**Phase-plate cryo-EM structure of a class B GPCR–G-protein complex**

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Class B G-protein–coupled receptors are major targets for the treatment of chronic diseases, such as osteoporosis, diabetes and obesity. Here we report the structure of a full–length class B receptor, the calcitonin receptor, in complex with a heterotrimeric Gαs–Gβγ–protein determined by Volta phase-plate single–particle cryo–electron microscopy. The peptide agonist engages the receptor by binding to an extended hydrophobic pocket facilitated by the large outward movement of the extracellular ends of transmembrane helices 6 and 7. This conformation is accompanied by a 60° kink in helix 6 and a large outward movement of the intracellular end of this helix, opening the bundle to accommodate interactions with the α5–helix of Gαs. Also observed is an extended intracellular helix 8 that contributes to receptor stability and functional G–protein coupling via an interaction with the Gβ3 subunit. This structure provides a new framework for understanding G–protein–coupled receptor function.

G-protein–coupled receptors (GPCRs) are the most abundant cell-surface receptor proteins, important for virtually all physiological processes. As major targets for treatment of disease, understanding GPCR structure and how this relates to their function is critically important for optimal exploitation of their therapeutic potential1. GPCRs allosterically transmit extracellular signals to the inside of cells by forming complexes with transducers, such as G proteins or arrestins2. To date, crystal structures of around 40 inactive class A GPCRs (of more than 800 encoded in the human genome) have been solved, with most being engineered to improve stability in detergents and/or crystal packing3. Fewer agonist-bound GPCR structures have been determined, and only one with a full heterotrimeric Gαs–Gβγ–protein4. Transmembrane domain structures of class B GPCRs have been especially refractory to crystallization and are currently limited to two inactive state structures5,6, with no reported full-length class B GPCR structures, to our knowledge.

Class B GPCRs bind physiologically and clinically important peptide hormones and are attractive targets for treatment of major chronic diseases8. These receptors have a large extracellular N-terminal domain (ECD) that is important for peptide binding. Multiple structures of the ECD fragments bound to short peptides have been reported9, but these do not inform on how bound peptides bind the receptor core for activation. The calcitonin receptor (CTR), a class B GPCR, is a therapeutic target for the treatment of a range of bone diseases, with salmon calcitonin and human calcitonin approved for clinical use8.

Single-particle cryo–electron microscopy (cryo–EM) provides a method to obtain high-resolution protein structure information without the need for crystallization. Notwithstanding the recent advances in electron detectors and cryo–EM data analysis, successful application of this technology for proteins/complexes smaller than 200 kDa remains challenging owing to the inherent low contrast that limits accurate alignment of projections to derive high resolution10,11,12. The recently introduced approach of using a Volta phase plate for data acquisition with increased contrast sidesteps many trade-offs that hamper structure determination of small proteins by cryo-EM13–15. In the current study, we used Volta phase plates to image particles of an approximately 150 kDa complex comprising an unmodified CTR bound to a peptide agonist in complex with a heterotrimeric Gαs protein. This enabled us to obtain the first near-atomic-resolution structure of an activated GPCR using cryo-EM.

**Structure determination**

To enable efficient expression and purification, the CTR was modified to replace the native signal peptide with haemagglutinin (HA), followed by a Flag epitope for affinity purification and an HRV 3C cleavage site, while the C terminus was modified by the addition of an HRV 3C cleavage site and histidine affinity tag (Extended Data Fig. 1). These modifications had no effect on receptor pharmacology (Extended Data Fig. 2).

To form an active, G-protein–coupled complex, the CTR was co-expressed with Gαs, His–Gβ1, and Gβ2 in HighFive insect cells and stimulated with an excess of the high-affinity agonist, salmon calcitonin (sCT) that has a very slow off-rate16,17. The complex was solubilized in MNG/cholesteryl hemisuccinate and purified using an anti-Flag antibody affinity column and size–exclusion chromatography (SEC) (Extended Data Fig. 3). Further complex stabilization was achieved with the addition of a camelid antibody, Nb35, which binds to the Gαs–Gβ3 interface and was used in the determination of the β3-adrenergic receptor (β3AR)–Gαs heterotrimer structure4. Addition of purified Nb35 to insect cell membranes expressing CTR, Gαs and β3AR before purification produced a monodispersed complex by SEC that remained stable at 4°C for at least 5 days; however, as noted for the β3AR–Gαs complex, the Gβ3 heterotrimer could no longer bind to GTPγS (Extended Data Figs 2e and 3e).

We recorded images of frozen-hydrated sCT–CTR–Gαs complex using Volta phase-plate cryo-EM, obtaining particle projections with high

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contiguous with transmembrane domain 1 (TM1) (Extended Data previously described\(^{23}\), mutation of either Asn125 or Asn130 to Asp was 130, corresponding to predicted glycosylation in this region. As pre-
Fig. 6a). There is also additional density in the ECD around residue
Data Bank (PDB) accession: 5II0)\(^{22}\) that could be fit to the density,
Data Fig. 4d). Although this did not permit accurate modelling, there
ECD is lower in resolution than the rest of the complex (Extended
sCT that extend secondary structure are detrimental to affinity and
flexible. This flexibility is likely to be important as modifications to
complex19 and light-activated rhodopsin complexed with Gi\(^20\). This is
interesting in the context of the CTR where two agonists, human and
salmon calcitonin, promote ternary complexes with distinct G-protein
conformations that exhibit different nucleotide affinities and have dis-
tinct efficacies\(^{27}\). The conformational heterogeneity of the sCT–CTR- 
complexed Go\(_s\) suggests that this may contribute to these observations.

We also observed multiple conformations of the receptor N-terminal ECD relative to the transmembrane core (Extended Data Fig. 5), indicating that, for the peptide-agonist-bound CTR, this domain remains flexible. This flexibility is likely to be important as modifications to sCT that extend secondary structure are detrimental to affinity and potency\(^{21}\). In the cryo-EM structure, the density corresponding to the ECD is lower in resolution than the rest of the complex (Extended Data Fig. 4d). Although this did not permit accurate modelling, there was strong agreement with the isolated ECD/sCT structure (Protein Data Bank (PDB) accession: 5II0)\(^{22}\) that could be fit to the density, contiguous with transmembrane domain 1 (TM1) (Extended Data Fig. 6a). There is also additional density in the ECD around residue 130, corresponding to predicted glycosylation in this region. As previously described\(^{21}\), mutation of either Asn125 or Asn130 to Asp was detrimental to sCT affinity and function, suggesting that this glyco-
sylation is required for normal agonist activity (Extended Data Fig. 6).

**Structure of the activated CTR transmembrane bundle**
The transmembrane bundle and the G-protein complex (minus the Go\(_s\), AHD) were resolved at a nominal resolution of 3.8 Å, with the quality of the cryo-EM density map highest within the G protein and at the Go\(_s\)–receptor interface. The local resolution varied within the CTR transmembrane bundle (Extended Data Fig. 4) with weaker density in the extracellular loops (ECLs), intracellular loops (ICLs), and the top of TM6. The limited resolution of the map in these regions suggests local flexibility that is probably required for receptor function. The CTR contains a very long extended helix 8, similar to that observed in the inactive state glucagon receptor (GCGR) structure\(^{7}\), implying that this may be a general feature for class B GPCRs. While the remaining CTR C terminus beyond helix 8 was present in the protein, it is not visible in the cryo-EM map, suggesting that this region remains mobile when the receptor is bound to a G protein.

sCT density indicates that the depth of the CTR orthosteric binding site (Figs 1 and 2a) is shallower than that predicted for some class B GPCRs\(^{2,24,25}\), with the N terminus residing approximately one helical turn above a network of conserved class B polar residues. Limited density was observed for sCT side chains, making peptide modelling ambiguous. Although specific interactions could not be identified, sCT is likely to form extensive contacts with the tops of all transmembrane domains (with the exception of TM4), as well as ECL2. The N termin-
us of the calcitonin family of peptides is distinct from that of other orthosteric class B GPCR peptide ligands, with a cyclized N terminus formed by a disulfide bond between residues 1 and 7. The apparent outward movements of TM6 and TM7 (relative to inactive structures, as discussed below) appear crucial to accommodate the peptide N terminus (Fig. 2a).

Although there are multiple possible modelling solutions for sCT, we have reported the most parsimonious, taking into account the cryo-EM map and experimental data. This model predicts that sCT maintains helicity up to residue 6, forming an amorphophilic helix with Val8, Leu12, Leu16 and Leu19 facing towards a hydrophobic receptor environment (Fig. 2b). This is in agreement with evidence that an amorphophilic helix contributes to peptide activity\(^{26}\) and consistent with that predicted from a solution NMR structure of sCT\(^{27}\). Gln14 is predicted to form hydro-
gen bond interactions with the ECL2 backbone, yet is in an orientation with solvent accessibility (Extended Data Fig. 7a). This is an essential requirement as Gln14 can be directly labelled or replaced with a Lys to conjugate bulky substituents (radiolabels or fluorophores), while
still maintaining peptide activity. In addition, two residues crucial for

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**Figure 1** The sCT–CTR–Gs cryo-EM structure. a, Orthogonal views of the cryo-EM map. The sharpened map with coloured densities (CTR transmembrane domain, blue; sCT, yellow; heterotrimeric Gs, copper; light blue and purple; Nb35, red) is overlaid with the non-sharpened map in transparency showing density for the ECD. b, Structure of the complex determined after refinement in the cryo-EM map. H8, helix 8. c, Snapshots of map versus model from transmembrane segments, Ras-like domain of Go\(_s\)–helix 5 and G3.

**Figure 2** The CTR transmembrane bundle orthosteric peptide-binding site. a, Cryo-EM density (yellow fill) for sCT in the CTR seven-transmembrane bundle (blue). The sCT N terminus sits one helical turn above a conserved polar network. Molecular models of the sCT backbone (yellow ribbon) align well with the density, side chain density, however, is not visible. b, Modelling suggests that the sCT hydrophobic face resides in a hydrophobic receptor environment formed by residues in TM1, TM2, TM3 and TM7.
Interactions in conserved residues

Class B GPCRs contain highly conserved transmembrane domain polar residues that have crucial roles in receptor integrity, high-affinity agonist interaction and/or receptor activation and downstream signalling. A central polar network (Asn194, Asn233, Glu355, and Glu383 in the CTR) is present in all inactive structures and in our inactive CTR homology model. This network is preserved in the active CTR transmembrane bundle, although the exact interactions and their relative strengths probably vary in the different structures (Fig. 3b and Extended Data Fig. 8c), in a receptor- and peptide-specific manner. Not predicted from previous studies, Tyr191 is also involved in this network. This residue is unique to the CTR, being a hydrophobic Phe in all other class B receptors (Supplementary Fig. 1), suggesting receptor-specific differences in how the binding energy is translated through the protein to promote G-protein activation.

At the cytoplasmic face, an important interaction between His5 and Glu6, considered to play an equivalent functional role in class B GPCRs to the DRY motif in class A GPCRs, is present in the inactive class B crystal structures and is maintained in the active CTR transmembrane bundle (Fig. 3b and Extended Data Fig. 8c). These residues form an extensive polar interaction network with Thr6 and Tyr7, locking the base of the receptor in an inactive conformation in our inactive CTR homology model, consistent with that of the two published GGRG inactive structures. In the CTR, these residues are further apart due to disruption by the thermostabilizing mutation Ala7 and binding of the small-molecule antagonist (Fig. 3b). This network is broken in the CTR–Gs structure, with Tyr7 forming new hydrogen bond interactions with the TM6 backbone (Fig. 3b and Extended Data Fig. 8c). This releases constraints on TM6 and probably assists in the large conformational transition of TM6 away from the core of the bundle. Consistent with this, mutation of these residues either results in constitutive activation or reduced receptor expression.

At the cytoplasmic face, Arg8, Arg/Lys, Asn7, and Glu4 (TM6–2–7–H8 network) form tight interactions in the GGRG inactive structure and our CTR homology model with two key salt bridges formed by Glu4 and Arg8 with Arg4 and Arg/Lys (Fig. 3b). These interactions are not present in the inactive CTR–R owing to the absence of helix 8, but have been predicted in other class B GPCRs and are likely conserved across the entire family. While the interaction between Glu4 and Arg4 is likely maintained in the active CTR-Gs structure, the salt bridge between Lys7 and Glu4 is broken with these residues residing 26 Å apart (Fig. 3b). The reordering of these side chains in the active structure releases ground state constraints on TM6.

The CTR–Gα interface

Extensive interactions formed between the CTR and Gα stabilize the active receptor conformation. The receptor–Gα interface is formed by agonist activity, Ser5 and Thr6, are predicted to interact with TM5, potentially forming polar interactions with His305 (superscript indicates class B GPCR numbering) (Extended Data Fig. 7b). This model is supported by mutation of His305 to Ala that reduced sCT potency in cAMP production and ERK1/2 phosphorylation, consistent with reduced sCT affinity (Extended Data Fig. 7c). The first three residues in sCT make minimal receptor contacts in this model, consistent with experimental data showing that these residues can be removed without altering peptide activity.

Comparison of active CTR to inactive class B GPCR TMs

Comparison of the CTR complex with inactive class B GPCR structures (GCGR and corticotropin-releasing hormone receptor 1 (CRF1R; ref. 5)) or inactive CTR homology models provides insight into transmembrane conformational transitions upon class B GPCR activation (Fig. 3a, Extended Data Fig. 8). The most striking observations relate to TM6 where there is a large outward movement at the cytoplasmic face in the activated CTR (approximately 15 Å) relative to the inactive structures when measured from the Cα of Phe X5.56b. An outward movement within TM6, as well as disordering and unwinding of this helix was observed at the extracellular face (9–11 Å when measured from the Cα of Phe X6.55b). This is correlated with an extremely sharp (around 60°) kink at the centre of TM6 formed around Pro6.47b–X–Gly6.50b. Importantly, residues within this motif influence agonist affinity, activation and function in both CTR and the related calcitonin receptor-like receptor and the very high conservation of the Pro6.47b–X–Gly6.50b motif in class B GPCRs (Supplementary Information Fig. 1) implies that these residues may be globally important for activation through the formation of a kink in TM6 that is much more pronounced than in any transmembrane helix in GPCR structures solved to date.

In comparison to both inactive structures, there is an inward movement at the top of TM1 in the CTR–Gc structure and a small outward movement at the top of TM5. TM7 movement differs when comparing individual inactive structures to the CTR–Gc complex (Fig. 3a). While the TM7 kink angle is similar in the inactive CRF1R and the CTR, TM7 is shifted towards TM6 in the CTR. By contrast, there is a large, 9 Å outward movement in the top of TM7 in the CTR structure relative to the GCGR. While the differences observed in location of the top of transmembrane helices may represent physiologically relevant differences, we interpret them with caution because the inactive structures do not include the receptor ECDs. There is increasing evidence for a dynamic role of class B ECD–transmembrane-core interactions in receptor quiescence and activation and the large outward movement of helices 6 and 7 required for peptide binding support a model of class B GPCR quiescence where the tops of TM6 and TM7 are potentially constrained by ECL3 interactions with the far N-terminal ECD, a theory supported by experimental data on the glucagon receptor. Nonetheless, in all structures, the top of TM6/ECL3 has a large degree of structural flexibility, supported by its weak corresponding density in the cryo-EM map of the CTR–Gc complex (Extended Data Fig. 4d) and the high crystallographic temperature factors exhibited by inactive structures in these regions.
residues located in TM2, 3, 5, 6, ICL2, ICL3 and helix 8 of the receptor with the α4- and α5-helices of the Ras-like domain of Goαs and the Goαs N–β1 junction of the G protein. The most extensive contacts consist of polar and hydrophobic van der Waals interactions between the receptor and the α5-helix of the Ras-like domain of Goαs (Fig. 4a and Extended Data Fig. 9a). Class B GPCRs all predominantly couple to Goαs and several of the CTR residues that form interactions with the Ras-like domain of Goαs are highly conserved and have been previously implicated in G-protein coupling.66,37

The buried interface between the receptor and the Ras-like domain of Goαs is 2,031 Å², while an interface of 663 Å² is also provided by the interaction of helix 8 of CTR with Gβ1. Notably, helix 8 is heavily buried within the detergent micelle, facilitated by bulky aromatic residues and additional polar charged residues that can interact with lipid/detergent head groups (Fig. 4b and Extended Data Fig. 9b). Sequential deletions of the CTR C terminus support a role for helix 8 lipid interactions in receptor stability at the cell surface (Fig. 4c). While truncation after Trp406 (Δ407) resulted in a greater reduction in cell-surface expression, further truncation after Gln399 (Δ400) further reduced cell-surface expression, highlighting that bulky, detergent-buried residues within helix 8 are crucial for CTR cell-surface localization. Middle, Δ407 and Δ400 had reduced maximal responses for cAMP production relative to wild type (WT). Right, calculation of cAMP efficacy via application of the Black–Leff operational model34 to cAMP accumulation data, followed by correction for alterations in cell-surface expression, reveals that Δ407 has reduced cAMP efficacy (log(ec)) that is not further reduced by additional truncation back to Gln399. This indicates that residues Thr400–Trp406 are crucial for CAMP efficacy that may be associated with their interaction with Gβ1. Pharmacological data are given as mean ± s.e.m. of five independent experiments performed in duplicate. *P < 0.05 compared to wild type using one-way analysis of variance followed by Dunnett’s test.

**Comparison of class A and class B GPCR–Goαs complex**

An overlay of the G-protein heterotrimer in the sC-T–CTR–Goαs structure, located above the first and seventh WD40 repeat (WD1 and WD7) domains of Gβ3, and within close proximity to the N-terminal α-helical domain (Nα) of the α-subunit (Extended Data Fig. 9c). As such, the I1+ (CTb) isoform is likely to sterically interfere with receptor–G-protein interactions, leading to the attenuation in signalling.

Figure 4 | CTR–G-protein interactions. a, α5-helix of the Ras-like domain of Goαs (orange) docks into a CTR intracellular face cavity (blue) by the opening of TM6, forming polar and non-polar interactions. Histidine 41 at the Goαs αN–β1 boundary interacts with the CTR ICL2 backbone. b, CTR helix 8 bulky aromatics heavily embed within the detergent micelle. Residues on the opposing face are in the vicinity of Gβ3, where they probably form polar interactions. c, CTR pharmacological characterization in COS-7 cells following gradual deletion of the C terminus. Left, deletion after Trp406 (Δ407) results in markedly reduced cell-surface expression. Further truncation after Gln399 (Δ400) further reduced cell-surface expression, highlighting that bulky, detergent-buried residues within helix 8 are crucial for CTR cell-surface expression. Middle, Δ407 and Δ400 had reduced maximal responses for cAMP production relative to wild type (WT). Right, calculation of cAMP efficacy via application of the Black–Leff operational model to cAMP accumulation data, followed by correction for alterations in cell-surface expression, reveals that Δ407 has reduced cAMP efficacy (log(ec)) that is not further reduced by additional truncation back to Gln399. This indicates that residues Thr400–Trp406 are crucial for CAMP efficacy that may be associated with their interaction with Gβ1. Pharmacological data are given as mean ± s.e.m. of five independent experiments performed in duplicate. *P < 0.05 compared to wild type using one-way analysis of variance followed by Dunnett’s test.
receptors revealing conserved global cytoplasmic changes upon activation. Two notable differences include a helical extension of TM5 in the \( \beta_2 \)-AR–G\(_s\) complex that was not evident for the CTR, and the long helix 8 that was not observed in the \( \beta_2 \)-AR.

At the extracellular face there are major differences in the conformation of the two receptors, reflective of their distinct activating ligands, with the class B structure that is required to accommodate a peptide, being more open (Extended Data Fig. 10). There are remarkable differences in TM6 and TM7, which contain kinks in both receptors, but are more pronounced in the CTR. A large shift inwards and towards TM7 in extracellular side of TM1 is also evident in the class B structure relative to the \( \beta_2 \)-AR, with this transmembrane domain also forming an extended helical structure (three additional turns), a feature reported previously for the inactive state structure of the GCGR\(_2\). A notable feature is the distinct location of TM4 in the CTR relative to the \( \beta_2 \)-AR (Fig. 5 and Extended Data Fig. 10). This is particularly interesting as TM4 is the predominant interface for class B GPCR dimerization, with disruption of this interface leading to attenuated G-protein signalling for all receptors that have been assessed to date, including the CTR\(^{41,42}\).

**Conclusions**

The cryo-EM structure of the sCT–CTR–G\(_s\) complex provides a near-atomic resolution view of a full-length class B GPCR and of an activated class B GPCR–G\(_s\)-protein ternary complex. Notably, the CTR is completely unmodified (with the exception of affinity tags) and is one of only a few structures of a wild-type GPCR, highlighting the potential of cryo-EM in solving structures of GPCR complexes. The structure also provides the first visualization of the binding site of the N terminus of a peptide agonist within the transmembrane bundle of a class B GPCR as well as some insight into the biological flexibility of the ternary complex. This study provides a framework to further investigate the mechanism of agonist interactions and activation of other class B GPCRs that may open up new avenues for rational design of novel therapeutics for this class of receptors.

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1. Congreve, M. & Marshall, F. The impact of GPCR structures on pharmacology and structure-based drug design. *Br. J. Pharmacol.* **159**, 986–996 (2010).
2. Kenakin, T. & Miller, L. J. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol. Rev.* **62**, 255–304 (2010).
3. Zhang, D., Zhao, Q. & Wu, B. Structural studies of G protein-coupled receptors. *Mol. Cells* **38**, 836–842 (2015).
4. Rasmussen, S. G. et al. Crystal structure of the (\( \beta_2 \) adrenergic receptor-Gs protein complex. *Nature* **477**, 549–555 (2011).
5. Hollenstein, K. et al. Structure of class B GPCR corticotropin-releasing factor receptor 1. *Nature* **499**, 438–443 (2013).
6. Jazayeri, A. et al. Extra-helical binding site of a glucagon receptor antagonist. *Nature* **533**, 274–277 (2016).
7. Su, F. Y. et al. Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **499**, 444–449 (2013).
8. Culhane, K. J., Liu, Y., Cai, Y. & Yan, E. C. Transmembrane signal transduction by peptide hormones via family B G-protein-coupled receptors. *Front. Pharmacol.* **6**, 264 (2015).
9. Pal, K., Melcher, K. & Xu, H. E. Structure and mechanism for recognition of peptide hormones by Class B-G-protein-coupled receptors. *Acta Pharmacol. Sin.* **33**, 300–311 (2012).
10. Poyner, D. R. et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol. Rev.* **54**, 233–246 (2002).
11. Bai, X. C., McMullan, G. & Scheres, S. H. How cryo-EM is revolutionizing structural biology. *Trends Biochem. Sci.* **30**, 49–57 (2015).
12. De Zorzi, R., Mi, W., Liao, M. & Walz, T. Single-particle electron microscopy in the study of membrane protein structure. *Microscopy* **65**, 81–96 (2016).
13. Daner, R., Tegunov, D. & Baumeister, W. Using the Volta phase plate with defocus for cryo-EM single particle analysis. *eLife* **6**, e23006 (2017).
14. Khoshouei, M., et al. Cryo-EM structure of haemoglobin at 3.2 Å determined with the Volta phase plate. Preprint at https://doi.org/10.1087/841 (2016).
15. Chartouhi, M. et al. Volta phase plate cryo-EM of the small protein complex Prx3. *Nat. Commun.* **7**, 10534 (2016).
16. Hilton, J. M., Dovton, M., Houssami, S. & Sexton, P. M. Identification of key components in the irreversibility of salmon calcitonin binding to calcitonin receptors. *J. Endocrinol.* **166**, 213–226 (2000).
17. Furness, S. G. et al. Ligand-dependent modulation of G protein conformation alters drug efficacy. *Cell* **167**, 739–749.e11 (2016).
18. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).
19. Westfield, G. H. et al. Structural flexibility of the G. s vs. helical domain in the \( \beta_2 \)-adrenoreceptor Gs complex. *Proc. Natl. Acad. Sci. USA* **108**, 16086–16091 (2011).
20. Van Eps, N. et al. Interaction of a G protein with an activated receptor opens the interdomain interface in the alpha subunit. *Proc. Natl. Acad. Sci. USA* **108**, 9420–9424 (2011).
21. Andreetti, G. et al. Structural determinants of salmon calcitonin bioactivity: the role of the Leu-based amphipathic \( \alpha \)-helix. *J. Biol. Chem.* **281**, 24193–24203 (2006).
22. Johansson, E. et al. Type II turn of receptor-bound salmon calcitonin revealed by X-ray crystallography. *J. Biol. Chem.* **291**, 13689–13698 (2016).
23. Ho, H. H., Gilbert, M. T., Nussenzweig, D. R. & Gershengorn, M. C. Glycosylation is important for binding to human calcitonin receptors. *Biochemistry* **38**, 1866–1872 (1999).
24. Dods, R. L. & Donnelly, D. The peptide agonist-binding site of the glucagon-like peptide-1 (GLP-1) receptor based on site-directed mutagenesis and knowledge-based modelling. *Biochem. J.* **36**, e00285 (2015).
25. Wooten, D. et al. The extracellular surface of the GLP-1 receptor is a molecular trigger for biased agonism. *Cell* **165**, 1632–1643 (2016).
26. Houssami, S. et al. Divergent structural requirements exist for calcitonin receptor binding specificity and adenylate cyclase activation. *Mol. Pharmacol.* **47**, 798–809 (1995).
27. Meadows, R. P., Nikonowicz, E. P., Jones, C. R., Bastian, J. W. & Gorenstein, D. G. Two-dimensional NMR and structure determination of salmon calcitonin in methanol. *Biochemistry* **30**, 1247–1254 (1991).
28. Feyen, J. H. et al. N-terminal truncation of salmon calcitonin leads to calcitonin antagonists. Structure activity relationship of N-terminally truncated salmon calcitonin fragments in vitro and in vivo. *Biochem. Biophys. Res. Commun.* **187**, 8–13 (1992).
29. Wootten, D., Simms, J., Miller, L. J., Christopoulos, A. & Sexton, P. M. Polar transmembrane interactions drive formation of ligand-specific and signal pathway-biased family B G protein-coupled receptor conformations. *Proc. Natl. Acad. Sci. USA* **110**, 5211–5216 (2013).
30. Bailey, R. J. & Hay, D. L. Agonist-dependent consequences of proline to alanine substitution in the transmembrane helices of the calcitonin receptor. *Br. J. Pharmacol.* **151**, 678–687 (2007).
Conner, A. C. et al. A key role for transmembrane prolines in calcitonin receptor-like agonist binding and signalling: implications for family B G-protein-coupled receptors. Mol. Pharmacol. 67, 20–31 (2005).

Koth, C. M. et al. Molecular basis for negative regulation of the glucagon receptor. Proc. Natl Acad. Sci. USA 109, 14393–14398 (2012).

Mukund, S. et al. Inhibitory mechanism of an allosteric antibody targeting the glucagon receptor. J. Biol. Chem. 288, 36168–36178 (2013).

Yin, Y. et al. An intrinsic agonist mechanism for activation of glucagon-like peptide-1 receptor by its extracellular domain. Cell Discov. 2, 16042 (2016).

Zhao, L. H. et al. Differential requirement of the extracellular domain in activation of class B G protein-coupled receptors. J. Biol. Chem. 291, 15119–15130 (2016).

Vohra, S. et al. Similarity between class A and class B G-protein-coupled receptors exemplified through calcitonin gene-related peptide receptor modelling and mutagenesis studies. J. R. Soc. Interface 10, 20120846 (2012).

Wootten, D. et al. A hydrogen-bonded polar network in the core of the glucagon-like peptide-1 receptor is a fulcrum for biased agonism: lessons from class B crystal structures. Mol. Pharmacol. 89, 335–347 (2016).

Wootten, D. et al. Key interactions by conserved polar amino acids located at the transmembrane helical boundaries in Class B GPCRs modulate activation, effector specificity and biased signalling in the glucagon-like peptide-1 receptor. Biochem. Pharmacol. 118, 68–87 (2016).

Conner, M. et al. Functional and biophysical analysis of the C-terminus of the CGRP-receptor; a family B GPCR. Biochemistry 47, 8434–8444 (2008).

Furness, S. G., Wootten, D., Christopoulos, A. & Sexton, P. M. Consequences of splice variation on Secretin family G protein-coupled receptor function. Br. J. Pharmacol. 166, 98–109 (2012).

Harikumar, K. G., Ball, A. M., Sexton, P. M. & Miller, L. J. Importance of lipid-exposed residues in transmembrane segment four for family B calcitonin receptor homo-dimerization. Regul. Pept. 164, 113–119 (2010).

Harikumar, K. G. et al. Glucagon-like peptide-1 receptor dimerization differentially regulates agonist signaling but does not affect small molecule allostery. Proc. Natl Acad. Sci. USA 109, 18607–18612 (2012).

Black, J. W. & Leff, P. Operational models of pharmacological agonism. Proc. R. Soc. Lond. B Biol. Sci. 220, 141–162 (1983).

Supplementary Information is available in the online version of the paper.

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Author Contributions Y.-L.L. developed the expression and purification strategy (with D.M.T., S.G.B.F., B.K.K., D.W., G.S. and P.M.S.), performed virus production, insect cell expression, purification, complex stability, negative-stain EM, data acquisition/analysis, prepared samples for cryo-EM and assisted with manuscript preparation. M.K. performed phase-plate imaging, data collection, EM data processing and analysis and assisted with manuscript preparation. M.R. assisted with negative-stain analysis and conception of cryo-imaging by Volta phase plate, performed cryo-sample preparation, preliminary screening imaging and analysis and assisted with manuscript preparation. Y.Z. calculated the cryo-EM map, performed model building and refinement, and contributed to manuscript preparation. A.G. performed GTP–S and radioligand binding, model building and refinement and contributed to manuscript preparation. J.T. assisted in negative-stain EM screening, D.M.T. contributed to purification strategy, model refinement and manuscript preparation. S.G.B.F. provided project strategy and protein purification advice, assisted data interpretation and manuscript preparation. G.C. performed cloning for baculovirus expression, T.C. performed homology modelling, assisted with model refinement and manuscript preparation. R.D. developed Volta phase plate development project. L.J.M. provided insights into class B GPCRs, assisted with data interpretation and reviewed the manuscript. A.C. assisted with data interpretation and manuscript preparation. B.K.K. provided advice on GPR–Gs complex formation and purification. D.W. was responsible for overall project strategy and management (along with P.M.S.) and performed pharmacological characterization, interpreted data and wrote the manuscript. G.S. provided feedback to guide cryo-EM, oversaw EM data processing, structure determination and refinement, data interpretation and manuscript writing. P.M.S. was responsible for overall project strategy and management, data interpretation and writing the manuscript.

Author Information 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. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to P.S. (patrick.sexton@monash.edu), D.W. (denise.wootten@monash.edu) or G.S. (skinioti@umich.edu).

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METHODS

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

Constructs. Wild-type human calcitonin receptor (CTR) was modified to include an N-terminal Flag tag epitope and a C-terminal 8× histidine tag, both tags are removable by 3C protease cleavage (Extended Data Fig. 1). These constructs were generated in both mammalian and insect cell expression vectors.

Insect cell expression. CTR, human Grs1 short, His-tagged human Gβi1, and Gβ2 were expressed in HighFive insect cells (Thermo Fisher Scientific) using baculovirus. Cell cultures were grown in ESF 921 serum-free media (Expression System) to a density of 4 million cells per ml and then infected with three separate baculovirus at a ratio of 1:2:2 for hCTR, Grs0, and Gβ3i3. Cultures were grown at 27 °C and harvested by centrifugation 45 h post infection.

Complex purification. Cells were suspended in 20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM MgCl₂ supplemented with COMplete Protease Inhibitor Cocktail Tablets (Roche). Complex formation was initiated by addition of 1 μM salmon calcitonin, Nb35–H1 (10 μg ml⁻¹) and Appryse (25 μm ml⁻¹, NEB); the suspension was incubated for 1 h at room temperature. Membranes were collected by centrifugation at 30,000 g for 30 min, and solubilized by 0.5% (w/v) lauryl maltose neopentyl glycol (LMNG, Anacrace) supplemented with 0.03% (w/v) cholesteryl hemisuccinate (CHS, Anacrace) for 2 h at 4 °C in the presence of 100 mM salmon calcitonin and Appryse (25 μm ml⁻¹, NEB). Insoluble material was removed by centrifugation at 30,000 g for 30 min and the solubilized complex was immobilised by batch binding to M1 anti-Flag affinity resin in the presence of 3 mM CaCl₂. The resin was packed into a glass column and washed with 20 column volumes of 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 3 mM CaCl₂, 100 mM sCT, 0.01% (w/v) MN and 0.006% (w/v) CHS before bound material was eluted in buffer containing 5 mM EGTA and 0.1 mg ml⁻¹ Flag peptide. The complex was then concentrated using an Amicon Ultra Centrifugal Filter (MWCO 100kDa) and subjected to size-exclusion chromatography on a Superdex 200 Increase 10/300 column (GE Healthcare) pre-equilibrated with 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 100 mM sCT, 0.01% (w/v) MN and 0.006% (w/v) CHS to separate complex from contaminants. Eluted fractions consisting of receptor and G-protein complex were pooled and concentrated. Final yield of purified complex was approximately 0.5 mg l⁻¹ insect cell culture.

The stability of the CTR–G₃ heterotrimer complex at 4 °C was monitored by analytical SEC. SEC-purified complex was incubated on ice and 50 μg was applied onto Superose 6 Increase 10/300 GL column at 24 h interval for a total period of 5 days. Protein was detected by measuring absorbance at 280 nm with a fluorescence detector (RF-10AXL, Shimadzu).

SDS–PAGE and western blot analysis. Samples collected from each purification step were analysed by SDS–PAGE and western blot. For SDS–PAGE, precast gradient TGX gels (Bio-Rad) were used. Gels were either stained by Instant Blue (Expedeon) or immediately transferred to PVDF membrane (Bio-Rad) at 100 V for 1 h. The proteins on the PVDF membrane were probed with two primary antibodies, rabbit anti-Gr, C-18 antibody (cat. no. sc-383, Santa Cruz) against Grs0, subunit and mouse penta-His antibody (cat. no. 34660, QIAGEN) against His tags. The membrane was washed and incubated with secondary antibodies, 680RD goat anti-mouse and 806CW goat anti-rabbit (LI-COR). Bands were imaged using an infrared imaging system (LI-COR Odyssey Imaging System).

Specimen preparation and data acquisition. Concentrated sample from Superdex 200 Increase 10/300 GL column was loaded onto a Superose 6 Increase 10/300 GL column (GE Healthcare). Eluted fractions were used to prepare specimens for EM imaging using conventional negative-staining protocol44. Negative-stained samples were prepared by mixing 1 ml of sample preparation for cryo-EM. Cells were harvested approximately 42 h after the viral infection. For crude membrane preparations cells were resuspended in membrane buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM MgCl₂, with protease inhibitors and benzonase), dounced 20 times followed by centrifugation (10 min, 30,000 g) to harvest the membranes. Membrane buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 10 mM MgCl₂; 0.1% BSA). First, membranes (1–2 mg per sample, where applicable) were incubated on ice and 50 μg was applied onto Superose 6 Increase 10/300 GL column at 24 h interval for a total period of 5 days. Protein was detected by measuring absorbance at 280 nm with a fluorescence detector (RF-10AXL, Shimadzu).
using a MicroBeta LumiJET counter (PerkinElmer). Non-specific binding was measured in the presence of 1 μM of sCT (8–32). Curves were fitted to one- or two-site competition binding equations in Prism 6.0 (GraphPad). Data from each experiment were normalized to the response of membranes prepared from cells expressing all the components of the CTR complex (CTR, Gαs, and G3γ3) in the absence of sCT (100%).

(35S)GTPγS binding on CTR expressed in insect cells. Measurement of (35S)GTPγS incorporation was performed in 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl2, 1 mM EDTA; 0.1% BSA; 30 μg ml−1 saponin. First, membranes (50 μg per sample) were pre-incubated with 5 μM GDP and increasing concentrations of sCT for 30 min at 22°C. For experiments investigating the effects of Nb35, indicated amounts of Nb35 were also added at this step. Reactions were started by the addition of (35S)GTPγS and ATP to final concentrations of 300 μM and 50 μM, respectively. After 1 h incubation at 30°C, the reaction was terminated by harvesting the membranes on Whatman UniFilter GF/C plates using Filtermate 196 harvester (Packard). Membranes were extensively washed with ice-cold 50 mM Tris pH 7.6, 10 mM MgCl2, 100 mM NaCl, dried, dissolved in 40 μl of Microscint-O scintillation cocktail (Packard) and counted using a MicroBeta LumiJET counter (PerkinElmer). Data from each experiment were normalized to the response of membranes prepared from cells expressing all the components of the CTR complex (CTR, Gαs, and G3γ3) in the presence of 1 μM sCT (100%).

Generation of mutant receptors and C-terminal deletion constructs in mammalian cell vectors. Mutagenesis and C-terminal deletions were generated in a c-Myc epitope-tagged receptor that displays the same pharmacological properties as the wild-type receptor and the construct used for purification studies. The desired mutations were introduced into the CTR in the pEFS/FRT/V5-DEST designation vector (Invitrogen) using oligonucleotides for site-directed mutagenesis purchased from GeneWorks (Hindmarsh) and the QuikChange site-directed mutagenesis kit (Stratagene). C-terminal deletions were generated using primers designed to amplify the CTR from the N terminus to the various points within the C terminus via standard PCR methods. PCR products were purified using a Qiagen gel purification kit (Stratagene). C-terminal deletions were generated using primers designed to amplify the CTR from the N terminus to the various points within the C terminus via standard PCR methods. PCR products were purified using a Qiagen gel purification kit following the manufacturer’s protocol. The primers for PCR contained sequences that incorporated sites into the PCR products for recombination into the destination vector pEFS/FRT/V5-DEST via Gateway technology. Sequences of receptor clones were confirmed by automated sequencing at the Australian Genome Research Facility.

Mammalian cell expression. COS-7 or 3T3 FlpIn cells (Invitrogen) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS) (Thermo Electron Corporation) at 37°C and 95% O2/5% CO2 in a humidified incubator. Cells were routinely tested for mycoplasma with 10% v/v fetal bovine serum (FBS) (Thermo Electron Corporation) at 37°C and 95% O2/5% CO2 in a humidified incubator. Cells were routinely tested for mycoplasma. Cells were transiently transfected, using polyethylenimine (PEI). DNA and PEI diluted in 150 mM NaCl were combined in a 1:6 ratio and incubated for 15 min, added to cell suspension and the cells plated.

Mammalian whole-cell binding experiments. Transfected COS-7 cells were seeded at a density of 3 × 104 cells per well into 96-well culture plates and incubated for 48 h at 37°C in 5% CO2, and radioligand binding carried out as previously described. For each cell line in all experiments, total binding was defined by 0.05 nM [35S]sCT (8–32) alone, and non-specific binding was defined by co-incubation with 1 μM sCT (8–32). For analysis, data are normalized to the specific binding for each individual experiment.

Mammalian cAMP assays. Transfected COS-7 or 3T3 FlpIn cells were seeded at a density of 3 × 104 cells per well into 96-well culture plates and incubated for 48 h at 37°C in 5% CO2, and cAMP detection carried out as previously described. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently normalized to the response of 100 nM forskolin in each cell line.

Mammalian ERK1/2 phosphorylation assays. Transfected 3T3 FlpIn cells were seeded at a density of 3 × 104 cells per well into 96-well culture plates and incubated for 48 h at 37°C in 5% CO2, and pERK1/2 assays were carried out using Alphascreen technology as previously described. FBS was used as a positive control. Data were subsequently normalized to the response of 100 10% FBS in each cell line.

Statistics. Where required, statistics for mutational studies were performed using one-way analysis of variance followed by Dunnett’s test using wild-type receptors as the control (P < 0.05). Sample sizes were based on those routinely used for mutational studies and determination of alterations in cell signalling.

Data availability. All relevant data are available from the authors and/or are included with the manuscript or Supplementary Information. Atomic coordinates and the cryo-EM density map have been deposited in the Protein Data Bank under accession number 5UZ7 and the Electron Microscopy Data Bank under accession number EMD-8623.
Extended Data Figure 1 | Schematic of the CTR used in the study. In our construct HA–Flag–3C–CTR–3C–8×His, the native signal peptide of the CTR (residues 2–24) was replaced with a HA signal peptide (red), Flag epitope (green) and a 3C cleavage site (yellow). The C terminus was modified with a 3C cleavage site (yellow) and a His epitope (blue). Also highlighted on the schematic are consensus glycosylation sites (purple) and class B GPCR conserved disulfide bonds. Residues highlighted in bold are the most conserved residue in each helix and represent residues ×50 for each helix according to the class B GPCR numbering. The location of the 16 amino acid insertion within ICL1 for a common splice variant of the CTR (CTRb) is shown. In addition, the locations of the truncation sites within the CTR C terminus/helix 8 assessed in this study are also highlighted.
Extended Data Figure 2 | Pharmacology of the CTR construct used in this study. a–d, Pharmacological assessment in mammalian COS-7 cells (a, b) and HiveFive insect cells (c, d) of the untagged CTR and the construct shown in Extended Data Fig. 1 (HA–Flag–3C–CTR–3C–8×His). The presence of purification tags does not alter receptor pharmacology. a, Radioligand competition binding for sCT in competition with the radiolabelled ligand \([^{125}\text{I}]\text{sCT}(8–32)\) in whole cells transiently expressing wild-type CTR or HA–Flag–3C–CTR–3C–8×His. Data are normalized to maximum \([^{125}\text{I}]\text{sCT}(8–32)\) with nonspecific measured in the presence of 1 μM unlabelled sCT(8–32). b, Concentration response curves assessing G\(_\text{s}\) activation via measurement of cAMP accumulation at wild-type CTR and HA–Flag–3C–CTR–3C–8×His in the presence of sCT. c, Radioligand competition binding for sCT or the radiolabelled ligand \([^{125}\text{I}]\text{sCT}(8–32)\) performed with HA–Flag–3C–CTR–3C–8×His in the presence of G\(_\text{s}\) protein heterotrimer reveals similar affinities in insect cells versus mammalian cells. The presence of Nb35 does not alter ligand affinity. d, Concentration response curves to assess G-protein activation by HA–Flag–3C–CTR–3C–8×His via GTP\(_\gamma\)S binding in the absence and presence of G\(_\text{s}\) protein heterotrimer reveals that the tagged CTR can robustly activate G\(_\text{s}\) in insect cells. e, GTP\(_\gamma\)S binding to HA–Flag–3C–CTR–3C–8×His in the presence of 1 μM sCT is inhibited by increasing concentrations of Nb35. All data are mean ± s.e.m. of four independent experiments, conducted in duplicate or triplicate.
Extended Data Figure 3 | Expression and purification of the sCT–CTR–
G complex. a, Flow chart of the purification steps for the human CTR (hCTR)–G complex. b, SDS–PAGE/western blot of samples obtained at various stages of hCTR–G purification. hCTR and the G heterotrimer were co-expressed in insect cell membrane. Addition of the agonist salmon calcitonin initiates complex formation and was solubilized by detergent. Solubilized hCTR and the hCTR–Gs complex were immobilized on Flag antibody resin. Flag-eluted fractions were further purified by SEC. An anti-His antibody was used to detect Flag–CTR–His, Gβ–His and Nb35–His (red) and an anti-Gα antibody was used to detect Gαs (green).
c, Representative elution profile of Flag-purified complex on Superdex 200 Increase 10/30 SEC (top). SEC fractions containing hCTR–G complex (within dashed lines) were pooled, concentrated and analysed by SEC on Superose 6 Increase 10/30 column (bottom). d, SDS–PAGE/Coomassie blue stain of the purified complex concentrated from the Superose 6 Increase 10/30 column. e, The stability of the purified hCTR–Gα was monitored by SEC following incubation at 4 °C for 5 days. All images and SEC profiles are representative of more than ten experiments, except for e, which was performed once.
Extended Data Figure 4 | Cryo-EM of the sCT–CTR–G\textsubscript{s} complex.

a, Representative Volta phase plate (of 2,780 recordings) cryo-EM micrograph of the sCT–CTR–G\textsubscript{s} complex (scale bar, 15 nm).

b, Reference-free two-dimensional averages of the complex in maltose-neopentyl glycol/cholesterol hemisuccinate micelle.

c, Gold-standard Fourier shell correlation (FSC) curves, showing the overall nominal resolution at 4.1 Å and 3.8 Å on the stable region including the transmembrane domain and G\textsubscript{s} protein complex without AHD.

d, Final three-dimensional density map coloured according to local resolution.

e, FSC curves of the final refined model versus the final cryo-EM map (full dataset, black), of the outcome of model refinement with a half map versus the same map (red), and of the outcome of model refinement with a half map versus the other half map (green). At FSC = 0.5, the resolution is 4.1 Å. f, EM density of TM1, TM5, TM6, TM7 and helix 8.
Extended Data Figure 5 | Flexibility of ECD and AHD in the sCT–CTR–Gα complex. Representative maps from three-dimensional classification showing the dynamics of the CTR ECD and Gα AHD. The overlaid maps are shown from top and side views. In the right panel the blue, green, purple and red density maps show the four 3D classifications. These are overlayed on the left to demonstrate the observed flexibility of the Gα AHD and the CTR ECD.
Extended Data Figure 6 | The N-terminal ECD of the CTR. a, Rigid body fitting of the structure of CTR ECD bound to sCT (PDB: 5I10)\textsuperscript{22} into the corresponding regions of the cryo-EM map revealed additional density (close to residue 130) that may be attributed to glycosylation. b–d, Asp mutation of four consensus glycosylation residues (N28D, N73D, N125D and N130D) reveals the relative unimportance of glycosylation on cell-surface expression (b), determined via cell-surface ELISA for the N-terminal epitope tag. c, Competition radioligand binding studies for sCT in competition with the radiolabelled ligand \( [^{125}\text{I}] \text{sCT}(8–32) \) revealed reduced affinity for N130D, and to a lesser extent N125D, compared to the wild-type CTR. d, Concentration response curves for cAMP accumulation for mutant receptors relative to wild type show that N130D, and to a lesser extent N125D, reduce the potency of sCT in functional experiments. All data are mean ± s.e.m. of five independent experiments, conducted in duplicate or triplicate.
Extended Data Figure 7 | Molecular modelling of sCT peptide reveals potential interactions between peptide and receptor. Cryo-EM density is shown in yellow fill, the sCT peptide model in yellow cartoon and the CTR in blue cartoon. a, Gln14 in sCT is predicted to form interactions with the backbone of ECL2. b, Ser5 and Thr6 are predicted to form hydrogen bonds with His302 in TM5 of the CTR, while Leu4 points down into the bundle towards TM6. c, Mutation of H302 to Ala (H302A) results in reduced potency for sCT in cAMP production (left) and phosphorylation of ERK1/2 (right) when expressed in 3T3-FlpIn cells. This supports a role H302 in sCT affinity. Data are mean ± s.e.m. of four independent experiments performed in duplicate.
Comparisons of an inactive CTR homology model and the active CTR structure. 

**a.** Side view of the activate sCT–CTR–Gs complex transmembrane structure (blue) relative to the inactive CTR homology model (red).

**b.** Tube representation for transmembrane domains showing extracellular (top) and cytoplasmic (bottom) views of the activate sCT–CTR–Gs complex transmembrane structure (blue) relative to the inactive CTR homology model (red).

In **a** and **b** large differences are observed at the extracellular ends of TM6 and TM7, with additional differences within TM1 and TM5. In addition, a very large outward movement is observed within TM6 of the active structure relative to the inactive homology model at the intracellular face.

**c.** The positions of class B conserved polar residues located within the inactive CTR homology model.
Extended Data Figure 9 | CTR-G protein interactions. 

a. The α5-helix of Goαs (orange) docks into a cavity formed on the intracellular side of the receptor (blue) by the opening of TM6. G-protein side chains within this cavity are supported by the cryo-EM map. 

b. Helix 8 of the CTR forms an amphipathic helix with multiple bulky aromatics heavily embedded within the detergent micelle that are evident in the map. Residues within the more polar face of helix 8 are in the vicinity of Gβ3, where they probably form polar interactions, although specific side chain density in this region is not evident. 

c. ICL1 is located in close proximity to the G protein. A common CTR splice variant contains a 16 amino acid insertion within this loop (between Arg174 and Ser175), an insertion that would sterically hinder G-protein interactions with the receptor.
Extended Data Figure 10 | Comparison of the activated β2AR and CTR viewed from the extracellular face. Tube representation of the transmembrane domains of the CTR (blue) and β2AR (green) viewed from the cytoplasmic face (based on overlay of the Gs protein from each structure). Despite similarities in the position of transmembrane tips at the intracellular face, there are substantial differences in the location of the extracellular transmembrane tips, highlighting marked differences in the ligand binding mode and initiation of receptor activation between class A and B GPCRs.