Structures of the glucocorticoid-bound adhesion receptor GPR97–G_o complex

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Adhesion G-protein-coupled receptors (GPCRs) are a major family of GPCRs, but limited knowledge of their ligand regulation or structure is available^{1–3}. Here we report that glucocorticoid stress hormones activate adhesion G-protein-coupled receptor G3 (ADGRG3; also known as GPR97)^{4–6}, a prototypical adhesion GPCR. The cryo-electron microscopy structures of GPR97–G_o complexes bound to the anti-inflammatory drug beclomethasone or the steroid hormone cortisol revealed that glucocorticoids bind to a pocket within the transmembrane domain. The steroidal core of glucocorticoids is packed against the ‘toggle switch’ residue W^{6,53}, which senses the binding of a ligand and induces activation of the receptor. Active GPR97 uses a quaternary core and HLY motif to fasten the seven-transmembrane bundle and to mediate G protein coupling. The cytoplasmic side of GPR97 has an open cavity, where all three intracellular loops interact with the G_o protein, contributing to the high basal activity of GPR97. Palmitoylation at the cytosolic tail of the G_o protein was found to be essential for efficient engagement with GPR97 but is not observed in other solved GPCR complex structures. Our work provides a structural basis for ligand binding to the seven-transmembrane domain of an adhesion GPCR and subsequent G protein coupling.

The orphan receptor GPR97 is a member of the adhesion GPCR (aGPCR) family^{1–3}. As one of the evolutionarily ancient families in the GPCR superfamily, aGPCRs are crucial molecular switches that regulate many physiological processes, including brain development, ion–water homeostasis, inflammation and cell-fate determination^{2,7–11}. Mutations in aGPCRs have been associated with specific human diseases, including vibratory urticaria, bilateral frontoparietal polymicrogyria, chondrogenesis, Usher syndrome and male infertility^{2,7,8}. Compared with other GPCR families, aGPCRs are well-known for the presence of a large ectodomain that contains the GAIN domain, which functions together with the seven-transmembrane (7TM) bundle as a pair, and subsequent activation of the receptor through tethered agonism, mechanical force or other mechanisms^{3,7,12–21}. Although substantial progress has been made in discovering the emerging functions of aGPCRs, the coupling of several aGPCR members to G proteins remains to be clarified, the structural basis for aGPCR activation is unclear and

whether the 7TM bundle of aGPCR constitutes a typical pocket to recognize a small chemical ligand is uncertain. In addition, because the aGPCR family does not share the conserved residues in class A or class B GPCRs for receptor activation and G protein coupling, aGPCRs may be activated through distinct sets of residue connections and coupling to G proteins via different motifs²². On the basis of this, the structural characterization of aGPCRs in complex with downstream signal transducers is of great value.

In the present study, we found that glucocorticoid stress hormones acutely inhibited the levels of cAMP via the activation of one aGPCR member, GPR97, which is involved in the development of experimental autoimmune encephalomyelitis, determination of B lymphocyte fate and the progression of acute kidney injury^{4,5,23,24}. We further determined the cryo-electron microscopy (cryo-EM) structures of active GPR97 in complex with the heterotrimeric G protein G_o and two glucocorticoids, the anti-inflammatory drug beclomethasone (BCM) and cortisol,

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without or with scFv16 stabilization, respectively. Our studies provide important structural insights into the ligand binding, receptor activation and G protein coupling of an aGPCR member.

Glucocorticoids activate GPR97

BCM dipropionate is an exogenous ligand of GPR97 (ref. ²⁵). In solution, BCM dipropionate may undergo hydrolysis to produce BCM, a synthetic glucocorticoid drug⁶ (Supplementary Fig. 2). We therefore suspected that BCM directly activates GPR97 and then verified this effect (Extended Data Fig. 1a–e, Supplementary Fig. 3, Supplementary Table 1). Dexamethasone, another glucocorticoid drug, showed greater potency (approximately threefold higher) than BCM (Extended Data Fig. 1d, Supplementary Fig. 4a–c, Supplementary Table 1). Both of these anti-inflammatory drugs share the same steroid core as endogenous steroid hormones. On the basis of this, we screened a panel of 23 endogenous steroid hormones and derivatives for the induction of GPR97 activity (Extended Data Fig. 1b, Supplementary Fig. 3, Supplementary Table 1). Glucocorticoid hormones, including cortisol (hydrocortisone), cortisone and 11-deoxycortisol, were found to be activating ligands for GPR97.

The administration of cortisol and cortisone to HEK293 cells overexpressing wild-type GPR97 elicited a dose-dependent decrease in the levels of forskolin-induced cAMP, with half maximal effective concentration (EC_{50}) values of 800 ± 10 pM and 2.61 ± 0.14 nM, respectively (Extended Data Fig. 1c–e, Supplementary Fig. 4a–c). The activation of GPR97 by glucocorticoids was further verified by G_{qo} dissociation assays (Extended Data Fig. 1f, Supplementary Fig. 4d, e). It has long been suspected that one or several GPCRs were unidentified glucocorticoid membrane receptors^{26–28}. Thus, G_o -coupled GPR97 was a candidate for this unidentified glucocorticoid membrane receptor. To explore this hypothesis, we administered cortisone to mouse Y-1 cells of the adrenal cortex, which resulted in an acute reduction in the cAMP-induced and adrenocorticotropic hormone-induced release of corticosterone. The knockdown of *Gpr97* expression abolished the effects of cortisone (Extended Data Fig. 1g–i). These data suggest that GPR97 may be involved in the acute effects of glucocorticoids.

Cryo-EM studies of GPR97

We set out to determine the structure of human GPR97 in complex with glucocorticoids and G_{oi} using single-particle cryo-EM. GPR97 contains a GAIN domain, which has an auto-cleavage site that produces two subunits: the α -subunit (N-terminal fragment (NTF)) and the β -subunit (C-terminal fragment) (Extended Data Fig. 1a). Initially, we attempted to form the complex that comprised wild-type GPR97 and G_{oi} ; however, the intrinsic tendency of the GPR97 α -subunit to dissociate from the β -subunit and the resulting instability of the complex during purification presented a challenge for structure determination using cryo-EM. Thus, we mutated the autoproteolysis motif²⁴⁸ HLT²⁵⁰ of GPR97 to²⁴⁸ALA²⁵⁰ to produce stabilized full-length GPR97 (designated GPR97-FL-AA) and used thermostabilized miniGo²⁹ to facilitate structure determination (Extended Data Figs. 1a, 2a, d, Supplementary Fig. 1). GPR97-FL-AA showed comparable activity towards various agonists and therefore would not cause significant structural perturbation (Extended Data Fig. 1d, Supplementary Fig. 4a). Accumulated data have suggested that the Stachel sequence that resides in the GAIN domain has a central role in governing aGPCR activation^{7,16–20}. The GPR97 β -subunit responded to BCM and cortisol stimulation with an 8–30-fold higher potency than GPR97-FL-AA or wild-type GPR97 (Extended Data Fig. 1d, Supplementary Fig. 4a). Removal of the Stachel sequence, which forms the tethered motif, from the N-terminal GPR97 β (designated GPR97- β -T) eliminated this effect, confirming the important role of the tethered sequence^{19,21} (Extended Data Fig. 1a, d, Supplementary Fig. 4a).

Fig. 1 | Cryo-EM structure of the GPR97- G_o complex. a, b, Orthogonal views of the density map for the BCM-GPR97- G_o (a) and the cortisol-GPR97- G_o -scFv16 (b) complexes. GPR97 is shown in light sea green, G α_o in salmon, G β in light blue, G γ in yellow, scFv16 in purple, BCM in slate blue and cortisol in pink. c, Orthogonal views of the model of the GPR97- G_o complex and the detailed ligand positions of BCM (slate blue) and cortisol (pink) in the structure.

Using a Titan Krios microscope, a total of 2,707 and 5,871 movies were collected for the BCM-GPR97-FL-AA- G_o and the cortisol-GPR97-FL-AA- G_o -scFv16 complexes reconstituted in vitro, respectively (Extended Data Fig. 2). The 2D averages showed clear density for the GPR97 transmembrane domain and the heterotrimeric G protein; however, the density of the NTF was visible only in certain directions and was very weak (Extended Data Fig. 2b, e, Supplementary Fig. 5). We therefore improved the quality of the cryo-EM map by applying a masked classification with the alignment focused on the GPR97 7TM bundle and the G_o protein subunits. The resulting cryo-EM maps after final refinement have overall resolutions of 3.1 Å and 2.9 Å for the BCM-bound and the cortisol-bound GPR97- G_o complexes, respectively (Fig. 1, Extended Data Fig. 2c, f).

The high-quality density map allowed accurate model building for receptor residues R270 to P527, the active core of BCM or cortisol, and most residues of the miniGo protein trimer (Fig. 1, Extended Data Fig. 2g, h, Extended Data Table 1). The structure of GPR97 adopted a unique 7TM bundle with several differences in both the spatial arrangement and the lengths of the transmembrane helices (see details in the following text) compared with other reported GPCR structures (Fig. 1c). Although previous experimental evidence indicated that the GAIN and 7TM domains function as a pair that serves as the hub for aGPCR activation^{7,16–20}, which leads to the hypothesis that the small-compound activator may also bind to the GAIN domain, we unexpectedly but unambiguously observed that glucocorticoids bound to a pocket within the 7TM domain of GPR97. At the cytoplasmic face, the 7TM bundle of GPR97 contains a large crevice to accommodate the G_o trimer. Unique to GPR97- G_o complexes, palmitoylation at the C terminus of G_o was observed to insert deeply into the 7TM core of GPR97, and the intracellular loop 1 (ICL1) of the receptor adopted a one-turn helical structure that formed direct contacts with G β (Fig. 1c).

The 7TM bundle and ECLs of GPR97

To date, the transmembrane domain structures of representative members from all GPCR families have been reported, with the sole

Fig. 2 | The glucocorticoid-binding pocket of GPR97. a, Vertical cross-section of the BCM-binding pocket in GPR97. **b**, Corresponding interactions that contribute to BCM binding in GPR97. The hydrogen bond is depicted as a dashed line. In contrast to cortisol, BCM interacts with I494, which is labelled in red. **c**, Vertical cross-section of the cortisol-binding (top) and the BCM-binding (bottom) pockets in GPR97. **d**, Corresponding interactions that contribute to cortisol binding in GPR97. The polar interaction is depicted as a dashed line. **e, f**, The effects of mutations in the ligand-binding pocket or structural motifs in GPR97 on BCM-induced (**e**) or cortisol-induced (**f**) cAMP inhibition. Values

are shown as the mean $\pm$ s.e.m. from three independent experiments performed in triplicate. *** $P < 0.001$; NS, not significant (comparison between the wild-type full-length GPR97 (GPR97-FL-WT) and its mutant). All data were analysed by two-sided, one-way analysis of variance (ANOVA) with Tukey's test ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P = 0.1034$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$ and $P = 0.1233$ from top to bottom for the BCM group; $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P = 0.5662$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$ and $P = 0.0646$ from top to bottom for the cortisol group).

exception of the aGPCR family. Comparison of the 7TM bundle of the GPR97–G_o complex with the structures of neurotensin receptor type 1 (NTSR1; class A), parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R; class B) and smoothened receptor (class F) in complex with their cognate G-protein-binding partners revealed similar topology (Extended Data Fig. 3a). For class C GPCRs, we used the recently reported structure of the active GABA_B receptor that was determined in the presence of G protein for comparison (Protein Data Bank (PDB) ID: 7C7Q)³⁰. However, structural comparison between these receptors suggested that there was greater separation between TM6 and TM7 in GPR97 than in the other four structures and that TM1 and TM7 were relatively close together (Extended Data Fig. 3a–c). In particular, GPR97 showed a relatively short TM6 (Extended Data Fig. 3d). The proximity of the extracellular portions of TM6 and TM7 provides additional space for ligand binding. Above the transmembrane bundle, an extended β -sheet in extracellular loop 2 (ECL2) created a flap on the top of TM6 and TM7 to constitute the top cover of the ligand-binding site (Fig. 1c, Extended Data Fig. 3e). Notably, the part of ECL2 that covers ECL3 (the top of TM6 and TM7) was constituted by consecutive hydrophilic residues, namely, R409^{ECL2}DRENRT415^{ECL2} (Extended Data Fig. 3e). Cryo-EM densities were unambiguously assigned only for the main chain of this ECL2 region. ECL2 consistently exhibited a very weak interaction with ECL3 (Extended Data Fig. 3f), serving as a flexible lid for the ligand-binding pocket, and might connect the conformational changes of the NTF to the 7TM bundle.

Ligand-binding pocket

The presence of a small ligand-binding pocket within GPR97 is unusual but is consistent with the hypothesis that aGPCRs could be activated via a Stachel-independent mechanism^{3,15}. The overall glucocorticoid-binding pocket of GPR97 within the 7TM bundle had a long ellipsoidal shape with a large, solvent-accessible channel that connected to the extracellular region (Fig. 2a–d). The cryo-EM maps enabled the unambiguous assignment of BCM and cortisol, with the A–B rings of the steroid core facing towards the solvent channel and the C–D rings oriented downwards against TM1, TM2 and TM7 (Fig. 2a–d, Extended Data Fig. 4a–f). The binding modes of the two glucocorticoids were further supported by molecular dynamics simulations (Extended Data Fig. 4g). The steroid cores of bound glucocorticoids were perpendicular to the plane of the plasma membrane, parallel to TM7 and at an angle of approximately 70° from the central TM3 (Extended Data Fig. 4e, f). The bound glucocorticoids fit into the centre of the pocket, which was deep and open, made up of residues from six transmembrane helices (TM1–TM3 and TM5–TM7) as well as residues from ECL2 and ECL3 (Fig. 2a–d). Compared to ligands in other ligand-bound GPCR structures (Extended Data Fig. 4h), BCM and cortisol were observed to pack relatively close to TM6 and TM7, with the bulky steroid core further pushing TM7 away from the core of the transmembrane domain, thus leading to a large separation between the extracellular ends of TM6 and TM7.

Glucocorticoids are recognized by extensive hydrophobic contacts and only one polar interaction. The residues in contact with BCM were

Fig. 3 | The active structure of GPR97. **a**, The position of the W490 toggle switch in GPR97, which directly interacts with BCM. **b**, Superimposition of G_o-coupled GPR97 with G_o-coupled 5-HT_{1B}R aligned at the 'PIF motif'. **c**, The detailed interactions of TM3–TM5–TM6 in G_o-coupled GPR97. **d**, The effects of mutations in transmembrane-helix tethering motifs in GPR97 on BCM-induced cAMP inhibition. Values are shown as the mean ± s.e.m. from three independent experiments performed in triplicate. ****P* < 0.001 (comparison between

GPR97-FL-WT and its mutant). All data were analysed by two-sided, one-way ANOVA with Tukey's test (from left to right: *P* < 0.0001, *P* < 0.0001, *P* < 0.0001, *P* < 0.0001, *P* = 0.0961 and *P* = 0.0881). **e**, Interactions mediated by H(N)^{3.53}L(M)^{3.54}Y^{3.55}. Both H^{3.53} and L^{3.54} of GPR97 directly interact with the G_o protein or the specific palmitoylation site. The hydrogen bond is depicted as a dashed line.

very similar to those in contact with cortisol, sharing ten identical residues and differing by only three hydrophobic residues (Extended Data Fig. 4i). The plane of the steroid core of these two glucocorticoids was defined by two hydrophobic patches: one side constituted by F323^{2.64}, F345^{3.36}, Y406^{ECL2} and W421^{ECL2}, and the other side by W490^{6.53}, I494^{6.57}, L498^{ECL3} and F506^{7.42} (the superscript text following the receptor residues is indicated according to the Wootten numbering system for the class B subfamily of GPCRs³¹; the lowercase letter 'a' after the superscript text indicates the use of the Ballesteros–Weinstein system for the class A subfamily of GPCRs³² (Fig. 2b, d, Extended Data Fig. 4a–d, Supplementary Table 2)). The polar contacts resided in the bottom of the pocket and were mediated by the interaction of the 18-carbonyl or the 19-hydroxyl group with the side chain of N510^{7.46} in glucocorticoid-bound GPR97 structures (Fig. 2b, d, Extended Data Fig. 4c, d).

The binding of a ligand to the 7TM bundle of class A receptors has been demonstrated to couple to specific conformational alterations in the extracellular domain³³. To exclude the constitutively bound glucocorticoids within GPR97, we used the intramolecular fluorescent arsenical hairpin bioluminescence resonance energy transfer (FIAsH-BRET) method to monitor the glucocorticoid-induced GPR97 conformational changes in the extracellular domain³⁴ (Extended Data Fig. 5a). The binding of BCM to GPR97 caused separation of the ECLs from the N terminus in a concentration-dependent manner, with the largest signal produced by labelling the FIAsH motif in ECL2 (Extended Data Fig. 5a–e, Supplementary Table 3). We next used both the cAMP assay and FIAsH-BRET-based extracellular conformational sensors to monitor the effects of mutations in ligand-binding pockets of GPR97. Consistent with the observations of the structures of GPR97 bound to glucocorticoids, mutations of the polar residue N510^{7.46} or of any five hydrophobic pocket residues to alanine impaired both agonist-induced cAMP inhibition and extracellular conformational changes (Fig. 2e, f, Extended Data Figs. 5f–j, 6a–e, Supplementary Tables 3, 4). Notably, seven out of nine hydrophobic residues and one polar residue involved in BCM interaction are highly conserved in all aGPCR G subfamily

members (Extended Data Fig. 5k), strongly implying that steroid hormones are probably ligands of other aGPCR G subfamily members.

Active structure of GPR97

Structural superimposition of the GPR97–G_o complex with other GPCR–G_o complexes corroborated the previous assignment of homologous residues at X.50 positions for each transmembrane helix by comparative studies³⁵. Residue W490^{6.53} of GPR97 aligned to roughly the same position as the homologous toggle switch residue W^{6.48a} in class A GPCRs^{29,36,37}. In GPR97, the potential toggle switch W490^{6.53} sensed the binding of glucocorticoids by direct interactions (Fig. 3a, Extended Data Fig. 7a, b). Residues I^{3.40a}, P^{5.50a} and F^{6.44a} form the core triad motif³⁷, which is located close to the toggle switch, and these were replaced by F353^{3.44}, T442^{5.46} and V486^{6.49} in GPR97 (Fig. 3b). T442^{5.46} of GPR97 did not form many interactions with F353^{3.44}, and these two residues were far from V486^{6.49} (Fig. 3b). Thus, there are no traditional PIF core triad interactions in the active structure of GPR97. Instead, TM3, TM5 and TM6 were tethered by packing interactions mediated by F353^{3.44}, M356^{3.47}, F439^{5.43} and the toggle switch residue W490^{6.53} in the upper region (Fig. 3c). F353^{3.44}, F439^{5.43}, M356^{3.47} and W490^{6.53} served as a newly identified 'upper quaternary core' (UQC) (Fig. 3c, Extended Data Fig. 7c). In the lower region, L363^{3.54} was packed against L450^{5.54} and L479^{6.42}, forming a specific 'lower triad core' (Fig. 3c). The importance of the tight packing of TM3–TM6 by these residues was further supported by the observation that mutations in the UQC of GPR97 reduced the potency of stimulation of GPR97 by BCM (Figs. 2e, 3d, Extended Data Fig. 6, Supplementary Table 4). Residues involved in W^{6.53} and the UQC were conserved in members of the G subfamily of aGPCRs (Extended Data Fig. 7d). Mutations of the UQC residues in GPR126, another aGPCR member, impaired Stachel peptide-induced activation (Extended Data Fig. 7e, f, Supplementary Table 5). These results indicated the potential structural importance of the conserved W^{6.53} and UQC residues, which could be further confirmed by the solution of additional aGPCR structures.

Fig. 4 | General features of G_o coupling to GPR97. **a**, A cytoplasmic view (left) and an orthogonal view (right) of the GPR97 7TM bundle compared with G_o -coupled 5-HT_{1B}R (PDB ID: 6G79) and M2R (PDB ID: 6OIK). GPR97 is shown in light sea green, 5-HT_{1B}R in wheat and M2R in salmon. **b**, The structures of G_o -coupled GPR97 (light sea green), 5-HT_{1B}R (wheat) and M2R (salmon) complexes were superimposed based on TM2, TM3 and TM4. This panel is

Another notable observation is that in all aGPCR G subfamily members, TM7 lacks a conserved N^{7.49a}P^{7.50a}XXY^{7.53a} motif, which mediates the packing of TM7 against TM3 in several active GPCR structures^{29,36,38}. No significant packing between TM3 and TM7 was observed in the GPR97- G_o complex structures. The mutation of I517^{7.53} to asparagine consistently had no significant effect on BCM-induced GPR97 activation (Fig. 3d, Supplementary Table 4). Therefore, GPR97 has a different transmembrane helix arrangement from other active class A GPCR structures.

On the cytoplasmic side, the separation of the TM3 and TM6 ends is approximately 14 Å (Extended Data Fig. 7g, h). The separation of the transmembrane ends is generally accompanied by a break in the ionic lock and the structural rearrangement of the D^{3.49a}R^{3.50a}Y^{3.51a} motif in active class A GPCR structures³⁷. The D^{3.49a}R^{3.50a}Y^{3.51a} motif was replaced by H(N)^{3.53}L(M)^{3.54}Y^{3.55} in the aGPCR family (Fig. 3e, Extended Data Fig. 7i, j). In GPR97, the conserved Y364^{3.55} residue formed contacts with residues from TM5 and ICL2 and stabilized the ICL2 conformation (Fig. 3e). H362^{3.53} and L363^{3.54} of the H(N)^{3.53}L(M)^{3.54}Y^{3.55} motif of GPR97 were shown to interact with L348^{G.H5.20} and a palmitoylation on the $\alpha 5$ helix of G_o (the superscripts following the G_{α} residues are according to the Common G_{α} numbering system³⁹) (Fig. 3e, Extended Data Fig. 7i). Mutations of any residue in the conserved HLY motif of GPR97 to alanine or mutation of L363^{3.54} to the corresponding R^{3.50a} in class A GPCRs significantly decreased the activation of GPR97 (Fig. 2e, f, Extended Data Figs. 6, 7k, Supplementary Table 4), suggesting the functional importance of the conserved H(N)^{3.53}L(M)^{3.54}Y^{3.55} motif in the aGPCR family.

Coupling of GPR97 with G_o

The cytoplasmic side of GPR97 displayed a wide cavity. Aligning the central TM3 (GPR97- G_o , 5-HT_{1B}R- G_o and M2R- G_o) allowed us to identify

shown with views at two different angles; the red arrows indicate the tilt of the $\alpha 5$ helix of $G_{\alpha o}$ from the GPR97- G_o complex compared to the 5-HT_{1B}R- G_o or M2R- G_o complexes. **c**, The residues in GPR97, 5-HT_{1B}R and M2R that contact G_o . **d-f**, The detailed interactions of ICL1 with $G\beta$ (**d**), ICL2 with the $\alpha 5$ helix and the $\beta 1$ and $\beta 3$ strands of $G_{\alpha o}$ (**e**) and of ICL3 with the $\beta 6$ strand and the $\alpha 4$ and $\alpha 5$ helices of $G_{\alpha o}$ (**f**). The hydrogen bonds are depicted as dashed lines.

a wider separation between TM5 and TM7 of GPR97 on the cytoplasmic side (Fig. 4a). This change led to an approximately 10° tilt of the $\alpha 5$ helix of G_o towards the plasma membrane in the GPR97- G_o complex structure (Fig. 4b). GPR97 has a significantly larger buried surface (1,116 Å²) for G_o than does 5-HT_{1B}R (822 Å²). The interface between GPR97 and G_o consisted of four transmembrane helices (TM3, TM5, TM6 and TM7) and three intracellular loops of GPR97 (Fig. 4c-f, Supplementary Tables 6–9). Specifically, residues of the C-terminal wavy hook of the $\alpha 5$ helix of G_o form hydrophobic and polar interactions with the cytoplasmic portion of TM6 (Extended Data Fig. 8a, b). C351^{G.H5.23} of G_o formed a polar interaction with the side chain of W520^{7.56}. The N terminus of C351^{G.H5.23} was the helical portion of the $\alpha 5$ helix of G_o , which fit into the cavity formed by residues from TM3, TM5 and TM6 (Extended Data Fig. 8a, b).

All three intracellular loops of GPR97 participated in direct binding to the G_o trimer. ICL1 formed both electrostatic and van der Waals interactions with the $G\beta$ subunit (Fig. 4d, Supplementary Table 7). Mutations of ICL1 residues significantly reduced BCM-induced GPR97 activity (Extended Data Figs. 6, 8c, Supplementary Table 4). ICL2 of GPR97 fit into the groove formed by the $\alpha 5$, αN , $\beta 1$ and $\beta 3$ strands of G_o (Fig. 4e). The hydrophobic side chains of V370^{ICL2} and F371^{ICL2} of GPR97 were packed against a hydrophobic cleft created by the $\alpha 5$ helix and $\beta 1$ – $\beta 3$ strands of G_o (Fig. 4e, Supplementary Table 8). ICL3 is unstructured in most GPCR structures solved to date, but the complete structure was visible in the cryo-EM density in GPR97, which was probably stabilized by many direct interactions with G_o (Fig. 4f, Extended Data Fig. 9). Several GPR97 ICL3 residues (A463, T464, V466, K471 and N472) interacted directly with $\alpha 4$, $\alpha 5$ and $\beta 6$ of G_o (Fig. 4f, Supplementary Table 9). Importantly, compared with all other solved GPCR structures, some of the extensive interactions of the three ICLs of GPR97 with G_o might contribute to the high basal activity observed for GPR97. Consistent with this hypothesis, mutations in key ICL1 and ICL3 residues that contact

Fig. 5 | Palmitoylation of G_{α_o} mediates GPR97 coupling. **a**, An unknown continuous electron density connected to C351 in the $\alpha 5$ helix of G_{α_o} was observed in the cortisol–GPR97– G_o complex structure. A similar cryo-EM density was also observed in the BCM-bound GPR97– G_o complex structure. **b**, The detailed interactions of TM3–TM5–TM6–TM7 with palmitoylated C351 in G_o -coupled GPR97 (PDB ID: 7D77). The hydrogen bond is depicted as a dashed line. **c**, The effects of mutations of C351 in the $\alpha 5$ helix of G_{α_o} on GPR97 or D2R activities. The bar graph represents the average pEC_{50} (that is, $-\log EC_{50}$)

the G_o trimer not only decreased the BCM-induced cAMP reduction but also greatly decreased the basal activity of GPR97 (Extended Data Fig. 8d–f). Therefore, the interactions mediated by the ICLs could be one determinant for the high basal activity of GPR97.

Palmitoylation at the C-tail of G_o

A strong cryo-EM density was observed in the crevice between TM3, TM5 and TM6 of GPR97 and was connected to C351^{G.H5.23} of the $\alpha 5$ helix end of G_o (Fig. 5a). Mass spectrometry enabled us to clarify this specific post-modification as a palmitoylation, which could be unambiguously assigned into the cryo-EM density (Extended Data Fig. 9a, b). Notably, the carbonyl group of the palmitoylation formed a well-defined hydrogen bond with H362^{3.53} of the conserved H^{3.53}L^{3.54}Y^{3.55} motif. The aliphatic chain of the palmitoylation formed contacts with 9 hydrophobic residues that were contributed by TM3 and TM5–TM7, inserting 17 Å deep into the 7TM core and finally reaching the UQC, thus further tethering the transmembrane helices in the active receptor conformation (Fig. 5a, b, Extended Data Fig. 9c). Importantly, a similar palmitoylation at C351^{G.H5.23} of G_o or G_i proteins would clash with other GPCRs in all solved GPCR– G_i or GPCR– G_o complex structures (Extended Data Fig. 9d). Mutations of C351A or C351S of G_o markedly impaired G_o coupling to GPR97 but not to D2R, a prototype G_o/G_i -coupled receptor (Fig. 5c, Extended Data Fig. 9e–h). Therefore, the palmitoylation of the C terminus of the G_o protein contributed to its specific coupling to GPR97 (Supplementary Table 10).

Conclusion

In summary, we have provided experimental evidence that endogenous steroid hormone glucocorticoids are high-affinity agonists for GPR97, an aGPCR, and presented the structures of GPR97– G_o in complex with two glucocorticoids. Our structure highlighted that the small steroid hormone could bind to the 7TM bundle of one aGPCR member for activation of the receptor. The ligand-binding pocket of GPR97 is within the top half of the 7TM bundle and is covered by the extended ECL2. The glucocorticoids bound at the centre of this pocket with their D rings packed against the toggle switch residue W490^{6.53}. Furthermore, our cryo-EM structure revealed that GPR97 has a unique 7TM architecture with an extended ECL2 β -sheet and a relatively short helix TM6. Although GPR97 has neither the traditional ‘PIF’ core triad nor the NPXXY motif that commonly mediates the activation of class A GPCRs, it senses ligand binding by the conserved toggle switch W^{6.53} and tethers TM3–TM5–TM6 by the newly identified and sequence-conserved UQC consisting of F353^{3.44}M356^{3.47}F439^{5.47}W490^{6.53} for the active receptor

measured from three independent experiments. The original dose–response data are shown in Extended Data Fig. 9g, h. The data represent the mean $\pm$ s.e.m. from three independent experiments performed in triplicate. *** $P < 0.001$, G_{α_o} C351 mutations versus G_{α_o} WT in activating GPR97; NS, G_{α_o} C351 mutations versus G_{α_o} WT in activating D2R. All data were analysed by two-sided, one-way ANOVA with Tukey’s test ($P < 0.0001$ and $P < 0.0001$ from left to right for the GPR97 group; $P = 0.0859$ and $P = 0.0744$ from left to right for the D2R group).

conformation. In the aGPCR family, the conserved D/ERY motif is replaced by H(N)L(M)Y, which forms specific interactions with the palmitoylation of G_o . In addition, all three ICLs of GPR97 are involved in direct interactions with the G_o subunits, which may contribute to the high basal activity. Several of these observed characteristics in the GPR97 structure, including the arrangement of the 7TM bundle, the ligand-binding mode^{40,41}, the ligand-induced receptor activation and the manner of G protein coupling, could also be shared by other aGPCR family members (Supplementary Fig. 6).

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-020-03083-w>.

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Methods

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

Cell lines

HEK293 cells were obtained from the Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences). *Spodoptera frugiperda* (Sf9) cells were purchased from Expression Systems (cat. 94-001S). Y-1 cells were originally obtained from the American Type Culture Collection (ATCC). The cells were grown in monolayer culture in RPMI 1640 with 10% FBS (Gibco) at 37 °C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

Constructs of GPR97 and miniGo heterotrimer

For protein production in insect cells, the human GPR97 (residues 21–549) with the autoproteolysis motif mutation (H248/A and T250/A) was sub-cloned into the pFastBac1 vector. The native signal peptide was replaced with the haemagglutinin signal peptide (HA) to enhance receptor expression, followed by a Flag tag DYKDDDK (China peptide) to facilitate complex purification. An engineered human G α_{o1} with G α_{o1} H domain deletion, named miniG α_{o1} , was cloned into pFastBac1 according to published literature²⁹. Human G $\beta 1$ with the C-terminal hexa-histidine tag and human Gy2 were subcloned into the pFastBacDual vector. scFv16 was cloned into pFastBac1 with the C-terminal hexa-histidine tag and the N-terminal GP67 signal peptide. To examine the activities of GPR97, the GPR97-FL-WT (wild-type full-length GPR97), GPR97-FL-AA (GPR97 GPS site mutation, H248/A and T250/A), GPR97 β (GPR97 with the NTF removed, residues 250–549) and GPR97- β -T (GPR97 β with the N-terminal tethered Stachel sequence removed, residues 265–549) were sub-cloned into the pcDNA3.1 plasmid. The GPR97 mutations E298A, R299A, F345A, F353A, H362A, L363A, Y364A, V370A, F371A, Y406A, W421A, W490A, A493G, I494A, L498A and N510A were generated using the Quikchange mutagenesis kit (Stratagene). The G protein BRET probes were constructed according to previous publications^{42,43}. Human G protein subunits (G α_q , G $\beta 1$ and Gy2) were sub-cloned into the pcDNA3.1 expression vectors. The G α_q -RlucII subunit was generated by amplifying and inserting the coding sequence of RlucII into G α_q between residue L97 and K98. The G α_q probe, in which the six amino acids of the C-terminal of G α_q -RlucII were substituted with those from G α_{o1} , was constructed by PCR amplification using synthesized oligonucleotides encoding swapped C-terminal sequences. The GFP10–Gy2 plasmid was generated by fusing the GFP10 coding sequence in frame at the N terminus to Gy2. All of the constructs and mutations were verified by DNA sequencing.

Protein expression

High titre recombinant baculoviruses were generated using Bac-to-Bac Baculovirus Expression System. In brief, 2 μ g of recombinant bacmid and 2 μ l X-tremeGENE HP transfection reagent (Roche) in 100 μ l Opti-MEM medium (Gibco) were mixed and incubated for 20 min at room temperature. The transfection solution was added to 2.5 ml Sf9 cells with a density of 1×10^6 per ml in a 24-well plate. The infected cells were cultured in a shaker at 27 °C for 4 days. P0 virus was collected and then amplified to generate P1 virus. The viral titres were determined by flow cytometric analysis of cells stained with gp64-PE antibody (1:200 dilution; 12-6991-82, Thermo Fisher). Then, Sf9 cells were infected with viruses encoding GPR97-FL-AA, miniG α_o , G $\beta\gamma$, and with or without scFv16, respectively, at equal multiplicity of infection. The infected cells were cultured at 27 °C, 110 rpm for 48 h before collection. Cells were finally collected by centrifugation and the cell pellets were stored at –80 °C.

GPR97–G_o complex formation and purification

Cell pellets transfected with virus encompassing the GPR97-FL-AA, miniGo trimer and scFv16 (only existed in cell pellets for purifying the

cortisol–GPR97-FL-AA–G_o–scFv16 complex) were resuspended in 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 10 mM MgCl₂ and 5 mM CaCl₂ supplemented with Protease Inhibitor Cocktail (B14001, Bimake) and 100 μ M TCEP (Thermo Fisher Scientific). The complex was formed for 2 h at room temperature by adding 10 μ M BCM (HY-B1540, MedChemExpress) or cortisol (HY-N0583, MedChemExpress), 25 mU/ml apyrase (Sigma), and then solubilized by 0.5% (w/v) lauryl maltose neopentylglycol (LMNG; Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate TRIS salt (CHS; Anatrace) for 2 h at 4 °C. Supernatant was collected by centrifugation at 30,000 rpm for 40 min, and the solubilized complex was incubated with nickel resin for 2 h at 4 °C. The resin was collected and washed with 20 column volumes of 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 2 mM MgCl₂, 25 mM imidazole, 0.01% (w/v) LMNG, 0.01% GDN (Anatrace), 0.004% (w/v) CHS, 10 μ M BCM (or cortisol) and 100 μ M TCEP. The complex was eluted with 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 2 mM MgCl₂, 200 mM imidazole, 0.01% (w/v) LMNG, 0.01% GDN, 0.004% (w/v) CHS, 10 μ M BCM (or cortisol) and 100 μ M TCEP. The elution of nickel resin was applied to M1 anti-Flag resin (Sigma) for 2 h and washed with 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 2 mM MgCl₂, 5 mM CaCl₂, 0.01% (w/v) LMNG, 0.01% GDN, 0.004% (w/v) CHS, 10 μ M BCM (or cortisol) and 100 μ M TCEP. The GPR97–G_o complex was eluted in buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 2 mM MgCl₂, 0.01% (w/v) LMNG, 0.01% GDN, 0.004% (w/v) CHS, 10 μ M BCM (or cortisol), 100 μ M TCEP, 5 mM EGTA and 0.2 mg/ml Flag peptide. The complex was concentrated and then injected onto Superdex 200 increase 10/300 GL column equilibrated in the buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 0.00075% (w/v) LMNG, 0.00025% GDN, 0.0002% (w/v) CHS, 10 μ M BCM (or cortisol) and 100 μ M TCEP. The complex fractions were collected and concentrated individually for EM experiments.

Cryo-EM grid preparation and data collection

For the preparation of cryo-EM grids, 3 μ l of purified BCM-bound and cortisol-bound GPR97–G_o complex at approximately 20 mg/ml was applied onto a glow-discharged holey carbon grid (Quantifoil R1.2/1.3). Grids were plunge-frozen in liquid ethane cooled by liquid nitrogen using Vitrobot Mark IV (Thermo Fisher Scientific). Cryo-EM imaging was performed on a Titan Krios at 300 kV accelerating voltage in the Center of Cryo-Electron Microscopy, Zhejiang University. Micrographs were recorded using a Gatan K2 Summit direct electron detector in counting mode with a nominal magnification of $\times 29,000$, which corresponds to a pixel size of 1.014 Å. Movies were obtained using serialEM at a dose rate of about 7.8 electrons per Å² per second with a defocus ranging from –0.5 to –2.5 μ m. The total exposure time was 8 s and intermediate frames were recorded in 0.2-s intervals, resulting in an accumulated dose of 62 electrons per Å² and a total of 40 frames per micrograph. A total of 2,707 and 5,871 movies were collected for the BCM-bound and cortisol-bound GPR97–G_o complex, respectively.

Cryo-EM data processing

Dose-fractionated image stacks for the BCM–GPR97–G_o complex were subjected to beam-induced motion correction using MotionCorr2.1⁴⁴. Contrast transfer function (CTF) parameters for each non-dose-weighted micrograph were determined by Gctf⁴⁵. Particle selection, 2D and 3D classifications of the BCM–GPR97–G_o complex were performed on a binned data set with a pixel size of 2.028 Å using RELION-3.0-beta⁴⁶.

For the BCM–GPR97–G_o complex, semi-automated particle selection yielded 2,026,926 particle projections. The projections were subjected to reference-free 2D classification to discard particles in poorly defined classes, producing 911,519 particle projections for further processing. The map of the 5-HT_{1B}R–miniGo complex (EMDB-4358)⁴⁷ low-pass filtered to 40 Å was used as a reference model for maximum-likelihood-based 3D classification, resulting in one

well-defined subset with 307,700 projections. Further 3D classifications focusing the alignment on the complex produced two good subsets that accounted for 166,116 particles, which were subsequently subjected to 3D refinement, CTF refinement and Bayesian polishing. The final refinement generated a map with an indicated global resolution of 3.1 Å at a Fourier shell correlation of 0.143.

For the cortisol–GPR97–G_o complex, particle selection yielded 4,323,518 particle projections for reference-free 2D classification. The well-defined classes with 2,201,933 particle projections were selected for a further two rounds of 3D classification using the map of the BCM-bound complex as reference. One good subset that accounted for 335,552 particle projections was selected for a further two rounds of 3D classifications that focused the alignment on the complex, and produced one high-quality subset with 75,814 particle projections. The final particle projections were subsequently subjected to 3D refinement, CTF refinement and Bayesian polishing, which generates a map with a global resolution of 2.9 Å. Local resolution for both density maps was determined using the Bsoft package with half maps as input maps⁴⁸.

Model building and refinement

For the structure of the BCM–GPR97–G_o complex, the initial template of GPR97 was generated using the module ‘map to model’ in PHENIX⁴⁴. The coordinate of the 5-HT_{1B}R–G_o complex (PDB ID: 6G79) was used to generate the initial models for G_o (ref.⁴⁴). Models were docked into the EM density map using UCSF Chimera⁴⁹, followed by iterative manual rebuilding in COOT⁵⁰ according to side-chain densities. BCM and lipid coordinates and geometry restraints were generated using phenix.elbow. BCM was built to the model using the ‘LigandFit’ module in PHENIX. The placement of BCM shows a correlation coefficient of 0.81, indicating a good ligand fit to the density. The model was further subjected to real-space refinement using Rosetta⁵¹ and PHENIX⁴⁴.

For the structure of the cortisol–GPR97–G_o complex, the coordinates of GPR97 and G_o from the BCM-bound complex and scFv16 from the human NTSR1–G_q complex (PDB ID: 6OS9) were used as initial model. Models were docked into the density map and then were manual rebuilt in COOT. The agonist cortisol was built to the model using the ‘LigandFit’ module as described, showing a good density fit with a correlation coefficient of 0.80. The model was further refined using Rosetta⁵¹ and PHENIX⁴⁴. The final refinement statistics for both structures were validated using the module ‘comprehensive validation (cryo-EM)’ in PHENIX⁴⁴. The goodness of the fit of the model to the map was performed for both structures using a global model-versus-map FSC (Extended Data Fig. 2). The refinement statistics are provided in Extended Data Table 1. Figures of the structures were generated using UCSF Chimera, UCSF ChimeraX⁵² and PyMOL⁵³.

Molecular dynamics simulation of the BCM–GPR97 and cortisol–GPR97 complexes

On the basis of the favour binding poses of BCM and cortisol with the receptor GPR97, which was calculated by the LigandFit program of PHENIX, the GPR97–agonist complexes were substrate from the two GPR97–agonist–mG_o complexes for molecular dynamics simulation. The orientations of receptors were calculated by the Orientations of Proteins in Membranes (OPM) database. Following this, the whole systems were prepared by the CHARMM-GUI and embedded in a bilayer that consisted of 200 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids by replacement methods. The membrane systems were then solvated into a periodic TIP3P water box supplemented with 0.15 M NaCl. The CHARMM36m Force Field was used to model protein molecules, CHARMM36 Force Field for lipids and salt with CHARMM General Force Field (CGenFF) for the agonist molecules BCM and cortisol.

Then, the system was subjected to minimization for 10,000 steps using the conjugated gradient algorithm and then heated and equilibrated at 310.13 K and 1 atm for 200 ps with 10.0 kcal mol^{−1} Å^{−2} harmonic

restraints in the NAMD 2.13 software. Next followed five cycles of equilibration for 2 ns each at 310.13 K and 1 atm, for which the harmonic restraints were 5.0, 2.5, 1.0, 0.5 and 0.1 kcal mol^{−1} Å^{−2} in sequence.

Production simulations were run at 310.13 K and 1 atm in the NPT ensemble using the Langevin thermostat and Nose–Hoover method for 200 ns. Electrostatic interactions were calculated using the particle mesh Ewald (PME) method with a cut-off of 12 Å. Throughout the final stages of equilibration and production, 5.0 kcal mol^{−1} Å^{−2} harmonic restraints were placed on the residues of GPR97 that were within 5 Å of G_o in the BCM (or cortisol)–GPR97–G_o complex to ensure that the receptor remained in the active state in the absence of the G protein. Trajectories were visualized and analysed using Visual Molecular Dynamics (VMD, version 1.9.3)

cAMP ELISA detection in Y-1 cells

Y-1 cells were transfected with *Gpr97* siRNA (si-97, GUGCAGGGAU-GUCUUUAA) or control siRNA (si-Con) for 48 h. After starvation for 12 h in serum-free medium, the cells were further stimulated with cortisone (8 nM), forskolin (5 μM) (Sigma-Aldrich) or control vehicle for 10 min. Then, cells were washed three times with pre-cooled PBS and resuspended in pre-cooled 0.1 N HCl containing 500 μM IBMX at a 1:5 ratio (w/v). The samples were neutralized with 1 N NaOH at a 1:10 ratio (v/v) after 10 min. The supernatants were collected after centrifugation of the samples at 600g for 10 min. The supernatants were then prepared for cAMP determination using the cAMP Parameter Assay Kit (R&D Systems) according to the manufacturer’s instruction. The *Gpr97* expression level under various conditions were further confirmed using quantitative real-time PCR.

Corticosterone measurements

Mouse adrenocorticotoma cell line Y-1 cells were transfected with *Gpr97* siRNA (si-97) or control siRNA (si-Con) for 48 h. Then, the cells were treated with serum-free medium for 12 h. After that, cortisone (16 nM) or ACTH (0.5 μM) were added to cells for 30 min. The supernatants of the cell culture medium were collected for measurements of corticosterone by ELISA according to the manufacturer’s instructions.

Quantitative real-time PCR

Total RNA of cells was extracted using a standard TRIzol RNA isolation method. The reverse transcription and PCR experiments were performed with the Revertra Ace qPCR RT Kit (TOYOBO FSQ-101) using 1.0 μg of each sample, according to the manufacturer’s protocols. The quantitative real-time PCR was conducted in the Light Cycler apparatus (Bio-Rad) using the FastStart Universal SYBR Green Master (Roche). The mRNA level was normalized to GAPDH in the same sample and then compared with the control. The forward and reverse primers for GPR97 used in the experiments were CAGTTTGGGACTGAGGGACC and GCCACACTTGGTGAAACAC. The mRNA level of GAPDH was used as an internal control. The forward and reverse primers for GAPDH were GCCTTCCGTGTTCTACC and GCCTGCTTCACCACCTTC.

cAMP inhibition assay

To measure the inhibitory effects on forskolin-induced cAMP accumulation of different GPR97 constructs or mutants in response to different ligands or constitutive activity, the GloSensor cAMP assay (Promega) was performed according to previous publications^{12,13}. HEK293 cells were transiently co-transfected with the GloSensor and various versions of GPR97 or vehicle (pcDNA3.1) plasmids using PEI in six-well plates. After incubation at 37 °C for 24 h, transfected cells were seeded into 96-well plates with serum-free DMEM medium (Gibco) and incubated for another 24 h at 37 °C in a 5% CO₂ atmosphere. Different ligands were dissolved in DMSO (Sigma) to a stock concentration of 10 mM and followed by serial dilution using PBS solution immediately before the ligand stimulation. The transfected cells were pre-incubated with 50 μl of serum-free DMEM medium containing GloSensor cAMP reagent

(Promega). After incubation at 37 °C for 2 h, varying concentrations of ligands were added into each well and followed by the addition of forskolin to 1 μ M. The luminescence intensity was examined on an EnVision multi-label microplate detector (Perkin Elmer).

The G_{qo} protein activation BRET assay

According to previous publications, the BCM dipropionate-induced GPR97 activity could be measured by chimeric G_{qo} protein assays²⁵. The G_{qo} BRET probes were generated by replacing the six amino acids of the C-terminal of G_q-RLucII with those from G_{oAl}, creating a chimeric G_{qo}-RLucII subunit⁴⁷. GFP10 was connected to Gy. The G_{qo} protein activation BRET assay was performed as previously described⁵⁴. In brief, HEK293 cells were transiently co-transfected with control D2R and various GPR97 constructs, plasmids encoding the G_{qo} BRET probes, incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. Cells were washed twice with PBS, collected and resuspended in buffer containing 25 mM HEPES, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.5 mM MgCl₂ and 0.37 mM NaH₂PO₄. Cells that were dispensed into a 96-well microplate at a density of 5–8 × 10⁴ cells per well were stimulated with different concentrations of ligands. BRET² between RLucII and GFP10 was measured after the addition of the substrate coelenterazine 400a (5 μ M, Interchim) (Cayman) using a Mithras LB940 multimode reader (Berthold Technologies). The BRET² signal was calculated as the ratio of emission of GFP10 (510 nm) to RLucII (400 nm).

Measurement of receptor cell-surface expression by ELISA

To evaluate the expression level of wild-type GPR97 and its mutants, HEK293 cells were transiently transfected with wild-type and mutant GPR97 or vehicle (pcDNA3.1) using PEI reagent in six-well plates. After incubation at 37 °C for 18 h, transfected cells were plated into 24-well plates at a density of 10⁵ cells per well and further incubated at 37 °C in a 5% CO₂ atmosphere for 18 h. Cells were then fixed in 4% (w/v) para-formaldehyde and blocked with 5% (w/v) BSA at room temperature. Each well was incubated with 200 μ l of monoclonal anti-FLAG (F1804, Sigma-Aldrich) primary antibody overnight at 4 °C and followed by incubation of a secondary goat anti-mouse antibody (A-21235, Thermo Fisher) conjugated to horseradish peroxidase for 1 h at room temperature. After washing, 200 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added. Reactions were quenched by adding an equal volume of 0.25 M HCl solution and the optical density at 450 nm was measured using the TECAN (Infinite M200 Pro NanoQuant) luminescence counter. For determination of the constitutive activities of different GPR97 constructs or mutants, varying concentrations of desired plasmids were transiently transfected into HEK293 cells and the absorbance at 450 nm was measured.

The FIAsH-BRET assay

HEK293 cells were seeded in six-well plates after transfection with GPR97-FIAsH with Nluc inserted in a specific N-terminal site. Before the BRET assay, HEK293 cells were starved with serum for 1 h. Then cells were digested, centrifuged and resuspended in 500 μ l BRET buffer (25 mM HEPES, 1 mM CaCl₂, 140 mM NaCl, 2.7 mM KCl, 0.9 mM MgCl₂, 0.37 mM NaH₂PO₄, 5.5 mM D-glucose and 12 mM NaHCO₃). The FIAsH-EDT2 was added at a final concentration of 2.5 μ M and incubated at 37 °C for 60 min. Subsequently, HEK293 cells were washed with BRET buffer and then distributed into black-wall clear-bottom 96-well plates, with approximately 100,000 cells per well. The cells were treated with a final concentration of BCM and cortisol at 10⁻⁵ to 10⁻¹¹ and then coelenterazine H was added at a final concentration of 5 μ M, followed by checking the luciferase (440–480 nm) and FIAsH (525–585 nm) emissions immediately. The BRET ratio (emission enhanced yellow fluorescent protein/emission Nluc) was calculated using a Berthold Technologies Tristar 3 LB 941 spectrofluorimeter. The procedure was modified from those described previously^{34,55,56}.

Statistical analysis

A one-way ANOVA test was performed to evaluate the statistical significance between various versions of GPR97 and their mutant in terms of expression level, potency or efficacy using GraphPad Prism. For all experiments, the standard error of the mean of the values calculated based on the data sets from three independent experiments is shown in respective figure legends.

Reporting summary

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

Data availability

The density maps and structure coordinates have been deposited to the Electron Microscopy Database (EMDB) and the PDB with the accession codes EMD-30602 and 7D76 for the BCM–GPR97–G_o complex. The cryo-EM density map and coordinates for the cortisol–GPR97–G_o complex have been deposited to the EMDB and PDB under the accession codes EMD-30603 and 7D77. The Orientations of Proteins in Membranes database is accessible at <http://opm.phar.umich.edu>. All other data are available on request to the corresponding authors.

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Article

Author contributions J.-P.S., Y.Z. and H.E.X. conceived, designed and supervised the overall project. J.-P.S., Y.Z., H.E.X., Y.-Q.P., C.M., X.Y., P.X. and Y.J. participated in data analysis and interpretation. Y.-Q.P. generated the GPR97 insect cell expression construct, established the BCM-GPR97-G_s and cortisol-GPR97-G_s-scFv16 complex purification protocol and prepared samples for the cryo-EM supervised by Y.J. Y.-Q.P., G.-Y.W., X.W., X.Y. and Y.-N.Z. generated viruses and infected Sf9 cells for large quantitative protein production. D.-D.S. and C.M. evaluated the sample by negative-stain electron microscopy. C.M. prepared the cryo-EM grids, collected the cryo-EM data with assistance from D.-D.S. and performed the cryo-EM map calculation, model building and refinement. Q.S. performed molecular dynamics simulations. H.Z. assisted in protein purification. J.-P.S., Y.Z., X.Y., J.Y., C.M. and P.X. designed all of the mutations for determination of ligand-binding sites. X.T., R.-J.Z., S.L. and F. Yang generated all of the GPR97 constructs and mutants for the cell-based G protein activity assays. R.-J.Z., P.X., S.L. C.Q. and Z.Y. performed the ELISA assays and cAMP inhibition assays. W.-T.A., X.Y., F.Yi and J.-P.S. designed the cellular functional assays for GPR97. W.-T.A. performed the cellular

functional assays. J.-P.S., P.X., X.Y. and Z.-J.L. designed the FIAsh-BRET assays. R.-J.Z. performed the FIAsh-BRET experiments. C.T. and J.Y. performed the mass spectrometry experiment. J.Y. manually characterized fatty acylation on miniGo protein. Y.-Q.P., P.X., C.M., Q.S. and Y.J. prepared the figures. J.-P.S., Y.Z. and H.E.X. wrote the manuscript with input from all authors.

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Additional information

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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Glucocorticoids are activating ligands of GPR97.

a, Schematic diagrams showing the sequence features of GPR97 constructs used in this study. Wild-type full-length GPR97 (GPR97-FL-WT) is comprised of the conserved 7TM region and an intact GAIN domain, whereas the autoproteolysis-deficient mutant (GPR97-FL-AA) introduces two alanines to replace the autoproteolysis catalytic residues H248 and T250. **b**, The inhibition rates of steroid ligands on forskolin (1 μ M)-induced cAMP accumulation in GPR97-FL-WT overexpressing HEK293 cells. Values are the mean $\pm$ SEM from three independent experiments. Statistical differences between different ligands and the control vehicle were determined by two-sided one-way ANOVA with Tukey's test. *** P < 0.001 (From top to bottom, P = < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, 0.0016). The data were generated according to data shown in Supplementary Fig. 3. The original data are referred to Supplementary Table 1. **c**, Chemical structures of representative exogenous and endogenous glucocorticoids used in this study. **d**, The inhibitory effects of BCM, cortisone, cortisol, and dexamethasone (DEX) on forskolin (1 μ M)-induced cAMP accumulation in GPR97-FL-WT, GPR97-FL-AA, GPR97- β and GPR97- β -T overexpressing HEK293 cells. Values are the mean $\pm$ SEM from three independent experiments, each performed in triplicate. The potency and efficacy are summarized from data presented in Supplementary Fig. 4a. **e**, The effect of the GPR97 mediated cAMP inhibition compared with other known Gi/o coupled receptors, including AT1R and GPR120. Summarized potency and efficacy for effects of dexamethasone (DEX), Angiotensin II or TUG891 on Gi mediated cAMP level inhibition of HEK293 cells overexpressing similar amounts of GPR97, AT1R or GPR120 (AT1R data are according to the data generated in Supplementary Fig. 4b, c). These data indicate that the potency of the dexamethasone for GPR97 is approximately 20 fold stronger than the AngII towards AT1R, the Emax of dexamethasone induced cAMP inhibition via GPR97 is similar to the AngII induced cAMP inhibition via AT1R, but is approximately 1/3 compared to the TUG891 (full agonist) induced GPR120 activity. Values are the mean $\pm$ SEM from three independent experiments, each performed in

triplicate. **f**, The potency and efficacy for effects of ligand induced Gqo/ G β y dissociation were summarized according to the data generated in Supplementary Fig. 4d, e. The Emax of the GPR97-FL-WT is similar to known Gi coupled dopamine receptor D2R. Values are the mean $\pm$ SEM from three independent experiments, each performed in triplicate. **g**, *Gpr97* knockdown in Y-1 cells abolished the acute inhibitory effects of cortisone on corticosterone. Y-1 cells were transfected with *Gpr97* siRNA (si-97) or control siRNA (si-con) for 48 h. Cells were then treated with cortisone (16 nM), ACTH (500 nM) or combined cortisone and ACTH. The level of corticosterone was measured at 30 min. Values are the mean $\pm$ SEM from three independent experiments, each performed in triplicate. *** P < 0.001; ns, no significance; cortisone stimulation compared with control vehicle. ### P < 0.001; ns, no significance; si-97 compared with si-con. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, P = 0.0865, 0.0016, 0.0502, 0.1888, 0.2126, 0.0043). **h**, Knock down of *Gpr97* in neuronal cell abolished the acute effects of cortisone induced cAMP inhibition. Adrenal cortex Y-1 cells transfected with *Gpr97* siRNA (si-97) or control siRNA (si-Con) were stimulated with cortisone (16 nM) or ACTH (500 nM). The levels of cAMP for each condition were measured at 10 min after cortisone, ACTH or control vehicle stimulation. Values are mean $\pm$ SEM from three independent experiments. * P < 0.05, ns, no significance; Comparison between cells treated with or without cortisone; ### P < 0.001; Cells transfected with or without *Gpr97* siRNA were compared under cortisone stimulation. All data were analysed using by two-sided One-way ANOVA with Turkey test (From left to right, P = 0.0108, 0.0007, 0.0002, 0.5445, 0.2659). **i**, The expression of *Gpr97* was measured using q-PCR. Values are mean $\pm$ SEM from three independent experiments. *** P < 0.001. Cells transfected with or without *Gpr97* siRNA were compared under various stimulation conditions. All data were analysed using by two-sided One-way ANOVA with Turkey test (From left to right, P = < 0.0001, < 0.0001, < 0.0001, < 0.0001).

Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | GPR97-Go complex purification, cryo-EM data processing, and overall resolution analysis of electron density of transmembrane helices, $\alpha 5$ -helix of G α o and the glucocorticoid ligands.

a, Representative elution profile of Flag-purified BCM-GPR97-FL-AA-miniGo complex and SDS-PAGE of the size-exclusion chromatography peak. For gel source data, see Supplementary Fig. 1. Experiments were repeated three times with similar results. **b**, Cryo-EM micrographs of BCM-GPR97-FL-AA-Go complex (scale bar: 30 nm) and 2D class averages (scale bar: 5 nm). Electron density of GPR97 N-terminal fragment (NTF) were labelled with red line. Representative cryo-EM micrograph from 2707 movies and representative two-dimensional class averages determined using approximately 2 million particles were shown. The 2D averaged electron density for NTF is significant weaker than the transmembrane helix or the G protein. The NTF is known to play important roles in aGPCR activation in many cases and also serves as a mechanosensor for extracellular force, potentially by engaging with the extracellular face of the 7TM bundle. The weak electron density of the NTF suggested high flexibility in this domain, which may be consistent with the highly dynamic nature of the mechanical stimuli, because the electrophoresis and cryo-EM experiments confirmed the integrity of the full-length receptor in our cryo-EM samples. Thus, the future determination of a high-resolution structure of full-length GPR97 by cryo-EM is important but may require additional stabilization of the

receptor by additional ligands or antibodies. **c**, Flow chart of cryo-EM data processing of BCM-GPR97-FL-AA-Go complex. It is also worth noting that the 7TM structure solved by our current study closely reflects its conformational state in the context of the presence of the NTF, despite being in a static mode. **d**, Representative elution profile of Flag-purified cortisol-GPR97-FL-AA-miniGo complex and SDS-PAGE of the size-exclusion chromatography peak. For gel source data, see Supplementary Fig. 1. Experiments were repeated three times with similar results. **e**, Cryo-EM micrographs of cortisol-GPR97-FL-AA-Go complex (scale bar: 30 nm) and 2D class averages (scale bar: 5 nm). Representative cryo-EM micrograph from 5871 movies and representative two-dimensional class averages determined using approximately 4 million particles were shown. Electron density of GPR97 N-terminal fragment (NTF) were labelled with red line. The 2D averaged electron density for NTF is significant weaker than the transmembrane helix or the G protein. **f**, Flow chart of cryo-EM data processing of cortisol-GPR97-FL-AA-Go complex. **g**, 3D density map of BCM and cortisol-bound complex colored according to local resolution ($\text{\AA}$). Fourier shell correlation (FSC) curves of the final reconstruction and the refined model versus the final map. **h**, Cryo-EM density of transmembrane helices of GPR97, $\alpha 5$ -helix of G α o, the ligand of BCM and cortisol in determined BCM-GPR97-Go and cortisol-GPR97-Go cryo-EM structure respectively.

Extended Data Fig. 3 | Detailed characteristics of the GPR97 7TM bundle.
a, Structural comparison of 7TM bundle (upper panel) and ECL2 (lower panel) of Go-bound GPR97 with Gi-bound NTSR1 (6OS9), Gs-bound PTH1R (6NBF), active GABA_B (7C7Q) GB2 subunit and Gi-bound Smoothen receptor (6OT0) (from left to right). **b**, **c**, Plot of C α distances of residues between the TM6 and TM7 (**b**) or between the TM1 and TM7 (**c**) of GPR97 in BCM-GPR97-Go complex structure and structures of NTSR1 (6OS9), PTH1R (PDB 6NBF), GABA_B (7C7Q) and SMO (PDB 6OT0) in complex with their corresponding G proteins. Residues

indicated in the X-axis were chosen based on Wootten numbering system. **d**, TM6 length of different GPCRs, including NTSR1 (6OS9), PTH1R (PDB 6NBF), GABA_B (7C7Q), and SMO (PDB 6OT0). The length of TM6 were measured according to the length between the first helix turn in the cytoplasmic side and the last helix turn at the extracellular side. **e**, Consecutive polar residues in ECL2 and their interactions with the ECL3, TM6, and TM7 in GPR97. **f**, The weak association of ECL2 with ECL3 and TM1 in GPR97.

Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Ligand binding pocket and 7TM bundle of GPR97 and its comparison with other GPCRs. **a, b**, High quality electron density enabled unambiguous assignment of BCM (**a**), cortisol (**b**) and several residues surrounding them in ligand binding pocket. Only one direction of BCM or cortisol could be fit correctly. **c, d**, Diagram of BCM (**c**) and cortisol (**d**) interaction in the ligand binding pocket of GPR97. Different interaction residues in BCM-GPR97-Go and cortisol-GPR97-Go complex were labelled with red and lemon circle respectively. In particular, F345A and I494A exhibited a more significant decrease in the potency of the BCM compared to cortisol, consistent with the observation of the stronger contacts of F345^{3,36} and I494^{6,57} with the chloride or B ring in BCM, respectively. **e, f**, The steroid core of BCM (**e**) and cortisol (**f**) are vertical relative to the plane of the plasma membrane, paralleling to TM7 and approximately 70-degree deviation from the central

TM3. **g**, r.m.s.d. analysis of GPR97-Beclomethasone and GPR97-cortisol 200 ns trajectories. (Upper panel) RMSDs of GPR97 ligands Beclomethasone (purple) or cortisol (pink). (Lower panel) RMSDs of the binding sites of GPR97 towards counterpart ligands. Values were calculated based on the initial complex state after equilibration (0 ns). **h**, Vertical view of BCM binding into GPR97 binding pocket and its comparison with ligands binding in 5-HT_{1B}R (PDB 6G79), NTSR1 (PDB 6OS9) and M2R (PDB 6OIK). **i**, Interaction residues in the ligand binding pocket of the BCM-GPR97-G_o and the cortisol-GPR97-G_o-scFv16 complexes. Residues that interact with both glucocorticoids are indicated by green circles, and those that show no interaction are indicated by blank circles. The different ligand interaction residues in the two binding pockets are indicated by red and lemon circles, respectively. Wootten's residue numbers are provided for reference.

Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | FIAsh insertion site screening and FIAsh-BRET

mutation experiment. **a**, Schematic representation of the FIAsh-BRET assay design. The Nluc was inserted at the N terminus of wild-type GPR97- β , and the FIAsh motif were inserted in the designated positions in the figure. **b**, Detailed description of the FIAsh motif incorporation site at the extracellular loops of GPR97. FIAsh motifs are labelled in red. The specific residues that interact with the ligand are highlighted in yellow. **c**, ELISA experiments to determine the expression levels of the wild-type GPR97 and five FIAsh motif incorporated FIAsh-BRET sensors. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. ns, no significance; Comparison between GPR97-WT-Nluc and its mutant. The original data are referred to Supplementary Table 3. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P = 0.5910, 0.1912, 0.7642, 0.3076, 0.5938$). **d**, The potency changes upon BCM activation by different GPR97 FIAsh-BRET sensors. These sensors were corresponding to those used in **e**. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. The original data are referred to Supplementary Table 3. $*P < 0.05$, $***P < 0.001$; ns, no significance; Comparison between site2 and other sites FIAsh-BRET sensors. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P = < 0.0001, < 0.0001, < 0.0001, < 0.0001$). **e**, Representative dose response curve of the BCM-induced BRET ratio in HEK293 cells overexpressing five FIAsh-BRET sensors using FIAsh-BRET assay. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates. **f**, ELISA experiments to determine the expression levels of the site2 FIAsh-BRET and corresponding mutations for glucocorticoids interaction sites. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. ns, no significance; Comparison between FIAsh-BRET sensor-2 wild type and corresponding interaction sites mutations. The original data are referred to Supplementary Table 3. All data were analysed by two-sided

one-way ANOVA with Turkey test (From left to right, $P = 0.5446, 0.6579, 0.6133, 0.3484, 0.5069, 0.6488, 0.6571$). **g**, The potency changes upon BCM activation by different site2 FIAsh-BRET sensor-2 mutants. These mutants were designed according to those used in **i**. The FIAsh-BRET assay and cAMP assay are two different functional assays to investigate the contribution of interaction residues observed in the cryo-EM structure. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. $**P < 0.01$, $***P < 0.001$; ns, no significance; Comparison between FIAsh-BRET sensor-2 wild type and corresponding mutant. The original data are referred to Supplementary Table 3. All data were analysed by two-sided one-way ANOVA with Turkey test (From top to bottom, $P = < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, 0.6514$). **h**, The potency changes upon cortisol activation by different site2 FIAsh-BRET sensor-2 mutants. These mutants were designed according to those used in **j**. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. $***P < 0.001$; ns, no significance; Comparison between FIAsh-BRET sensor-2 wild type and corresponding mutant. The original data are referred to Supplementary Table 3. All data were analysed by two-sided one-way ANOVA with Turkey test (From top to bottom, $P = < 0.0001, < 0.0001, < 0.0001, < 0.0001, 0.0002, 0.0005, 0.5028$). **i, j**, Representative dose response curves of the BCM- (**i**) or cortisol-induced (**j**) BRET ratio in HEK293 cells overexpressing FIAsh-BRET sensor-2 wild type and corresponding mutant. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates. **k**, Sequence alignment of residues involved in BCM and cortisol binding in GPR97 with other members of aGPCR G subfamily. The residues involved in the ligand binding of GPR97 and their counterparts in other aGPCR G subfamily members are highlight in different background. The same interaction residue in BCM-GPR97-Go and cortisol-GPR97-Go are highlight in green background. Different interaction residues are highlight in yellow or red background respectively.

Extended Data Fig. 6 | See next page for caption.

Extended Data Fig. 6 | Effects of mutations in the different structural motifs in GPR97 on BCM-induced cAMP inhibition. **a**, ELISA experiments to determine the expression levels of the GPR97 wild type and indicated mutants in HEK293 cells. The mutant receptor expression was normalized to comparable levels as wild-type receptor expression in our ELISA assay by adjusting the amount of plasmid transfected. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. ns, no significance; Comparison between GPR97-FL-WT and its mutant. The original data are referred to Supplementary Table 4. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P=0.1072, 0.7137, 0.1804, 0.0661, 0.8105, 0.7227, 0.1521, 0.2276, 0.1118, 0.0907, 0.2280, 0.2112, 0.3975, 0.1672, 0.7230, 0.7904, 0.1302, 0.0646, 0.1433, 0.0848, 0.6376, 0.9843$). **b**, The potency changes upon BCM activation by different GPR97 mutants. These mutants were corresponding to those used in Figs. 2e, 3d and Extended Data Fig. 8c. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. *** $P < 0.001$; ns, no significance; Comparison between GPR97-FL-WT and its mutant. The original data are referred to Supplementary Table 4. All data were analysed by two-sided one-way ANOVA with Turkey test. (From left to right, $P=0.8274, 0.0009, 0.0006, <0.0001, <0.0001, <0.0001,$

$<0.0001, 0.1967, <0.0001, <0.0001, 0.0002, <0.0001, <0.0001, 0.8059, 0.0177, <0.0001, 0.0010, <0.0001, <0.0001, 0.2317, <0.0001, <0.0001$). **c**, Representative dose response curve of the BCM-induced cAMP inhibition in HEK293 cells overexpressing wild type or mutant GPR97 using Glosensor assay. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates. Mutation of either V466^{ECL3} or K471^{ECL3} reduced the potency of BCM stimulation of GPR97, indicating an essential role of ICL3-mediated GPR97-Go coupling. **d**, The potency changes upon cortisol activation by different GPR97 mutants. These mutants were corresponding to those used in Fig. 2f. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. *** $P < 0.001$; Comparison between GPR97-FL-WT and its mutant. The original data are referred to Supplementary Table 4. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P=0.0036, 0.0271, 0.0038, 0.0455, 0.0007, 0.5604, 0.0104, 0.0012, 0.0002, 0.0004, 0.0014, 0.0009$). **e**, Representative dose response curve of the cortisol-induced cAMP inhibition in HEK293 cells overexpressing wild type or mutant GPR97 using Glosensor assay. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates.

Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | The structural motifs in the active state of GPR97.

a, The toggle switch W327^{6.48a} in 5-HT_{1B}R (6G79) directly interacts with ligand EP5. **b**, The toggle switch W400^{6.48a} in M2R (6OIK) interacts with ligand IXO. **c**, The schematic representation of quaternary motif in GPR97 tethering TM3, TM5, and TM6. **d**, The sequence alignment of adhesion GPCR ADGRG subfamily members, 5-HT_{1B}R, and M2R was created using GPCRdb (<http://www.gpcrdb.org>) and Jalview software. Coils represent α -helices. The conserved residues (quaternary core) tethering TM3, TM5 and TM6 in GPR97 are highlighted in green. The toggle switch W, TFV (PIF), and HLY (DRY) motifs are indicated by purple, red and blue dots, respectively. **e**, ELISA experiments to determine the expression levels of the GPR126 wild type and indicated mutants in HEK293 cells. The mutant receptor expression was normalized to comparable levels as wild-type receptor expression in our ELISA assay by adjusting the amount of plasmid transfected. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. ns, no significance; Comparison between GPR126 and its mutant. The original data are referred to Supplementary Table 5. All data were analysed by two-sided one-way ANOVA with Turkey test ($P = 0.2558, 0.5436, 0.3412$). **f**, Representative dose response curves of S13L (a modified stachel peptide of GPR64 functions as an agonist)-induced cAMP accumulation in HEK293 cells overexpressing GPR126 wild type

or mutant using Glosensor assay. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates. **g**, Comparison of TM3 and TM6 in Go-coupled GPR97, Go-coupled 5-HT_{1B}R, and inactive 5-HT_{1B}R. The Go-coupled GPR97 is in green, the inactive 5-HT_{1B}R is in grey (PDB 5V54), and Go-coupled 5-HT_{1B}R is in yellow (PDB 6G79). The toggle switch of Go-bound GPR97 is located at a position similar to that of Go-bound 5-HT_{1B}R. The separation of TM3 and TM6 in GPR97 was smaller than that in 5-HT_{1B}R-Go but larger than that in the inactive 5-HT_{1B}R structure. **h**, Plot of C α distances of residues between the TM3 and the TM7 of GPR97 in BCM-GPR97-Go complex structure and the active structures of the M2R (6OIK) or 5-HT_{1B}R (6G79) with Go, or inactive 5-HT_{1B}R (5V54) structure. Residues indicated in the X-axis were chosen based on Wootten numbering system. **i**, Interaction of H362 in HLY motif of GPR97 with the palmitoylated C351 α 5-helix of Go. **j**, Interaction of R147 in DRY motif of 5-HT_{1B}R (PDB 6G79) with the α 5-helix of Go. **k**, Effects of the mutation of L363R in HLY motif of GPR97 on BCM induced cAMP inhibitory activity (mutating the key HLY motif residue in a GPCR family corresponding to DRY motif residue in other GPCR family). The original data are referred to Supplementary Table 4. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. *** $P < 0.001$; All data were analysed by two-sided one-way ANOVA with Turkey test ($P = < 0.0001$).

Extended Data Fig. 8 | Molecular mechanisms of Go coupling to GPR97.

a, Close-up view of the interactions between the C-terminal $\alpha 5$ helices of Go and GPR97 (light sea green), 5-HT_{1B}R (wheat), and M2R (salmon). Hydrogen bonds were highlighted by dashed lines. **b**, Close up view of interactions between C-terminal $\alpha 5$ helix of Go and GPR97. **c**, Effects of mutations in ICLs of GPR97 on BCM-induced cAMP inhibition. Values are the mean $\pm$ SEM from three independent experiments performed in triplicate. * $P < 0.05$; *** $P < 0.001$; ns, no significance; Comparison between GPR97-FL-WT and its mutant. All data were analysed by two-sided one-way ANOVA with Tukey's test (From left to right, $P = < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001$). **d, e**, Effects of mutations of ICL1, ICL2 or ICL3 on the basal activities of GPR97. Inhibitory effects of forskolin (1 μ M)-induced cAMP accumulation on the constitutive activities of GPR97 wild-type and its ICL region mutants were tested in HEK293 cells. All data are presented as the percentage of activities of each GPR97 mutant vs. that of wild-type GPR97 at different relative receptor expression level. Overexpression of wild-type GPR97 led to an elevated inhibitory effect on forskolin-induced cAMP accumulation, whereas mutations in either ICL1 or ICL3 significantly decreased this effect. The mutant receptor expression was normalized to comparable levels as wild-type receptor expression in our ELISA

assay by adjusting the amount of plasmid transfected (see f). Data represent the mean $\pm$ s.d. from three independent experiments performed in triplicate. **d**, *** $P < 0.001$, ### $P < 0.001$, \$\$\$ $P < 0.001$, GPR97 mutants ICL1-R299A, ICL1-E298A or ICL1-R297S versus GPR97-FL-WT; **e**, *** $P < 0.001$, ### $P < 0.001$, ns, no significance. ICL2/V370A or ICL3-L460A versus GPR97-FL-WT. All data were analysed by two-sided one-way ANOVA with Tukey's test. ($P = 0.6262, < 0.0001, < 0.0001, < 0.0001, < 0.0001$ from left to right for R297S curve; $P = 0.8416, < 0.0001, < 0.0001, < 0.0001, < 0.0001$ from left to right for E298A curve; $P = 0.9125, < 0.0001, < 0.0001, < 0.0001, < 0.0001$ from left to right for R299A curve; $P = > 0.9999, 0.2254, 0.0014, 0.1836, 0.3143$ from left to right for V370A curve; $P = 0.6291, 0.0003, 0.0002, 0.0001, 0.3372$ from left to right for L460A curve). **f**, ELISA experiments to determine the expression levels of GPR97 wild type and indicated mutants in HEK293 cells. Values are mean $\pm$ SEM from three independent experiments performed in triplicates. ns, no significance; Comparison between GPR97-FL-WT and its mutant. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P = 0.9496, 0.2058, 0.2316, 0.7907, 0.2100, 0.9558, 0.4538, 0.1063, 0.3675, 0.5311, 0.9426, 0.6767, 0.1491, 0.3360, 0.5109, 0.9700, 0.6505, 0.7398, 0.7890, 0.1970, 0.9809, 0.4458, 0.1032, 0.2440, 0.2231$).

Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Palmitoylation of Go mediates GPR97 coupling.

a, MS-MS spectrum identified that the S-palmitoylation occurred at C351 in the $\alpha 5$ -helix of G α o in the cortisol-GPR97-Go complex. **b**, Table of mass to charge ratio of different ion type in mass spectrometry. The increase m/z from the b_{14}^{+} to b_{15}^{+} is 341.24, which larger than the cysteine molecular weight and close to the palmitoylation of the C351. **c**, Cross-sectional view showing that the palmitoylation of C351 is inserted approximately 17Å deep into the 7TM bundle (PDB 7D77). **d**, Structure comparison of GPR97-Go complex (PDB 7D77) with Go-bound M2R (PDB 6OIK), Go bound 5HT_{1B}R (PDB 6G79), Gi-bound Mu-Opioid (PDB 6DDF), Gi bound CB2 (PDB 6PT0). A presumed palmitoylation at the C351 of Go or Gi will clash with these GPCRs in the complex structures. **e**, The potency and efficacy for effects of ligand induced Gqo/G $\beta\gamma$ dissociation was summarized according to the data generated in **f–h**. Values are the mean $\pm$ SEM

from three independent experiments, each performed in triplicate. **f**, Effects of mutations in C351 in $\alpha 5$ -helix of G α o on GPR97 or D2R activities. The C351S and C351A mutations impaired the activation of GPR97 but not D2R. The original dose response data were shown in **g** and **h**. Bar graph represents the mean $\pm$ SEM value of E_{max} from three independent experiments performed in triplicates. *** $P < 0.001$; Comparison between G α o C351 mutations and G α o-WT in activating GPR97; ns, no significance; Comparison between G α o C351 mutations and G α o-WT in activating D2R. All data were analysed by two-sided one-way ANOVA with Turkey test (From left to right, $P = 0.0053, 0.0006, 0.0795, 0.1868$). **g**, **h**, Representative dose–response curves for BCM (**g**) or dopamine (**h**) induced G α o/G $\beta\gamma$ dissociation from their corresponding receptors using wild type or C351 mutations of G α o. Values are mean $\pm$ s.d. from three independent experiments performed in triplicates.

Extended Data Table 1 | Cryo-EM data collection, model refinement and validation statistics

	BCM-GPR97-Go	Cortisol-GPR97-Go
Data collection and processing		
Magnification	4,9310	4,9310
Voltage (kV)	300	300
Electron exposure (e ⁻ /Å ²)	62	62
Defocus range (μm)	-0.5 ~ -2.5	-0.5 ~ -2.5
Pixel size (Å)	1.014	1.014
Symmetry imposed	C1	C1
Initial particle projections (no.)	2,026,926	4,323,518
Final particle projections (no.)	166,116	75,814
Map resolution (Å)	3.1	2.9
FSC threshold	0.143	0.143
Map resolution range (Å)	2.5-5.0	2.5-5.0
Refinement		
Initial model used	6G79	6OS9
Model resolution (Å)	3.2	3.0
FSC threshold	0.5	0.5
Map sharpening <i>B</i> factor (Å ²)	-96.98	-83.49
Model composition		
Non-hydrogen atoms	7015	8785
Protein residues	874	1105
Ligand	1	1
Lipids	6	6
<i>B</i> factors (Å²)		
Protein	74.39	82.24
Ligand	85.79	101.29
Lipids	71.44	87.66
R.m.s. deviations		
Bond lengths (Å)	0.009	0.010
Bond angles (°)	0.788	0.796
Validation		
MolProbity score	1.77	1.69
Clashscore	8.10	8.28
Rotamer outliers (%)	0.00	0.21
Ramachandran plot		
Favored (%)	95.25	96.33
Allowed (%)	4.75	3.67
Disallowed (%)	0	0

Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided
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☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
<i>Give P values as exact values whenever suitable.</i>
☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection: Cyro-EM data were automatically collected using serialEM 3.7 on a Titan Krios.

Data analysis: The following softwares were used in this study: MotionCor2.1, gCTF v1.18, RELION 3.0-beta2, UCSF Chimera 1.13, UCSF ChimeraX 1.0, Phenix 1.16, Coot 0.8.9, Graphpad Prism 8.0, Pymol 1.7.0.0, NAMD 2.13, VMD 1.9.3, Bsoft package 2.0.3, Rosetta 2018.33.

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Data

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The density maps and structure coordinates have been deposited to the Electron Microscopy Database (EMDB) and the Protein Data Bank (PDB) with accession number of EMD-30602, PDB ID 7D76 for the BCM-GPR97-Go complex. The cryo-EM density map and coordinates for the cortisol-GPR97-Go complex have been deposited in the EMDB and PDB under accession codes EMD-30603 and 7D77. The Orientations of Proteins in Membranes (OPM) database is accessible at <http://opm.phar.umich.edu>. All other data are available upon request to the corresponding authors.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For structural determination, 2707 movies of BCM-GPR97-Go complex and 5871 movies of cortisol-GPR97-Go complex were collected using an Titan Krios equipped with a Gatan K2 Summit direct electron detector. For cAMP accumulation and Elisa assays, three biologically independent experiments (n=3) were performed as depicted in related Figure legends. Data were analysed by fitting various ligand concentrations and readouts using appropriate equations in GraphPad Prism 8.0.
Data exclusions	No data were systematically excluded from the analysis apart badly picked or low resolution particles from cryo-EM data. This is implemented in RELION 3.0beta.
Replication	Each experiment was reproduced at least three times independently. Experimental findings were reliably reproduced.
Randomization	Randomization was not relevant for this study, as the independent variables to be tested were sufficient for the functional interpretation within this study.
Blinding	Blinding is not necessary or valid for the purposes of structural determination. For cryo-EM study, purified ligand bound-GPR97-Go complex samples were applied onto a glow-discharged holey carbon grid and subsequently vitrified using a Vitrobot Mark IV. Cryo-EM imaging was performed on a Titan Krios equipped with a Gatan K2 Summit direct electron detector. The microscope was operated at 300 kV accelerating voltage, at a nominal magnification of 29,000 $\times$ in counting mode, corresponding to a pixel size of 1.014 Å. In total, 2707 movies of BCM-GPR97-Go complex and 5871 movies of cortisol-GPR97-Go complex were obtained at a dose rate of about 7.8 electrons per Å ² per second with a defocus range of -0.5 to -2.5 μ m. The total exposure time was 8 s and intermediate frames were recorded in 0.2 s intervals, resulting in an accumulated dose of 62 electrons per Å ² and a total of 40 frames per micrograph. For functional analysis, blinding was not necessary due to the quantitative nature of the experiment. All experimental data acquired or analyzed in this study are included in this published article, and subjected to statistical analysis whenever necessary.

Behavioural & social sciences study design

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Study description	Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study).
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Sampling strategy	Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed.
Data collection	Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.
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Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Non-participation	State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation.
Randomization	If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

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Study description	Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.
Research sample	Describe the research sample (e.g. a group of tagged <i>Passer domesticus</i> , all <i>Stenocereus thurberi</i> within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.
Sampling strategy	Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.
Data collection	Describe the data collection procedure, including who recorded the data and how.
Timing and spatial scale	Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Reproducibility	Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.
Randomization	Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.
Blinding	Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.
Did the study involve field work?	☐ Yes ☐ No

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Field conditions	Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).
Location	State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).
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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	For measurement of receptor cell surface expression, monoclonal anti-FLAG primary antibody (Sigma Aldrich, Catalog # F1804) and goat anti-mouse secondary antibody (Thermo Fisher, Catalog # A-21235) were used. The primary antibody was used in 1:1000 dilution, and the secondary antibody in 1:5000 dilution. For determination of the viral titers, the anti-gp64-PE monoclonal antibody (Thermo Fisher, Catalog # 12-6991-82, in 1:200 dilution) was used.
Validation	All antibodies used are commercially purchased and have been validated by the vendors. All antibodies are well characterized and were applied according to data sheet information details. Monoclonal anti-FLAG: https://www.sigmaaldrich.com/catalog/product/sigma/f1804 ; Goat anti-Mouse secondary antibody: https://www.thermofisher.com/cn/zh/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21235 ; gp64-PE antibody: https://www.thermofisher.com/cn/zh/antibody/product/Baculovirus-Envelope-gp64-Antibody-clone-AcV1-Monoclonal/12-6991-82

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	HEK293 cells were purchased from Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). sf9 cells were obtained from Expression Systems (Cat 94-001S), and Y-1 cells were purchased from the American Type Culture Collection (ATCC).
Authentication	All of the cell lines are maintained by the supplier. No additional authentication was performed by the authors.
Mycoplasma contamination	The above cell lines were negative for Mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Palaeontology and Archaeology

Specimen provenance	<i>Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).</i>
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☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.	
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Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Human research participants

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Population characteristics	<i>Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic</i>
----------------------------	---

Population characteristics

information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight

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Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

Study protocol

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Data collection

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Outcomes

Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

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Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

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ChIP-seq

Data deposition

- ☐ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
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Provide a list of all files available in the database submission.

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Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

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- ☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
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Methodology

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Design type

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Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures

State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)	<i>Specify: functional, structural, diffusion, perfusion.</i>
Field strength	<i>Specify in Tesla</i>
Sequence & imaging parameters	<i>Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.</i>
Area of acquisition	<i>State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.</i>
Diffusion MRI	☐ Used ☐ Not used

Preprocessing

Preprocessing software	<i>Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).</i>
Normalization	<i>If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.</i>
Normalization template	<i>Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.</i>
Noise and artifact removal	<i>Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).</i>
Volume censoring	<i>Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.</i>

Statistical modeling & inference

Model type and settings	<i>Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).</i>
Effect(s) tested	<i>Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.</i>
Specify type of analysis:	☐ Whole brain ☐ ROI-based ☐ Both
Statistic type for inference (See Eklund et al. 2016)	<i>Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.</i>
Correction	<i>Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).</i>

Models & analysis

n/a	Involved in the study
☐	☐ Functional and/or effective connectivity
☐	☐ Graph analysis
☐	☐ Multivariate modeling or predictive analysis
Functional and/or effective connectivity	<i>Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).</i>
Graph analysis	<i>Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).</i>
Multivariate modeling and predictive analysis	<i>Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.</i>