Probing the correlation between ligand efficacy and conformational diversity at the α_{1A}-adrenoreceptor reveals allosteric coupling of its microswitches

Received for publication, January 31, 2020, and in revised form, April 14, 2020. Published Papers in Press, April 17, 2020, DOI 10.1074/jbc.RA120.012842

© 2020 Wu et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

Feng-Jie Wu,§ Lisa M. Williams,§ Alaa Abdul-Ridha,§ Avanka Gunatilaka,§ Tasneem M. Vaid,§ Martina Kocan,§ Alice R. Whitehead,§ Michael D. W. Griffin,7§ Ross A. D. Bathgate,§ Daniel J. Scott,§ and Paul R. Gooley §‡

From the §Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville 3052, VIC, Australia, the Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville 3052, VIC, Australia and The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville 3052, VIC, Australia

Edited by Henrik G. Dohlman

G protein–coupled receptors (GPCRs) use a series of conserved microswitches to transmit signals across the cell membrane via an allosteric network encompassing the ligand-binding site and the G protein-binding site. Crystal structures of GPCRs provide snapshots of their inactive and active states, but poorly describe the conformational dynamics of the allosteric network that underlies GPCR activation. Here, we analyzed the correlation between ligand binding and receptor conformation of the α_{1A}-adrenoreceptor, a GPCR that stimulates smooth muscle contraction in response to binding noradrenaline. NMR of [13C_{3}]methionine-labeled α_{A}-adrenoreceptor variants, each exhibiting differing signaling capacities, revealed how different classes of ligands modulate the conformational equilibria of this receptor. [13C_{3}]Methionine residues near the micro-switches exhibited distinct states that correlated with ligand efficacies, supporting a conformational selection mechanism. We propose that allosteric coupling among the microswitches controls the conformation of the α_{1A}-adrenoreceptor and underlies the mechanism of ligand modulation of GPCR signaling in cells.

This work was supported by National Health and Medical Research Council (NHMRC) project Grants 1081801 (to D. J. S.), 1081844 (to P. R. G., D. J. S., and R. A. D. B.), and 1141034 (to D. J. S., P. R. G., and R. A. D. B.), and a NHMRC Boosting Dementia Research Leadership Fellowship (to D. J. S.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S11 and Table S1.

1 To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biology, The University of Melbourne, Bio21 Molecular Science and Biotechnology Institute. Tel.: 61-0-3-8344-2273; E-mail: prg@unimelb.edu.au.

‡ To whom correspondence may be addressed: The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville 3052, VIC, Australia. Tel.: 61-0-3-9035-7584; E-mail: daniel.scott@florey.edu.au.

¶ This article contains Figs. S1–S11 and Table S1.

The abbreviations used are: GPCR, G protein–coupled receptors; β_{2}-AR, β_{2}-adrenergic receptor; μ-OR, μ-opioid receptor; BPH, benign prostatic hyperplasia; DDM, dodecyl-β-D-maltopyranoside; HMQC, heteronuclear multiple quantum coherence; MD, molecular dynamics; TMD, transmembrane domain; IP, inositol phosphate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; QAPB, quinazoline piperazine BODIPY; PDB, Protein Data Bank; α_{1A}-AR, α_{1A}-adrenoreceptor.

This is an Open Access article under the CC BY license.
inactive and active conformations, all existing in equilibrium. Ligands preferentially bind to particular receptor states, depending on their pharmacological characteristics, thus shifting the conformational equilibrium toward these preferred states and modulating the signaling output of the system. The extracellular orthosteric ligand-binding site in adrenoceptors is connected to the intracellular G protein-binding site through a series of conserved microswitches (24–27) (Fig. 1): a central transmission switch (also called the connector region, CWXP motif or PIF motif (28)), the NPXXY switch, and the intracellular G protein-binding site, characterized by the DRY motif (or switch). How these microswitches coordinate the transmission of the extracellular signal is not clear, but molecular dynamics (MD) simulations and NMR data have led to a mechanistic description of “loose allosteric coupling” (28).

This mechanism refers to each microswitch as conformationally independent from the others, which as an active DRY motif state is not significantly dependent on an active state in the transmission switch. That said, an active state in the transmission switch does increase the probability of the DRY motif (and thus the receptor) to sample active states (thus, loose allosteric coupling) (28). Put simply, the conformational changes that occur in the microswitches are thought to drive the overall equilibrium state of the receptor system. Despite recent work, it is not well-understood how the binding of ligands such as inverse agonists influence the microswitch state equilibrium to decrease basal receptor activity.

α1-Adrenoceptors (α1-ARs) comprise three Gq-coupled GPCR subtypes (α1A-, α1B-, and α1D-) that bind and sense the endogenous catecholamines, adrenaline and noradrenaline, to modulate a range of physiological processes. In the periphery, postsynaptic α1-AR stimulation by catecholamines mediates smooth muscle contraction, thus α1-AR antagonists and inverse agonists are clinically prescribed to treat hypertension and benign prostatic hyperplasia (BPH) (29). α1-ARs are also widely expressed in the central nervous system, but the lack of subtype-selective antibodies and ligands limits the understanding of their role in neuroplasticity and neurodegeneration (30). Currently there are no available crystal structures of an α1-AR family member, which limits the rational design of more selective compounds to probe the physiological role of α1A-adrenoceptor (α1A-AR) in the central nervous system.

Recombinant α1A-AR expresses poorly and the resultant protein is particularly unstable when purified in detergent (31), which has hindered biochemical studies of this GPCR. Recently, we engineered an α1A-AR variant, α1A-AR-A4, that can be expressed in Escherichia coli and exhibits improved stability when purified in detergents (32). When expressed in COS-7 cells α1A-AR-A4 exhibits no signaling efficacy in response to adrenaline stimulation (32). In the present study, α1A-AR-A4 was labeled with [13C3]methionine at the five naturally occurring methionine residues, providing NMR probes to assess how inverse agonists, partial agonists, and full agonists influence receptor conformational equilibria. Three of these methionines are excellent probes of the ligand-binding site and the microswitches proposed to be markers of signal transmission: Met-2926.55 (superscript denotes GPCRdb numbering (33)) is located in the ligand-binding site; Met-1153.41 is proximal to the transmission switch (Ile-1143.40, Pro-1965.50, Leu-1975.51, Phe-2816.44, and Trp-2856.48); and Met-2035.57 sits above the tyrosine of the DRY motif (Asp-1303.49, Arg-1313.50, and Tyr-1323.51). Using the inactive α1A-AR variant, α1A-AR-A4, and by reverse mutation to an active receptor (α1A-AR-A4–active) we show that for Met-1153.41 and Met-2035.57 the chemical shifts and line widths of the 13C3H3 groups are dependent on ligand efficacy (from strong inverse agonist to full agonist), suggesting that α1A-AR activation proceeds primarily through a conformational selection mechanism.
Correlation of conformation with ligand efficacy for α1A-AR

Results

**7^3^C^1^H_3 Methionine labeling and NMR signal assignment**

α1A-AR-A4 is a thermostabilized variant of the human α1A-AR that contains 15 amino acid substitutions over wildtype (WT) human α1A-AR (Fig. S1). Excluding Met-1, α1A-AR-A4 possesses six methionine residues, five of which are naturally occurring (Met-1153.41, Met-1454.44, Met-2035.57, Met-248ICL3, and Met-2926.55) and one, Met-802.58, is a thermostabilizing mutation previously selected for using directed evolution for detergent stability (32) (Fig. S1). Homology models of α1A-AR (Fig. 1) built on in inactive and active states of X-ray structures of β2-AR show that three of these methionines were particularly interesting as conformational probes as they are located either within the adrenaline-binding site (Met-2035.57), immediately adjacent to the highly conserved Ile1143.40 of the transmission switch (Met-1153.41), or sitting above Tyr-1253.51 of the DRY motif within the G protein-binding site (Met-2926.55). These homology models of α1A-AR suggest that each of these regions undergo significant local rearrangements between inactive to active conformations (Fig. 1).

α1A-AR-A4 was expressed and labeled with [13C3]methionine using an adapted E. coli methionine biosynthesis pathway inhibition protocol that we have previously used to generate [13C3]methionine-labeled neurotensin receptor 1 (NTS1) samples labeled with 96% incorporation efficiency (34, 35). Using this method α1A-AR-A4 expressed well and could be purified, solubilized in n-dodecyl-β-d-maltopyranoside (DDM), with a yield of (0.5–1 mg/liter of culture). 40–60 μM samples of [13C3]methionine-labeled α1A-AR-A4 were subsequently used to record 2D 1H-13C SOFAST-HMQC spectra in the apo state, and in the bound states for prazosin (full inverse agonist), WB-4101 (partial inverse agonist), phentolamine (partial agonist), oxymetazoline (partial agonist), silodosin (or KMD-3213, neutral antagonist), oxymetazoline (partial agonist), and adrenaline (full agonist) (Fig. 2 and Fig. S2). Individual [13C3]methionine resonances were assigned by expressing and analyzing α1A-AR-A4 M80L, α1A-AR-A4 M1151, α1A-AR-A4 M203L, α1A-AR-A4 M248I, and α1A-AR-A4 M292I mutants in the same way. 1H-13C SOFAST-HMQC spectra enabled clear assignment of mutated methionines as the remaining five resonances in these spectra showed only small chemical shift differences in the presence of the mutation (Fig. S3). The [13C3]methionine of the apo state of α1A-AR-A4 showed clear single resonances for each methyl with no significant heterogeneity (Fig. 2, in contrast to many previously studied GPCRs (5, 8, 14, 20–22). Met-1454.44 and Met-248ICL3 exhibited intense signals with 1H and 13C chemical shifts of the methyl group indicative of solvent-exposed, unrestrained methyl groups. Met-248ICL3, located within ICL3 (Fig. 1A), showed strong signal intensity most likely due to the mobility of this loop and exposure to the bulk solvent. Met-1454.44 is at the C-terminal intracellular end of TM4, predicted to be exposed on the surface of the helix (Fig. 1A) and thus also highly mobile. Met-802.58 was not unambiguously assigned (Fig. S3, A and F) as it either is significantly broadened and difficult to resolve in all receptor states or may overlap with Met-1454.44 and under some conditions with Met-2926.55 (Fig. S3E). The remaining methionines, Met-1153.41, Met-2035.57, and Met-2926.55, were readily assigned (Fig. S3, B, C, E, G, H, and J) and exhibited resolved chemical shifts for the 13C3H3 that were sensitive to the bound ligand (Fig. 2, B–D).
Correlation of conformation with ligand efficacy for \(\alpha_{1A}\)-AR

Based on homology models, Met-292\(^{6.55}\) projects into the orthosteric ligand-binding pocket (Fig. 1A) and mutational studies support a role for this residue in ligand binding (36). Thus, the \[^{13}\text{C}CH_3\] chemical shifts of Met-292\(^{6.55}\) likely reflect a direct interaction with chemical groups of each ligand. Interestingly, the resonance intensities of Met-292\(^{6.55}\) increased in the presence of antagonists and inverse agonists relative to the apo state (Fig. 2B), indicating that binding of these ligands reduces conformational dynamics in the orthosteric binding site. Met-115\(^{3.41}\) and Met-203\(^{3.57}\) are distant from the orthosteric site, but both the chemical shifts and line widths of their \[^{13}\text{C}CH_3\] groups were sensitive to ligand binding (Fig. 2, C and D), likely reflecting receptor conformational changes in the transmission switch and G protein-binding site, respectively (Fig. 1, C and D). The \(^1\text{H}\) chemical shift of the methyl of Met-203\(^{3.57}\) was shifted upfield from typical small-peptide positions (2.1 ppm) to 1.58 ppm in agreement with our models, which predict ring-current induced effects from Tyr-125\(^{3.51}\) of the DRY motif (Fig. S4). Met-203\(^{3.57}\) therefore serves as a probe of conformational changes within this region. Indeed, the resonances of the \[^{13}\text{C}CH_3\] of Met-203\(^{3.57}\) exhibited a significant linear chemical shift change depending on which ligand was bound, demonstrating that allosteric coupling between the ligand-binding site and the G protein-binding site is retained in the inactive \(\alpha_{1A}\)-AR-A4 in solution. Such a linear chemical shift change is also expected for ligands modulating the receptor state via conformational selection. We postulate that the Met-203\(^{3.57}\) signal reflects the average, equilibrium signal, between inactive and active states undergoing fast exchange. Inverse agonists preferentially bound to inactive states, shifting the Met-203\(^{3.57}\) equilibrium to an upfield position (inactive state) compared with the apo state receptor, which can sample active-like states to a certain degree.

We were interested to see if an opposite trend could be observed for receptor agonists, which we hypothesized would shift the position of the Met-203\(^{3.57}\) resonance downfield. For \(\alpha_{1A}\)-AR-A4, however, the binding of the full agonist adrenaline to \(\alpha_{1A}\)-AR-A4 resulted in complete line broadening of the Met-115\(^{3.41}\) and Met-203\(^{3.57}\) resonances despite the promotion of a distinct chemical shift for Met292\(^{6.55}\) in the binding site. Binding of the partial agonist oxymetazoline resulted in substantial broadening of Met-203\(^{3.57}\) and Met-292\(^{6.55}\), but not Met-115\(^{3.41}\). The loss of these chemical shifts upon agonist binding was likely due to the significantly weaker agonist affinities at \(\alpha_{1A}\)-AR-A4 compared with unmutated, WT \(\alpha_{1A}\)-AR, as a result of the F312L-stabilizing mutation (32). Thus, NMR experiments were repeated on \(\alpha_{1A}\)-AR-A4 (L312F), for which agonist affinities were largely restored to that of WT \(\alpha_{1A}\)-AR (Fig. S5 and Table S1) (32).

Agonist-induced chemical shifts of Met-115\(^{3.41}\) and Met-203\(^{3.57}\) resonances

Despite the reduced thermostability of \(\alpha_{1A}\)-AR-A4 (L312F) (32), we were able to \[^{13}\text{C}CH_3\]methionine label and record \(^1\text{H}-^{13}\text{C}\) SOFAST-HMQC spectra for this receptor in the apo state and bound to adrenaline (full agonist), phenylephrine (full agonist), A-61603 (full agonist), and oxymetazoline (partial agonist) in addition to the inverse agonists and neutral antagonists tested on \(\alpha_{1A}\)-AR-A4 (Fig. 3A). Overall, the \(^1\text{H}-^{13}\text{C}\) SOFAST-HMQC spectra of the apo, antagonist, and inverse agonist-bound states of \(\alpha_{1A}\)-AR-A4 (L312F) were similar to those of \(\alpha_{1A}\)-AR-A4. Again, single resonances for the \[^{13}\text{C}CH_3\]methionine groups of \(\alpha_{1A}\)-AR-A4 (L312F) were observed for all ligands. The chemical shifts of Met-292\(^{6.55}\) induced by each ligand in \(\alpha_{1A}\)-AR-A4 (L312F) were slightly different to those of \(\alpha_{1A}\)-AR-A4, most likely due to orthosteric binding site changes after the L312F reversion. Inverse agonist binding increased the intensity of the Met-292\(^{6.55}\) resonance in \(\alpha_{1A}\)-AR-A4 (L312F), as was seen with \(\alpha_{1A}\)-AR-A4; whereas the neutral antagonist silodosin significantly decreased the peak intensity and the partial agonist oxymetazoline and full agonist A-61603 highly broadened the resonance of Met-292\(^{6.55}\) in \(\alpha_{1A}\)-AR-A4 (L312F) (Fig. S6).

The recovered agonist affinity for \(\alpha_{1A}\)-AR-A4 (L312F) allowed the measurement of \(^1\text{H}-^{13}\text{C}\) SOFAST-HMQC spectra where we were confident of full receptor-agonist saturation. Binding of the full agonist adrenaline to \(\alpha_{1A}\)-AR-A4 (L312F) produced a similar Met-292\(^{6.55}\) chemical shift to that seen with \(\alpha_{1A}\)-AR-A4 (Fig. 3A), and also weak peaks were now observed for Met-115\(^{3.41}\) and Met-203\(^{3.57}\) (Fig. 3, B and C), which were completely broadened in adrenaline-bound \(\alpha_{1A}\)-AR-A4. Importantly, the binding of all agonists, adrenaline, phenylephrine, and A-61603 to \(\alpha_{1A}\)-AR-A4 (L312F) induced distinct chemical shift and line broadening changes to Met-115\(^{3.41}\) and Met-203\(^{3.57}\) compared with neutral antagonists and inverse agonists (Fig. 3, B and C). The agonist-induced Met-115\(^{3.41}\) resonances cluster together as potentially indicative of an active transmission switch conformation (Fig. 3B). Binding of the partial agonist oxymetazoline induced a chemical shift of Met-115\(^{3.41}\) falling between the inverse agonist and full agonist clusters, consistent with partial agonists promoting a weaker shift in the inactive-active transmission switch state equilibrium. The linear change in the Met-203\(^{3.57}\) chemical shift position upon inverse agonist binding seen with \(\alpha_{1A}\)-AR-A4 was retained in \(\alpha_{1A}\)-AR-A4 (L312F), but as hypothesized, agonist binding promoted opposite, downfield resonance shifts in the \(^{13}\text{C}\) dimension along the same vector (Fig. 3C). This change in \[^{13}\text{C}CH_3\]methionine chemical shift in the \(^{13}\text{C}\) dimension reflects a change in the \(\chi_3\) dihedral angle. The \(^{13}\text{C}\) chemical shift dependence of this angle is about 19 ppm for trans and 16 ppm for \(\pm\) gauche (37). For the apo and antagonist states the chemical shift of 18.25 to 18.5 ppm suggests a trend toward trans, whereas in the full-inverse agonist state the resonance shifts up-field to 17.7 ppm, indicative of an averaging between gauche and trans. Consistent with our homology models (Fig. S4) for full agonist a further downfield shift between 19 and 19.25 ppm infers an increase in the trans conformer. Interestingly, the partial agonist oxymetazoline induced a small upfield \(^{13}\text{C}\) shift of Met-203\(^{3.57}\), similar to the inverse agonist phenylephrine. The fact that full agonists induced Met-203\(^{3.57}\) chemical shifts to move in the opposite direction to inverse agonists suggests an equilibrium shift away from inactive to active conformational states of the DRY motif. Furthermore, the resonance intensities of both Met-115\(^{3.41}\) and Met-203\(^{3.57}\) in \(\alpha_{1A}\)-AR-A4 (L312F), relative to the ligand-insensitive Met-145\(^{7.44}\) resonance, were weakened upon agonist binding compared with the
Correlation of conformation with ligand efficacy for $\alpha_{1A}$-AR

intensity increases seen with antagonists and inverse agonists (Fig. 3D). The intensities of Met-115$^{3,41}$ and Met-203$^{5,57}$ upon binding of the partial agonist oxymetazoline fell between the antagonist- and agonist-induced intensities. The behavior of the $^{13}$C$^3$H$_3$ of Met-115$^{3,41}$ and Met-203$^{5,57}$ is consistent with the current concept that agonists increase conformational heterogeneity in GPCRs, where agonists increase microsecond timescale transitions to active receptor states, to increase the probability of engaging and activating effector proteins (5, 8, 10, 14, 15, 23).

**Chemical shift changes of Met-203$^{5,57}$ correlate with ligand efficacy**

In mammalian cells, $\alpha_{1A}$-AR exhibits basal activity in the absence of bound ligands (38). Such basal activity is unaffected by the binding of neutral antagonists but is reduced by the binding of inverse agonists to the receptor. In the case of $\alpha_{1A}$-AR, by probing the ability of various antagonists to reduce the signaling of a constitutively active receptor mutant, the rank order of inverse agonist efficacies has been found to be: prazosin (strongest); WB-4101, phentolamine (weakest), and silodosin being a neutral antagonist (38). To understand how the NMR signals of Met-203$^{5,57}$ in $\alpha_{1A}$-AR-A4 (L312F) relate to receptor conformational equilibria, the changes to the chemical shifts for the $^{13}$C$^3$H$_3$ of Met-203$^{5,57}$ were plotted against the previously published relative efficacy values for the inverse agonists, revealing a strong linear correlation ($R^2 = 0.99$, Fig. 4A). To test if this correlation is retained when probing inverse agonism at the WT $\alpha_{1A}$-AR, we determined the relative inverse agonist efficacies of these ligands using a NanoBiT split luciferase assay (39). In this assay the 18-kDa Large BiT (LgBiT) fragment was fused to the N terminus of G$_\alpha_q$ and the 1.3-kDa Small BiT (SmBiT) was fused to the N terminus of G$_\gamma_2$. When co-expressed with G$_\beta_1$, the formation of a G$_\alpha_q$(LgBiT)-G$_\beta_1$-G$_\gamma_2$(SmBiT) heterotrimer results in bright luminescence. GPCR-induced stimulation of this G protein complex causes dissociation of the heterotrimer and thus reduction in luminescence output, whereas inhibition of basal GPCR activation would be predicted to increase luminescence output. COS-7 African green monkey kidney cells stably expressing WT $\alpha_{1A}$-AR were transfected with G$_\alpha_q$(LgBiT), G$_\beta_1$, and G$_\gamma_2$(SmBiT) encoding expression plasmids, incubated with luminescence substrate, and then treated with various $\alpha_{1A}$-AR ligands, while monitoring cellular luminescence. A-61603 induced $\alpha_{1A}$-AR activation led to heterotrimer dissociation of the G$_\alpha_q$(LgBiT)-G$_\beta_1$-G$_\gamma_2$(SmBiT) complex and thus a reduction in luminescence output (Fig. 4B). Inverse agonists on the other hand reduced basal activation of $\alpha_{1A}$-AR, maintaining the G$_\alpha_q$(LgBiT)-G$_\beta_1$-G$_\gamma_2$(SmBiT) complex leading to increased luminescence output from the cells (Fig. 4B). The specificity of these responses was probed by conducting the same experiments on COS-7 cells not expressing $\alpha_{1A}$-AR (Fig. S7, A–C). The observed changes in luminescence after $\alpha_{1A}$-AR ligand treatments were specific to $\alpha_{1A}$-AR expressing cells except for WB-4101, which induced a short (5 min) increase in luminescence in the control cells (Fig. S7A). To exclude this nonspecific effect the net luminescence change for each sample group was calculated as the area under the luminescence curves between

---

[Image of chemical structures and spectra related to the text]
Correlation of conformation with ligand efficacy for α_{1A}AR

5 and 10 min after ligand addition. A strong linear correlation was found between the chemical shift changes for the $^{13}$C/H$_3$ of Met-203$^{5,57}$ in α_{1A}AR-A4 (L312F) and the net luminescence increase generated by each inverse agonist over the 5-min period in the WT α_{1A}AR-expressing cells ($R^2 = 0.72$, Fig 4C). Importantly, the Met-203$^{5,57}$ chemical shift positions of α_{1A}AR-A4 (L312F) did not correlate with the net luminescence changes in COS-7 cells not expressing WT α_{1A}AR (Fig S7B). Critically, the correlation between chemical shift changes of $^{13}$C/H$_3$Met-203$^{5,57}$ in α_{1A}AR-A4 (L312F) and WT α_{1A}AR–specific luminescence increases in the NanoBit assay remained when the analysis window was extended to include the full 10 min after ligand addition (Fig S7D).

Improving signaling competency in α_{1A}AR-A4

When expressed in COS-7 cells, α_{1A}AR-A4 is incapable of stimulating cellular increases in inositol phosphate 1 (IP$_1$) in response to adrenaline binding, or activation of a cAMP response element (CRE) reporter gene after treatment with another α_{1}AR agonist, phenylephrine (32). To ensure biological relevance of our NMR studies we thus sought α_{1A}AR-A4 back-mutants that were able to stimulate canonical signaling pathways in mammalian cells upon agonist treatment. Seven thermostabilizing mutations within the TMD of α_{1A}AR-A4 were back-mutated (Y67N, M80L, A127G, K322N, L327P, and Y329S) was able to facilitate significant oxymetazo-line-induced cellular accumulation of IP$_1$ compared with α_{1A}AR-A4 and WT α_{1A}AR (Fig S8A) it expressed poorly in bacteria. The back-mutant α_{1A}AR–AR–A4 (Y67N, K322N), termed α_{1A}AR–AR–A4–active, however, was able to stimulate IP$_1$ accumulation in response to both phenylephrine and oxymetazoline treatment (Fig S8A) and it expressed well in bacteria. Importantly, α_{1A}AR–A4 contains the N322K-stabilizing mutation in the NPXXY switch (25), which is hypothesized to form a stabilizing salt bridge with Asp-$^{72}$-$^{50}$ to lock the NPXXY switch in an inactive and stable state. We thus expected that reversion of this mutation (K322N) would restore the function of the NPXXY switch and the signaling activity of α_{1A}AR–A4. Interestingly, the Y67N mutation was required on top of K322N to restore signaling activity in α_{1A}AR–A4–active. Asn-$^{62}$-$^{45}$ is distant from the NPXXY switch and its importance is not clear.

Using an intracellular calcium mobilization assay, α_{1A}AR–A4–active was able to be activated by the full agonists adrenaline and A-61603, as well as the partial agonists oxymetazoline and PF-3774076 (Fig. S8, B–E). The affinity of QAPB for α_{1A}AR–AR–A4–active was retained upon purification of the receptor in DDM (Fig S9A). Competition binding assays revealed, however, that the affinities of agonists for α_{1A}AR–A4–active (Fig. S9B and Table S1) were weaker than WT α_{1A}AR due to the F312L stabilizing mutation, but stronger than at α_{1A}AR-A4 (32). When purified in DDM α_{1A}AR–AR–A4–active was significantly less stable than α_{1A}AR–A4 (Fig S9C) and thus back-mutation of F312L to recover agonist potency was not pursued as it was deemed unlikely that the resultant receptor would be stable enough for NMR experiments.

α_{1A}AR–A4–active was labeled with $^{13}$C/methionine and 2D $^{1}$H/$^{13}$C SOFAST-HMQC spectra were acquired as above (Fig 5, A and D). Overall the ligand-ruptured chemical shifts of the $^{13}$C/methionine resonances in α_{1A}AR–A4–active were similar to those in α_{1A}AR–A4 and α_{1A}AR–AR–A4 (L312F), except for several key differences with the Met-115$^{3,41}$ and Met-203$^{5,57}$ resonances. We acquired spectra of four independent preparations of apo α_{1A}AR–AR–A4–active (four biologi-

**Figure 4. Correlation between the chemical shift positions of the $^{13}$C/H$_3$ in Met-203$^{5,57}$ and inverse agonists efficacy.** A, linear regression analysis of the average chemical shift differences ($\Delta \delta$) for the $^{13}$C/H$_3$ of Met-203$^{5,57}$ in α_{1A}AR-A4 (L312F) when bound to prazosin (black circles), WB-4101 (orange circles), and phentolamine (purple circles) compared with silodosin (blue circles) and the published efficacy of each ligand in reducing the signaling of a constitutively active mutant of α_{1A}AR (38). Published data were extracted using WebPlotDigitizer (RRID: SCR_013996). Testing the resultant equation against the null hypothesis of a slope of zero resulted in a p value of <0.0001. B, NanoBit G protein activity assay demonstrating inverse agonism of prazosin, WB-4101, phentolamine, and silodosin at WT α_{1A}AR–expressing COS-7 cells. Each of these inverse agonist experiments were repeated in three independent biological replicate experiments, with the mean ± S.E. of the resultant luminescence plotted for each time point. To demonstrate the response from an agonist, A-61603 treatment was performed in two independent biological replicate experiments. Each biological replicate comprised three technical replicates measured in parallel. The gray shaded region indicates where the area under each biological replicate curve was calculated for C. C, linear regression analysis of the average chemical shift differences ($\Delta \delta$) for the $^{13}$C/H$_3$ of Met-203$^{5,57}$ in α_{1A}AR-A4 (L312F) and the increase in luminescence seen in the NanoBit assay for each inverse agonist and neutral antagonist. Ligands are colored as listed above and the p value testing against a slope of 0 was 0.011. D, linear regression analysis of the average chemical shift differences ($\Delta \delta$) for the $^{1}$H of Met-203$^{5,57}$ in α_{1A}AR-A4 (L312F) and the affinities of each inverse agonist and neutral antagonist. Ligands are colored as listed above and the p value testing against a slope of 0 was 0.89. In A, C, and D, $\Delta \delta$ is plotted for two independent titrations of prazosin and silodosin, and single experiments for WB-4101 and phentolamine. Average chemical shift differences ($\Delta \delta$) were normalized using the equation $\Delta \delta = (\sqrt{\Delta \delta_{\text{H}}}) + (\Delta \delta_{\text{C}}/3.5)$, and error was calculated by the formula $\sqrt{(\Delta \delta_{\text{H}}^2 \times \Delta \delta_{\text{C}}^2)}$.
**Correlation of conformation with ligand efficacy for $\alpha_{1A}$-AR**

Figure 5. $^1$H-$^1$C SOFAST-HMQC spectra of $\alpha_{1A}$-AR-A4–active. A, Overlay of 2D $^1$H-$^1$C SOFAST-HMQC spectra of ([13C]Met$^-$) $\alpha_{1A}$-AR-A4–active bound to prazosin (black, inverse agonist), WB-4101 (orange, inverse agonist), phenotamine (purple, inverse agonist), and silodosin (blue, neutral antagonist). B, Close-up of the Met-2035 $^1$H resonance. C, Linear regression analysis of the average chemical shift differences ($\Delta\delta$) for the $^1$H$^1$C of Met-2035 $^1$H in $\alpha_{1A}$-AR-A4–active when bound to prazosin (black circles), WB-4101 (orange circles), and phenotamine (purple circles) compared with silodosin (blue circles) and the increase in luminescence seen in the NanoBit assay with $\alpha_{1A}$-AR-A4–active expressing COS-7 cells treated with the same antagonist (from Fig. S11A). Testing the resultant equation against the null hypothesis of a slope of zero resulted in a $p$ value of 0.0041. D, Overlay of 2D $^1$H-$^1$C SOFAST-HMQC spectra of ([13C]Met$^-$) $\alpha_{1A}$-AR-A4–active bound to prazosin (black, inverse agonist), silodosin (blue, neutral antagonist), oxymetazoline (cyan, partial agonist), PF-3774076 (magenta, partial agonist), A-61603 (maroon, full agonist), and adrenaline (green, full agonist). E, Close-up of the Met-115 $^1$H resonance. F, Linear regression analysis of the average chemical shift differences ($\Delta\delta$) for the $^1$H$^1$C of Met-115 $^1$H in $\alpha_{1A}$-AR-A4–active when bound to oxymetazoline (cyan circles), PF-3774076 (pink circles), A-61603 (dark red circles), adrenaline (green circles), and silodosin (blue circles) and the efficacy of each agonist in triggering Ca$^{2+}$ mobilization in $\alpha_{1A}$-AR-A4–active expressing COS-7 cells (from Fig. S8, B–E). The resultant equation against the null hypothesis of a slope of zero resulted in a $p$ value of 0.0154. C and F, $\Delta\delta$ are plotted for two independent titrations of prazosin, silodosin, and oxymetazoline, and single experiments for other ligands. In A, B, D, and E, spectra were acquired on $-50 \mu M$ $\alpha_{1A}$-AR-A4–active dissolved in 0.02–0.1% DDM micelle, pH 7.5, and 25°C. Average chemical shift differences ($\Delta\delta$) were normalized using the equation $\Delta\delta = (\Delta\delta^{H_1}) + (\Delta\delta^{13C}/3.5)R^{1H}R^{13C}/(3.5)^2$ and errors were calculated by the formula $R^{1H} = R^{13C}/(3.5)$, where $R^{1H}$ and $R^{13C}$ are the digital resolutions in ppm in the $^1$H and $^1$C dimensions respectively (S).

cal replicates) and found that in the absence of bound ligand the data were not easily reproduced (Fig. S10A). The well-resolved spectra were not as distinct as now observed for $\alpha_{1A}$-AR-A4 (L312F) did not show two discrete peaks for Met-115 $^1$H and Met-2035 $^1$H. Such apo state sample-to-sample heterogeneity may suggest the presence of misfolded contaminants, but upon the addition of prazosin or silodosin each of these samples gave identical spectra (Fig. S10A), supporting the binding competency of the $\alpha_{1A}$-AR-A4–active samples. The diversity of apo state spectra likely reflects diversity of conformational states of similar free energy. The addition of agonist again resulted in a single resonance for $[13C]^{1H}$-Met115 $^1$H that shifts upfield in $^1$H and downfield in $^1$C (Fig. 5E). The trend in shifts of these resonances, however, suggests they follow in a linear manner evolving from the downfield (basal) signal of the apo state and reflects the selection of the active-like state.

A major difference between the spectra of $\alpha_{1A}$-AR-A4 and $\alpha_{1A}$-AR-A4–active, however, was significantly increased line broadening of the Met-2035 $^1$H signal of $\alpha_{1A}$-AR-A4–active in the apo state (Fig. S10A) and when bound to antagonists (Fig. 5B). This broadening suggests that the DRY motif near the G protein-binding site of $\alpha_{1A}$-AR-A4–active is more dynamic compared with $\alpha_{1A}$-AR-A4, consistent with a receptor that more readily transitions between inactive and active-receptor states. Importantly, similar to $\alpha_{1A}$-AR-A4 and $\alpha_{1A}$-AR-A4–active
Correlation of conformation with ligand efficacy for \(\alpha_{1A}-\text{AR}\)

(L312F) variants, the \(^{13}\text{C}H_3\) of Met-203\(^{5.57}\) shows an efficacy-dependent linear \(^{13}\text{C}H_3\) chemical shift change in the presence of inverse agonist and neutral antagonist, trending to an upfield \(^{13}\text{C}\) position (\(\chi^3\) of \(\pm \text{gauche}\)) for the more potent inverse agonist (Fig. 5, A and B). Unexpectedly, the addition of silodosin (neutral antagonist) resulted in a significant \(^{13}\text{C}\) downfield shift to near 19.5 ppm consistent with a trans \(\chi^3\) angle for Met-203\(^{5.57}\). Furthermore, similar to \(\alpha_{1A}-\text{AR}\), the Met-203\(^{5.57}\) \(^{13}\text{C}H_3\) resonance of \(\alpha_{1A}-\text{AR}\)-A4–active was near completely broadened in the presence of agonists.

NanoBiT \(\text{G protein activity assays were performed on COS-7 cells expressing } \alpha_{1A}-\text{AR-A4–active to determine relative inverse agonist efficacies. The inverse agonists reduced basal } G_q \text{ activity in } \alpha_{1A}-\text{AR-A4–active in a similar way as WT } \alpha_{1A}-\text{AR expressing cells (Fig. 4A and Fig. S1A)}. The net luminescence change induced by each inverse agonist at \(\alpha_{1A}-\text{AR-A4–active expressing cells correlated well, in a linear fashion, with the } 13\text{C}_2 \text{chemical shift changes of Met-203}^{5.57} \text{that each ligand induced at purified } \alpha_{1A}-\text{AR-A4–active, when analyzed over two separate time periods (Fig. 5C and Fig. S1B). Interestingly, the agonist-induced chemical shift changes of } [^{13}\text{C}H_3]\text{Met-115}^{3.41} \text{showed a linear correlation with the efficacy of each agonist in Ca}^{2+} \text{mobilization assays on } \alpha_{1A}-\text{AR-A4–active expressing COS-7 cells (Fig. 5F) although the partial agonist PF-3774076 was a notable outlier. Overall, these cell-based assays with the ligand efficacy-correlated chemical shift changes of Met-115}^{3.41} \text{and Met-203}^{5.57} \text{clearly demonstrate that a conformational selection mechanism underlies receptor function in cells.}

Discussion

Recent spectroscopic studies have demonstrated that different classes of GPCR ligands distinctly alter the population of receptor states within the GPCR conformational equilibrium (23). GPCR conformational changes are driven by defined structural changes in the microswitches (24–26, 28) (Fig. 1) and, thus, how particular ligands affect the GPCR microswitch states likely underlies their pharmacological output as inverse, partial, full, or biased agonists. Observing these effects, however, remains challenging. \(\alpha_{1A}-\text{AR}\) was one of the first GPCRs to be cloned and pharmacologically characterized (40) and is clinically targeted with agonists as nasal decongestants and antagonists for hypertension and BPH. Despite the importance of this receptor there are currently no three-dimensional structures of \(\alpha_{1A}-\text{AR}\), reflecting the inherent instability of this protein. Here, we demonstrate that prototypical ligands modulate the conformational equilibrium, as measured at the microswitches, of \(\alpha_{1A}-\text{AR}\) in defined and predictable ways by \(^{13}\text{C}H_3\) methionine labeling \(\alpha_{1A}-\text{AR}\) variants and monitoring the \(^1\text{H}\) and \(^{13}\text{C}\) chemical shifts of these methyl resonances in the presence of ligands of different efficacy.

It is well-accepted that the NMR signals of methionine methyl groups are sensitive to the local environment and the conformation of the methionine side chain (5, 8). Many of the conclusions made in this study rely on Met-115\(^{3.41}\), a probe for the conformation of the transmission switch, and Met-203\(^{5.57}\) as a probe of the DRY motif that signifies intracellular TMD rearrangements for G-protein binding. In our model of \(\alpha_{1A}-\text{AR}, \text{Met-203}^{5.57} \text{sits over Tyr-125}^{3.51}\) of the DRY motif but is distant from Arg-124\(^{5.50}\), which is expected to undergo significant rotameric changes within this motif (2) (Fig. 1D). Met-115\(^{3.41}\) is sequential to the Ile-114\(^{3.46}\) in TM3 but it points away and is distant to the transmission switch residue Phe-281\(^{6.44}\) located on TM6, which is expected to undergo significant rotameric changes (Fig. 1C). Although, in the thermostabilized inactive \(\alpha_{1A}-\text{AR}-\text{A4}\) mutant the residues of the transmission switch and DRY motif are retained, the asparagine of a third microswitch, the NPXXY motif, is mutated to lysine, which likely forms a salt bridge with Asp-72\(^{7.56}\) to lock this switch in an inactive state. The transmission switch and NPXXY motif are proximal to each other and therefore Met-115\(^{3.41}\), whereas distant to NPXXY, is likely to be sensitive to conformational changes involving both switches. In the reported active-state GPCR structures, three conserved residues (Arg-124\(^{5.50}\) of the DRY motif, Tyr-3.53 of the NPXXY motif, and Tyr-5.58) adopt near identical positions and connect these microswitches through water-mediated hydrogen bonds (1, 2). Furthermore, in our model of active \(\alpha_{1A}-\text{AR}\), which is based on structures of \(\beta_2-\text{AR}, \text{Arg-124}^{5.50}\) of the DRY motif is in contact with Tyr-326\(^{2.53}\) of the NPXXY motif (Fig. 1D).

In our NMR experiments for all ligands the \(^{13}\text{C}H_3\) group of both Met-115\(^{3.41}\) and Met-203\(^{5.57}\) show significant directional chemical shift and line width changes that are correlated with ligand efficacy, not affinity. As a distinct peak is observed for the addition of each ligand the chemical shift likely reflects an average population exchanging on a fast to intermediate timescale. The chemical shift differences, however, reflect a shift in the equilibrium, and specifically for the \(^{13}\text{C}H_3\) of Met-203\(^{5.57}\), from a \(\chi^3\) of a gauche-trans average (inverse agonist) toward a trans (agonist) average (Fig. 6A). An NMR study using \(^{15}\text{N}\)-labeled, thermostabilized \(\beta_2-\text{AR}\) observed substantial ligand efficacy-correlated backbone chemical shift changes for Val-226\(^{5.57}\) which is in the same position as Met-203\(^{5.57}\) in \(\alpha_{1A}-\text{AR}\) (13). The authors speculated that these changes were caused by TM5 bending toward the active receptor state (13), an idea that may also apply to \(\alpha_{1A}-\text{AR}\) and other GPCRs. Here, the linear chemical shift changes of Met-203\(^{5.57}\), and to a lesser degree Met-115\(^{3.41}\), in response to ligands of different efficacy is strong evidence that agonists activate \(\alpha_{1A}-\text{AR}\) via a conformational selection mechanism. The line broadening of Met-115\(^{3.41}\) and Met-203\(^{5.57}\) upon agonist binding supports an efficacy-driven shift in dynamics, and thereby the equilibrium of conformational states, communicated allosterically by the microswitches and sensed by these methionine residues (Fig. 6).

For our signaling incompetent receptors, \(\alpha_{1A}-\text{AR-A4}\) and \(\alpha_{1A}-\text{AR-A4 (L312F)}\), the NPXXY microswitch has been mutated (N322K) in a way that would bias the NPXXY switch toward inactive states (Lys-322–Asp-72 salt bridge). A consequence of this mutation is that for \(\alpha_{1A}-\text{AR-A4 (L312F)}\) the DRY motif probe, Met-203\(^{5.57}\), gives relatively intense chemical shifts in the apo and antagonist-bound states (conformational equilibrium biased toward inactive states) (Fig. 6B). On restoration of the NPXXY microswitch in \(\alpha_{1A}-\text{AR-A4–active}\), however, the Met-203\(^{5.57}\) chemical shifts broaden and shift toward the agonist-bound position (as defined for \(\alpha_{1A}-\text{AR-A4 (L312F)}\), even with neutral antagonist bound. Therefore,
Correlation of conformation with ligand efficacy for \( \alpha_{1A}^{-}\text{AR} \)

restoring the NPXXY microswitch enables the DRY motif of \( \alpha_{1A}^{-}\text{-AR-A4} \)--active to more readily sample active-like states. The full trans (19.5 ppm in \( ^{13}\text{C} \)) populated by silodosin indicates that we may observe the full-active state, although previous studies showed that the full-active state is only populated in the presence of both agonist and nanobody (8, 10, 14, 19, 22).

Our two methionine probes, Met-1153.41 and Met-2035.57 retain similar ligand-induced behavior in \( \alpha_{1A}^{-}\text{-AR-A4} \)--active compared with \( \alpha_{1A}^{-}\text{-AR-A4} \) and \( \alpha_{1A}^{-}\text{-AR-A4} \) (L312F), where the latter are both essentially inactive. The chemical shift and line-broadening trends of Met-1153.41 and Met-2035.57 suggest that the transmission switch and DRY motif, in the presence of an inactive NPXXY motif, can independently adopt conformations representative of active and inactive states. To fully adopt the conformational signatures of an active receptor, a functional NPXXY motif is required (in \( \alpha_{1A}^{-}\text{-AR-A4} \)--active) thus increasing the dynamics of the transmission switch and DRY motif, suggesting that the interdependence of the three microswitches is a consequence of their dynamic nature, and that this is required for full receptor function.

Although the striking linear chemical shift dependence for the \( ^{13}\text{C}H_3 \) of Met-2035.57 on ligand efficacy is consistent with a smooth change in equilibria from inactive to active, linear trends for \( ^{13}\text{C}H_3 \) of Met-1153.41 were less clear. In the inactive variants \( \alpha_{1A}^{-}\text{-AR-A4} \) and \( \alpha_{1A}^{-}\text{-AR-A4} \) (L312F) the resonance for apo, inverse agonist, and neutral antagonist shows little variation, but clear chemical shift changes and broadening are observed for agonists. The most distinct changes for the \( ^{13}\text{C}H_3 \) of Met-1153.41 were for \( \alpha_{1A}^{-}\text{-AR-A4} \)--active, where in the apo state two peaks were consistently observed, suggesting slow exchange (> millisecond) between two distinct states, to which we attribute to restoring the NPXXY microswitch. On the basis of chemical shift, we propose that the upfield peak of apo \( \alpha_{1A}^{-}\text{-AR-A4} \)--active represents fully inactive receptor (state A in Fig. 6), expected for full inverse agonists, and the downfield peak with a basal state receptor that is stabilized by the neutral antagonist (state B in Fig. 6). This downfield "basal" peak shows approximate linear efficacy-dependent chemical shift changes with agonist titrations. Therefore, the \( ^{13}\text{C}H_3 \) of Met-1153.41 reflects three states, inactive (inverse agonist), an intermediate (basal), and active states (state(s) C in Fig. 6), where the latter progressively shift from partial to full agonist states. Interestingly, in a \( ^{13}\text{C}H_3 \) methionine-labeled study on the M2R, Met-1123.41, which is equivalent to Met-1153.41 in \( \alpha_{1A}^{-}\text{-AR} \), did not display efficacy-dependent chemical shift changes. In the presence of ligands, however, M2R Met-1123.41 was resolved as two separate resonances, consistent with a slow exchanging microswitch (22) and may highlight some differences between how different rhodopsin family GPCRs function.

In this NMR study, by starting with a signaling incompetent variant of \( \alpha_{1A}^{-}\text{-AR} \) and subsequently restoring signaling activity through back-mutations, we were able to study the functional dynamics of the key GPCR microswitches and how different ligands modulate this. Our NMR data for the transmission and DRY microswitches revealed ligand efficacy-dependent changes to the microswitch conformational equilibria, supporting a conformational selection mechanism for \( \alpha_{1A}^{-}\text{-AR} \) modulation. This and the agonist-driven line broadening for both microswitches suggest similar mechanistic actions on the different microswitches, supporting ligand-driven allosteric communication between the microswitches. MD simulations (41) of \( \beta_3\text{-AR} \) suggest that these microswitches behaved independently of each other, with only loose allosteric coupling. Although this may be true over the relatively short timescales of
MD, we believe that over the course of an NMR experiment such loose allosteric coupling culminates in significant coupled shifts to the microswich conformations and that this is likely how ligands modulate GPCR signaling in cells.

**Experimental procedures**

**α1A-AR constructs**

The α1A-AR-A4 variant is a thermostabilized human α1A-AR, containing 15 stabilizing mutations (32). Met-802.58 in the spectra was introduced through the stabilization process in lieu of the naturally occurring amino acid leucine. As compared with WT, the C termini of the α1A-AR-A4 variant was modified by truncation at Ser-351 and addition of a deca-His tag to facilitate purification (Fig. S1). For expression, the α1A-AR variants sequences were subcloned into the pQE30-derived vector, pDS15, with a maltose-binding protein and a methionine-free monomeric ultrasbtable GFP (−Met-muGFP) (42) attached, respectively, to the N and C termini of the receptor via HRV 3C protease-cleavage sites. For the purpose of KingFisher-binding assays, α1A-AR variants were subcloned into a similar vector, pDS11, in which muGFP was replaced with mCherry because the excitation and emission wavelengths of fluorescent QAPB were overlapping with those of GFP. The final sequence of α1A-AR-A4 after purification (with residues left from HRV 3C cleavage) is: GPGSVFLSGNASDSSNSIQPPAPVNISKAILLGVILG-AR-A4 after purification (with residues left from HRV 3C cleavage) is: GPGSVFLSGNASDSSNSIQPPAPVNISKAILLGVILG-AR-A4 after purification (with residues left from HRV 3C cleavage) is: GPGSVFLSGNASDSSNSIQPPAPVNISKAILLGVILG-

**α1A-AR purification**

The frozen cell pellet was thawed at room temperature for 30 min. A 10-ml pellet was gently resuspended in 40 ml of ice-cold solubilization buffer (25 mM HEPES, pH 7.5, 200 mM NaCl, 10% glycerol, 1% DDM (Anatrace), 0.12% cholesterol hemisuccinate (Anatrace), 0.6% CHAPS (Sigma), 50 mg of lysozyme, 5 mg of DNase, one tablet of EDTA-free complete protease inhibitor mixture (Roche Applied Science), 0.2–0.4 mM phenylmethyl-sulfonyl fluoride, and incubated on a turning wheel for 30 min at 4 °C. The cell membranes were then disrupted by a sonication device (Diagenode Bioruptor Plus, high power, 10 s on/20 s off for 30 cycles) followed by another 1-h incubation at 4 °C on the turning wheel. The cell debris was removed by centrifugation (12,000 × g, 4 °C, 40 min) and the supernatant was filtered using a 45-μm Durapore syringe filter (Merck Millipore). The cleared cell lysate was incubated with 3 ml of Talon metal affinity resin pre-equilibrated with 45 ml of equilibrium buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 0.05% DDM). After 1.5 h incubation at 4 °C, the resin retaining the receptor was washed three times with washing buffer 1 (20 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, 0.05% DDM) and then the full-length protein was eluted by 30 ml of elution buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 0.05% DDM, 250 mM imidazole). The eluate was concentrated down to 0.5–1 ml using a 100-kDa cut-off centrifugal filter device (Amicon Ultra, Millipore). Imidazole was removed by using a PD10 desalting column (GE Healthcare). Cleaveage of fusion proteins from the receptor was carried out overnight at 4 °C by adding 100 mM Na2SO4, 1 mM tris(2-carboxyethyl)phosphine, and 300 pmol of glutathione S-transferase-tagged HRV 3C protease (made in house).

The cleaved mixture was incubated for 1 h with 2 ml of pre-equilibrated Talon resin. The resin was washed using 30 ml of washing buffer 2 (20 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 0.05% DDM, 30 mM imidazole) and the receptor was eluted by 20 ml of elution buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol, 0.05% DDM, 250 mM imidazole). The eluate was concentrated down to 450 μl by a 30-kDa cut-off centrifugal filter device (Amicon Ultra, Millipore) and was loaded onto a Superdex 200 10/300 increase column (GE Healthcare) equilibrated with size exclusion chromatography buffer (50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 0.02% DDM). Size exclusion chromatography was carried out at a flow rate of 0.5 ml/min. The peak
Correlation of conformation with ligand efficacy for \( \alpha_{1A} \)-AR

NMR spectroscopy

NMR samples were prepared to 130 \( \mu l \) at 40 – 60 \( \mu M \) receptor in 3-mm Shigemi NMR tubes (Shigemi Inc., Allison Park, PA). Ligands were added at saturating concentrations that were 2 \( \mu M \) adrenaline for \( \alpha_{1A} \)-AR-A4 and \( \alpha_{1A} \)-AR-A4 (active), 400 \( \mu M \) prazosin, and 1 \( \mu M \) of other ligands to all mutants (Supporting Table S1). Adrenaline was supplemented with 1 \( \mu M \) of the antioxidant ascorbic acid. Samples containing low affinity agonists (phenylephrine and adrenaline) were recycled via competition with high affinity ligands, exchange was judged via the chemical shift of the Met-2926.55 resonance. Experiments on apo \( \alpha_{1A} \)-AR-A4 and \( \alpha_{1A} \)-AR-A4-L312F, apo, and all ligands were performed at least twice on independent receptor samples (biological replicates), except WB-4101 and phenolamine, which were acquired once. Experiments on apo \( \alpha_{1A} \)-AR-A4 – active and bound to prazosin, silodosin, and oxytemozolamine were performed at least twice on independent receptor samples, and for other ligands were performed once.

All NMR spectra were collected at 25 °C on an 800-MHz Bruker Avance II spectrometer equipped with a triple resonance cryoprobe. 2D \(^1H\)-\(^13C\) SOFAST-HMQC (43) spectra were recorded by excitation with a 2.25-ms PC9 120° \(^1H\) pulse and refocusing with a 1-ms r-SNOB shaped 180° \(^1H\) pulse. The spectral widths were set to 12 and 25 ppm for \(^1H\) and \(^13C\) dimensions, respectively. For the spectra recorded for \( \alpha_{1A} \)-AR-A4 variant (Fig. 2 and Fig. S3), 1024 \times 128 complex points were recorded with a 25% Poisson-gap sampling schedule and 2048 scans; an acquisition time of 8.5 h. For the other spectra, 1024 \times 200 complex points were recorded with either traditional or 60% Poisson-gap sampling schedule and 368 scans resulting in acquisition times of 10 and 6 h, respectively. Spectra were reconstructed with compressed sensing using qMDD and processed using NMRpipe (44) where data were multiplied by cosine bell functions and zero-filled once in each dimension. Spectra were analyzed in NMRFAM-Sparky (45) (Goddard, T. D. and Kneller, D. G., University of California, San Francisco).

The average chemical shift differences, \( \Delta \delta \), were normalized using the equation \( \Delta \delta = [(\Delta \delta_{1H})^2 + (\Delta \delta_{13C}/3.5)^2]^{1/2} \). The error values were calculated by the formula \([\Delta \delta_{1H} \times R_{1H} + \Delta \delta_{13C} \times R_{13C}/(3.5)]/\Delta \delta \), where \( R_{1H} \) and \( R_{13C} \) are the digital resolutions in ppm in the \(^1H\) and \(^13C\) dimensions, respectively (5).

Saturation and competition-binding assays

1 nmol of purified full-length \( \alpha_{1A} \)-AR variant (mCherry attached) was resuspended in 10 ml of assay buffer (20 \( \mu M \) HEPES, pH 7.5, 100 \( \mu M \) NaCl, 0.02% DDM) and immobilized onto 200 \( \mu l \) of Dynabeads (Streptavidin T1) for 30 min at 4 °C. 100 \( \mu l \) of the suspension-containing beads with immobilized receptor were aliquoted to a 96-Deep Well plate from which the beads were transferred to another 96-Deep Well plate containing 100 \( \mu l \) of ligand solution using a KingFisher Flex magnetic particle processor. For saturation binding, immobilized receptors in each well were incubated with 100 \( \mu l \) of assay buffer containing increased concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 \( \mu M \)) of QAPB (quinazoline piperazine BODIPY) for 2 h at 22 °C. Nonspecific binding was determined by repeating the experiment in the presence of 10 \( \mu M \) prazosin. For competition binding, immobilized receptors were incubated with 100 \( \mu l \) of assay buffer containing 10 \( \mu M \) QAPB with the addition of ligands at various concentrations, as shown in supporting Figs. S5 and S8, for 2 h at 22 °C. Immobilized receptors were subsequently washed with 200 \( \mu l \) of assay buffer and resuspended in 100 \( \mu l \) of assay buffer. 90 \( \mu l \) of the final beads solution was transferred to a 96-well Greiner Bio-One nonbinding black plate. Fluorescence of bound QAPB was measured using a POLARstar OMEGA plate reader (BMG Labtech, Ortenburg, Germany) and normalized to mCherry fluorescence, which was detected simultaneously. Data represent the mean ± S.D. of three independent biological replicate experiments each performed in duplicate technical measurements. To compare ligand-binding affinities at \( \alpha_{1A} \)-AR-A4 (L312F) and \( \alpha_{1A} \)-AR-A4 – active of \( \alpha_{1A} \)-AR-A4, raw data from our previously published paper (32), were reanalyzed and presented in Supporting Table S1.

Thermostability assay

1 \( \mu M \) purified full-length \( \alpha_{1A} \)-AR-A4 or \( \alpha_{1A} \)-AR-A4–active (mCherry attached) was prepared in base buffer (20 \( \mu M \) HEPES, 100 \( \mu M \) NaCl, 0.1% DDM). To measure thermostability of receptors in the apo state, 100 \( \mu l \) of receptor solution was aliquoted into 24 wells of a 96-well PCR plate. 10 of the 12 duplicates were heated in gradient temperatures for 30 min and the two remaining duplicates were left at 4 °C for normalization. After thermo-treatment, the receptors were transferred to a KingFisher 96-Deep Well plate containing 2 \( \mu l \) of paramagnetic Dynabeads per well (streptavidin T1, ThermoFisher Scientific). The following few steps were automatically performed by using a KingFisher 96 magnetic particle processor. The receptor was first incubated with magnetic beads for 30 min at 4 °C. Then, magnetic beads were transferred to another 96-Deep Well plate containing 100 \( \mu l \) of ligand solution (20 \( \mu M \) HEPES, 100 \( \mu M \) NaCl, 0.1% DDM, 100 \( \mu M \) QAPB). The non-specific binding was determined by competing QAPB with 100 \( \mu M \) of prazosin. After 1.5 h incubation, immobilized receptors were subsequently washed with 200 \( \mu l \) of assay buffer and resuspended in 100 \( \mu l \) of assay buffer. 90 \( \mu l \) of the final beads solution was transferred to a 96-well Greiner Bio-One nonbinding black plate. Fluorescence of bound QAPB was measured using a POLARstar OMEGA plate reader (BMG Labtech, Ortenburg, Germany) and normalized to mCherry fluorescence, which was detected simultaneously. To measure the thermostability of \( \alpha_{1A} \)-AR variants in the presence of ligand, receptors were preincubated with 100 nm QAPB for 1 h on ice prior to being heated at varying temperatures. The remaining steps were carried out as described for apo state thermostability assay. Data represent the mean ± S.D. of three independent biological replicate experiments each performed in duplicate technical replicate measurements.
IP₁ assay

Gα₁q/11 signaling assays were carried out using the IP-One HTRF® Assay Kit (Cisbio Bioassays, France) measuring IP₁ using the manufacturer’s protocol. COS-7 cells were seeded at 25,000 cells per well in a 96-well plate and incubated overnight at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (ScientificLife, Melbourne, Australia), 1% l-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). Cells were transfected with pcDNA3.1 constructs of WT or mutant α₁A-ARs using Lipofectamine 2000 (Invitrogen) at 0.25 μg of DNA/well. 24 h later, cells were stimulated with ligands for 2 h at 37 °C in 40 μl of Stimulation Buffer, then frozen at −80 °C. 14 μl of thawed sample were transferred to a white HTRF® 384-well Optiplate (PerkinElmer Life Sciences), incubated with development reagents in the dark for 1 h with shaking, and analyzed by time-resolved fluorescence using a POLARstar OMEGA plate reader (BMG Labtech, Ortenburg, Germany). Data were analyzed against the kit’s standard curve. Data represent mean ± S.D. of three independent biological replicate experiments (except for some samples in the screening assay in Fig. S8, where some receptors were measured only once or twice). For each biological replicate samples were measured in triplicate technical replicate measurements, unless otherwise stated in the figure legends (as in Fig. S8).

NanoBiT G protein activity assay

COS-7 cells grown in 10% FBS, 1% l-glutamine, 1% penicillin/streptomycin, DMEM were seeded at 250,000 cells/well on a 6-well–plate. Cells were then transiently co-transfected in the lin/streptomycin, DMEM were seeded at 25,000 cells/well in a 96-well plate and incubated overnight. On the day of the assay, plates were preincubated with 10 μM Stimulation Buffer, then stimulated with ligands for 2 h at 37 °C in 40 μl of Stimulation Buffer, then frozen at −80 °C. 14 μl of thawed sample were transferred to a white HTRF® 384-well Optiplate (PerkinElmer Life Sciences), incubated with development reagents in the dark for 1 h with shaking, and analyzed by time-resolved fluorescence using a POLARstar OMEGA plate reader (BMG Labtech, Ortenburg, Germany). Data were analyzed against the kit’s standard curve. Data represent mean ± S.D. of three independent biological replicate experiments (except for some samples in the screening assay in Fig. S8, where some receptors were measured only once or twice). For each biological replicate samples were measured in triplicate technical replicate measurements, unless otherwise stated in the figure legends.

Intracellular Ca²⁺ mobilization assays

COS-7 cells were seeded in 10-cm culture dishes at 3 × 10⁶ cells/dish and allowed to grow overnight at 37 °C, 5% CO₂ in DMEM supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin (Life Technologies). For the next day, the cells were transfected with 30 μg of receptor DNA construct (pcDNA3.1 expression vector containing WT or mutant α₁A-ARs) using Lipofectamine 2000 (Invitrogen) transfecting reagent per dish. The following day, cells were transferred to 96-well–culture plates (5 × 10⁴ cells/well) and allowed to grow overnight. On the day of the experiment cells were washed twice with Ca²⁺ assay buffer (150 mM NaCl, 2.6 mM KCl, 1.2 mM MgCl₂, 10 mM d-glucose, 10 mM HEPES, 2.2 mM CaCl₂, 0.5% (w/v) BSA, and 4 mM probenecid, pH 7.4) and incubated in Ca²⁺ assay buffer containing 1 mM Fluo-4 AM for 1 h in the dark at 37 °C and 5% CO₂. After two washes with Ca²⁺ assay buffer, fluorescence was measured for 1.5 min upon the addition of ligands in a Flexstation 3 (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 485 nm and emission wavelength of 520 nm. Data were normalized to the peak response elicited by 3 μM ionomycin (Life Technologies). Data represent the mean ± S.D. of three independent biological replicate experiments each performed in triplicate technical replicate measurements, unless otherwise stated in the figure legends.

Homology modeling

Homology models of inactive and active state α₁A-AR were built with I-TASSER (46) using crystal structures of β₂-AR as the templates, which show 23% identity and 39% similarity. For inactive state models, the structure of β₂-AR bound to the antagonist carazolol and the inactive state stabilizing nanobody, Nb60 (PDB ID 5JQH) (47), was used as a template. For the active state models, the crystal structure of a β₂-AR–Gs protein complex bound to the agonist BI-167107 (PDB ID 3SN6) (48) was used as a template. The N- and C-terminal regions as well as the ICL3 regions, which have no sequence similarity to the template, were deleted from the model. Energy minimization was performed using Minimize tool in Maestro version 11.7.012 (Schrödinger, Inc.) under OPLS 2005 (49) forcefield.

Data availability

All data generated or analyzed during this study are included in the published article (and supporting information).

Author contributions—F.-J. W., A. A.-R., R. A. B., D. J. S., and P. R. G. data curation; F.-J. W. formal analysis; F.-J. W., L. M. W., A. A.-R., A. G., T. M. V., M. K., A. R. W., M. D. G., R. A. B., D. J. S., and P. R. G. investigation; F.-J. W. methodology; F.-J. W. writing-original draft; F.-J. W., L. M. W., A. A.-R., A. G., T. M. V., M. K., A. R. W., M. D. G., R. A. B., D. J. S., and P. R. G. writing-review and editing; A. A.-R., A. R. W., M. D. G., R. A. B., D. J. S., and P. R. G. supervision; A. A.-R., R. A. B., D. J. S., and P. R. G. funding acquisition; A. A.-R., R. A. B., D. J. S., and P. R. G. project administration.
Correlation of conformation with ligand efficacy for $\alpha_{1A}$-AR

Acknowledgments—We thank Dr. Fabian Bumbak (The Florey Institute of Neuroscience and Mental Health) for assistance with optimizing the expression and purification of receptor samples; Prof. Dmitry Veprintsev (University of Nottingham) and Dr. Franziska Heydenreich (Stanford University) for suggesting back-mutations for the generation of $\alpha_{1A}$-AR-A4–active; Prof. Asuka Inoue (Tohoku University) for supplying plasmids for the G protein activity assays; Sharon Layfield (The Florey Institute of Neuroscience and Mental Health) and Dr. Ashish Sethi (The University of Melbourne) for assistance with cell-based assays and NMR data analysis, respectively; Dr. David Chalmers (Monash University) and the Monash University Medicinal Chemistry Computational Chemistry Facility for the assistance with computational modeling and The Bio21 NMR facility for access to spectrometers.

References

1. Manglik, A., and Kruse, A. C. (2017) Structural basis for G protein-coupled receptor activation. Biochemistry 56, 5628–5634 CrossRef Medline

2. Carpenter, B., and Tate, C. G. (2017) Active state structures of G protein-coupled receptors highlight the similarities and differences in the G protein and arrestin coupling interfaces. Curr. Opin. Struct. Biol. 45, 124–132 CrossRef Medline

3. García-Nafría, J., and Tate, C. G. (2019) Cryo-EM structures of GPCRs coupled to Gs, Gi, and Go. Mol. Cell. Endocrinol. 488, 1–13 CrossRef Medline

4. Bokoch, M. P., Zou, Y., Rasmussen, S. G., Liu, C. W., Nygaard, R., Rosenbaum, D. M., Fung, J. J., Choi, H. I., Thian, F. S., Koblika, T. S., Puglisi, J. D., Weis, W. I., Pardo, L., Prosser, R. S., Mueller, L., and Koblika, B. K. (2010) Ligand-specific regulation of the extracellular surface of a G protein-coupled receptor. Nature 463, 108–112 CrossRef Medline

5. Kofuku, Y., Ueda, T., Okude, J., Shiraishi, K., Kondo, K., Maeda, M., Tsujishita, H., and Shimada, I. (2012) Efficacy of the $\beta_2$-adrenergic receptor is determined by conformational equilibrium in the transmembrane region. Nat. Commun. 3, 1045 CrossRef Medline

6. Liu, J. J., Horst, R., Kattitch, V., Stevens, R. C., and Wüthrich, K. (2012) Biased signaling pathways in $\beta_2$-AR characterized by $^{19}$F-NMR. Science 335, 1106–1110 CrossRef Medline

7. Horst, R., Liu, J., Stevens, R., and Wüthrich, K. (2013) $\beta_2$-adrenergic receptor activation by agonists studied with 19F NMR spectroscopy. Angew. Chem. 125, 10962–10965 CrossRef

8. Nygaard, R., Zou, Y., Dror, R. O., Mildorf, T. J., Arlow, D. H., Manglik, A., Pan, A. C., Liu, C. W., Fung, J. J., Bokoch, M. P., Thian, F. S., Koblika, T. S., Shaw, D. E., Mueller, L., Prosser, R. S., and Koblika, B. K. (2013) The dynamic process of $\beta_2$-adrenergic receptor activation. Cell 152, 532–542 CrossRef Medline

9. Kofuku, Y., Ueda, T., Okude, J., Shiraishi, K., Kondo, K., Mizumura, T., Suzuki, S., and Shimada, I. (2014) Functional dynamics of deuterated $\beta_2$-adrenergic receptor in lipid bilayers revealed by NMR spectroscopy. Angew. Chem. Int. Ed. Engl. 53, 13376–13379 CrossRef Medline

10. Manglik, A., Kim, T. H., Masureel, M., Altenbach, C., Yang, Z., Hilger, D., LeVine, J. V., Khelashvili, G., Rasmussen, S. G., Gardner, K. H., and Rosenbaum, D. M. (2017) Ligand modulation of sidechain dynamics in a wild-type human GPCR. Elife 6, e28505 CrossRef Medline

11. Ye, L., Neale, C., Sjøka, A., Lyda, B., Puchigun, D., Tsuchimura, N., Larda, S. T., Pomés, R., García, A. E., Ernst, O. P., Sunahara, R. K., and Prosser, R. S. (2014) Mechanistic insights into allosteric regulation of the A2A adenosine G protein-coupled receptor by physiological cations. Nat. Commun. 9, 1372 CrossRef Medline

12. Eddy, M. T., Lee, M. Y., Gao, Z. G., White, K. L., Didenko, T., Horst, R., Audet, M., Stanczak, P., McClary, K. M., Han, G. W., Jacobson, K. A., Stevens, R. C., and Wüthrich, K. (2018) Allosteric coupling of drug binding and intracellular signaling in the A2A adenosine receptor. Cell 172, 68–80.e12 CrossRef Medline

13. Sounier, R., Mas, C., Steyaert, J., Laermens, T., Manglik, A., Huang, W., Koblika, B. K., Déméné, H., and Granier, S. (2015) Propagation of conformational changes during $\mu$-opioid receptor activation. Nature 524, 375–378 CrossRef Medline

14. Okude, J., Ueda, T., Kofuku, Y., Sato, M., Nobuyama, N., Kondo, K., Shiraishi, Y., Mizumura, T., Onishi, K., Natsume, M., Maeda, M., Tsujishita, H., Kuranaga, T., Inoue, M., and Shimada, I. (2015) Identification of a conformational equilibrium that determines the efficacy and functional selectivity of the $\mu$-opioid receptor. Angew. Chem. Int. Ed. Engl. 54, 15771–15776 CrossRef Medline

15. Casiraghi, M., Damian, M., Lescop, E., Point, E., Moncoq, K., Morelet, N., Levy, D., Marie, J., Guittet, E., Banères, J. L., and Catoire, L. J. (2016) Functional modulation of a G protein-coupled receptor conformational landscape in a lipid bilayer. J. Am. Chem. Soc. 138, 11170–11175 CrossRef Medline

16. Xu, J., Hu, Y., Kaindl, J., Risel, P., Hübiner, H., Maeda, S., Niu, X., Li, H., Gneiiner, P., Jin, C., and Koblika, B. K. (2019) Conformational complexity and dynamics in a muscarinic receptor revealed by NMR spectroscopy. Mol. Cell 75, 53–65.e7 CrossRef Medline

17. Shimada, I., Ueda, T., Kofuku, Y., Eddy, M. T., and Wüthrich, K. (2019) GPCR drug discovery: integrating solution NMR data with crystal and cryo-EM structures. Nat. Rev. Drug Discov. 18, 59–82 Medline CrossRef Medline

18. Deupi, X., and Standfuss, J. (2011) Structural insights into agonist-induced activation of G-protein-coupled receptors. Curr. Opin. Struct. Biol. 21, 541–551 CrossRef Medline

19. Trzaskowski, B., Latek, D., Yuan, S., Ghoshdastider, U., Debinski, A., and Filipek, S. (2012) Action of molecular switches in GPCRs: theoretical and experimental studies. Curr. Med. Chem. 19, 1090–1109 CrossRef Medline

20. Ahuja, S., and Smith, S. O. (2009) Multiple switches in G protein-coupled receptor activation. Trends Pharmacol. Sci. 30, 494–502 CrossRef Medline

21. Bhattacharya, S., and Vaidehi, N. (2014) Differences in allosteric communication in the inactive and active states of a GPCR. Biophys. J. 107, 422–434 CrossRef Medline

22. Latorraca, N. R., Venkatakrishnan, A. J., and Dror, R. O. (2017) GPCR dynamics: structures in motion. Chem. Rev. 117, 139–155 CrossRef Medline

23. Akina, I., García-Sainz, J. A., and S. Pupo, A. (2019) Updates in the function and regulation of $\alpha_1$-adrenoceptors. Br. J. Pharmacol. 176, 2343–2357 Medline

24. Perez, D. M., and Doze, V. (2011) Cardiac and neuroprotection regulated by $\alpha_1$-adrenergic receptor subtypes. J. Recept. Signal. Transduct. Res. 31, 98–110 CrossRef Medline

25. Scott, D. J., and Plückthun, A. (2013) Direct molecular evolution of detergent-stable G protein-coupled receptors using polymer encapsulated cells. J. Mol. Biol. 425, 662–677 CrossRef Medline

26. Yong, K. J., Vaid, T. M., Shilling, P. J., Wu, F. J., Williams, I. M., Deluigi, M., Plückthun, A., Bathgate, R. A. D., Gooley, P. R., and Scott, D. J. (2018) Determinants of ligand subtype-selectivity at $\alpha$-adrenoceptors. J. Mol. Biol. 425, 2343–2357 Medline

7416 J. Biol. Chem. (2020) 295(21) 7404–7417
Correlation of conformation with ligand efficacy for α1A-AR

using saturation transfer difference (STD) NMR. ACS Chem. Biol. 13, 1090–1102 CrossRef Medline

33. Isberg, V., de Graaf, C., Bortolato, A., Cherezov, V., Katritch, V., Marshall, F. H., Mordalski, S., Pin, J.-P., Stevens, R. C., Vriend, G., and Gloriam, D. (2015) Generic GPCR residue numbers: aligning topology maps while minding the gaps. Trends Pharmacol. Sci. 36, 22–31 CrossRef Medline

34. Bumbak, F., Keen, A. C., Gunn, N. J., Gooley, P. R., Bathgate, R. A. D., and Scott, D. J. (2018) Optimization and 13CH3 methionine labeling of a signaling competent neurotensin receptor 1 variant for NMR studies. Biochim. Biophys. Acta 1860, 1372–1383 CrossRef

35. Bumbak, F., Bathgate, R. A. D., Scott, D. J., and Gooley, P. R. (2019) Expression and purification of a functional E. coli 13CH3-methionine-labeled thermostable neurotensin receptor 1 variant for solution NMR studies. Methods Mol. Biol. 1947, 31–55 CrossRef Medline

36. Hwa, J., Graham, R. M., and Perez, D. M. (1995) Identification of critical determinants of 13A-1-adrenergic receptor subtype selective agonist binding. J. Biol. Chem. 270, 23189–23195 CrossRef Medline

37. Butterfoss, G. L., DeRose, E. F., Gabel, S. A., Perera, L., Krahn, J. M., Muller, G. A., Zheng, X., and London, R. E. (2010) Conformational dependence of 13C shielding and coupling constants for methionine methyl groups. J. Biomol. NMR 48, 31–47 CrossRef Medline

38. Zhu, J., Taniguchi, T., Takaui, R., Suzuki, F., Tanaka, T., and Muramatsu, I. (2000) Inverse agonism and neutral antagonism at a constitutively active 13A-1 adrenoceptor. Br. J. Pharmacol. 113, 546–552 CrossRef Medline

39. Inoue, A., Raimondi, F., Kajdi, F. M. N., Singh, G., Kishi, T., Uwamizu, A., Ono, Y., Shinjo, Y., Ishida, S., Arang, N., Kawakami, K., Gutkind, J. S., Aoki, J., and Russell, R. B. (2019) Illuminating G-protein-coupling selectivity of GPCRs. Cell 177, 1933–1947.e25 CrossRef Medline

40. Cotecchia, S., Schwinn, D. A., Randall, R. R., Lefkowitz, R. J., Caron, M. G., and Koblika, B. K. (1988) Molecular cloning and expression of the cDNA for the hamster α1-1-adrenergic receptor. Proc. Natl. Acad. Sci. U.S.A. 85, 7159–7163 CrossRef Medline

41. Dror, R. O., Arlow, D. H., Maragakis, P., Mildorf, T. J., Pan, A. C., Xu, H., Borhani, D. W., and Shaw, D. E. (2011) Activation mechanism of the β2-adrenergic receptor. Proc. Natl. Acad. Sci. U.S.A. 108, 18684–18689 CrossRef Medline

42. Scott, D. J., Gunn, N. J., Yong, K. J., Wimmer, V. C., Veldhuis, N. A., Challis, L. M., Haidar, M., Petrou, S., Bathgate, R. A. D., and Griffin, M. D. W. (2018) A novel ultra-stable, monomeric green fluorescent protein for direct volumetric imaging of whole organs using CLARITY. Sci. Rep. 8, 667 CrossRef Medline

43. Schanda, P., Kupce, E., and Brutscher, B. (2005) SOFAST-HMQC experiments for recording two-dimensional heteronuclear correlation spectra of proteins within a few seconds. J. Biomol. NMR 33, 199–211 CrossRef Medline

44. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293 Medline

45. Lee, W., Tonelli, M., and Markley, J. L. (2015) NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. Bioinformatics 31, 1325–1327 CrossRef Medline

46. Zhang, J., Yang, J., Jiang, R., and Zhang, Y. (2015) GPCR-I-TASSER: a hybrid approach to G protein-coupled receptor structure modeling and the application to the human genome. Structure 23, 1538–1549 CrossRef Medline

47. Staus, D. P., Strachan, R. T., Manglik, A., Pan, B., Kahlai, A. W., Kim, T. H., Wingler, L. M., Ahn, S., Chatterjee, A., Masoudi, A., Kruse, A. C., Pardon, E., Steyaert, J., Weis, W. I., Prosser, R. S., et al. (2016) Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation. Nature 535, 448–452 CrossRef Medline

48. Rasmussen, S. G., Devree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Koblika, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., et al. (2011) Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477, 549–555 CrossRef Medline

49. Sui, S. W., Pluhackova, K., and Böckmann, R. A. (2012) Optimization of the OPLS-AA force field for long hydrocarbons. J. Chem. Theory Comput. 8, 1459–1470 CrossRef Medline