METHODS

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

Peptide design of NNC1702. NNC1702, a low-potency partial agonist of GCGR, was designed to have reduced agonist activity but to maintain relatively high binding affinity to the receptor by deleting the N-terminal residue H1 of glucagon and introducing the mutation D9E[24] (Extended Data Fig. 1), aiming for better stability of the GCGR–peptide complex compared to that of the full agonist-bound receptor. Two more mutations, Q24K(4×γE) and M27L, were included to improve the solubility and stability of the peptide at neutral pH.

Cloning and insect-cell expression of GCGR. The codon-optimized human *GCGR* gene (Genewiz) was cloned into a modified pFastBac1 vector with a haemagglutinin signal sequence at the N terminus and a PreScission protease site followed by a 10×His tag and a Flag tag at the C terminus. The native signal peptide, M1–A26, was removed from the N terminus of the receptor. T4-lysozyme was fused into the second intracellular loop (ICL2) of GCGR between residues T257 and E260. To further improve protein thermostability, 45 residues were truncated at the C terminus and the mutation R173$^{2.46b}$A was introduced. Our ligand-binding assay showed that the binding affinity of the engineered GCGR to both glucagon and NNC1702 is close to that of the wild-type receptor (Extended Data Fig. 1d, e). The engineered receptor displayed a higher binding affinity to NNC1702 compared to glucagon, in agreement with the fact that the construct was optimized to improve protein stability of the GCGR–NNC1702 complex.

GCGR expression was performed using the same procedure as previously described[16]. The optimized GCGR construct was expressed in *Spodoptera frugiperda* (*Sf*9) insect cells (Invitrogen) using the Bac-to-Bac Baculovirus Expression System (Invitrogen).

Purification of GCGR–NNC1702 complex. The cells expressing the GCGR–T4-lysozyme protein were lysed in a lysis buffer containing 10 mM HEPES, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$ and EDTA-free protease inhibitor cocktail tablets (Roche), then washed three times with a high salt buffer containing 10 mM HEPES, pH 7.5, 1 M NaCl, 20 mM KCl and 10 mM $MgCl_2$. Purified membranes were resuspended in 10 ml lysis buffer supplemented with 40% glycerol and stored at −80 °C until use.

Prior to solubilization, the purified membranes were thawed in 30 ml buffer containing 10 mM HEPES, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 13% glycerol, 40 μM NNC1702 and EDTA-free protease inhibitor cocktail (Roche) at 4 °C for 1 h. The receptor was then solubilized in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM, Anatrace) and 0.2% (w/v) cholesteryl hemisuccinate (CHS, Sigma) at 4 °C for 3 h. The supernatant was isolated by ultra-centrifugation at 160,000*g* for 30 min. The supernatant was incubated with TALON resin (Clontech) overnight at 4 °C.

The TALON resin was washed with 25 column volumes of wash buffer 1 containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% glycerol, 10 μM NNC1702 and 30 mM imidazole, and followed by 10 column volumes of wash buffer 2 containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% glycerol, 20 μM NNC1702 and 15 mM imidazole. The GCGR–NNC1702 complex was eluted with 5 column volumes of 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% glycerol, 50 μM NNC1702 and 300 mM imidazole. The PD MiniTrap G-25 column (GE Healthcare) was used to remove imidazole. The sample was treated overnight with custom-made His-tagged PreScission protease to remove the C-terminal His-tag and Flag tag, and custom-made His-tagged PNGase F was also added to the sample to deglycosylate the receptor. The His-tag cleaved GCGR–NNC1702 complex was collected after flowing through a Ni-NTA column (Qiagen), and then concentrated to 20–30 mg ml^{-1} with a 100-kDa molecular weight cut-off concentrator (Millipore).

Crystallization in lipidic cubic phase. Crystallization was performed using the lipidic cubic phase (LCP) method[28] at 20 °C. The protein sample (20–30 mg ml^{-1}) was mixed with lipid (7.8 MAG/cholesterol 9:1 by mass) at a ratio of 1:1 (v/w) using a syringe mixer. The LCP mixture was dispensed onto 96-well glass sandwich plates (Shanghai FAstal, BioTech) in 35–40-nl drops and overlaid with 800 nl precipitant solution using a Gryphon robot (Art Robbins). Protein reconstitution in LCP and crystallization trials were performed at room temperature. Plates were incubated and imaged at 20 °C using an automated incubator–imager (RockImager, Formulatrix). The crystals of the GCGR–NNC1702 complex grew in 100 mM Tris, pH 8.0, 70–120 mM potassium phosphate dibasic, 27–33% (v/v) PEG 200, and reached a maximum size of 200 μm × 10 μm × 10 μm after four days. Crystals were collected using 75–100-μm MiTeGen micromounts (M2-L19-50/150, MiTeGen) and immediately flash-frozen in liquid nitrogen.

X-ray diffraction data collection and structure determination. Data collection was performed at the SPring-8 beam line 41XU, Hyogo, using a Pilatus3 6M detector (X-ray wavelength 1.0000 Å). The crystals were exposed with an 11-μm × 9-μm mini-beam for 0.2 s and 0.2° oscillation per frame. Owing to radiation damage, data collection was limited to 5–10° per crystal. Diffraction data from 10 crystals were integrated and scaled using XDS[29].

The structure of the GCGR–NNC1702 complex was solved by molecular replacement implemented in Phaser[30] using the models of GCGR TMD in the structure of the GCGR–NNC0640–mAb1 complex, GCGR ECD in the structure of the GCGR-ECD–mAb1 complex and T4-lysozyme (PDB IDs: 5XEZ, 4LF3 and 2RH1, respectively). One molecule of GCGR TMD, one molecule of GCGR ECD and one molecule of T4-lysozyme were found sequentially by molecular replacement search. The structure was initially solved and refined to an R_{free} of approximately 40% with REFMAC[31]. The model maps from the data were of sufficient quality to interpret the overall structure of the GCGR–NNC1702 complex; the stalk, ECL1 and the peptide NNC1702 were built on the basis of electron density map. The model then underwent iterated cycles of manual building into $|2F_o| - |F_c|$ maps with Coot[32] and refinement with REFMAC[31] and BUSTER[33]. The structure was carefully refined, and Ramachandran plot analysis indicates that 100% of the residues are in favourable (94.0%) or allowed (6.0%) regions (no outliers).

The final model of the GCGR–NNC1702 complex contains 398 residues (Q27–T257 and E260–E426) of GCGR, 28 residues (S2–T29) of NNC1702 and 160 residues (N2–Y161) of T4-lysozyme. The 4×γE tail of the mutation Q24K(4×γE) in NNC1702 was not traced owing to poor electron densities. There is no residue from the receptor or neighbouring molecules adjacent to this mutation, which reduces the possibility that this tail had an effect on the structure. The ECD of the receptor forms contacts with the T4-lysozyme fusion proteins from two neighbouring molecules in the crystal lattice with buried surface areas of 150 Å^2 and 340 Å^2, which are much smaller than the buried surface area between the ECD, TMD and the peptide ligand (4,760 Å^2). This indicates that the lattice interactions of the ECD are considerably weaker than the interactions between the ECD and the TMD or peptide, which have key roles in stabilizing the ECD conformation. This suggests that crystal packing is unlikely to have an effect on the conformational change of the GCGR ECD.

Ligand-binding assay. To determine the binding affinities of human glucagon and NNC1702 to GCGR, we performed scintillation proximity assay (SPA) binding using plasma membranes from BHK cells expressing the human GCGR. The BHK cell line was stably transfected with GCGR and CRE luciferase. Cells were routinely tested for mycoplasma contamination. Plasma membranes were prepared by washing cultured cells in PBS before lysis in ice-cold 25 mM HEPES, 2 mM $MgCl_2$ and 1 mM EDTA (HME) buffer. Tubes with lysed cells were frozen in liquid nitrogen and quickly thawed again. The thawed cell lysate was vortexed at maximum speed for 20 s and centrifuged at 20,000*g* for 10 min at 4 °C. Pellets were re-suspended in HME buffer and protein concentrations determined by BioRad protein assay (Bradford; BioRad). Membranes (5 μg per well) were combined with 2.5 mg ml^{-1} wheat germ agglutinin (WGA)-coated SPA beads (Perkin Elmer), diluted ligand (highest final concentration, 1 μM) and ^{125}I-labelled glucagon–NH_2 (60 pM) in binding buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.02% Tween-20 and 0.1% ovalbumin) and incubated for 2 h at 25 °C. Assay plates (OptiplateTM-96, Perkin Elmer) were centrifuged for 10 min at 1,500 r.p.m. at room temperature before counting using a Topcounter (Perkin Elmer). Half maximal inhibitory concentration (IC_{50}) values were calculated with the GraphPad Prism software (version 7.0a, GraphPad).

Ligand-potency assay. To determine the potencies of glucagon and NNC1702, we activated human GCGR in transfected BHK cells. The BHK cell line was stably transfected with the human GCGR and CRE luciferase. Assay was performed in DMEM medium without phenol red (Gibco 11880-028, Thermo Fisher Scientific), 10 mM HEPES (Gibco 15630), 1× Glutamax (Gibco 35050), 1% ovalbumin and 0.1% Pluronic F-68. Ligands were dissolved into 300 μM stocks in 80% DMSO, and serial dilutions were prepared in medium with 1 μM as the highest final concentration. Before incubation in Black Microwell 96-well plates (Thermo Fisher Scientific), cells were washed twice in PBS and adjusted to 100,000 cells per ml. The assay plate was incubated for 3 h in 5% CO_2 at 37 °C. Aliquots of Steadylite Plus were added to each well and shaken for 30 min at room temperature before the plate luminescence was read on a BioTek Synergy2 reader (BioTek). Half maximal effective concentration (EC_{50}) values were calculated with Prism software.

Whole-cell ligand-binding assay. To determine the binding affinity of glucagon and NNC1702 to the engineered GCGR used for crystallization, we performed a whole-cell ligand-binding assay. CHO-K1 cells (obtained from American Type Culture Collection) were seeded onto 96-well cell-culture plates (PerkinElmer) treated with poly-D-lysine, at a density of 3 × 10^4 cells per well. The cells were routinely tested for mycoplasma contamination. After overnight culture, the cells were transiently transfected with wild-type or the engineered GCGR using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were collected 24 h after transfection, washed twice and incubated with a blocking buffer (F12 supplemented