PtdIns(4,5)P₂ stabilizes active states of GPCRs and enhances selectivity of G–protein coupling

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G-protein-coupled receptors (GPCRs) are involved in many physiological processes and are therefore key drug targets. Although detailed structural information is available for GPCRs, the effects of lipids on the receptors, and on downstream coupling of GPCRs to G proteins are largely unknown. Here we use native mass spectrometry to identify endogenous lipids bound to three class A GPCRs. We observed preferential binding of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) over related lipids and confirm that the intracellular surface of the receptors contain hotspots for PtdIns(4,5)P₂ binding. Endogenous lipids were also observed bound directly to the trimeric Gα₃βγ protein complex of the adenosine A₂A receptor (A₂A) in the gas phase. Using engineered Gα subunits (mini-Gαs, mini-Gαi, and mini-Gα12), we demonstrate that the complex of mini-Gαs, with the β₁ adrenergic receptor (β₁AR) is stabilized by the binding of two PtdIns(4,5)P₂ molecules. By contrast, PtdIns(4,5)P₂ does not stabilize coupling between β₁AR and other Gα subunits (mini-Gαi or mini-Gα12) or a high-affinity nanobody. Other endogenous lipids that bind to these receptors have no effect on coupling, highlighting the specificity of PtdIns(4,5)P₂. Calculations of potential of mean force and increased GTP turnover by the activated neortensin receptor when coupled to trimeric Gα₃βγ complex in the presence of PtdIns(4,5)P₂ provide further evidence for a specific effect of PtdIns(4,5)P₂ on coupling. We identify key residues on cognate Gα subunits through which PtdIns(4,5)P₂ forms bridging interactions with basic residues on class A GPCRs. These modulating effects of lipids on receptors suggest consequences for understanding function, G–protein selectivity and drug targeting of class A GPCRs.

The emerging view from biophysical studies of GPCRs is that they exist as ensembles of discrete conformations that can be influenced by ligands, regulatory proteins, pH, ions and, potentially, lipid molecules. The complex roles of these conformational ensembles in signalling pathways are further compounded by the combinatorial effects of the multiple distinct heterotrimeric complexes formed from 21 Gα, 6 Gβ3 and 12 Gγ subunits. Investigating the relationship between GPCRs, small molecule modulators and numerous binding partners is therefore challenging, owing to the difficulty of observing the complexity of these interactions directly. A previous study characterized interactions of lipids with the β₁ adrenergic receptor (β₁AR) in high-density lipoparticles to which phospholipids were added exogenously, but did not address the selectivity and effects of different phosphatidylinositol (PI) phosphate lipids on coupling with downstream effectors. In this study, we develop and apply high-resolution native mass spectrometry to interrogate endogenous lipid–receptor interactions of three class A GPCRs: the β₁ adrenergic receptor (β₁AR), the adenosine A₂A receptor (A₂A), and neurotensin receptor 1 (NTSR1). We reveal effects of PtdIns(4,5)P₂ that stabilize these receptors in active states, increase GTPase activity and enhance selectivity of coupling to G proteins.

First, we considered the endogenous lipids that bind directly to β₁AR and the stabilized NTSR1 (HTGH4–ΔIC3BY¹), which were expressed in and purified from insect cells and Escherichia coli, respectively. Peaks corresponding to lipid adducts were observed for β₁AR and for NTSR1 (Fig. 1a and Extended Data Fig. 1a). Collisional dissociation of protein–lipid complexes allowed us to identify two major classes of lipids bound to β₁AR, the phosphatidylycerines (PS) (34:2 and 36:2) and PI phosphates (42:5), as well as phosphatidic acid (PA) (36:2), which bound to NTSR1 (Extended Data Fig. 1b, c and Extended Data Table 1). To investigate this selectivity, we incubated NTSR1 with PA and other anionic lipids (PS and PI), a zwiterionic lipid (phosphatidylcholine (PG)), and a neutral lipid (diacylglycerol (DAG)). Analysis of the resulting native mass spectra show that NTSR1 interacts preferentially with PA, PS and PI (Extended Data Fig. 2a–e). We did not observe apparent binding of phosphatidylglycerol (PG) to NTSR1, although PG has been reported to increase G-protein activation of NTSR1 in a nanodisc. It is possible that PG affects the local net charge at the receptor–lipid interface. Similarly, β₁AR, when incubated with detergent-solubilised PS (16:0–18:1) or phosphatidylinositol-4 phosphate (PtdIns(4)P) (18:1–18:1), showed higher affinity towards PtdIns(4)P than to PS (Fig. 1a and Extended Data Fig. 2f, g).

To probe the selectivity of different PI derivatives we incubated β₁AR with equimolar ratios of PI, PtdIns(4)P, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃), all containing the same acyl chains (18:1–18:1). Plotting intensity of peaks corresponding to lipid-bound states in the mass spectrum, relative to those of the apo protein, showed that PtdIns(4,5)P₂ had a higher affinity than PtdIns(4)P for β₁AR (Fig. 1b). In the case of PtdIns(3,4,5)P₃, which contains one more phosphate group than PtdIns(4,5)P₂, binding to β₁AR was reduced to a similar level as observed for PI. This demonstrates that binding is selective for the head group of PtdIns(4,5)P₂. We performed similar experiments for NTSR1 and A₂A, and in both cases (PtdIns(4,5)P₂) was found to bind with the highest affinity (Extended Data Fig. 3), implying that all three class A GPCRs contain preferential binding sites for PtdIns(4,5)P₂.

We performed coarse-grained molecular dynamics (CGMD) simulations (Extended Data Fig. 4) to characterize the molecular nature of GPCR–PtdIns(4,5)P₂ interactions in a phospholipid bilayer environment. PtdIns(4,5)P₂ molecules bound at the interface formed by the cytoplasmic loops linking tranmsembrane helix (TM1), TM2, TM4 and TM7 of NTSR1; this binding was mediated via interactions between the phosphorylated inositol head group and basic protein side chains (Fig. 1c and Extended Data Fig. 4a). Simulation of NTSR1–PI interactions indicated that these were lower-intensity, diffuse interactions that did not compete with PtdIns(4,5)P₂ (Extended Data Fig. 4c). Similar interactions were seen with β₁AR, which also exhibited the capacity to interact with PtdIns(4,5)P₂ via the positively charged intracellular surfaces of TM5, TM6 and TM7 (Extended Data Fig. 4b). A more extensive comparison of simulations for nine class A GPCRs (Extended Data
Identification of endogenous lipids, preferential binding of PI(4,5)P2, molecular dynamics simulation and site-directed mutagenesis define intracellular PtdIns(4,5)P2-binding hotspots.

Fig. 1 | Identification of endogenous lipids, preferential binding of PI(4,5)P2, molecular dynamics simulation and site-directed mutagenesis define intracellular PtdIns(4,5)P2-binding hotspots. a, Mass spectrum of β1AR (agonist free, green; charge state is shown) and β1AR adducts (red, orange). Peaks (highlighted yellow) are selected in the quadrupole and analysed by tandem mass spectrometry. Phosphatidylserine (PS) and PtdIns(4)P (PIP) were identified in the resulting mass spectra. Binding curves plotted against lipid concentration confirm preferential binding of PtdIns(4,5)P2 over PS. PIP was identified as forming contacts with PtdIns(4,5)P2 (Fig. 1d) to residues that are mutated in NTSR1(TM86V-IC3B) embedded in a lipid bilayer containing mixed PC and PtdIns(4,5)P2. Green spheres represent basic residues with high levels of interaction with lipids; purple surfaces represent regions with high density of occupation by PtdIns(4,5)P2.

To locate preferential binding sites for PtdIns(4,5)P2, we performed site-directed mutagenesis on NTSR1, mutating residues that we identified as forming contacts with PtdIns(4,5)P2 (Fig. 1d) to residues that retain the expression and folding state of the receptor. We developed a mass-spectrometry-based strategy to analyse the effect of these mutations on PtdIns(4,5)P2 binding (Extended Data Fig. 5a). Mutating selected Lys or Arg residues to residues of lower mass decreased the binding affinity of β1AR (Extended Data Fig. 5a). Mass spectra confirm preferential binding of PtdIns(4,5)P2. 

On the basis of the location of these sites on the cytoplasmic surface, we hypothesized that PtdIns(4,5)P2 binding influences downstream G-protein coupling. To investigate this, we developed a mass-spectrometry-based assay in which the pentameric complex of A2AR (A2AR-mini-Gαsβγ-Nb35; Nb35 is a stabilizing nanobody) was preserved in vacuum. The heteropentamer separated into several subcomplexes following collision-induced dissociation, and PS and PI were observed to be directly bound to A2AR at higher abundance than they were before G-protein coupling (Fig. 2a). We reasoned that in receptor–Gαiβγ complexes, these lipids may have a stabilizing role, thereby, in turn, increasing signalling. To investigate these effects, we measured the GTPase activity of Gαiβγ when coupled to active NTSR1 (bound to neurotensin, Fig. 2b). We found that GTP hydrolysis was enhanced to 1.3-fold in the presence of PtdIns(4,5)P2. Therefore, PtdIns(4,5)P2 enhances both G-protein coupling and GTPase activity.

Because of the instability of the trimeric G-protein complex, it is not possible to explore the effects of lipids on coupling in an unbiased way. We therefore investigated receptor complexes formed with engineered mini-G subunits that recapitulate the increase in agonist affinity observed upon coupling with the native heterotrimeric G protein (Fig. 2c). We recorded mass spectra of thermolysin-stabilized β1AR in complex with mini-Gs. We found increased association of lipids when
β1AR was in a complex with mini-Gi (Fig. 2d). The stability of the receptor–mini-G complex allowed us to investigate the selectivity towards different subtypes of Gα subunits (Gαs, Gαi/o and Gα12/13). We investigated the coupling of agonist-bound β1AR to mini-Gi(ο) which was engineered from mini-Gi by introducing nine mutations on the β helix to the corresponding residues on Gαi. We performed a similar experiment with the analogous mutant of Gαi2 in which we transferred the mutations from mini-Gi to Gαi2. In comparison to mini-Gi, there was a reduced degree of coupling with mini-Gi and virtually no coupling with mini-Gi(ο) (Fig. 2d).

To investigate the effect of PtdIns(4,5)P2 on GPCR–mini-Gα interactions, we incubated agonist-bound β1AR with mini-Gi, in the presence of lipid and compared the mass spectrometry peaks corresponding to the lipid-bound protein. Although the complex can form in the absence of lipids, or with only one bound PtdIns(4,5)P2 complex formation is markedly enhanced (2.7- or 4.5-fold compared to the receptor without lipid, respectively) in the presence of two or three PtdIns(4,5)P2 molecules (Fig. 3a, g). We observed a similar effect in a time-course experiment in which coupling of mini-Gi to β1AR increased by 21 ± 6% when two PtdIns(4,5)P2 molecules were bound and by a further 12 ± 5% when three PtdIns(4,5)P2 molecules were bound (Extended Data Fig. 6a).

We examined the effect of PS, an anionic lipid that was endogenously bound to β1AR (Fig. 1a), on coupling of mini-Gi. We performed analogous experiments using a threefold higher concentration of PS than that used in the experiments with PtdIns(4,5)P2 to reflect the reduced affinity of β1AR for PS (Fig. 3b and Extended Data Fig. 2). Mass spectra showed only a slight increase in the extent of mini-Gi coupling as a function of PS binding. This reduced effect in comparison to PtdIns(4,5)P2 suggests that the electrostatic interactions of the polyanionic lipid headgroups in PtdIns(4,5)P2, which have multiple basic sidechains, are necessary for receptor coupling (as observed for KIR channels, for example), and that such interactions do not occur with PS.

These data indicate that additional PtdIns(4,5)P2, but not PS, stabilize the complex once receptor coupling has occurred. Therefore, we used potential of mean force (PMF) calculations to explore the effect of PtdIns(4,5)P2 binding on the free-energy landscape of A2AR–mini-Gα interactions. Comparison of PMFs for PtdIns(4,5)P2-bound versus PS-bound receptor in a lipid bilayer indicates that the interaction of mini-Gi with A2AR is stabilized significantly (50 ± 10 kJ mol⁻¹) in the presence of PtdIns(4,5)P2 compared with PS (Fig. 3c and Extended Data Fig. 6b). The presence of PtdIns(4,5)P2 at the interface between the receptor and mini-Gi in the PMF calculation implies that PtdIns(4,5)P2 molecules form bridging interactions to stabilize the complex.

The increase in PtdIns(4,5)P2 binding to β1AR when it is coupled to mini-Gi could be a result of either (i) active conformations of receptors binding more PtdIns(4,5)P2 than their inactive counterparts, or (ii) positively charged residues in mini-Gi, at the receptor–G protein interface, recruiting additional PtdIns(4,5)P2 molecules following coupling. To investigate the dependence of PtdIns(4,5)P2 binding on receptor conformation, we incubated PtdIns(4,5)P2 with β1AR (co-purified with the agonist isoproterenol) containing an E130W mutation to stabilize ligand-free β1AR without affecting G-protein coupling. We observed a 31 ± 1% increase in PtdIns(4,5)P2 binding to the β1AR–isoproterenol complex versus ligand-free β1AR (Extended Data Fig. 6c). Whereas in general, transition to active states is thought to involve substantial movements of TM5 and TM6, intracellular loop (ICL)2 was also found to undergo significant changes during activation of the μ-opioid receptor. These results are consistent with PtdIns(4,5)P2 stabilizing active states of receptors via binding hotspots directly on ICL2, and, more generally, via diffuse intracellular PtdIns(4,5)P2-binding sites.

To explore the second possibility, in which additional PtdIns(4,5)P2-binding sites form following coupling, we carried out CGMD simulations for A2AR–mini-Gi, which is, to our knowledge, the only available structure of a receptor–mini-G complex. In addition to the contacts described above, PtdIns(4,5)P2 interacted with residues of mini-Gi proximal to the lipid contacts in TM3, TM4 and TM5 of A2AR (Fig. 3e). To investigate the significance of these additional binding sites we used a nanobody (Nb6B9), in which the lipid-binding residues identified in mini-Gi are absent (Extended Data Fig. 7). Structures of receptors bound to Nb6B9 or to mini-Gi are virtually identical.

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The effect of PtdIns(4,5)P₂ on coupling to mini-Gₛ, and comparison with PS, Nb6B9 and mini-Gₛ. a, Representative mass spectra of β₁AR and β₁AR-mini-Gₛ (n = 3 independent experiments) in the presence of PtdIns(4,5)P₂ and the agonist isoprenaline. Coloured peaks highlight β₁AR lipid-bound states (top) and β₁AR-mini-Gₛ lipid-bound states (bottom). b, Representative mass spectra of β₁AR and β₁AR-mini-Gₛ (n = 3 independent experiments) in the presence of PS and the agonist isoprenaline. There is no marked difference in PS binding between β₁AR and β₁AR-mini-Gₛ. c, Snapshots of steered molecular dynamics simulations to separate mini-Gₛ and A₂A R in the presence of PtdIns(4,5)P₂ (green) and PS (pink). Orange outlines highlight the different binding modes of PtdIns(4,5)P₂ and PS to the receptor. The interaction of mini-Gₛ with A₂aR is stabilized by ~50 kJ mol⁻¹ in the presence of PtdIns(4,5)P₂ relative to PS (Extended Data Fig. 6b). d, Representative mass spectra following incubation of β₁AR with PtdIns(4,5)P₂ and isoprenaline in the absence or presence of Nb6B9 (Nb6B9: receptor, 0.3; n = 3 independent experiments). e, PtdIns(4,5)P₂ contacts on A₂AR-mini-Gₛ are shown on the receptor (purple) and mini-Gₛ (Thr40, His41, Arg42, Lys216 and Arg380; green), and juxtaposed to basic residues on the β₁AR–Nb80 complex (Nb80, purple). f, Representative mass spectra following incubation of β₁AR with PtdIns(4,5)P₂ and isoprenaline in the absence or presence of mini-Gₛ (n = 3 independent experiments). No difference was detected between peaks in the presence or absence of PtdIns(4,5)P₂. g, Normalized intensity of different lipid-bound states of the apo state of isolated receptor or receptor complexes. *P < 0.05; one-way ANOVA with Dunnett’s multiple comparison test. Bars show mean ± s.d., points show data from three independent experiments.

As the local concentration of PtdIns(4,5)P₂ in the membrane has the potential to be modulated by different signalling pathways, such as receptor tyrosine kinases or Ca²⁺ signalling, crosstalk with GPCRs through PtdIns(4,5)P₂ may represent an additional mode of regulation.
in the cell. Further, the potential to stabilize the active conformation of G-protein-coupled receptors through the binding of potent small molecules that mimic the bridging effects of the PtdIns(4,5)_{2} head group provides a further avenue for stabilizing active states of GPCRs for therapeutic purposes. As PtdIns(4,5)_{2} is able to discriminate between different G-protein subunits, and is likely to also influence binding to β-arrestin, there are potential benefits in developing novel compounds that bind specifically to different G-protein-coupled or β-arrestin-bound states, thereby providing a new perspective for rational design of novel biased allosteric agonists.

**Online content**

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0325-6.
METHODS

Constructs and proteins. We used expression plasmids for two stabilized variants of rat NTSR1.2,3 NTSR1-HTGH4-ΔIC3B contains the protein sequence from amino acids 50 to 390 with deletion of ICL3 (residues 273–290) and 26 thermostabilizing point mutations. It should be noted that this construct is only 80% identical to the wild-type. NTSR1-HTGH4-ΔIC3B contains the intact protein sequence from residues 43 to 421, with the same stabilizing mutations as NTSR1-HTGH4-ΔIC3B. Purified thermostabilized turkey (Meleagris gallopavo) β3AR, human wild-type α2Aβ, engineered G90 (mini-G) and nanobody Nb6B9 were used for mass spectrometry analysis.4,5,26,27 The following point mutations on β3AR were used throughout: R68S, M90V, F327A, F338M (thermostabilizing); C116L (to stabilize α2Aβ). Purified thermostabilized turkey (Meleagris gallopavo) β3AR, human wild-type α2Aβ, engineered G90 (mini-G) and nanobody Nb6B9 were used for mass spectrometry analysis.4,5,26,27

Protein expression and purification. The human α2Aβ construct (residues 1–308) was expressed in E. coli, Thioredoxin fused to the N-terminus of TM1 and the mutations C116L to improve expression and C358A to prevent potential palmitoylation. Both constructs were expressed in S9 insect cells using recombinant baculoviruses prepared using the transfer vector pAcGP67B (BD Biosciences) and BacPAK6 linearized baculovirus DNA (Oxford Expression Technologies). The membrane containing the expressed receptor was solubilized and purified in 2% and 0.02% dodecylmaltoside (DDM, Generon), respectively, as described previously.28–29 For (316), the final purification step was competitive elution from an alprenolol sepharose ligand-affinity column in 20mM Tris-HCl, pH 7.4, 350 mM NaCl and 0.02% DDM supplemented with 1mM isoproterenol, so that the receptor was prepared with bound agonist ligand. The purified receptor was finally concentrated to 15 mg/ml in the alprenolol sepharose elution buffer. (311) used the purification buffer and elution buffer of (313) with addition of 0.02% DDM. The protein was further purified by a Superdex 200 Increase PC 3.2/300 column (GE Healthcare) and the protein tag was removed by incubation with human rhinovirus 3C protease (produced in house) overnight. Following buffer exchange to storage buffer (20 mM HEPES pH 7, 100 mM NaCl, 10 mM MgCl2, 25 mM imidazole pH 8, 10 μM GDP and 0.1 mM TCEP). The complex with trimeric G protein was concentrated to at least 2 mg/ml for experimental use.

NTSR1 expression. BL21 E. coli cells were transformed with the expression plasmid encoding NTSR1-HTGH4-ΔIC3B and grown overnight at 37 °C in 20 ml 2YT medium supplemented with 1% (w/v) glucose and 100 μg/ml ampicillin. Two flasks, each containing each 1.2 ml, were inoculated with 10 ml pre-culture and grown to an A600 nm of 0.5 with shaking at 37 °C. Culture induction was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were cultivated at 28 °C overnight. Cells were harvested after overnight expression and E. coli cell pellets were resuspended in 10 ml buffer (500 mM NaCl, 10 mM MgCl2, 25 mM HEPES, pH 7.5, 1% (w/v) lysozyme, and 20 ml detergent mixture (0.2% (w/v) cholesteryl hemisuccinate (CHS) and 2% (w/v) dodecylmaltoside (DDM, Generon)), respectively, as described previously.28–29 The mixture was loaded into a PD10 column (GE Healthcare) and was washed with three column volumes of buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl2, 100 mM GDP, 2 mM β-mercaptoethanol, and Complete protease inhibitor (Roche)). The membranes were pelleted by ultracentrifugation at 108,000g for 35 min and solubilized in solubilisation buffer (50 mM HEPES pH 7, 150 mM NaCl, 10 mM MgCl2, 100 mM GDP, 2 mM β-mercaptoethanol, 1% decyl-β-maltopyranoside (DM) (w/v), 10% (v/v) glycerol, and Complete protease inhibitor (Roche)) for 3 h. The supernatant was collected after centrifugation at 108,000g for 35 min and incubated with 1.2 ml TALON beads (GE Healthcare) overnight. The beads were collected and washed with ten column volumes buffer (30 mM HEPES pH 7, 300 mM NaCl, 10 mM MgCl2, 25 mM imidazole pH 8, 10 μM GDP, 2 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) DM) and reverse immobilized metal affinity chromatography (IMAC) by Ni-NTA superflow beads (GE Healthcare). The protein complex was concentrated to at least 2 mg/ml for experimental use.

Preparation of receptor–G-protein complexes. Several receptor–G-protein complexes were prepared for mass spectrometry analysis. α2Aβ-mini-G,β3 was prepared by incubating and co-purifying α2Aβ, containing a TrxA fusion at the N-terminal, with N-acetylcarboxamidoadenosine (NECA). The complex with trimeric G proteins complex contained of mini-G, G3, G12 and nanobody Nb35 with receptor:G proteins:Nb35 at a 1:2.4 molar ratio to stabilize the complex. The complex was further purified by gel-filtration chromatography after overnight incubation. α2Aβ-mini-G was prepared by incubating α2Aβ-co-purified with isoprenaline and purified from the different mini-G proteins (mini-G, mini-G30 and mini-G12) at 1:1.2 molar ratio. The incubation time was varied to capture the equilibrium of complex formation.

Purification of heterotrimetric G protein. Baculovirus encoding the desired subunits (α2Aβ,γγ) was used to express the heterotrimeric G protein in S9 cells as previously described.25 Cells from a 1-1 expression culture were suspended and lysed in lysis buffer (10 mM HEPES pH 7, 20 mM KCl, 10 mM MgCl2, 10 μM GDP, 2 mM β-mercaptoethanol, and Complete protease inhibitor (Roche)). The membranes were pelleted by ultracentrifugation at 108,000g for 35 min and solubilized in solubilisation buffer (50 mM HEPES pH 7, 150 mM NaCl, 10 mM MgCl2, 100 mM GDP, 2 mM β-mercaptoethanol, 1% decyl-β-maltopyranoside (DM) (w/v), 10% (v/v) glycerol, and Complete protease inhibitor (Roche)) for 3 h. The supernatant was collected after centrifugation at 108,000g for 35 min and incubated with 1.2 ml TALON beads (GE Healthcare) overnight. The beads were collected and washed with ten column volumes buffer (30 mM HEPES pH 7, 300 mM NaCl, 10 mM MgCl2, 25 mM imidazole pH 8, 10 μM GDP, 2 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) DM). The protein was further purified by a Superdex 200 Increase PC 3.2/300 column (GE Healthcare) and the protein tag was removed by incubation with human rhinovirus 3C protease (produced in house) overnight. Following buffer exchange to storage buffer (20 mM HEPES pH 7, 100 mM NaCl, 0.1 mM MgCl2, 4 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) DM) and reverse immobilized metal affinity chromatography (IMAC) by Ni-NTA superflow beads (GE Healthcare), G-protein complex was concentrated to at least 2 mg/ml for experimental use.
SEC using a Superdex 200 10/300 GL column (GE Healthcare), which had been pre-equilibrated with 10 mM HEPES pH 8, 150 mM NaCl, and 0.01% (w/v) LMNG. Peak fractions corresponding to NTSR1(HTGH4-ΔIC3B) were pooled (final volume 3–4 ml) and concentrated in an Amicon-4 Ultra-concentrator with a 50-kDa cut-off to a final protein concentration of approximately 50 μM. Purified and concentrated NTSR1-H4 was mixed with 10 mM HEPES pH 8, 150 mM NaCl, 0.01% (w/v) LMNG, and 50% (v/v) glycerol to yield a final glycerol concentration of 25%. Aliquots were frozen in liquid nitrogen and stored at –80 °C for later use.

Preparation of phospholipids and titration experiment. Phospholipids were purchased from Avanti (Avanti Polar Lipids) and prepared as 3 mM stock solutions in 200 mM ammonium acetate buffer pH 7.5 containing the detergent-mixed micelle preparation, containing DDM and foscholine as previously described. Phosphate analysis was performed to determine the concentration of phospholipids in solution. For the titration experiment, 5 μM buffer-exchanged receptors in 200 mM ammonium acetate buffer pH 7.5 containing the detergent mixtures (DDM, LMNG, and foscholine for β2AR and A2A, R) were mixed with lipids at various concentration points followed by equilibration at 4 °C for 5 min, by which time lipid binding had stabilized according to our time course measurements. Following mass spectrometry analysis, UniDec (Universal Deconvolution) software was used to quantify the relative abundance of each lipid-bound state and statistical analysis was performed using GraphPad Prism, assuming a one-site total binding model.

Lipidomics analysis. Co-purified lipids from recombinant GPCRs were extracted by chloroform–methanol (2:1, v/v) and lyophilized and re-dissolved in 60% acetonitrile (ACN). For LC–MS/MS analysis, the extracted lipids were separated on a C18 column (Acclaim PepMap 100, 15 μm, Thermo Scientific) using a Dionex UltiMate 3000 RSLC nano LC System. The buffers and gradient are adapted from a previous protocol. In brief, the lipids were separated using a binary buffer system at 40 °C using a gradient of 32–99% B buffer at a flow rate of 300 nl/min over 30 min. (Buffer A: acetonitrile: H2O (60:40), 10 mM ammonium formate, 0.1% formic acid) and buffer B (propan-2-ol:acetonitrile (90:10), 10 mM ammonium formate, 0.1% formic acid)). The column eluent was delivered via a dynamic nanospray source to a hybrid LTQ Orbitrap mass spectrometer (Thermo Scientific). Typical mass spectrometry conditions were: spray voltage (1.8 kV) and dynamic nanospray source to a hybrid LTQ Orbitrap mass spectrometer (Thermo Scientific). An activation time of 30 ms.

GPase assay. The GPase activity of trimeric Gα16Pl was measured with the GPase-Glo assay (Promega). The assay was performed in white 96-well plates (Corning costar black/clear bottom). POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) was used in a 2:1 molar ratio. The reaction mixture containing trimeric G proteins under the condition described above. Following incubation at room temperature (1 h) following the manufacturer’s protocol to indicate the level of residual GTP. To analyse the impact of PDIns(4,5)P2, we used NTSR1(HTGH4-ΔIC3B) co-purified with recombinant neurotensin, following the method described previously. The receptor was pre-incubated with detergent-solubilised PDIns(4,5)P2 (1:3 molar ratio (receptor:lipid) in the protein buffer (10 mM HEPES pH 8, 150 mM NaCl, 0.01% (w/v) LMNG, containing 100 mM neurotensin for 15 min on ice. The activated receptor was then added to the reaction mixture containing trimeric G proteins under the condition described above.

Native mass spectrometry of GPCRs. Purified GPCRs were buffer exchanged into 200 mM ammonium acetate buffer pH 7.5 containing the mixed micelle preparation optimized for GPCR analysis as described previously. The concentration of DDM, foscholine and CHS required to form a mixed micelle range from 0.006–0.02%, 0–0.002%, and 0.001–0.01%, respectively, and are optimized for each receptor preparation. The samples were immediately introduced into a modified Q-Exactive mass spectrometer (Thermo Scientific). Typical mass spectrometry conditions were: spray voltage (1.8 kV) and dynamic nanospray source to a hybrid LTQ Orbitrap mass spectrometer (Thermo Scientific). An activation time of 30 ms.

α-γ-Mu opioid receptor linkage analysis. The interaction of 1,5 μM κ opioid receptor, 100 nM µ opioid receptor and 0.01% (w/v) LMNG, and 50% (v/v) glycerol to yield a final glycerol concentration of 25%. Aliquots were frozen in liquid nitrogen and stored at –80 °C for later use. A gentle voltage gradient (injection flatapole, inter-flatapole lens, bent flatapole, transferred into the higher-energy collisional dissociation (HCD) cell following a gentle voltage gradient (injection flatapole, inter-flatapole lens, bent flatapole, transferred multiple: 7.9, 6.94, 5.9, 4 V, respectively). An optimized acceleration voltage (100–130 V) was then applied to the HCD cell to remove the detergent micelle from the protein ions. Backing pressure was maintained at ~1.00 × 10−9 mbar and data was analysed using Xcalibur 2.2 SP148. The bound-lipid identification experiments were performed with a modified SynaptoTagger (Waters) equipped with a 2-spray source. The typical instrumental setting was source pressure (4.5–5.0 mbar), capillary voltage (1.2–1.5 kV) and cone voltage (100–200 V). An extraction voltage of 1–5 V was applied and 80–150 V was used as the collision voltage with argon as the collision gas at a pressure of 0.2–0.3 MPa. To strip the detergent from protein ions in the source region, instrument values were optimized to capillary voltage (1.5 kV), cone voltage (200 V) and extraction voltage (3 V). A collision voltage ramp (from 20–100 V) was applied to dissociate protein–lipid complexes after quadrupole selection.

Identification of preferential PDIns(4,5)P2-binding sites on NTSR1. Unmodified NTSR1 and NTSR1 variants were pre-incubated at 1:1 molar ratio to produce a total protein concentration of 12 mM in protein buffer (10 mM HEPES pH 8, 150 mM NaCl, 0.01% (w/v) LMNG and 25% (v/v) glycerol). Detergent solubilised PI(4,5)P2 was then added to the protein mixture at a final molar ratio of 1:2.5:1 lipid:receptor. The reaction mixture was incubated at 4 °C for 5 min and analysed by mass spectrometry after buffer exchanging to 200 mM ammonium acetate buffer containing the mix of detergents of DDM, LMNG and foscholine as described previously.

The ratio of PDIns(4,5)P2 binding to the receptor was calculated by normalizing the intensity of the receptor in PDIns(4,5)P2-bound states to the unbound state using UniDec software. The results were evaluated by comparing the ratio of PDIns(4,5)P2 binding between mutants and the unmodified receptor and plotted as a bar chart using GraphPad Prism.

Modelling and simulation system setup. Simulations were performed using the GROMACS 4.6.3 simulation package. Initial protein coordinates were obtained using PDB ID 4BUO (NTSR1) and PDB ID 2Y03 (β3AR), with missing atoms added using MODELLER. In the case of β2AR, a model was also constructed using pdb2gmx. The N and C termini were treated with neutral charge. Each protein structure was then energy minimized using the steepest descents algorithm implemented in GROMACS, before being converted to a coarse-grained representation using the MARTINI 2.2 force field. The energy minimized coarse-grained structure was centred in a periodic simulation box with dimensions 70 × 70 × 70 Å3. POPC lipids were randomly exchanged to create a mixed-coarse grained lipid molecules 49 . POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) molecules were randomly placed around the protein and the system was solvated and neutralised to a concentration of 0.15 M NaCl. An initial 50 ns of coarse-grained simulation was applied to permit the self-assembly of a POPC lipid bilayer around the GPCR. POPC lipids were randomly exchanged to create a mixed-species bilayer of specified composition (Extended Data Table 2). A cut-off distance of 2.5 nm was applied, with only molecules outside this distance being subject to exchange. The exchange protocol was conducted independently for each repeat simulation, such that different random initial configurations of lipids around the protein were generated for each simulation repeat. A summary of simulations performed is provided in Extended Data Table 2.

Simulation details. The MARTINI force field was used to describe all system components. An ELNEDYN network was applied to the protein using a force constant of 500 kJ/mol/nm2 and a cut off of 1.5 nm. Simulations were performed as an NPT ensemble, with temperature maintained at 310 K using a Berendsen thermostat47 using a coupling constant of τT = 4 ps, and semi-isotropic pressure controlled at 1 bar using a Berendsen barostat48 with a coupling constant of τP = 4 ps and a compressibility of 5 × 10−9 bar−1. Electrostatics were modelled using the reaction field coulomb type, and smoothly shifted between 0 and 1.2 nm. Van der Waals interactions were treated using a shifting function between 0.9 and 1.2 nm. Covalent bonds were constrained to their equilibrium values using the LINCS algorithm49. Equations of motion were integrated using the leap–frog algorithm, with a 20-fs time step. All simulations were run in the presence of conventional MARTINI water, and neutralised to a concentration of 0.15 M NaCl.

Analysis of simulation data was conducted using VMD, PyMOL, tools implemented in GROMACS, and in-house protocols. Protein–lipid contact analysis was performed using a cut-off distance of 0.6 nm, based on radial distribution functions for coarse-grained structure was centred in a periodic simulation box with dimensions 70 × 70 × 70 Å3. POPC lipids were randomly exchanged to create a mixed-coarse grained lipid molecules 49 . POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) molecules were randomly placed around the protein and the system was solvated and neutralised to a concentration of 0.15 M NaCl. An initial 50 ns of coarse-grained simulation was applied to permit the self-assembly of a POPC lipid bilayer around the GPCR. POPC lipids were randomly exchanged to create a mixed-species bilayer of specified composition (Extended Data Table 2). A cut-off distance of 2.5 nm was applied, with only molecules outside this distance being subject to exchange. The exchange protocol was conducted independently for each repeat simulation, such that different random initial configurations of lipids around the protein were generated for each simulation repeat. A summary of simulations performed is provided in Extended Data Table 2.

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we first ran ten coarse-grained molecular dynamics simulations on receptor embedded in an asymmetric complex membrane, each lasting 8 µs (Extended Data Table 2). The r.m.s.d. to the crystal structure of Aβ3–R mini-Gs complex (PDB ID 5G53) was calculated for the protein in these ten simulations, and the protein complex with the lowest r.m.s.d. was saved together with the membrane bilayer. The coarse grained mini-Gs was then docked back to the membrane-embedded receptor based on the Aβ3–R mini-Gs crystal structure to generate the starting configuration of a steered molecular dynamics (SMD) simulation. In the SMD, the mini-Gs was pulled away from the receptor along the z axis (normal to the membrane plane) at a rate of 0.05 nm/ns using a force constant of 1000 kJ/mol/nm² while the receptor was restrained in place using a harmonic force of 1000 kJ/mol/nm². The distance between the centre of mass of the receptor and the mini-Gs was defined as the ID reaction coordinate and the pulling process covered a distance of 3 nm. The initial configurations of the umbrella sampling were extracted from the SMD trajectory spacing 0.05 nm apart along the reaction coordinate. Fifty umbrella sampling windows were generated, and each was subjected to 1-µs molecular dynamics simulation, in which a harmonic constraint of 1000 kJ/mol/nm² was imposed on the distance between the centre of mass of the receptor and the mini-Gs to maintain the separation of the two. The PMF was extracted from the umbrella sampling using the weighted histogram analysis method (WHAM) provided by the GROMACS g_wham tool. A Bayesian bootstrap was used to estimate the statistical error of the energy profile. The PMF of the binding process in the absence of PtdIns(4,5)P₂ was calculated following the same protocol, with the only change made to the lipid composition of the membrane lower leaflet. PtdIns(4,5)P₂ was taken out from the membrane and instead the concentrations of POPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were increased by 2.5% to make up for the vacancy left by the absence of PtdIns(4,5)P₂.

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

**Data availability.** All relevant data are available from corresponding authors on request.
Extended Data Fig. 1 | Identification of lipids bound to NTSR1(HTGH4-ΔIC3B). a, Endogenous lipids bound to NTSR1(HTGH4-ΔIC3B), isolated from E. coli, are identified as PA following m/z selection in the mass spectrometry quadrupole of the NTSR1:lipid 11+ charge state (highlighted yellow) and collisional activation to dissociate PA and its homologues (m/z, 700–760 Da). b, Lipidomics analysis of purified NTSR1 with three technical replicates reveals peaks at low m/z. MS/MS spectra of the precursor ion (M-H-1) at m/z 699.32 highlighted yellow, leads to definitive fragment ions at m/z 281 and 417 consistent with the structure of PA (36:2). c, Analogous lipidomics analysis of purified β1AR from insect cells with three technical replicates. MS/MS spectra of the two [M-H-1] precursor ions (m/z 758.50 and 786.53) identified the lipids as PS (34:2) and PS (36:2) respectively with diagnostic fragments indicated.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Lipid-binding preference of NTSR1 and β\textsubscript{1} AR. a–c, The binding of NTSR1(HTGH4-ΔIC3B), measured by mass spectrometry (n = 3 independent experiments), to the phospholipids PA (a), PS (b), PI (c), PC (d) and DAG (e). The measurements were performed at different lipid concentrations (0 to 160 μM) and the percentages of individual lipid-binding peaks (sum of apo protein and all lipid adducts obtained in the region of the mass spectrum under study) were plotted against lipid concentrations in solution. The lipid-binding curves were deduced from fitting to one-site total binding. Values of s.d. were calculated from three independent replicate experiments at each concentration. The results show that NTSR1 interacts preferentially with anionic phospholipids (PA and PS), as no binding was observed for neutral (DAG) and zwitterionic (PC) lipids. f, g, Exogenous POPS (f) and PtdIns(4)P (g) were added to β\textsubscript{1} AR at different final concentrations (10 μM is shown here). Spectra were recorded for a range of lipid concentrations from 0 to 80 μM for PS and 0 to 20 μM for PtdIns(4)P. Peak intensities of the individual PtdIns(4)P-bound species were measured and plotted against lipid concentration to yield a relative affinity for one PtdIns(4)P binding (1 ×), two PtdIns(4)P molecules binding (2 ×) or three PtdIns(4)P molecules binding (3 ×); only the first PtdIns(4)P molecule binds with high affinity (see Fig. 1a). Data are mean ± s.d. from three independent experiments.
Extended Data Fig. 3 | Investigation of the phospholipid preferences of A2AR and NTSR1. 

a, A representative mass spectrum of purified A2AR from three independent experiments revealed truncations of the N-terminal sequence (MPIM). The arrows between species indicate the mass differences corresponding to truncated amino acids (M, PI and M).  
b, A competitive binding assay (n = 3 independent experiments) in which A2AR was incubated with a mixture of lipids (PI, PtdIns(4)P, PI(4,5)P2 and PtdIns(3,4,5)P3) before mass spectrometry, indicated that PtdIns(4,5)P2 binds with a higher affinity than the other phospholipids to A2AR.  
c, The analogous competitive binding assay, in which NTSR1 was incubated with a mixture of lipids (PI, PtdIns(4)P, PI(4,5)P2 and PtdIns(3,4,5)P3) before mass spectrometry. Ratio to apo is plotted as a function of concentration and defined as the ratio of the intensity corresponding to individual PI phosphate adducts to the receptor in the apo state (inset). The same data analysis methods are used for Fig. 1b. PtdIns(4,5)P2 binds with a higher affinity than the other phospholipids to A2AR. Data are shown as mean ± s.d. from three independent replicates.  
d, A representative mass spectrum of A2AR (n = 3 independent experiments) used for preparation of the G-protein complex reveals lower abundance of PS and PI adducts prior to coupling to G proteins.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | NTSR1–PtdIns(4,5)P$_2$ and $\beta_1$AR–PtdIns(4,5)P$_2$ interactions within CGMD simulations, and comparison of PtdIns(4,5)P$_2$ contacts among different GPCRs. a, Volumetric density surfaces showing the average spatial occupancy of PtdIns(4,5)P$_2$ lipids around a crystal structure of NTSR1(TM86V–ΔIC3B) (PDB: 4BUO), which shares a greater sequence identity to the wild-type receptor (91%) than NTSR1(HTGH4–ΔIC3B) (86%), contoured to show the major PtdIns(4,5)P$_2$-interaction sites. Density surfaces were calculated over 5 μs of CGMD (blue surface, n = 10 independent experiments), and 100 μs of CGMD (magenta, n = 1 experiment). The cytoplasmic side of NTSR1 structure is coloured from white (low PtdIns(4,5)P$_2$ interaction) to red (high PtdIns(4,5)P$_2$ interaction). Extending a simulation to 100 μs revealed no overall change in the patterns of PtdIns(4,5)P$_2$ interaction. Less specific, and hence more dynamic, interaction was seen for the acyl chain moieties of PtdIns(4,5)P$_2$, which yielded more diffuse probability densities. b, $\beta_1$AR–PtdIns(4,5)P$_2$ interactions within CGMD simulations. Contact patterns are shown for simulations containing 5% PtdIns(4,5)P$_2$ in the lipid bilayer and thermostable $\beta_1$AR (PDB: 2Y03, top), 10% PtdIns(4,5)P$_2$ and thermostable $\beta_1$AR (middle), and 10% PtdIns(4,5)P$_2$ and $\beta_1$AR(S68R) construct (bottom). In each case PtdIns(4,5)P$_2$ contacts were calculated over 5 μs of CGMD (n = 10 independent experiments; error bars, s.d.), with each repeat simulation initiated from different random system configurations. c, PS and PtdIns(4,5)P$_2$ contacts with NTSR1 as a function of residue position, for PC:PS membranes (top left), PC:PS:PtdIns(4,5)P$_2$ membranes (top right), PC:PtdIns(4,5)P$_2$ membrane (bottom left) and PC:PS:PtdIns(4,5)P$_2$ (bottom right). The position of helices is denoted by horizontal grey bars. Lipid contact is calculated as the mean number of contacts between each residue and a given lipid species per frame, using a 6 Å distance cut-off. n = 3; error bars, s.d. d, PtdIns(4,5)P$_2$ contacts seen in CGMD simulations for nine class A GPCRs: histamine H1 receptor, PDB 3RZE; $\beta_1$ adrenergic receptor, 2VT4; $\beta_3$ adrenergic receptor, 2RH1; C$\beta_1$ cannabinooid receptor, 5TGZ; M4 muscarinic acetylcholine receptor, 5DSG; adenosine A$_2$A receptor, 3EML; dopamine D3 receptor, 3PBL; sphingosine 1-phosphate receptor, 3V2W; rhodopsin, 1F88. GPCR sequences are shown, with TM helices, intracellular loops (ICL) and H8 helices indicated by horizontal bars, and with amino acids coloured according to the mean number of contacts per simulation frame with the PtdIns(4,5)P$_2$ molecules. Green boxes correspond to the high frequency of PtdIns(4,5)P$_2$ interactions discussed in the main text for the TM1, TM4, and TM7/H8 motifs of NTSR1. Contacts were computed over 1 μs CGMD simulations (n = 3 independent experiments) for each GPCR, using a 6 Å cut-off. Sequences were aligned using T-Coffee$^{32}$ and mapping of protein–lipid contact data onto the sequence alignment used ALINE$^{33}$. © 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Fig. 5 | Site-directed mutagenesis attenuates PtdIns(4,5)P$_2$ binding to NSTR1. a, Schematic representation of the experimental protocol designed to combine mass spectrometry with mutagenesis to produce mutants of lower molecular mass than wild type, which, when incubated with PtdIns(4,5)P$_2$, yield a direct readout of the effect of mutations in specific regions. b, PtdIns(4,5)P$_2$ binding of NTSR1 mutants on residues that exhibit the highest frequency of PtdIns(4,5)P$_2$ interaction in molecular dynamics simulation. Mutation of NTSR1(HTGH4-ΔIC3B) residues on TM1 (R46G, K47G and K48G (R43G, K44G and K45G in NTSR1(TM86-ΔIC3B); R91G, K92G, K93G in wild type)), TM4 (R138I, R140I, K142L and K143L (R135I, R137T, K139L and K140L in NTSR1(TM86-ΔIC3B); R183I, R185T, K187L and K188L in wild type)) and TM7-H8 (R316N (R311N in NTSR1(TM86-ΔIC3B); R377N in wild type)) attenuate PtdIns(4,5)P$_2$ binding, and indicate that the TM4 interface is a preferential binding site over TM1 and TM7-H8 interfaces. Selection of residues for mutations was guided by molecular dynamics (Extended Data Fig. 4) and previous studies in which binding of a fluorescently labelled agonist, BODIPY neurotensin, to NTSR1, was screened and used to monitor efficient production, insertion, and folding$^{10}$. © 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Fig. 6 | PtdIns(4,5)P_2 binds preferentially to β_1 AR in an active state and stabilizes β_1 AR coupled to mini-G_s and A_2A R-mini-G_s complex. 

a, A time-course experiment was performed to monitor the formation of active β_1 AR–mini-G_s complex. The coupling efficiency (percentage) was calculated from the relative intensity of peaks assigned to β_1 AR–mini-G_s coupling in the appropriate lipid-bound state. The plot indicates that mini-G_s coupling is enhanced by PtdIns(4,5)P_2 when more than two lipid molecules are bound to the receptor. Error bars represent s.d. from at least three independent experiments.

b, Plot of PMF for the interaction of mini-G_s with A_2A R in the presence of PtdIns(4,5)P_2 (green) or PS (grey). The PMF is calculated along a reaction coordinate (Δz) corresponding to the centre–centre separation of the mini-G_s and receptor proteins along the z axis (normal to the bilayer plane). The interaction of mini-G_s with the A_2A R is stabilized in the presence of PtdIns(4,5)P_2 by 50 ± 10 kJ mol⁻¹ relative to PS. Error bars (which are < 10 kJ mol⁻¹) are from bootstrap sampling of the PMFs and therefore represent the ‘statistical’ errors in estimating the well depth from a given set of simulations and PMF calculation (n = 3 independent experiments). We therefore estimate a minimum error of ≤ 10 kJ mol⁻¹.

c, Mass spectra were recorded for a 1:1 equimolar mix of an inactive unliganded β_1 AR variant, E130W, and its unmodified active counterpart (co-purified with the agonist isoprenaline) in the presence of PI(4,5)P_2. Lipid binding occurred on both receptors, but following normalization to account for differences in ionization efficiency, a clear preference for PtdIns(4,5)P_2 binding to the active receptor was observed. Bars represent mean ± s.d.
Extended Data Fig. 7 | Detection of nanobody coupling to β1AR.
Peaks in the mass spectrum assigned to Nb6B9 binding to β1AR to form an equimolar β1AR–Nb6B9 complex are highlighted in orange, and demonstrate complete complex formation, implying that nanobody has a higher affinity than mini-Gα for β1AR. n = 3 independent experiments.
Extended Data Fig. 8 | Structural comparison of class A and class B GPCRs in complex with trimeric Gαβγ complexes. The PtdIns(4,5)P₂ contacts of the Gα₃ subunit observed in molecular dynamics simulations (green spheres) are highlighted on the structures of trimeric G-protein interactions with β₂AR (PDB: 3SN6), the glucagon-like peptide-1 receptor (GLP-1) (PDB: 5VAI) and the calcitonin receptor (CTR) (PDB: 5UZ7). Basic residues on the interface adjacent to the cytoplasmic end of TM4 are highlighted as purple spheres. Lower panels show an expanded view, highlighting the conserved pattern of PtdIns(4,5)P₂ bridging in class A GPCRs (β₂AR and A₂AR (Fig. 3e)), both of which have basic residues on TM4 (Lys140 and Arg107/111) that are not present in the class B GPCRs GLP-1R and CTR.
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Lipids were symmetrically distributed between leaflets.

Extended Data Table 2 | Simulations run

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Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
  - An indication of 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
    - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
  - A description of all covariates tested
  - A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
  - A full description of the statistics 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
    - Give P values as exact values whenever suitable.
  - 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
  - Clearly defined error bars
    - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about availability of computer code

Data collection: We have used MassLynx V4.1 and Xcalibur 2.2 mass spectra acquisition. GROMACS43 was used for performing molecular dynamics simulation.

Data analysis: UniDec was used mass spectra analysis. VMD50, PyMOL V1.3r1 and tools implemented in GROMACS43 were used for analysis of simulation data. All other data were plotted used Prism. These software are ready available.

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- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

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

| Sample size | No sample size calculations were performed. All proteins were selected based on underlining biology and availability |
| Data exclusions | No data were excluded form the analysis |
| Replication | All measurements were done in triplicate and all attempts at replication were successful and presented |
| Randomization | There was no allocation into experimental groups involved |
| Blinding | Blinding is not relevant to this study, no in vivo studies were used |

Reporting for specific materials, systems and methods

### Materials & experimental systems

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### Methods

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<td>ChIP-seq</td>
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<td>☑</td>
<td>Flow cytometry</td>
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<td>☑</td>
<td>MRI-based neuroimaging</td>
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**Unique biological materials**

Policy information about availability of materials

Obtaining unique materials: All unique materials are readily available from authors, with a reasonable request

**Antibodies**

Antibodies used: Nanobodies were expressed in E.coli strain BL21(DE3)RIL (Agilent Technologies), using synthetic genes

Validation: The validation of the nanobodies is shown by Ring 2013, Rasmussen 2011

**Eukaryotic cell lines**

Policy information about cell lines

Cell line source(s): SF9 and Tni (High 5™) cells were obtained from Invitrogen

Authentication: The cell line was authenticated by the supplier. None of the cell line used have been authenticated by the authors.

Mycoplasma contamination: Cell lines tested negative for mycoplasma contamination

Commonly misidentified lines (See ICLAC register): No commonly misidentified cell lines were used