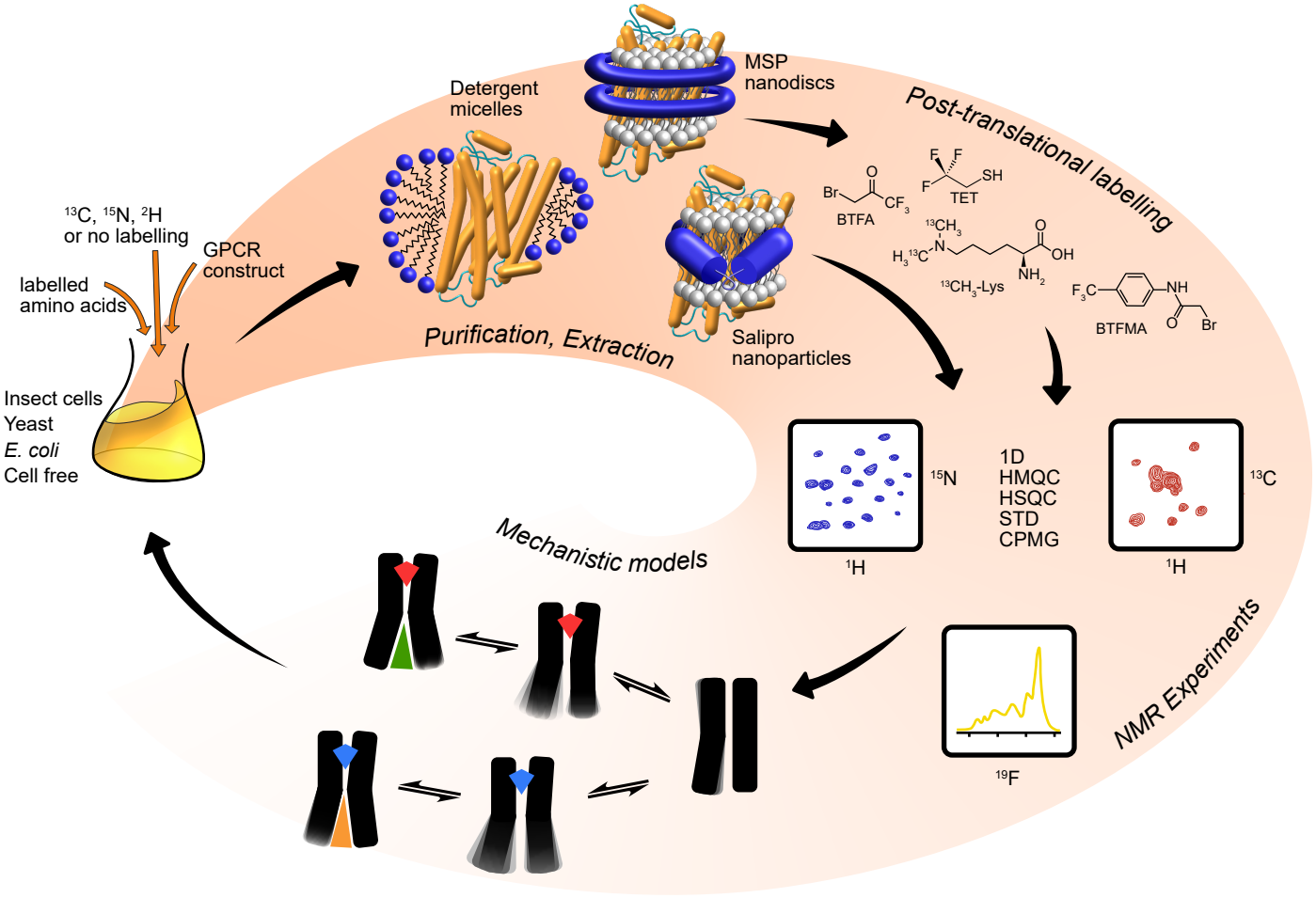
Figure 2 | Conformational equilibria observed by NMR related to adrenergic receptor activation:

A) Locations of the investigated residues of β_1AR and β_2AR mapped onto the crystal structure of β_1AR in the inactive state (PDB accession code: 2RH1). Residue positions use the GPCRdb numbering scheme [66]. **B-G)** Representative regions of NMR spectra from studies on β_1- and β_2AR emphasizing the dynamic nature of GPCRs and the conformational response of the receptors to ligands and IBPs. **B**) Overlay of a ^{1}H,^{13}C HMQC spectra region of β_2AR showing the response of the M82^{2x53} signal to binding of ligands of increasing efficacy: carazolol (black), alprenolol (cyan), tulobuterol (green), clenbuterol (purple), formoterol (red). With M82^{2x53} located in the proximity of the orthosteric binding pocket, a gradual change from the inverse agonist carazolol bound receptor to the full-agonist formoterol bound state is observed, indicating a conformational equilibrium between inactive (M82^{U}) and pre-active (M82^{A}) receptor forms. A second inactive, less populated conformation (M82^{D}) is observed in the carazolol bound state. **C)** The combined ^{1}H,^{13}C position of the signal of M223^{5x54} on TM5 of β_1AR correlates with the efficacy of the agonist - apo receptor (blue), carvedilol (red), 7-methylcyanopindolol (orange), cyanopindolol (green), xamoterol (yellow), salbutamol (purple), isoprenaline (pink) - suggesting that this area of the receptor

is exchanging rapidly between two states, corresponding to inactive (I) and pre-active (A) receptor in equilibrium. The population of the two states is determined by the ligand efficacy. In the full-agonist, isoprenaline bound state (A) a decrease in signal intensity occurs and variable temperature experiments (308 K, 298 K, 288 K) reveal multiple resolved peaks for M223 at lower temperature. This implies additional slower μs-ms timescale exchange processes for the pre-active state (A), revealing increased plasticity (A',A'',A''') of the full-agonist bound receptor (see Fig. 3A). **D)** Overlay of ^{1}H,^{15}N TROSY spectra showing the response of β_1AR V226^{5x57} backbone amide to agonists (apo (black), atenolol (ATE, cyan), carvedilol (CAR, red), alprenolol (ALP, purple), cyanopindolol (CYA, green), dobutamine (DOB, orange), isoprenaline (ISO, blue)). Correlation between the combined ^{1}H,^{15}N chemical shifts and the efficacy of the bound ligands is indicative of the propagation of the ligand stimulus from the orthosteric binding pocket towards the cytoplasm. **E)** ^{19}F NMR signals of β_2AR C265^{6x27} and C327^{7x54} probe conformational changes on the cytoplasmic side of the receptor. Deconvolution of the experimental data (black) in the apo and e.g. isoetharine bound state show a slow exchanging equilibrium between inactive ((I), cyan) and active ((A), red) receptor conformations. The slow exchange is in agreement with the large conformational displacements affecting this region of the receptor. The population of the two conformations (I) and (A) is sensitive to the orthosteric ligand bound, with more (A) state being populated with higher efficacy of the agonist. **F)** Overlay of ^{1}H,^{13}C HMQC spectra of β_1AR coupled to the G protein mimetic nanobody Nb6B9, showing the active state of the receptor in ligand-free form or bound to full-agonist isoprenaline, respectively. Residues M178^{4x62} on TM4/EL2 and M296^{6x41} on TM6 show distinct changes, indicating conformational differences for the two active receptor forms displayed. The insert shows an overlay zoomed in on M296^{6x41} with the Nb6B9 coupled receptor bound to a range of agonists: apo (blue), cyanopindolol (green), salbutamol (purple), isoprenaline (pink), adrenaline (brown). Signal positions correlate with the efficacy of the ligand similar to the ligand-only bound receptor (Fig. 2A), indicating that the ternary complex is rapidly exchanging between two conformations (AG^{-} and AG^{+}) (Figure 3). **G)** Dynamics of β_1AR M223^{5x54} and M296^{6x41} (TM5 and TM6 respectively) expressed as normalized intensity variations relative to M153 (ICL2) Apo (blue), agonist bound (orange; 7mC, 7-methyl-cyanopindolol; ADR, adrenaline; CVD, carvedilol; CYA, cyanopinodolol; ISO, isoprenaline; SLB, salbutamol) and ternary complex receptor states (red) are compared. Lower values indicate increased mobility on the μs-ms timescale. Increased dynamics of the full-agonist bound receptors (ISO and ADR) are observed. Ternary complex full-agonist bound states are much less dynamic although maintain a residual level of mobility relative to the reference, M153. Part B is adapted with permission from [15], Springer Nature Limited. Parts C, F, and G are adapted with permission from [14], licensed under Creative Commons 4.0. Part D is adapted with permission from [10], Springer Nature Limited. Part E is adapted with permission from [34], Science/AAAS.

Figure 3 | **Conformational dynamics of GPCRs: A)** Representative GPCR signalling model for a class A receptor based on NMR data for the β_1AR and β_2AR (see Fig. 2 for selected NMR spectra) [10,14,15,34]. The majority of the depicted equilibria conformational states are in agreement with the extended ternary model of activation [44]. Receptor states are shown colour coded with the inactive (I) form in blue, the pre-active (A) receptor in orange and the

fully active (AG) receptor coupled to an IBP shown in purple, illustrating the large outward movement of the cytoplasmic half of TM6 in the active state which enables binding of IBPs. The equilibrium between the (I) and the (A) forms represents orthosteric ligand-dependent activation. Low-level population of the (A) form in the absence of orthosteric ligand (apo state) represents the basal activity level of the receptor. The ligand-bound inactive receptor is shown in two forms, (I_1) and (I_2), that are stabilized to different amounts by inverse agonists. (I_1) is in a ligand-efficacy dependent equilibrium with the highly dynamic pre-active state (A), preferentially populated when bound to full-agonists. The increased plasticity of this full-agonist bound state, as revealed by ^{13}C NMR, is indicated by multiple conformations (A'), (A'') and (A''') that are interchanging on the μs-ms timescale. The activation model suggests that different conformations of the pre-active state vary in their ability to engage with a range of IBPs, resulting in signaling via G protein, β arrestin and other IBPs. For simplicity only the cytoplasmic interaction of (A') with G protein is shown. The agonist bound, fully active G protein coupled receptor is in equilibrium between (AG^-) and (AG^+) states with the populations determined by the efficacy of the agonist. It is suggested that (AG^-) and (AG^+) correspond to less and more active forms of the ternary complex. Thus, partial agonism manifests in both the (I)/(A) ligand-efficacy dependent equilibrium and in the equilibrium between a less and a more active ternary receptor form, with full-agonists increasing the population of the more active form. **B)** Based on ^{13}C methyl methionine NMR data on β_1AR the less (AG^-), and more active (AG^+) coupled receptor forms show conformational differences on TM5 and TM6 (Fig. 2E) [14]. These differences are hypothesized to modulate the receptor's ability to interact with Gα, which may regulate signalling via altering the GEF activity of the receptor, affecting the rate of GDP release. Thus partial agonists increase the population of (AG^-) which may reduce the turnover of GTP and hence receptor signaling. In contrast, full-agonists maximize the population of (AG^+) and hence may accelerate the release of GDP, leading to higher signaling efficacy via the G protein pathway.


Detergent micelles
MSP nanodiscs
Post-translational labelling
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GPCR construct
labelled amino acids
Insect cells
Yeast
E. coli
Cell free
Purification, Extraction
Salipro nanoparticles
BTFA
TET
$^{13}CH_3$-Lys
BTFMA
^{15}N
^{1}H
1D
HMQC
HSQC
STD
CPMG
^{13}C
^{1}H
Mechanistic models
^{19}F
NMR Experiments

Figure 1

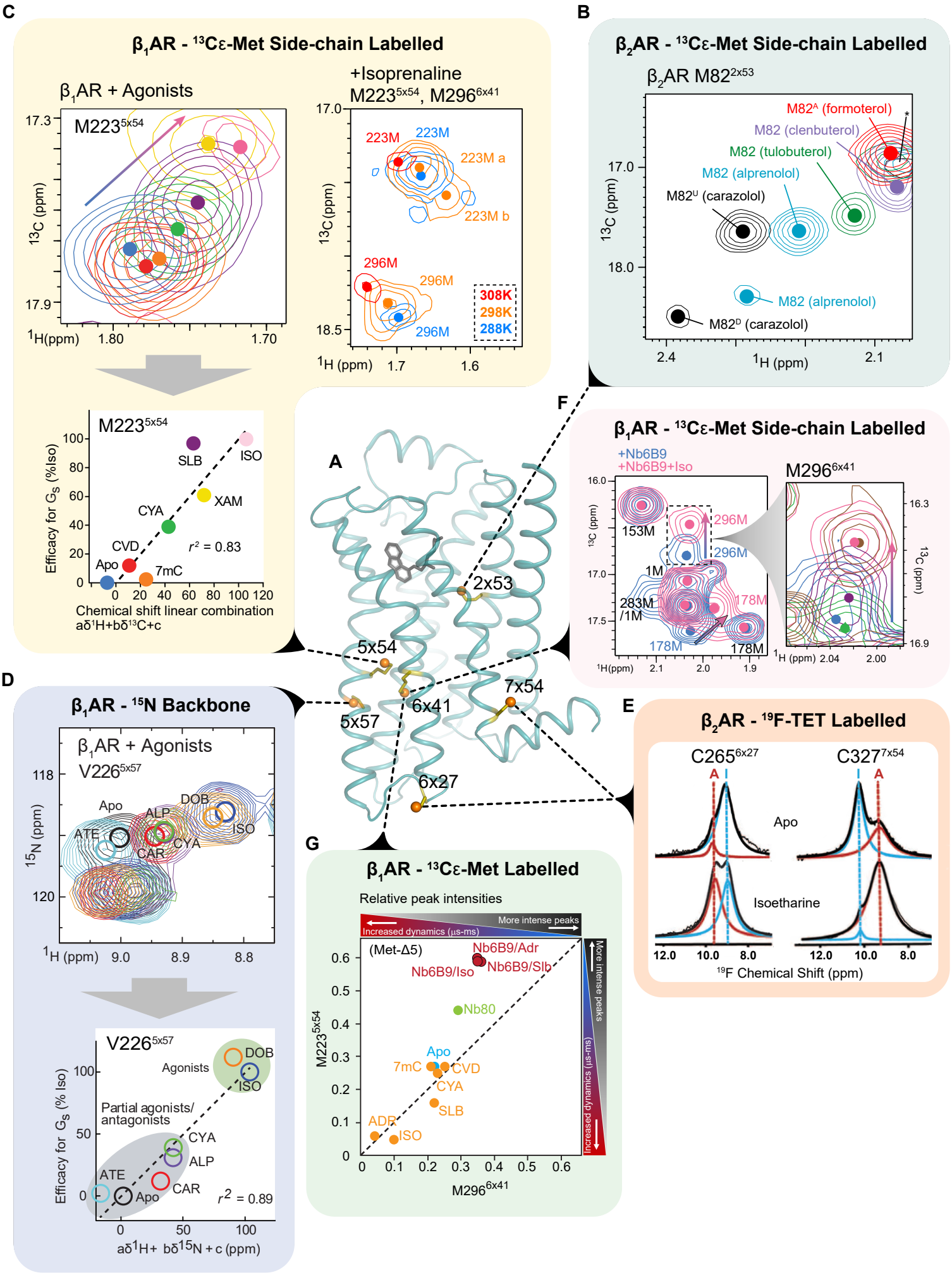
C
β1AR - 13Cε-Met Side-chain Labelled
β1AR + Agonists
M223 5x54
17.3
17.9
13C (ppm)
1H(ppm) 1.80 1.70
+Isoprenaline
M223 5x54, M296 6x41
17.0
18.5
223M
223M
223M a
223M b
296M
296M
296M
308K
298K
288K
1H (ppm) 1.7 1.6
M223 5x54
SLB
ISO
XAM
CYA
CVD
Apo
7mC
r2 = 0.83
Efficacy for Gs (%Iso)
0 20 40 60 80 100 120
Chemical shift linear combination
aδ1H+bδ13C+c
B
β2AR - 13Cε-Met Side-chain Labelled
β2AR M82 2x53
M82A (formoterol)
M82 (clenbuterol)
M82 (tulobuterol)
M82 (alprenolol)
M82U (carazolol)
M82 (alprenolol)
M82D (carazolol)
17.0
18.0
13C (ppm)
2.4 1H (ppm) 2.1
A
2x53
5x54
6x41
7x54
5x57
6x27
F
β1AR - 13Cε-Met Side-chain Labelled
+Nb6B9
+Nb6B9+Iso
153M
296M
296M
1M
283M
/1M
178M
178M
178M
M296 6x41
1H(ppm) 2.1 2.0 1.9
1H (ppm) 2.04 2.00
13C (ppm)
D
β1AR - 15N Backbone
β1AR + Agonists
V226 5x57
Apo
ALP
DOB
ATE
ISO
CAR
CYA
118
120
15N (ppm)
1H (ppm) 9.0 8.9 8.8
V226 5x57
Agonists
DOB
ISO
Partial agonists/
antagonists
CYA
ALP
ATE
CAR
Apo
r2 = 0.89
Efficacy for Gs (% Iso)
0 50 100
aδ1H+ bδ15N + c (ppm)
E
β2AR - 19F-TET Labelled
C265 6x27
C327 7x54
A
A
Apo
Isoetharine
12.0 10.0 8.0
12.0 10.0 8.0
19F Chemical Shift (ppm)
G
β1AR - 13Cε-Met Labelled
Relative peak intensities
More intense peaks
Increased dynamics (µs-ms)
(Met-Δ5)
Nb6B9/Adr
Nb6B9/Iso
Nb6B9/Slb
Nb80
Apo
7mC
CVD
CYA
SLB
ADR
ISO
0 0.1 0.2 0.3 0.4 0.5 0.6
M223 5x54
M296 6x41
More intense peaks
Increased dynamics (µs-ms)

Figure 2

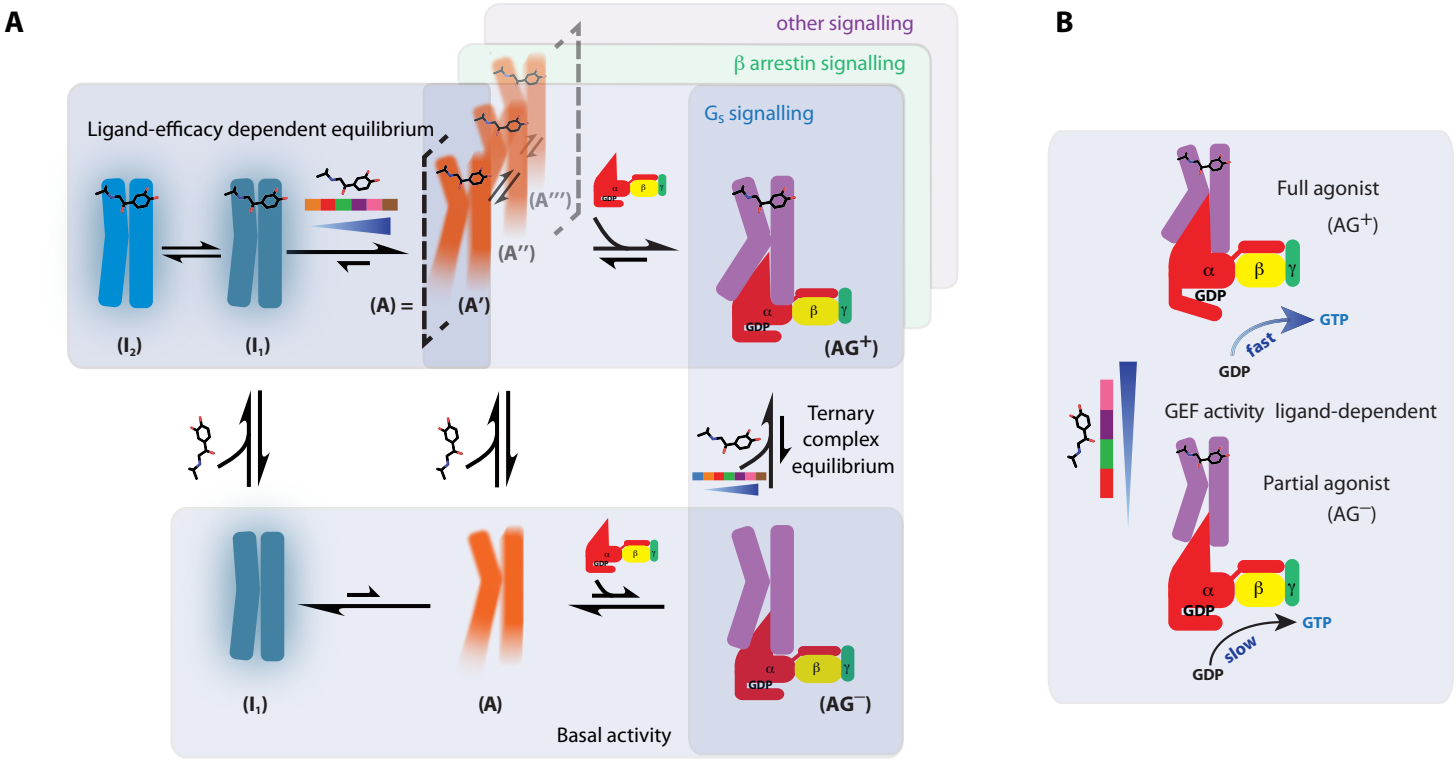
A
other signalling
β arrestin signalling
Ligand-efficacy dependent equilibrium
G_s signalling
(I_2)
(I_1)
(A) =
(A′)
(A″)
(A‴)
(AG^+)
Ternary complex equilibrium
(I_1)
(A)
Basal activity
(AG^-)
B
Full agonist
(AG^+)
GDP
GTP
fast
GEF activity ligand-dependent
Partial agonist
(AG^-)
slow

Figure 3