Conformational plasticity of ligand-bound and ternary GPCR complexes studied by $^{19}$F NMR of the $\beta_1$-adrenergic receptor

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G-protein-coupled receptors (GPCRs) are allosteric signaling proteins that transmit an extracellular stimulus across the cell membrane. Using $^{19}$F NMR and site-specific labelling, we investigate the response of the cytoplasmic region of transmembrane helices 6 and 7 of the $\beta_1$-adrenergic receptor to agonist stimulation and coupling to a $G_s$-protein-mimetic nanobody. Agonist binding shows the receptor in equilibrium between two inactive states and a pre-active form, increasingly populated with higher ligand efficacy. Nanobody coupling leads to a fully active ternary receptor complex present in amounts correlating directly with agonist efficacy, consistent with partial agonism. While for different agonists the helix 6 environment in the active-state ternary complexes resides in a well-defined conformation, showing little conformational mobility, the environment of the highly conserved NPxxY motif on helix 7 remains dynamic adopting diverse, agonist-specific conformations, implying a further role of this region in receptor function. An inactive nanobody-coupled ternary receptor form is also observed.
**G protein-coupled receptors (GPCRs)** are a family of plasma membrane-embedded, seven transmembrane sensors that respond to a wide range of extracellular ligands. GPCRs act as signal transducers stimulating a response on the cytoplasmic side of the membrane via a range of intracellular binding partners (IBPs), including G proteins and β-arrestins. With just over 800 GPCRs in humans they are the largest class of membrane proteins. Signal transduction occurs following ligand binding, which leads to an allosteric conformational signal response, whereupon changes induced by the binding event at the extracellular side of the receptor drive conformational rearrangements at the cytoplasmic face, leading to coupling with IBPs. Due to their central role in signal transduction GPCRs are involved in a wide range of physiological processes and consequently, are key proteins in many disease pathways. Class A or rhodopsin-like GPCRs are the targets for many disease pathways.}

Receptor regulation of the signalling process occurs at multiple levels, involving binding of orthosteric agonists as well as allosteric modulators such as lipids. Our understanding of GPCR signalling has been greatly enhanced by crystal structures of receptors in the inactive, ligand-bound and fully active (coupled) states. In our recent 13C methionine NMR study, we report information about the role of the highly conserved NPxxY motif at the end of TM7 in combination with fluorescence-tagging to explore the cytoplasmic side of TM7 upon ligand binding and nanobody coupling (Fig. 1, Supplementary Fig. 1) using 19F NMR. Furthermore, we introduce Cys82825.27 at the equivalent position to C26526,27 in the β2AR for a more detailed mechanistic investigation of the intracellular region of TM6 (Fig. 1, Supplementary Fig. 1). 19F NMR studies have been widely used to investigate the conformational equilibria of GPCRs including the β2AR and z2AR receptors. Using these two reporter cysteines combined with an NMR active 19F-TET tag (Supplementary Fig. 2), we investigate the dynamic response of previously unexplored regions of the cytoplasmic face of the turkey β2AR to ligand binding and ternary complex formation, using a G protein-mimetic nanobody. Allosteric signal transduction from the orthosteric ligand-binding pocket on the extracellular side of β2AR to the cytoplasmic IBP interface, reveals a shifting equilibrium between inactive and active states, correlating with ligand efficacy. β2AR coupling to nanobody results in the formation of active as well as inactive ternary complexes, with the amount of active ternary complex correlating with the efficacy of the bound ligand. Hence, our study provides direct structural evidence for the formation of the active-state ternary complex in proportions reflecting the ligand efficacy, which in turn defines the subsequent level of downstream signalling β2AR. Interestingly, the conformational response in the two cytoplasmic regions on TM6 and TM7 of the ternary receptor complex is different. The response of TM6 indicates the formation of a single, well-ordered active-state ternary complex in this region of the receptor, determined by the coupling partner, which is independent of the agonist type and shows little additional conformational dynamics. In contrast, TM7 in the vicinity of the NPxxY motif shows ligand-dependent conformational variability in the complex with extensive μ-to-ms dynamics and conformational features at the cytoplasm that are determined by the bound orthosteric ligand. Beyond the stabilisation of the active state, our observations suggest an involvement of the NPxxY microdomain during receptor activation in a manner that is predominantly related to the properties of the orthosteric ligand bound. This suggests a mechanistic role of this receptor region that might be independent of the coupled G protein, enabling the binding of further IBPs and resulting in a change in signalling bias or strength.

**Results**

**Ligand binding conformational equilibria of TM6 and TM7.** To obtain information on the response of the cytoplasmic region of turkey β2AR to ligand binding, individual samples of β2AR-m-TM6-CysΔ2 (Fig. 2a, Supplementary Note 1, Supplementary Figs. 4, 6a) and β2AR-m-CysΔ2 (Fig. 2b, Supplementary Note 1, Supplementary Figs. 4, 5, 6b) solubilized in LMNG detergent micelles were investigated by 1D 19F NMR upon addition of saturating amounts of agonists, including in order of increasing efficacy for TM6 atenolol, carvedilol, alpenolol, xamoteroi, isoprenaline and for TM7 atenolol, carvedilol, alpenolol, cyano-pindolol, bucinolol, xamoterol, isoprenaline and the natural ligand adrenaline (Supplementary Table 1). Bucindolol and carvedilol are known to be biased agonists for β2AR33–36. In the ligand-free apo state, A282C-TET 6.27 on TM6 appeared as a single sharp peak, P1 (Fig. 2a). Only minor changes in chemical shift and linewidth were observed upon binding to the different agonists (Supplementary Fig. 6a, Supplementary Table 2). Although
there was no systematic correlation with ligand efficacy, line- widths were slightly increased when bound to the higher efficacy agonists (Supplementary Fig. 7), suggesting some exchange broadening when bound to these latter ligands. For each of the current 19F spectra the signal P1 could be deconvoluted with a single Lorentzian line (Supplementary Fig. 8, Supplementary Table 2). Comparing the $R^2$ values of apo $\beta_1$AR and isoprenaline bound receptor substantiated the presence of a small exchange contribution to the linewidth of P1 (Supplementary Table 2). The failure to detect a more substantial response at the cytoplasmic side of TM6 parallels recent observations made in our 13C methionine NMR study using the immediately neighbouring residue on TM6, M2836.28, as a probe17. Despite demonstrating via other residues e.g. M2966.41 located on TM6 closer to the ligand-binding pocket that $\beta_1$AR-m responds in an efficacy dependent manner to stimulation by agonists, the detergent exposed M2836.28 showed only small changes17.

To examine whether P1 was undergoing sub-second conformational exchange with other very low populated states, we conducted a series of saturation transfer experiments with the apo receptor and the isoprenaline bound receptor, respectively. We scanned a region from $-1100$ to $+1100$ (±2 ppm) relative to the main peak P1 in steps of 100 Hz but found no evidence of exchange.

Comparable 19F NMR experiments were conducted with $\beta_1$AR-m-CysΔ2 and agonists using TETC3447.54 on TM7 to probe the conformational response of the region immediately adjacent to the NPxxY7.53 motif (Fig. 1). A single signal P2 (I1 state) was observed for the receptor apo form that shifted gradually towards higher field as receptor samples were bound to

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**Fig. 1 Structure overlay illustrating conformational changes in $\beta_1$AR upon activation.**

**a** Side-on view of $\beta_1$AR bound to cyanopindolol, representative of the inactive state (magenta, PDB code 2YCY), and the nanobody Nb80 coupled receptor bound to isoprenaline, showing $\beta_1$AR in the fully active state (blue, PDB code 6H7J). TM6, TM7 and helix 8 are shown in the colour of their respective state, with the Gs mimetic nanobody omitted for purposes of clarity. The 19F tagged cysteines A282C TET, 6.27 and TET C3447.54 are shown with their side chains represented as sticks and the $S_\gamma$ atom as coloured spheres. The structure overlay highlights the outward movement of TM6 and the rotation of TM7 upon formation of the ternary complex. **b** Enlarged view showing the NPxxY7.53 motif on TM7 containing the highly conserved Y3437.53 that upon formation of the ternary complex rotates behind TM6 and makes a water-mediated hydrogen bond with Y2275.58 on TM5 that stabilises the active state. Both Y3437.53 and TET C3447.54 rotate inwards upon formation of the ternary complex. **c** Cytoplasmic view of the region shown in (b), which illustrates the clockwise rotation of Y3437.53 and TETC3447.54 on TM7.
ligands of increasing efficacy (Fig. 2b, Supplementary Fig. 6b). Although the chemical shift changes were small (<0.06 ppm), they were accompanied by a considerable broadening of the signal linewidth on binding to agonists of higher G<sub>e</sub> efficacy (Fig. 2c, d, Supplementary Fig. 6b). This was particularly noticeable for xamoterol, isoprenaline and adrenaline, suggesting growing contributions to the receptor linewidth due to μs-to-ms timescale exchange. Signal positions and linewidths both correlated with the efficacy of the ligand bound (Fig. 2c, d). All signals for P2 were deconvoluted with a single Lorentzian line with no evidence of multiple overlapping peaks (Supplementary Figs. 8, 20, Supplementary Table 2). Hence, the line broadening with the receptor bound to high efficacy agonists was further investigated through 19F NMR CPMG experiments that were performed at 564 and 658 MHz (19F) and showed the effective removal of exchange broadening for TETC3447.54 as the pulsing rate ν<sub>CPMG</sub> was increased from 100 to 5000 Hz (Fig. 2e). No measurable exchange contribution was detected for the apo receptor. By fitting the CPMG data for xamoterol and isoprenaline and reconstructing the corresponding spectral shifts of P2 relative to apo.
the apo receptor position, exchange parameters were determined for xamoterol ($k_{ex} = 1.913 \pm 299 \text{s}^{-1}$, $p_{32} = 0.11 \pm 0.01$) and isoprenaline ($k_{ex} = 2.878 \pm 317 \text{s}^{-1}$, $p_{32} = 0.29 \pm 0.04$) bound receptor (Fig. 2e, Supplementary Fig. 6b, Supplementary Table 2).

Applying the saturation transfer technique to a region surrounding the P2 peak (Fig. 3) a substantial drop in the P2 intensity revealed the presence of a previously invisible receptor signal, P3 (A state), at a position 300 Hz upfield from the corresponding signal P2 (Fig. 3c). With the saturating field positioned on P3, a saturation time course was recorded for the apo receptor and for the receptor bound to isoprenaline (Fig. 3d). A reference series with the position of the saturating field held at 0 Hz demonstrated that the signal was indeed due to a change in exchange dynamics upon nanobody binding.

The individual helices were studied by monitoring A282CTET (Supplementary Table 2). All studies conducted in the presence of nanobody showed a new peak P5 for TETC344 that was shifted downfield from P2 by approximately 0.7 ppm and was attributed to the active ternary receptor complex ($A^G+$). In contrast to TM6, the TM7 region of the receptor in its apo form as well as when bound to the different agonists responded differently to nanobody binding, and resulted in P5 signals that varied in their position over a range of ~0.3 ppm, suggesting conformational differences on TM7 amongst the ternary complexes ($A^G+$) formed (Fig. 4b, Supplementary Fig. 9c, d). In contrast to the reduction in linewidth between P1 and P4 for A282CTET, 6.27 (Fig. 4a, Supplementary Table 2), the signals of P5 for TETC344 were substantially broadened when compared to their corresponding P2 signal (Fig. 4b, Supplementary Table 2). Again, the relative signal area of the active ternary peaks P5 showed excellent correlation with the efficacy of the ligands bound to the receptor (Fig. 4d), and a similar saturation behaviour as observed for TM6 upon addition of 5- and 10-fold excess of nanobody, respectively (Supplementary Fig. 9a).

Careful inspection of the signal area in the vicinity of the ligand-bound uncoupled receptor peak P2 near $-65.5 \text{ ppm}$ revealed the presence of an additional, broader peak P6 ($A^–$) superimposed at a position very similar to P2 (Supplementary Fig. 9b). At the larger excess of nanobody the relative size of this broad P6 signal rapidly overtook the signal intensity of the sharper P2 signal of the uncoupled receptor. The presence of P6 was easily inferred from the increasingly broader appearance of the signal at the 5- and 10-fold excess of nanobody, indicating that the signal envelope at $-65.5 \text{ ppm}$ was increasingly dominated by the presence of more P6 as the remaining free receptor (P2) eventually bound to nanobody (Supplementary Fig. 9b). Except for isoprenaline the region around P2 required two Lorentzian signals for a satisfactory deconvolution resulting in a sharper component for P2 and a broader component for P6. The broader component became increasingly more intense in the presence of larger excess in nanobody (Supplementary Fig. 8, Supplementary Table 2). With the shift positions of P2 and P6 being very similar and due to a lower signal-to-noise ratio the deconvolution of the isoprenaline receptor region around P2 returned a single Lorentzian with a linewidth between the one of the sharper P2 and the broader P6 (Supplementary Table 2). Accordingly, all studies conducted in the presence of nanobody...
confirmed the presence of a second, alternative Nb6B9-coupled receptor form.

Saturation transfer was used to assess a possible slow sub-second exchange between the signals P5 and P6. A saturation transfer time course with the saturation field centred on P6 of TM7 TET C344 (384 Hz upfield from P5) in the presence of a two-fold excess in Nb6B9 showed that P5 is in slow exchange with P6, with $k_{ex} = 8.1 \pm 1.6 \text{ s}^{-1}$ (Supplementary Fig. 10a). A second pre-saturation experiment was conducted with the irradiation position further upfield from P6, at a sufficient distance (684 Hz upfield from P5) not to saturate P2 while still irradiating the broad foot of the P6 signal (Supplementary Fig. 10b). This resulted in a reduced but still noticeable response on P5 that is in agreement with the broad nature of P6 and confirmed the slow exchange process to be taking place between P5 and P6, with $k_{ex} = 8.0 \pm 1.5 \text{ s}^{-1}$ showing a similar exchange rate (Supplementary Fig. 10).

Solvent-accessibility during ternary complex formation was assessed with a soluble Gd$^{3+}$ reagent (Supplementary Note 2).

**Discussion**

We investigated the cytoplasmic region of β$_1$AR using $^{19}$F NMR via TET fluoro-tagging of the native C344$^{7,34}$ in TM7 and of a separately introduced A282C$^{6,27}$ in TM6. These reporters were used to study β$_1$AR in the apo form and with a range of agonists of varying efficacy (in order of increasing efficacy: atenolol, carvedilol, alprenolol, cyanopindolol, bucindolol, xamoterol, isoprenaline and the natural ligand adrenaline) as well as using a G$_{i}$ protein mimetic nanobody Nb6B9 to form ternary receptor complexes (Supplementary Table 1).

Our $^{19}$F NMR experiments show that the ligand-free β$_1$AR is mostly in an inactive state (I$_1$), as indicated by a single, well-defined signal, P1, for TM6 (Fig. 2a) and P2 for TM7 (Fig. 2b). This supports previous studies that focused on the transmembrane region of β$_1$AR near the ligand binding pocket and showed this region of the apo receptor to be much less dynamic than when bound to full agonist$^{16,17}$. In view of the inherent low basal activity of the β$_1$AR, the apo form is likely to be representative of an inactive state, (I$_1$).
Probing the receptor with a range of agonists varying in efficacy revealed that the P1 signal for A282C\textsuperscript{TET}, showed only a relatively small response to ligand binding (Supplementary Note 3, Fig. 2a, Supplementary Figs. 6a, 12, 13).

In contrast, while the TM7 signal P2 of TETC34 indicated the apo receptor residing in an inactive form (I\textsubscript{1}), the signal P2 gradually moved further upfield with increasing efficacy of the bound agonist (Fig. 2b, c, Supplementary Fig. 6b). At the same time P2 also displayed increasing line-broadening (Fig. 2d). \textsuperscript{19}F CPMG relaxation dispersion experiments conducted at two fields for the receptor bound to isoprenaline revealed the presence of a fast exchange process with the receptor interconverting between two conformations, (I\textsubscript{1}) and (I\textsubscript{2}), with a rate $k_{ex} = 2,878 \pm 317$ s\textsuperscript{−1} (Fig. 2e). For xamoterol the exchange rate was reduced to

\textbf{NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-14526-3 ARTICLE}
k_{ex} = 1.913 ± 299 s^{-1}, while for the apo form of the receptor no additional exchange contribution to the linewidth was detected in the dispersion curve (Fig. 2e), in agreement with ligand-free β2AR mostly populating an inactive state (I1). However, for xamoterol and isoprenaline-bound receptor the second conformation (I2) was increasingly populated. The low population of this second state (I2) when in the apo form (p_{I2} = 0.02), with a slight increase when bound to xamoterol (p_{I2} = 0.11) and isoprenaline (p_{I2} = 0.29), indicates that (I2) is likely a further inactive form of β2AR (Fig. 2e). The timescale of the process 1I = 1I is too rapid to relate to substantial conformational rearrangements of the TM7 environment as e.g. observed crystallographically in the transition from the inactive state (PDB code 2CYC) to the fully active state adopted in the ternary complex structure (PDB code 6H77) (Fig. 1). Therefore, this process is most likely related to a smaller change in the side chain conformation, potentially a reorientation of the aromatic side chain of Y3437-53, one of the conserved residues consistently observed to be involved in receptor activation and IBP coupling (Fig. 5)11. An increase in the population of (I2) with strong agonists such as isoprenaline suggests that this conformational change might be linked to the early stages of receptor activation, with (I2) possibly being an on-pathway intermediate to a further downstream activated state. Aspotes would therefore shift the 1I = 1I equilibrium towards (I1), facilitating subsequent activation.

In addition to the 1I = 1I equilibrium, the existence of a further state (A) in slow exchange with the inactive (I1, I2) states of the receptor (signal P2) was found indirectly through saturation transfer experiments, which revealed the presence of a strongly broadened peak at position P3, not directly detectable by 19F NMR (Fig. 3). While only investigated for the apo and the isoprenaline bound receptor it became clear that the population of this state increased with ligand efficacy from p_{ex} = 0.15 in the apo form to p_{ex} = 0.20 when isoprenaline bound. Substantial broadening of this relatively low populated state prevented its direct observation by 1D 19F NMR and suggested the occurrence of substantial amounts of μs-to-ms conformational dynamics in the vicinity of the N-PxyT7.53 motif once the receptor was bound to full agonists. We assume that the signal P3 corresponds to a pre-active receptor state (A) that is competent to bind nanobody, subsequently upon IBP coupling leading to the fully active state as seen in the structures of the ternary complex of the receptor (Fig. 5)37. The slow rate of exchange (ISO: k_{ex} = 6.9 ± 1.8 s^{-1}, APO: k_{ex} = 3.6 ± 1.6 s^{-1}) between the states (I1, I2) and (A) is indicative of major structural rearrangements taking place in this region of the receptor. The exchange process (I1, I2) = (A) happens on a similarly slow sub-second timescale as observed in β2AR for the move of TM6 away from the helix bundle when exchanging between inactive and active-like states, suggesting that cytoplasmic TM6 and TM7 rearrangements might be related and due to steric reasons occur in parallel44. Based on the reorienta-

of aromatic side chains in the immediate environment of C3447-54 we calculated an increase in shielding of 0.16 ppm due to changes in ring current shifts when moving from the inactive structure of β2AR (approximated by the cyanopindolol bound structure) (PDB code 2CYC) to the ternary complex with Nb80 (as an approximation of the pre-active receptor state) (PDB code 6H77), suggesting a move of TETC3447-54 into a more hydrophobic environment upon reaching the pre-active state (Supplementary Fig. 14, Supplementary Table 3). This is in reasonable agreement with our experimental observation of P3 at a position 0.5 ppm upfield from the apo receptor signal P2 (Fig. 3), and suggests that the slow exchange process relates to the rotation and inward movement of the intracellular part of TM7 that allows Y7.53 following a change in the side chain rotamer conformation to slot behind the displaced TM6 (Fig. 1). As expected, the population of the (A) state increases with ligand efficacy, reaching 20% for isoprenaline. This is in a similar range to the 25% population found for the pre-active state using ^13C methionine NMR, as assessed by the reporters M2235.54 and M2966.4117. At the same time the exchange rate seems to increase with ligand efficacy as well (k_{ex} ISO > k_{ex} APO) (Supplementary Fig. 2e).

Our observation of multiple inactive receptor states (1I, 1I) for β2AR agrees with several NMR studies and MD simulations on β2AR15,19,38-40. The latter have suggested various intermediates between active and inactive states during deactivation simulations of β2AR, which in some cases showed a TM7 conformation different from the inactive or active state41. Different metastable states for β2AR were found, with TM6 adopting active-like outward as well as inward inactive-like conformations with TM7 not having reached its active-state like conformation.

MD simulations into the formation of a continuous internal water network during GPCR class A activation found such a network to be interrupted in the inactive state by the presence of two water-free layers of hydrophobic amino acid residues residing above the N-PxyT7.53 motif and below the conserved Y7.53 that opened upon agonist activation to form a continuous water channel connecting the orthosteric binding site to the G protein interaction region42. Y7.53 was found to transition between three rotamer conformations, representative of inactive (closed water channel), meta state (water channel closed at cytoplasm) and active state (open water channel). In the meta state, Y7.53 remained in a hydrophobic layer that breaks upon reaching the fully active state as a continuous water channel is formed42. In agreement with a potential meta state and the postulated model of water accessibility, the environment of TETC3447-54 adjacent to Y7.53 in our postulated pre-active (A) state is more hydrophobic than in the (1I, 1I) states as suggested by the upfield shift of P3 compared to P2 (Fig. 3, Supplementary Fig. 14)43. This agrees with the suggestion that in the (A) state the hydrophobic layer next to Y7.53 is still intact (Fig. 5). Accordingly, Y7.53 in the (A) state might have already partly rotated into an alternative conformation while not yet reaching the fully active state. In agreement with our experimental data this process is bound to be slow
as it involves the rotation and inward shift of TM7 and depends
on the outward movement of TM6 having already occurred.
Therefore, we suggest that the pre-active (A) state is competent to
bind nanobody but has not transitioned into the fully opened
conformation yet, retaining Y7.53 (and accordingly TETC3447.54)
in a hydrophobic environment.

Nanobodies such as Nb6B9 have been used to stabilise the
active state of receptors44,45. In the β2AR ternary complex they
maintain the receptor in a conformation very similar to the one in
the heterotrimeric Gs protein-bound complex46,47. Due to the
smaller size of the ternary nanobody complexes and their better
stability in detergents, we used Nb6B9 for our19F NMR studies of

Fig. 5 Schematic overview of β1AR ligand activation and ternary complex formation. Cartoon cross sections spanned by TM6, TM7 and H8 with the
TM7 TETC3447.54 probes shown as yellow spheres, adjacent to Y3437.53 of the NPxxY motif. The receptor exists in an equilibrium of inactive states (I1,2)
and a pre-active state (A), with the latter populated in growing amounts with increasing efficacy of the bound ligand. Nanobody Nb6B9 addition leads to
the formation of a fully active ternary complex (A>G+) in amounts proportional to the ligand efficacy. Nanobody binding also occurs with the inactive form
of the receptor, resulting in the formation of (A>G−). The latter can be considered as a pre-coupled inactive form, with inactive and active ternary complexes
in slow exchange with each other. The binding interface in the (A>G−) complex is shown faded, emphasising that Nb6B9 has not yet fully engaged the epitope
characteristic of the active receptor state. The (I1) ⇌ (I2) interchange takes place on a μs-to-ms timescale, while the (I2) ⇌ (A) interchange as well as the
(A>G−) ⇌ (A>G+) exchange occurs on a slower sub-second timescale. Exchange rates, where measured, are indicated below the equilibrium arrows. In the
ternary state (A>G+) the cytoplasmic region of TM6 is rigid while TM7 remains dynamic on the μs-to-ms timescale, implied by TM7 showing partly blurred.
In (A>G+) the conformation of TM7 in the vicinity of the NPxxY motif reveals agonist-dependent conformational differences, emphasised by TM7 showing
in different colours in the enlarged region marked with (*). The grey slider below each receptor cartoon approximates the relative hydrophobic/hydrophilic
extent of the TM7 19F NMR probe surrounding in that particular state. Lipid bilayer hydrophobic regions are shown in light grey. Dark grey areas indicate
transmembrane regions of the receptor with residues rich in hydrophobic side chains. These form hydrophobic gates above and below the NPxxY region
(e.g. in I1 and I2) that shield the receptor interior against bulk water access. Blue dots on a grey background (e.g. in I1, I2 and A>G−) indicate ordered internal
water molecules, separated from the bulk water through the hydrophobic gates (dark grey). Conformational changes upon activation disrupt the two
hydrophobic side chain layers, resulting in the gradual opening of a continuous internal water pathway with cytoplasmic influx of bulk water, as indicated by
the speckled grey/blue area between TM6 and TM7 in (A) and (A>G+).
β1,AR. Several crystal structures of β1,AR-nanobody complexes are now available (Supplementary Fig. 15)37. Addition of a two-fold excess of Nb6B9 to samples of β1,AR either bound to one of the diverse partial or full agonists used or in the receptor apo form led to the formation of an active state ternary complex (A(G+)) for each of the samples investigated, as evidenced by the appearance of a new 19F NMR signal P4 for the TM6 probe (Fig. 4a) and a signal P5 for TM7 (Fig. 4b). The P4 and P5 peaks were shifted substantially downfield from the signals of the uncoupled receptors. We were unable to rationalise the observed changes in the polarity of the local environment with A282CTET down variations in polarity were investigated by Ye et al. where a conformation that is unique to the active state by forming polar changes result mainly from variations in the solvent exposure of Figs. 11, 17, 18). It is likely, therefore, that the observed 19F shift changes result mainly from variations in the solvent exposure of A282CTET and TETC344 when adopting the ternary state. Although chemical shift changes for 19F are difficult to predict, the effects of variations in polarity were investigated by Ye et al. where a downfield shift was found to be indicative of an increase in solvent polarity or augmented solvent exposure43. Full activation into the ternary state results in the outward movement of TM6 and rotation of TM7 with the inward movement of the NPxxY7.53 motif (Fig. 1, Supplementary Fig. 16), that allows Y7.53 to adopt the conformation that is unique to the active state by forming polar contacts with Y5.58 on TM5 and water-mediated polar contacts to other residues, slotting behind TM6 (Fig. 1)11. The large downfield shifts observed upon formation of the ternary states suggest changes in the polarity of the local environment with A282CTET and TETC344 experiencing greater solvent exposure in the fully active state of the ternary complex. This can be understood as A282CTET moves outwards away from the TM core, while for C344T, the hydrophobic layer adjacent to the cytoplasm is now opened forming a continuous water channel between the ligand-binding pocket and cytoplasm (Fig. 5). Further discussion on the solvent exposure of the 19F probe can be found in the supplementary information (Supplementary Note 4, Supplementary Figs. 11, 17, 18).

For each agonist-bound receptor sample, the relative amount of ternary nanobody complex (A(G+)) formed was determined by integration of the corresponding NMR signal for both A282CTET (signal P4) (Fig. 4c) and TETC344 (signal P5) (Fig. 4d), the amounts of active ternary complex (A(G+)) in solution correlated very well with the efficacy of the bound agonist in the complex. Although ligand efficacy values are typically derived from cell-based assays, our experiments illustrate that agonist Gs directed signalling efficacy and the phenomenon of partial agonism closely relate to the total molecular amount of ternary receptor complex formed, as assessed here in vitro by two independent probes located at the cytoplasmic ends of TM6 and TM7.

For TM6, all active ternary complexes (A(G+)) showed P4 at the same chemical shift position indicating a strong similarity in the conformational environment of A282CTET across the different complexes (Fig. 4a), regardless to which ligand the receptor was bound. The signals were sharp with narrower linewidths than for the ligand-only bound receptor signals P2 (Fig. 4a, Supplementary Table 2), indicating the absence of any dynamics near A282CTET that would broaden the NMR signal. This is in agreement with previous observations with full-agonist isoprenaline bound β1,AR, which resulted in a sharper signal upon coupling to Nb80, as was assessed by a BTFMA probe attached to C2659.27 19. Interestingly, our previous 13C NMR study on β1,AR indicated that the region of TM6 below the binding pocket was still mobile, with the dynamics showing a dependency on the type of ligand bound, as assessed by M2968,41 F1. This suggests that even in the ternary state increased mobility persists in the regions of the allosteric network that are closer to the orthosteric binding pocket. In contrast, based on our 19F NMR data, TM6 seems likely to be more ordered near the cytoplasm, once the conformation that couples with the nanobody has been engaged. Accordingly, a single conformation for TM6 near A282CTET is adopted (Fig. 4a). This agrees with the cytoplasmic side of TM6 in its active signalling conformation guiding the positioning of the IBP, while the type of binding partner dictates the extent of displacement of the cytoplasmic end of TM6 relative to the receptor core. Therefore, a more rigid arrangement at the cytoplasm is suggested to increase the efficiency of the signalling transfer onto the coupling partner, while the residual dynamics below the orthosteric binding pocket in the receptor core maintain the allosteric signal transmission initiated by the orthosteric ligand bound.

In contrast, the appearance of the signal P5 from the cytoplasmic region of TM7 in the same complexes varied over a wide range of 0.3 ppm with the position of P5 determined by the orthosteric ligand bound (Fig. 4b, Supplementary Fig. 9d). This indicates that TETC344T4 reveals succinct ligand-dependent conformational differences in the adjacent NPxxY7.53 motif and/or helix 8 amongst the different agonist-bound ternary receptor complexes. Furthermore, all the ternary complex P5 signals of TM7 TETC344T54 were strongly broadened, supporting the presence of substantial ms-to-ms dynamics as this receptor region continues to sample multiple conformations (Fig. 4b, Supplementary Table 2). We tested whether the signal positions of P5 correlate with the efficacy of the ligands bound in the respective ternary complexes. A correlation would suggest that partial agonism is not only controlled via the amount of signalling complex formed but also via the adopted conformation at the cytoplasm of TM7. The weak correlation (R^2 = 0.51) between ligand Gs efficacy and chemical shift position of P5, however, indicated that this is not the case (Supplementary Fig. 9c). Therefore, the observed conformational variability of the NPxxY motif on TM7 seems unrelated to Gs protein signalling (Supplementary Note 5, Supplementary Figs. 15, 19).

The difference in the conformational response of TM6 and TM7 to Nb6B9 binding is remarkable and suggests a potential role for the TM7 NPxxY7.53 motif that extends beyond the stabilisation of the fully active state. Possibly this might indicate a tendency for this receptor region to engage alternative signalling pathways, be related to biased signalling, be relevant for IBP coupling specificity or reveal an additional layer of Gs independent signalling control that is determined by the orthosteric ligand. It is remarkable to note in this context that the carvedilol-bound complex resulted in the most downfield shifted peak and the largest difference to the balanced full agonist isoprenaline (Fig. 4b, Supplementary Fig. 9d). Previously, carvedilol has been associated with β1,AR biased signalling33,35,36. For the biased agonist bucindolol the effect is less pronounced with a shift similar to cyanopindolol. Accordingly, the biased agonist carve-dilol might induce a conformation on TM7 of β1,AR upon Gs protein binding that facilitates binding of further IBPs such as e.g. β-arrestin, leading to distinct cellular signalling outputs. Indeed, such complexes of GPCRs, β-arrestin and a G protein have been reported148.

19F NMR studies of the β1,AR have previously reported a semi-independent response of the TM6 and TM7 conformational equilibria following binding of orthosteric ligands of differing bias18,49. It was postulated that arrestin biased ligands preferentially activated TM7 over TM6, suggesting an involvement of TM7 in biased signalling. Our 19F NMR data for the ligand-bound β1,AR do not show such a response, possibly hindered through the inaccessibility of the P3 signal. In contrast, we observe a ligand dependent variability of the signal position for TM7 in ternary complexes of β1,AR (Fig. 4b) that seems largely decoupled from the
response of TM6. As for the β2AR this might indicate therefore that TM7 in β2AR plays a role in signal bias.

In order to assess the maximal amount of ternary complex that can be formed with nanobody we supplemented β1AR bound to carvedilol, xamoterol or isoprenaline, with a 2-fold, a 5-fold and a 10-fold excess of Nb6B9. Similar looking ligand-efﬁcacy dependent saturation curves were obtained for TM6 and TM7 (Supplementary Fig. 9a). From our previous 13C NMR study, it was known that at such an excess of nanobody there is no uncomplexed β1AR left in solution. Hence, the P2 signal (I1, I2 state) of the uncoupled receptor should have vanished. Upon closer inspection of the spectrum near the position of the P2 signal of TE3C344 we realised the gradual appearance of a new, broad peak P6 located in a very similar position to P2 that grew with the amount of Nb6B9 added (Supplementary Fig. 9b). In contrast the sharper, original P2 signal of the free receptor was gradually disappearing upon nanobody addition. Deconvolution of the P2/ P6 region of the spectra using two Lorentzian signals conﬁrmed the presence of the sharper P2 signal and the broader P6 signal (Supplementary Table 2), with β1AR in the latter increased and P2 decreasing as more nanobody was added (Supplementary Fig. 8g, h). Due to its similarly broad lineshape to the active ternary signal, P5, we conclude that in P6 the receptor is also nanobody-bound and hence also in a ternary complex. However, its signal position, similar to P2, suggests an inactive state. The active ternary signal, P5, we conclude that in P6 the receptor is not as clearly manifested except for the presence of the sharper P2 signal and the broader P6 signal (Supplementary Fig. 9a). From our previous13C NMR study, it was indicated by the position of the yellow sphere on the slider below the active ternary state is rigid, the TM7 environment remains unchanged. The active complexes with different ligands display distinct conformational variability at the frequency of the NPxxY motif. These gates are disrupted in the β1AR bound to carvedilol, xamoterol or isoprenaline, with a 2-fold, a 5-fold and a 10-fold excess of Nb6B9 added. In contrast the TM7 in β2AR this might indicate therefore that TM7 in β2AR plays a role in signal bias.

In conclusion, we investigated the response of the under-explored cytoplasmic region of turkey β1AR to ligand activation and nanobody binding using 19F NMR in combination with TET labelling of two cysteine probes located on TM6 A282C6.27 and TM7 C3447.54. We show that allosteric signal transmission initiated by agonist binding leads to the population of different inactive and pre-active receptor states that are in equilibrium with each other, gradually shifting the receptor towards a more active state as higher efﬁcacy ligands are used. Coupling of a G protein mimetic nanobody to receptors bound to agonists of varying efﬁcacy results in the formation of an active ternary receptor complex. The total amount of the latter relates to the efﬁcacy of the ligand bound, indicating that signalling output is proportional to the amount of this species formed, providing a molecular link to the concept of partial agonism. The responses of TM6 and TM7 upon nanobody binding, however, are very different. The cytoplasmic side of TM6 displays an invariable conformation, determined by the IBP coupling partner, shows no evidence of μs-to-ms dynamics and reveals the same conformation for different agonists bound. In contrast, TM7 displays distinct conformational variability in the vicinity of the NPxxY motif that depends on the agonist bound and in addition is dynamic on a μs-to-ms timescale. These agonist-dependent conformational differences in the NPxxY motif in the ternary complexes suggest a further mechanistic role of this receptor region and indicate that the response of TM6 and TM7 are partly decoupled from each other even in the ternary states. The conformational changes of TM7 are particularly pronounced between the ternary complexes with unbiased agonists and the one with the biased agonist carvedilol, suggesting a functional role.

Methods

β1AR construct generation. The turkey β1AR-m-TM6 construct was modiﬁed from the previously published β1AR-Met2-Δ5 construct by introducing two cysteine substitution mutations at position C6V2.48 and C163E4.47. Primer sequences are given in Supplementary Table 4. Differences between the β1AR-m-TM6 and the wildtype β1AR are truncations at the N-terminus, C-terminus and IL3, three thermostabilising mutations (R68L1.35, E130W3.41, F327A7.37), five methionine substitution mutations (M44L1.35, M48L1.57, M179L2.02, M281L4.3, M338A7.48) and four cysteine substitution mutations (C85V2.48, C163E4.47, C163L4.47, C163F4.47) for yield improvement and C58A to remove a palmitoylation site. The methionine modifications are not required for this work but were maintained for comparison purposes with our earlier work. We used amino acid sequence β1AR-m-Cys2 construct for β1AR expressions.

β1AR expression, puriﬁcation and 19F labelling. All receptor expressions were performed using the Flashbac baculovirus insect cell expression system. Transfection reactions for baculovirus generation were prepared by mixing 0.2 µg of pBacA9 plasmid containing the β1AR expression constructs with 4 µL Cefectin II (Thermo Fisher Scientiﬁc), 2 µL Fishbac DNA (Oxymed Expression Technologies) and 100 µL of Insect Xpress medium (Lonza). The transfection reaction was incubated for 30 min at room temperature and then applied directly onto adherent

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-14526-3 | www.nature.com/naturecommunications

NATURE COMMUNICATIONS | (2020) 11:669 | https://doi.org/10.1038/s41467-020-14526-3 | www.nature.com/naturecommunications

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-14526-3 ARTICLE

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S9 insect cells seeded at a density of 0.5 × 10^6 cells per ml in a culture volume of 1 ml. The transfection reaction was incubated at 27 °C for 5 days shaking at 90 rpm. After incubation, the cells were visually inspected for signs of viral infection. The resulting cell supernatant containing the recombinant baculovirus was harvested (P0 virus generation) and was used for further rounds of viral amplifications.

For expression, S9 insect cell cultures at a density of 1 to 1.5 × 10^6 cells per ml were infected with 4 ml virus stock per 1 L S9 cell culture. The expression was carried out for 48 to 72 h at 27 °C shaking at 160 rpm and the cells were subsequently harvested by centrifugation (4000g, 10 min, 4 °C). Resulting cell pellets were either used directly for βAR purification or stored at −20 °C. Extraction of the βAR from insect cell membranes was performed by resuspension of the cell pellet with solubilisation buffer (20 mM Tris pH 8, 8350 mM NaCl, 1% LMNG, 3 mM imidazole, Complete protease inhibitor (Roche)). The solubilisation was incubated for 1 h at 4 °C with stirring and the resulting suspension was cleared by centrifugation (175,000 g, 45 min, 4 °C). The cleared supernatant was applied onto a Hitrap FF 5 ml. Nickel affinity column on an AKTA Pure (GE Healthcare) pre-washed with equilibration buffer (20 mM Tris pH 8, 350 mM NaCl, 0.02% LMNG, 3 mM imidazole). After protease binding, the Ni column was washed in steps with the same buffer containing 3 mM, 50 mM and 250 mM imidazole, the latter eluting βAR.

The labelling of the βAR with the 19F probe 2,2,2-Trifluoroethanol (TET) was performed according to Supplementary Fig. 2B. In brief, the nickel affinity chromatography elution fractions were combined and concentrated (Amicon Ultra-15 spin concentrator with 50 kDa molecular weight cutoff) to 10 µM protein concentration. To activate cysteine side chains for 19F labelling, 4-F radical was added and the solution was incubated for 30 min at 4 °C with stirring. The TET and oxidised glutathione were removed by 1000× dilution from the supernatant and the resulting solution was stirred at 4 °C for 20 min. After incubation, Aldrithiol-4TM and oxidised glutathione were removed by 10000× dilution and the labelled βAR was further purified by alpropenol ligand affinity chromatography and the functional receptor eluted with either 1 M atenolol or 0.1 M alpropranol (Supplementary Figs. 21, 22).

Although not monitored routinely as untagged receptor is invisible to our investigations, the efficiency of the 19F labelling reaction was estimated from small-scale receptor preparations to be on the order of 80−95%. The estimate is based on the intensity comparison of the 19F NMR signal of free TET, released from the receptor following reduction of the S–S bond with DTT, relative to an external TET standard and the intensity of the SEC A280 signal of βAR prior to the labelling reaction.

Similar labelling reactions were also attempted with the reagents 3-bromo-1,1,1-trifluoroacetone (BTFMA) or 2-bromo-N-(4-(trifluoromethyl)phenyl)acetamide (BTFMA), respectively (Supplementary Methods, Supplementary Fig. 3).

Expression and purification of Nbe89. The expression and purification of Nbe89 followed established protocols17. In brief, the nanobody Nbe89 was expressed in BL21 (DE3) E. coli cells and the cell pellets were lysed before clearing the lysate by centrifugation (75,600 g, 4 °C, 30 min). The cleared lysate was applied onto a Hitrap FF 5 ml. Nickel affinity column on an AKTA Pure (GE Healthcare) pre-washed with equilibration buffer (20 mM Tris pH 8, 150 mM NaCl) and washed with the same buffer containing 15 mM imidazole before eluting the protein bound with 200 mM Tris pH 8, 350 mM NaCl and 0.02% LMNG. The labelled βAR was further purified by alpropenol ligand affinity chromatography and the functional receptor eluted with either 1 M atenolol or 0.1 M alpropranol (Supplementary Figs. 21, 22).

MD simulations. The ternary structure model of βAR bound to xamoterol in complex with Nbe89 (PDB ID 6H7N) was modified by coupling the cysteine side chain S3 position of C344 and A282C to TET and prepared for MD simulations with the Schrödinger 6 protein preparation wizard under the OPLS 2005 force field (pGlide). The structure was embedded in a fully hydrated POPC bilayer coupled to TIP3P water molecules, using the OPL3S force field for building the system and the steepest descent algorithm for energy minimisation. MD simulation time was set to 1 ns at constant volume and temperature, followed by 1 ns at constant pressure and temperature for initial system equilibration, while further extending the simulation time to 12 ns at 300 K.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that relevant data supporting the findings of this study are available within the article and its Supplementary Information files or on reasonable request from the corresponding author. The source data underlying Figs. 2–4 and Supplementary Figs. 10, 11 are provided in a Source Data file.

Received: 27 September 2019; Accepted: 10 January 2020; Published online: 03 February 2020

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Acknowledgements
This work was funded through a Hershell Smith studentship to J.N.F. and a BBSRC research grant to D.N. (BB/K01983 X/1). F.G. is the recipient of a DAAD scholarship. A.T. is grateful for financial support from the Trinity Henry Barlow Foundation, Newnham College and the Cambridge Trust. We are grateful for access to the Biomolecular NMR facility of the Department of Biochemistry (U. Cambridge) and the MRC Biomolecular NMR Centre of The Francis Crick Institute (London).

Author contributions
J.N.F. and D.N. designed the research. J.N.F. made constructs, performed molecular biology, expressed and purified proteins and prepared N.M.R. samples. A.S., B.S., A.J.T.Y., F.G. and M.J.B. conducted chemical shift calculations. J.N.F. and D.N. collected and processed N.M.R. data and analysed the spectra. R.W.B. wrote in-house software for data analysis and performed relaxation data fitting. D.N., J.N.F. and M.J.B. prepared the manuscript. D.N. supervised the project.

Competing interests
The authors declare no competing interests.
