ONLINE METHODS

Expression and purification

We generated a *Mus musculus* δ-OR construct with features designed to enhance crystallogenesis. A tobacco etch virus (TEV) protease recognition site was introduced after residue 35, and the carboxy terminus was truncated after P342. T4 lysozyme residues 2–161 were inserted in the third intracellular loop of δ-OR between residues 244 and 251. A FLAG epitope tag was added to the amino terminus and an octa-histidine tag was appended to the carboxy terminus. The mouse and human δ-OR share 94% sequence identity, with most sequence differences in the disordered amino and carboxy termini. The final crystallization construct (δ-OR–T4L) is shown in Supplementary Fig. 1.

The δ-OR–T4L construct was expressed in Sf9 cells using the pFastBac (Invitrogen) baculovirus system in the presence of 10 μM naloxone. Cell cultures were grown to a density of 4×10^6 cells per ml, infected with baculovirus containing the δ-OR–T4L gene, shaken at 27°C for 48 hr, and cell pellets were harvested and stored at -80 °C. To purify the protein, insect cells were first lysed by osmotic shock in a buffer comprised of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 μM naltrindole and 2 mg/ml iodoacetamide to block reactive cysteines. This was followed by an extraction step, in which Sf9 membranes were homogenized with a glass dounce homogenizer in a solubilization buffer comprised of 1.0% lauryl maltose neopentyl glycol (MNG), 0.3% sodium cholate, 0.03% cholesterol hemisuccinate (CHS), 20 mM HEPES pH 7.5, 0.5M NaCl, 30% v/v glycerol, 2 mg/ml iodoacetamide, and 1 μM naltrindole. This extraction reaction was mixed at 4 °C for 1 hr, then centrifuged at high speed to remove cell debris. Nickel-NTA agarose was then added to the supernatant and stirred for 2 hr. The Nickel-NTA resin was washed three times in batch with a washing buffer of 0.1% MNG, 0.03% sodium cholate, 0.01% CHS, 20 mM HEPES pH 7.5, 0.5 M NaCl and 1 μM naltrindole. The resin was transferred into a wide-bore glass column and bound receptor was eluted in washing buffer supplemented with 300 mM imidazole. Ni-NTA purified δ-OR-T4L was then loaded over anti-FLAG M1 affinity resin and the salt concentration was gradually lowered from 0.5 M to 0.1 M in a

buffer otherwise comprised of 0.1% MNG, 0.01% CHS, 20 mM HEPES pH 7.5 and 1 μM naltrindole. The receptor was then washed with a buffer containing 0.01% MNG, 0.001% CHS, 20 mM HEPES pH 7.5, 0.1 M NaCl and 1 μM naltrindole and eluted from the anti-FLAG M1 affinity resin with the same buffer containing 0.2 mg/ml FLAG peptide and 2 mM EDTA. To remove flexible amino and carboxy termini, TEV protease was added at a 1:3 TEV:δ-OR–T4L ratio by weight. The sample was incubated at room temperature (23°C) for 1h followed by treatment with carboxypeptidase A (1:100 w/w) at 4°C overnight. We used size exclusion chromatography to remove TEV and carboxypeptidase A. Size exclusion chromatography was performed on a Sephadex S200 column (GE Healthcare) in a buffer of 0.01% MNG, 0.001% CHS, 100 mM NaCl, 20 mM HEPES pH 7.5 and 1 μM naltrindole. After size exclusion, naltrindole was added to a final concentration of 10 μM. The resulting receptor preparation was pure and monodisperse (Supplementary Fig. 6).

Crystallization and data collection

Purified δ-OR–T4L receptor was concentrated to 50 mg/ml using a Vivaspin sample concentrator with a 50 kDa molecular weight cut-off (GE Healthcare). As for other GPCR-T4L fusion proteins crystallized to date, we utilized the *in meso* method to obtain crystals of δ-OR–T4L. Briefly, δ-OR–T4L was reconstituted into a mixture of monoolein and cholesterol (Sigma) by the two-syringe method. By weight, one part δ-OR–T4L was mixed with 1.5 parts of a 10:1 mixture of monoolein:cholesterol until the resulting phase was optically transparent. We used a Gryphon LCP robot (Art Robbins Instruments) to accurately dispense 20 to 55 nl mesophase drops onto glass plates. These lipidic boluses were overlaid with 700 nl precipitant solution. Crystals grew in precipitant solution consisting of 29-33% PEG 400, 100 mM HEPES pH 7.5, 120-180 mM sodium citrate (tribasic) and 350 mM magnesium chloride. Crystals were observed after 2 hrs and grew to full size after 5 days. Crystals used for data collection are shown in Supplementary Fig. 7.

Diffraction data were collected at Advanced Photon Source GM/CA-CAT beamline 23ID-B using a beam size of 10 μm. Due to radiation damage, the diffraction quality decayed during exposure. Wedges of 5 - 15 degrees were collected and merged from 20 crystals using HKL2000[21]. Diffraction quality ranged from 3.0 - 3.5 Å in most cases. Due to anisotropic diffraction (see Supplementary Table 1) the highest shell $\langle I \rangle / \langle \sigma I \rangle$ value was slightly lower than is typical for isotropically diffracting crystals.

The structure of the δ-OR was solved by molecular replacement in Phaser[22] using the μ-OR receptor as a search model. The lattice for δ-OR–T4L shows alternating lipidic and aqueous layers, with receptor molecules arranged in anti-parallel associations (Supplementary Fig. 8). We improved the initial model by iteratively building regions of the receptor in Coot[23] and refining in Phenix[24]. To assess the quality of the final structure, we used MolProbity[25]. As with μ-OR and κ-OR, electron density was clear, and allowed confident placement of the ligand and binding site residues (Supplementary Fig. 9). The resulting statistics for data collection and refinement are shown in Supplementary Table 1. Figures were prepared in PyMOL[26].

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