with 33 mM HEPES, pH 7.4, and 0.1% BSA) for 2 h at 37 °C. Cells were then washed twice with PBS and incubated in a binding buffer (PBS supplemented with 10% BSA, pH 7.4) with a constant concentration of ^{125}I-labelled glucagon (60 pM) and varying concentrations of unlabelled glucagon and NNC1702 (17.86 pM–5 μM) at room temperature for 3 h. Cells were washed three times with ice-cold PBS and lysed by 50 μl lysis buffer (PBS supplemented with 20 mM Tris-HCl, 1% Triton X-100, pH 7.4). The plates were subsequently counted for radioactivity (counts per minute) in a scintillation counter (MicroBeta^2 Plate Counter, PerkinElmer) using a scintillation cocktail (OptiPhaseSuperMix, PerkinElmer).

Molecular dynamics simulation. To investigate the binding of glucagon to GCGR and the role of glucagon in receptor activation, we conducted long-time molecular dynamics simulations on the basis of the crystal structure of GCGR–NNC1702. The prepared GCGR structure was obtained by back-mutating the R173A mutation to its wild-type residue, omitting T4-lysozyme, completing ICL2 and adding A26 to the N terminus of GCGR. To obtain the prepared glucagon structure, the residue H1 was added with an α-helical secondary structure to the N terminus of NNC1702 in PyMOL (The PyMOL Molecular Graphics System, version 1.8), and without space conflict with neighbouring residues. The other three mutations—D9E, Q24K(4×γE), M27L—were back-mutated to their wild-type residues. This GCGR–glucagon model was used as the starting structure for molecular dynamics simulations. The chain termini of GCGR and glucagon were all charged, except for the C terminus of GCGR, which was capped with neutral groups. Most notably, all titratable residues were left in the dominant protonation state at pH 7.0, which was calculated using the H++ server (http://biophysics.cs.vt.edu/H++), and H1 was protonated during all simulations.

The GCGR–glucagon model was then embedded in a 90 Å × 90 Å palmitoyl oleoyl phosphatidyl choline (POPC) bilayer and the lipids located within 1 Å of the receptor were removed. The system was solvated in a box (90 Å × 90 Å × 156 Å) with TIP3P water model and 0.15 M NaCl, including 241 lipid molecules, 28,622 water molecules, 92 chloride ions and 80 sodium ions, for a total of 125,237 atoms. Three parallel 1-μs molecular dynamics simulations were performed using the GROMACS 5.1.4 package[34] with isothermal–isobaric ensemble and periodic boundary condition. The CHARMM36–CAMP force field[35] was used for the protein, glucagon, the POPC phospholipids, ions and water molecules. First, energy minimizations were performed to relieve unfavourable contacts in the system; this was followed by equilibration steps to a total of 50 ns to equilibrate the lipid bilayer and the solvent, with restraints on the main chain or Cα atoms of GCGR. Subsequently, three 1-μs production runs were performed. The temperature of the systems was maintained at 310 K using the v-rescale method[36] with a coupling time of 0.1 ps. The pressure was kept at 1 bar using the Parrinello–Rahman method[37] with $\tau_p = 1.0$ ps and a compressibility of 4.5×10^{-5} per bar. SETTLE[38] constraints were applied to the hydrogen-involved covalent bonds in water molecules, and LINCS[39] constraints were applied to the hydrogen-involved covalent bonds in other molecules; the time step was set to 2 fs. Electrostatic interactions were calculated with the Particle–Mesh Ewald algorithm[40] with a real-space cut-off of 1.2 nm.

DEER spectroscopy of GCGR. The GCGR mutant with H89 replaced by cysteine and C171 replaced by serine was generated by removing a single reactive cysteine residue at position 171 and introducing a reactive cysteine at position 89. The native cysteine C287 was used as a reference for GCGR conformational changes. The mutant was expressed as described above, and then purified in the absence of any ligand or in the presence of a ligand (NNC1702 or NNC0640), following the same protocol that was used to prepare protein samples for crystallization. For DEER measurements, the apo mutant and the mutant–ligand complexes were reacted with the sulfhydryl-specific label (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl) methanethiosulfonate (MTSSL, Toronto Research Chemicals) to generate R1 nitroxide side chains at positions 89 and 287, following standard procedures[41]. The spin-labelled samples of the apo receptor and the receptor–ligand complexes were concentrated to 50–70 μM. For the receptor–ligand complexes, 50 μM NNC1702 or 30 μM NNC0640 was added to the buffer to increase protein stability. Deuterated glycerol (20%) was added to the samples as a cryoprotectant. The spin-labelled mutants were loaded into quartz capillaries (1.5-mm ID and 1.8-mm OD) and flash-frozen using a dry-ice–ethanol bath. After freezing, they were loaded into an ER 5107D2 Q-band flexline resonator, and Q-band measurements were performed at 80 K on a Bruker Elexsys 580 spectrometer (at the University of Toronto) with a Super Q-FTu Bridge. A 32-ns π-pump pulse was applied to the low-field peak of the nitroxide field swept spectrum, and the observer π/2 (16-ns) and π (32-ns) pulses were positioned 50-MHz (17.8-G) upfield, which corresponds to the nitroxide centre line. Distance distributions were obtained from the raw DEER data using the LabVIEW program 'LongDistances' (v.593, by C. Altenbach, http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/). Background correction was performed using a quadratic background. The primary DEER data were fitted via a 'model-free' algorithm as implemented in the 'LongDistances' software. Nitroxide labels were modelled into the GCGR–NNC1702 crystal structure using the Multiscale Modelling of Macromolecular systems (MMM) software package (http://www.epr.ethz.ch/software/mmm-older-versions.html). Common nitroxide rotamers were used for the modelling[42].

Data availability. Atomic coordinates and structure factor files for the GCGR–NNC1702 structure have been deposited in the RCSB Protein Data Bank with identification code 5YQZ. All other data are available from the corresponding authors upon reasonable request.

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