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Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation

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G-protein-coupled receptors (GPCRs) modulate many physiological processes by transducing a variety of extracellular cues into intracellular responses. Ligand binding to an extracellular orthosteric pocket propagates conformational change to the receptor cytosolic region to promote binding and activation of downstream signalling effectors such as G proteins and β-arrestins. It is well known that different agonists can share the same binding pocket but evoke unique receptor conformations leading to a wide range of downstream responses ("efficacy"). Furthermore, increasing biophysical evidence, primarily using the β2-adrenergic receptor (β2AR) as a model system, supports the existence of multiple active and inactive conformational states2–5. However, how agonists with varying efficacy modulate these receptor states to initiate cellular responses is not well understood. Here we report stabilization of two distinct β2AR conformations using single domain camelpid antibodies (nanobodies)—a previously described positive allosteric nanobody (Nb80)6,7 and a newly identified negative allosteric nanobody (Nb60). We show that Nb60 stabilizes a previously unappreciated low-affinity inactive receptor state which corresponds to one of two inactive receptor conformations as delineated by X-ray crystallography and NMR spectroscopy. We find that the agonist isoprenaline has a 15,000-fold higher affinity for β2AR in the presence of Nb80 compared to the affinity of isoprenaline for β2AR in the presence of Nb60, highlighting the full allosteric range of a GPCR. Assessing the binding of 17 ligands of varying efficacy to the β2AR in the absence and presence of Nb60 or Nb80 reveals large ligand-specific effects that can only be explained using an allosteric model which assumes equilibrium amongst at least three receptor states. Agonists generally exert efficacy by stabilizing the active Nb80-stabilized receptor state (R80). In contrast, for a number of partial agonists, both stabilization of R80 and destabilization of the inactive, Nb60-bound state (R60) contribute to their ability to modulate receptor activation. These data demonstrate that ligands can initiate a wide range of cellular responses by differentially stabilizing multiple receptor states.

The allosteric behaviour of GPCRs is responsible for the complex signalling properties associated with these important regulators of human physiology. GPCR allostery, defined here as a linkage between the extracellular orthosteric ligand pocket and the intracellular G-protein-binding pocket, has long been analysed by pharmacological methods8–10 (see Supplementary Information). Conformational changes within a GPCR induced by agonist binding can enhance the affinity and binding of intracellular signalling transducers, such as G proteins and β-arrestins. Conversely, transducer coupling further enhances agonist affinity, resulting in the formation of the ternary complex of receptor, intracellular signalling transducer, and ligand (Fig. 1a). The conceptual framework of the ternary complex model equates the magnitude of these affinity changes with the strength of transducer activation in cells11,12, as demonstrated for several GPCR systems13–16. However, the structural basis underlining these allosteric relationships and how they relate to ligand efficacy is not well understood.

Ligand-dependent GPCR activation has traditionally been conceptualized as a conversion between a single inactive and a single active receptor state. However, recent studies using various spectroscopic techniques have identified multiple inactive and active receptor states, suggesting that the mechanisms underlying receptor activation may be more complex than previously thought2–5. To better understand how ligands with varying efficacies may differentially regulate these conformations, we sought to develop reagents to stabilize specific inactive and active conformations of the β2AR. We and others have previously used Nb80, a Gs mimetic nanobody, to stabilize an active conformation of the β2AR6,7. Indeed, competition radioligand binding assays using iodinated cyanopindolol ([125I]CYP) and β2AR reconstituted into high-density lipoprotein (HDL) particles (nanodiscs) demonstrated that Nb80 increases the affinity of the agonist isoprenaline by 75-fold (Fig. 1b), which is similar, but not identical, to the 33-fold increase seen in the presence of purified heterotrimeric Gβγ (Fig. 1b). To investigate the pharmacological properties of the inactive receptor, we identified a nanobody (Nb60) that preferentially bound inverse-agonist-bound β2AR7. Remarkably, though the affinity of the receptor for agonist in the absence of G or Nb80 was presumed to reflect the pharmacological properties of the inactive state, the presence of Nb60 reduced agonist affinity by approximately 70-fold (Fig. 1b). The effects of Nb60 and Nb80 on radiotracer affinity were negligible and could not account for the large changes in affinity (Extended Data Table 1).

To further quantify the allosteric effects of Nb60, Nb80, and Gs on agonist binding, we measured isoprenaline affinity with radioligand competition binding over a range of nanobody/Gs concentrations (Extended Data Fig. 1). As the concentration of allosteric modulator increases, the effect on isoprenaline affinity becomes saturable, reaching two opposite plateau values with Nb60 and Nb80 or Gs (Fig. 1c). This is a hallmark pattern of true allosteric interactions, with
Figure 1 | Allosteric nanobodies have opposing effects on agonist affinity for the β2AR. a, Schematic of the ternary complex model. Ligand (L) affinity to receptor (R) increases in the presence of transducer (T), this allosteric linkage is denoted by dashed line with arrows. b, Compared to the absence of modulator, Nb60 decreases isoprenaline affinity (negative cooperativity) and Nb80 and Gs increases affinity (positive cooperativity) as assessed by radioligand competition assays using β2AR HDL particles. c, The effects of Nb60 and Nb80 or Gs on isoprenaline affinity are

the net log-change in isoprenaline affinity at saturating concentration of nanobody/Gs gauging the extent of cooperativity between nanobody and agonist binding (negative for Nb60 or positive for Nb80 and Gs) (see Supplementary Information). As predicted by the ternary complex model, this coupling energy, termed α, must be constant—the effect of bound nanobody on agonist binding is reciprocal to the effect of bound agonist on nanobody binding. To verify such a prediction, we measured the affinity of Nb60 for the β2AR in the absence and presence of agonist (isoprenaline) using isothermal titration calorimetry (Fig. 1d, e). As expected, the affinity of Nb60 for β2AR decreased in the presence of isoprenaline. Consistent with its preference for the inactive state, Nb60 dose-dependently increased binding of the radio-labelled inverse antagonist [3H]ICI-118,551 to the β2AR, whereas binding was decreased in the presence of Nb80 (Fig. 1f). Together, these data show that Nb60 and Nb80 are potent allosteric modulators that can be used to stabilize inactive and active β2AR states.

The decrease in isoprenaline affinity observed in the presence of Nb60 reveals a previously unappreciated ‘very-low-affinity’ state (KVL) in competition binding experiments. The affinity of agonist for an uncoupled GPCR has traditionally been referred to as the ‘low-affinity’ (KL) state; however, our results show that KL values reflect binding of an ensemble of conformations that exchange rapidly over the course of the binding reaction. This phenomenon is probably conserved among GPCRs, as a similar KVL state has been observed with the A2A adenosine receptor using an antibody fragment. This conformational heterogeneity is consistent with recent spectroscopic and computational studies, which have shown that the β2AR exists in multiple inactive, intermediate, and active conformations that exchange within milliseconds. To assess which receptor state Nb60 stabilizes, we conducted 19F fluorine NMR spectroscopy of β2AR labelled with a trifluoroacetanilide probe at the endogenous residue C265 located at the cytoplasmic end of transmembrane 6 (TM6). As shown previously, the unliganded β2AR exists in two equally distributed inactive states (termed S1 and S2) that exchange on a fast timescale (700 ± 137 μs), and complete conversion into the active S4 state requires both agonist and transducer binding. Using structural insights from double electron–electron resonance studies, S1 was identified as an inactive state with an interaction between TM3 residue R1313.50 and E2686.30 (superscripts indicate Ballesteros–Weinstein numbering for GPCRs and E2684.30 in TM6 (ref. 5), commonly termed the ionic lock. The ionic lock has previously been shown to be important in maintaining the inactive conformation of β2AR, as charge-neutralizing mutations at these positions increase receptor constitutive activity. Additionally, the S2 conformation was also identified as an inactive state but with a disengaged ionic lock (Fig. 2b). The binding of G protein or other positive allosteric modulators such as Nb80 lowers the energy of the active receptor states, driving the receptor from S2 towards active conformations. The 19F NMR spectra showed that the addition of Nb60 to β2AR bound to the inverse agonist carazolol shifted the S1–S2 equilibrium towards the inactive S2 state (Fig. 2c), providing a mechanism for its negative cooperative effects on isoprenaline affinity. Given the broad NMR line shape (Fig. 3c, red line) of the β2AR when bound to carazolol and Nb60, we conducted Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion measurements to measure potential conformational heterogeneity. We found that the β2AR when bound to Nb60 and carazolol interconverts (860 ± 530 s−1) between S1 and S2, but is predominantly found (75–90%) in the S2 inactive state (Extended Data Fig. 1d).

To further decipher how Nb60 induces a negative cooperative effect on agonist binding, we determined a 3.2 Å X-ray crystal structure of a ternary complex comprised of β2AR, Nb60, and the inverse agonist carazolol (Fig. 2d–f, Extended Data Fig. 2 and Extended Data Table 2). The complementary determining region 3 (CDR3) of Nb60 inserts into a similar β2AR allosteric pocket as G protein and Nb80, located between the cytoplasmic ends of TM3, TM4, and TM6 (Fig. 2d, e). We found that T102 and Y106 in Nb60 bridge an interaction between residues R1313.50 and E2684.30. This interaction does not exist in the absence of Nb60 (Protein Data Bank (PDB) accession code 2RH1), indicating that Nb60 stabilizes an inactive conformation through interactions with the β2AR ion lock (Fig. 2e). However, this polar network appears ‘disengaged’ compared to the fully closed ion lock in the β1-adrenergic receptor (PDB accession code 2YCW) (Fig. 2e), thus supporting the 19F NMR data showing that Nb60 specifically stabilizes the S2 inactive state. The insertion of Nb60 F103 into a hydrophobic pocket in the β2AR may also contribute to the affinity and/or negative allosteric properties of Nb60 (Extended Data Fig. 2c). Confirming the importance of T102 and F103 for the β2AR–Nb60 interaction, alanine mutations at these positions inhibited Nb60 binding to the β2AR and the negative cooperative effects on isoprenaline binding.
Data Fig. 2e, f). Other than changes within the ionic lock, the overall structure of β2AR bound to Nb60 is highly similar to the previously determined inactive β2AR structure bound to carazolol alone (root mean squared deviation (r.m.s.d.) of 0.3 Å for the transmembrane domains and orthosteric binding pocket, Fig. 2f). Taken together, the pharmacological, biophysical, and crystallographic studies show that Nb60 exerts its negative allosteric effect on agonist binding by stabilizing the S2 inactive β2AR conformation.

Our observation that isoprenaline bound to the Nb80-stabilized active β2AR with approximately 15,000-fold greater affinity than to the Nb60-stabilized inactive β2AR (Fig. 1b, c) provides a measurement of the full allosteric power of an agonist to activate a GPCR. The large free energy difference (−24 kJ mol$^{-1}$) between these states is probably important for GPCR function, allowing agonist-stimulated activity to be significantly higher than that of the basal activity. As simulated for a full agonist in Fig. 3a, the overall affinity shift from inactive to active receptor (black arrow) is comprised of two components, the negative cooperative (α) effects of Nb60 (αNb60, blue) and positive effects of Nb80 (αNb80, red). Given the complexities and limitations of using NMR and crystallography to gain mechanistic insights into ligand activation of a GPCR, we used a pharmacological approach to quantify αNb60 and αNb80 for 17 β2AR ligands of varying efficacy (Fig. 3b and Extended Data Fig. 3).

We first identified a significant positive correlation ($r = 0.8514$, $P = 0.004$) between αNb80 values ($K_i/K_D$ ratios) and the relative intrinsic efficacies ($\tau$ values) of various ligands obtained from cellular G-protein assays (Extended Data Fig. 4a, b). This finding provides additional support for the claim that Nb80 exerts allosteric effects that mimic those of a G protein. It also confirms our previous reports that ligand efficacy is not a product of modified downstream signalling events but is rather achieved at the level of ternary complex interactions, reflecting the allosteric interactions between different ligands and transducers.12 Interestingly, we find no significant correlation between αNb80 and Nb60 (αNb80-Cz–Nb60).

Figure 3 | Nb60 and Nb80 have varying effects on the affinity of different β2AR ligands. a, Schematic depicting the use of equilibrium radioligand binding studies to quantify the cooperativity (α) between Nb60 or Nb80 binding and ligand affinity (see Methods and Supplementary Information). c.p.m., counts per minute. b, Cooperativity values for Nb60 (αNb60) and Nb80 (αNb80) for β2AR ligands with varying efficacies. Ligands are ordered by magnitude of αNb80. c, Correlation plot of αNb60 and αNb80; regression shown as solid red line with 95% confidence interval (dotted red line). All α values derived from at least three independent radioligand binding experiments with the deviation depicted as standard error. Adr, adrenaline; alp, alprenolol; cary, carvedilol; caraz, carazolol; clen, clenbuterol; fen, fenoterol; form, formoterol; hbi, hydroxybenzyl isoproterenol; ICI, ICI-118,551; iso, isoprenaline; isoe, isoevethane; pin, pindolol; proc, procaterol; sal, salbutamol; salm, salmeterol; zint, zintrolerol.
αNb60 and ligand efficacy, providing the first evidence, to our knowledge, that ligands perceive the Nb60 and Nb80 stabilized receptor states differently (Extended Data Fig. 4c). We observed a significant negative correlation (r = 0.7417, P = 0.0013) between αNb60 and αNb80 values (Fig. 3c) across all ligands, suggesting these nanobodies stabilize functionally opposite conformations. However, the relationship between αNb60 and αNb80 was unexpectedly complex (Fig. 3b). For example, several full agonists exhibited comparable levels of positive (Nb80) and negative (Nb60) cooperativity (noradrenaline, adrenaline, and isoprenaline), whereas some partial agonists displayed patterns with surprising discrepancies (clenbuterol, salbutamol, and zinterol) (Fig. 3b). Importantly, for a subset of these ligands we confirmed that the allosteric effects of Nb80 are consistent with those elicited by the physiological transducer heterotrimeric Gs (Extended Data Fig. 5a, b). Moreover, the surprisingly divergent, ligand-specific effects of Nb60 were also observed with another inactive state-stabilizing nanobody, A11 (Extended Data Fig. 5c). Together, these data indicate that the ligand-specific effects of Nb80 and Nb60 are not nanobody-specific, but rather reflect how ligands perceive specific receptor conformations.

We next tested two different allosteric models to try to explain how the different conformations stabilized by Nb60 and Nb80 can generate the observed cooperativities. First we tested whether the dynamics of receptor states can be sufficiently modelled as a simple interconversion between two allosteric conformations, despite biophysical evidence for multiple inactive and active receptor states. Accordingly, we attempted to fit the experimentally observed αNb60 and αNb80 values for all ligands using the two-state model of receptor activation (Fig. 4a). In this simulation, the equilibrium J represents the distribution of inactive (R60) and active (R80) receptor states in the absence of ligand. The variable β describes the effect that each ligand has on the distribution of receptor states (J), with agonists displaying larger β values (that is, they stabilize more R80 relative to R60). As shown in Fig. 4a, the theoretical curve generated by varying β in the two-state model (dotted black line) failed to accurately predict the experimentally determined αNb60 and αNb80 values for 30% of ligands tested, consisting primarily of partial agonists (dotted red oval). A different equilibrium J constant would be required to explain the cooperativities of these ligands with the same two-state model. These findings argue that ligands must modulate more than these two states to control receptor activation.

Biophysical evidence supports the existence of at least three predominant receptor states; therefore we hypothesized that a three-state model (Fig. 4b) may better explain the experimentally observed αNb60 and αNb80 values. In this model, the equilibria of R60 (J1) and R80 (J2) can be regulated separately by ligands, described by the allosteric factors β1 and β2, respectively. We found that the observed cooperativities for 12 ligands (Fig. 4b, class I, orange), which encompasses all the full agonists, can be predicted if these ligands primarily stabilize the active R80 state, while having negligible effects on the inactive R60 state. The efficacy of these ligands is thus directly proportionally related to their effect on the R80 equilibrium (β2), such that highly efficacious ligands have a large β2 (adrenaline), whereas partial agonists have a lower β2 (clenbuterol). In contrast, the observed cooperativity of other partial agonists (classes II and III) could only be predicted by differentially modulating the R60 and R80 equilibria, suggesting these ligands regulate multiple receptor states to control receptor activation. For example, even though clenbuterol (class I), zinterol (class II), and procaterol (class III) are similar partial agonists (Extended Data Fig. 4a), the mechanism by which they promote receptor activation varies based on their ability to differentially stabilize and destabilize the active (R80) and inactive (R60) states, respectively (Fig. 4b). Importantly, divergences in receptor activation mechanisms can only be uncovered by studying ligands (dashed red oval). However, the observed cooperativity values can be accurately predicted using an allosteric model in which ligands can differentially modulate three independent receptor states (three-state). Certain ligands (orange) primarily stabilize the active R80 state, whereas others (purple or green) can stabilize R60 but simultaneously destabilize the inactive R60 state. All α values are derived from at least three independent radioligand binding experiments with the deviation depicted as standard error.
ligand effects at both the Nb60-stabilized and Nb80-stabilized β2AR states. The inability to accurately predict efficacy (G-protein activation) based on ligand class supports the notion that molecular efficacy is a complex process that probably involves many more unresolved receptor states.

These findings reshape our current understanding of GPCR activation. For almost 40 years, studies of these receptors have been guided by the understanding that they can exist in two forms, one of high affinity for agonists (active, coupled to the G protein) and one of low affinity (inactive, not coupled to the G protein), which are interconverted by the cooperative interaction of agonists and guanine nucleotides. We used conformationally sensitive nanobodies (Nb60 and Nb80) to reveal that Nb60-bound β2AR has affinity ~100-fold lower than the previously described low-affinity inactive state, which is in fact an ‘average’ of multiple, rapidly interconverting inactive and active states. The implication is that the full allosteric potential of the β2AR is orders of magnitude greater than formerly appreciated. Moreover, binding cooperativity between ligands of varying efficacy and Nb60 and Nb80 could only be explained by models featuring multiple (at least three) receptor states, which are differentially stabilized and/or destabilized by various agonists. Thus these data demonstrate a previously unsuspected heterogeneity in the mechanisms by which different agonists stimulate biological responses. In view of the general conservation of GPCR activation mechanisms, and a report of an A2A adenosine receptor 'Nb60-like' state, these findings will probably apply to many GPCRs.

Given the primacy of GPCRs as therapeutic targets, these new findings and concepts may also provide a basis for the design of drugs with potentially new therapeutic properties.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions D.P.S. and R.T.S. conceived the project. Pharmacological assessment of the interactions between Nb60 and Nb80 with the β2AR were designed, performed and analysed by D.P.S., R.T.S., B.P., S.A., and A.C. Formulation, purification, and crystallization of the β2AR–Nb60–carazolol complex was conducted by D.P.S. and A. Manglik. Data collection, refinement, and structural analysis was done by A. Manglik, A.K.C., and A. Masoudi, and W.I.W. NMR spectroscopy was executed by A. Manglik, T.H.K, and supervised by R.S.P. Isothermal titration calorimetry was conducted by A.W.K. Nanobody reagents were provided by E.P. and J.S. Detailed allosteric analysis of radioligand binding data was implemented by T.C. Figures were created by D.P.S., A. Manglik, T.C., L.M.W., R.T.S., and A. Masoudi. The manuscript was written by D.P.S., T.C., R.T.S., L.M.W., A. Manglik, A.K.C., and R.J.L. Overall research was supervised by B.K.K. and R.J.L.

Author Information Coordinates and structure factors for the β2AR–Nb60–carazolol complex are deposited in the Protein Data Bank (accession code 5JQH). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.J.L. (lefko001@receptor-biol.duke.edu), B.K.K. (kobilka@stanford.edu) or T.C. (tommaso.costa@iss.it).

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METHODS

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

β2AR reconstitution in HDL particles. Human β2AR containing an amino-terminal Flag epitope tag, carboxyl-terminal His-tag, and a N187E glycosylation allocation during experiments and outcome assessment. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Crystallography of β3AR–carazolol–Nb60 complex. For crystallography trials, we used a previously described fusion protein of human β3AR with an amino-terminal Flag–carboxyl-terminal His6 tag, biotinylated complex model data analysis. Briefly, 50 ng of carboxypeptidase-treated (to remove β2AR) and Nb60, purified as described above, were both dialysed against a buffer consisting of 20 mM HEPES pH 7.5, 100 mM NaCl, 0.01% MNG, and 0.001% CHS. The detergent was gradually exchanged to 0.01% lauryl maltose neopentyl glycol (MNG, Anatrace). The purified complex was concentrated to 37.5 mg ml⁻¹, and flash frozen in liquid nitrogen.

Data collection and refinement. X-ray diffraction was collected at the Advanced Photon Source GM/CA beamline 23-ID-B. As with previous in mero crystallography of GPCRs, crystals suffered considerable radiation damage during data collection. Typically, 20–60 degree wedges of data were collected using a 20 μm beam. The resulting diffraction data from 10 crystals were processed in HKL2000 (ref. 28). The structure of the β3AR–carazolol–Nb60 complex was solved by molecular replacement using carazolol-bound β3AR with the T4 lysozyme removed (PDB accession code 2RH1) and a structure of Nb60 solved to 1.8 Å as search models in Phaser. The model was refined iteratively in Phenix with manual rebuilding in Coot with and without averaging of non-crystallographic symmetry. The quality of the final model was assessed using MolProbity and refinement statistics are presented in Extended Data Table 2. Figure were prepared using PyMOL (Schrödinger). The r.m.s.d. analysis for the orthosteric binding pocket was performed in PyMOL by measuring residues within 4 Å of the ligand carazolol between β3AR–Nb60–Cz and the Protein Data Bank accession code 2RH1.

19F NMR of β3AR. 19F fluorine NMR studies were conducted as previously described. Briefly, full-length β3AR with four cysteine mutations (C77V, C327S, C178A, and C406A) was expressed in S9 cells, extracted using dodecylmaltoside, and purified initially by M1 Flag affinity chromatography. The receptor sample was subsequently labelled with a bromotrifluoroacetanilide probe at a fivefold stoichiometric excess and purified by alprenolol–sepharose and M1 affinity chromatography. NMR experiments were performed in PyMOL by measuring residues within 4 Å of the ligand carazolol between β3AR–Nb60–Cz and the Protein Data Bank accession code 2RH1.

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\[ [R_j] = [R_f] \cdot \left[ 1 + M[N]_j + \sum_{i=1}^{n} K_i[L_i]_j(1 + \alpha_i M[N]_j) \right]^{-1} \]

\[ [N]_j = [N_f] \cdot \left[ 1 + M[R]_j + \left(1 + \sum_{i=1}^{n} \alpha_i K_i[L_i]_j \right) \right]^{-1} \]

\[ [L_i]_j = [L_i]_t \cdot (1 + K_i[R]_j(1 + \alpha_i M[N]_j))^{-1} \]

where subscripts \( f \) and \( t \) indicate respectively free and total concentrations and the equilibrium binding constants of ligands (\( K \)) and nanobody (\( M \)) are defined as in supplementary (analysis of binding cooperativity). Exact numerical solutions were obtained using a globally convergent iterative algorithm\(^3^4\), which was coded as add-in function for Excel\(^3^5\). The built-in optimizer ‘solver’ was used to minimize the sum of squares difference between predicted and experimental data (choosing the Newton search option and setting convergence threshold at \( 10^{-12} \)). Sets of 3 curves representing bound radiotracer as a function of increasing concentrations of unlabelled ligand, obtained in the absence and presence of Nb80 or Nb60, were fitted simultaneously. Known parameters are the \( M \) of each Nb (Extended Data Fig. 6), the \( K \) and \( \alpha \) value of \([^{125}\text{I}]\text{cyanopindolol} \) (Extended Data Fig. 1). Unknown fitted parameters are: non-specific tracer binding, receptor concentration and \( K \) value of the competing ligand (both shared across the 3 curves), and the individual \( \alpha \) values for each ligand/Nb pair. For data measured in the presence of Gs (the \( M \) value of which was not available), all ligands curves were fitted globally and constrained to share a common best-fitting \( M \). This fitted value was close to that experimentally measured for Nb80. See supplemental information and Extended Data Figures 7 and 8 for additional information on allosteric modeling.
Extended Data Figure 1 | Characterization of Nb60 interaction with β2AR. a–c, Competition equilibrium binding studies using[^25I] cyanopindolol (CYP), the cold competitor agonist isoprenaline (ISO), β2AR in HDL particles, and the indicated concentration of Nb80 (a), Gs (b), or Nb60 (c). The dotted vertical line represents log IC50 in absence of modulator, and the change in ligand affinity is depicted with coloured arrows. d, 19F NMR CPMG relaxation dispersion experiment with β2AR–Nb60–carazolol (Cz). Kex, exchange rate. e, Competition equilibrium binding studies using[^25I]cyanopindolol, the non-labelled competitor agonist isoprenaline, β2AR in HDL particles, and 1μM wild-type Nb60 or Nb60(T102A/F103A). f, ELISA depicting capture of β2AR by wild-type Nb60 or the T102A/F103A variant. Inset: Coomassie stain of nanobody input. Radioligand binding and ELISA experiments were performed at least three times with deviation shown as standard error.
Extended Data Figure 2 | Characterization of β2AR–Nb60–carazolol crystals. a, Monodispersity of T4L–β2AR–Nb60–carazolol (β2AR–Nb60–Cz) complex as assessed by size exclusion chromatography. Inset, Coomassie stain illustrating presence of β2AR and Nb60 in fractions combined for crystallography. b, Representative picture of β2AR–Nb60–Cz lipidic cubic phase (LCP) crystals. c, Insertion of F103 (green) from Nb60 CDR3 (purple) into hydrophobic β2AR pocket, nitrogen and oxygen shown as blue and red shaded surfaces, respectively. d, Example of β2AR–Nb60–Cz crystal lattice. e, Electron density 2Fo–Fc map (Sigma: 1) of carazolol binding pocket (top panels) Nb60 CDR3 binding pocket (bottom panels) within β2AR.
Extended Data Figure 3 | Differential effects of Nb60 and Nb80 on the affinity of 12 different β2AR ligands. Competition equilibrium binding studies using [125I]cyanopindolol, the indicated non-labelled competitor, β2AR in HDL particles, and 1μM of Nb60 or Nb80. Data represent at least three independent experiments with deviation depicted as standard error.
Extended Data Figure 4 | Agonist-induced G-protein activation in cellulo correlates with the magnitude of affinity change mediated by Nb80 in vitro. a, Table representing cell signalling and ligand affinity data. Ligand-dependent G-protein activation was quantified by measuring cAMP levels (GloSensor, Promega) from HEK293 cells overexpressing β2AR. Ligand affinity was measured in membranes prepared from the same cells as above using competition binding assays with [125I] cyanopindolol. Ligand efficacy (log τ) was calculated as previously described. See methods and Supplementary Information for cooperativity (α) determination. b, c, Correlation plot of log τ and αNb80 (b), or αNb60 (c). All data represent at least three independent experiments with deviation shown as standard error.
Extended Data Figure 5 | Positive correlation between allosteric properties of Nb80 and Gs. a, Equilibrium binding studies using HDL β2AR, [125I]cyanopindolol, the indicated unlabelled competitor, and 100 nM purified heterotrimeric Gs protein. b, Correlation plot of cooperativity values (α) for Nb80 and Gs. c, Sequence alignment of Nb60 and NbA11. Radioligand competition binding studies with Nb80, Nb60 or NbA11, [125I]cyanopindolol, the unlabelled competitor isoprenaline or clenbuterol, and HDL β2AR. All data represent at least three independent experiments with deviation shown as standard error.
Extended Data Figure 6 | Affinity determination for Nb60 and Nb80 for unliganded β2AR. ELISA assay detecting capture of increasing concentrations of Nb60 or Nb80 by immobilized HDL β2AR in the absence of ligand. All data represent at least three independent experiments with deviation shown as standard error.
Extended Data Figure 7 | Theoretical framework illustrating the two views of allostery. **a.** Nested reaction schemes at equilibrium indicating the correspondence (arrowed light-blue shades) between binding site cooperativity (ternary complex model in outer box) and changes of allostERIC conformations (inner cubes). Arrows stand for reversible equilibrium interactions. **b.** Change of the macroscopic dissociation constant (1/K) of a ligand L (shifting the equilibrium towards r₁) induced by increasing the concentrations of nine different N-ligands with diverse allosteric effects (γ₁, γ₂) on receptor states. Simulations were made using a three-state model based on the parameter values listed on the right side of the plot (curves on the left side are colour coded in red/blue tones corresponding to the boxes on the right). The change in K (that is, log difference between presence and absence of N) is calculated from equation 1 in the Supplementary Information (analysis of nanobody allostery).
Extended Data Figure 8 | Comparison of experimental and theoretical cooperativities predicted according to a two-state or three-state allosteric model. See also the Supplementary Information section on analysis of nanobody allostery. a–d, Theoretical log $\alpha$ values were computed according to a two-state model for a series of hypothetical ligands (L) ($\log(\beta_1)$ range: 4/8) and a positive (PAN, $\log(\gamma_1) > 0$) or negative (NAN, $\log(\gamma_1) < 0$) nanobody. a–d, Observed data overlaid on values simulated at $J_1 = 8.9 \times 10^{-4}$ in histogram form (with experimental bars drawn on the closest theoretical $\log(\beta_1)$ bin value) (a), or superimposed (b), on the $\log(\alpha_{\text{NAN}})$ versus $\log(\alpha_{\text{PAN}})$ relationships predicted for different $J_1$ values. The same data are replotted as separate graphs for lower $J_1$ (c) and larger $J_1$ (d) values, to show the sigmoidal relationships existing between macroscopic log $\alpha$s and log $\beta_1$. e, f, Simulations made according to the three-state allosteric model. e, Predicted (lines) and observed (circles) log $\alpha$ values plotted as functions of log ($\beta_1/\beta_2$). Three groups of ligands (I to III, defined by the table of $a_0$ and $m$ parameters) produce increasingly stronger reductions of $r_2$ equilibrium. f, Same data plotted as $\log(\alpha_{\text{Nb60}})$ versus $\log(\alpha_{\text{Nb80}})$ relationships (see Fig. 4). All $\alpha$ values derived from at least three independent radioligand binding experiments with deviation depicted as standard error.
|                  | $[^{125}]$-Cyanopindolol Affinity | Kd Ratio |
|------------------|----------------------------------|----------|
|                  | Kd (pM)  | SEM     | Control / Nb | SEM   |
| Control          | 737.3    | 92.9    | ----         | ----   |
| Nb80             | 315.6    | 62.4    | 2.5          | 0.35   |
| Nb60             | 663.0    | 17.7    | 1.1          | 0.12   |
Extended Data Table 2  |  Data collection and refinement statistics (molecular replacement)

| **Data collection** | **β_2_AR-Carazolol-Nb60^a** |
|---------------------|-------------------------------|
| Space group         | *P*2₁₂₁₂₁                      |
| Cell dimensions     |                               |
|  \( a, b, c (\text{Å}) \) | 43.9, 164.5, 218.8             |
|  \( \alpha, \beta, \gamma (^\circ) \) | 90.0, 90.0, 90.0            |
| Resolution (Å)      | 32.9-3.2 (3.30-3.20)           |
| \( R_{\text{merge}} \) (%) | 17.5 (89.6)                  |
| \( <I/\sigma I> \) | 5.3 (1.5)                     |
| \( \text{CC}_{1/2} \) (%) | 98.6 (82.4)                   |
| Completeness (%)    | 98.6 (95.5)                   |
| Redundancy          | 5.5 (4.6)                     |

**Refinement**

| Resolution (Å) | 32.9-3.2 (3.30-3.20) |
| Number of reflections | 26,778              |
| \( R_{\text{work}}/R_{\text{free}} \) (%) | 24.7/29.0 (38.3/43.9) |
| Number of atoms |                               |
| Protein         | 7,703                       |
| Ligand (Carazolol) | 44               |
| Solvent (lipid, water, other) | 56             |
| B-factors (Å²)  |                               |
| Protein         | 129.81                      |
| Ligand (Carazolol) | 116.36             |
| Solvent (lipid, water, other) | 157.51          |
| R.M.S. deviation from ideality |                     |
| Bond lengths (Å) | 0.003                      |
| Bond angles (°)  | 0.429                      |
| Ramachandran statistics^c (%) |                   |
| Favored         | 96.8                       |
| Allowed         | 3.2                        |
| Outliers        | 0                          |

^aDiffraction data from 10 crystals were merged into a complete data set. ^bHighest resolution shell statistics are shown in parentheses. ^cAs calculated by MolProbity.