

Structure of the δ -opioid receptor bound to naltrindole

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The opioid receptor family comprises three members, the μ -, δ - and κ -opioid receptors, which respond to classical opioid alkaloids such as morphine and heroin as well as to endogenous peptide ligands like endorphins. They belong to the G-protein-coupled receptor (GPCR) superfamily, and are excellent therapeutic targets for pain control. The δ -opioid receptor (δ -OR) has a role in analgesia, as well as in other neurological functions that remain poorly understood¹. The structures of the μ -OR and κ -OR have recently been solved^{2,3}. Here we report the crystal structure of the mouse δ -OR, bound to the subtype-selective antagonist naltrindole. Together with the structures of the μ -OR and κ -OR, the δ -OR structure provides insights into conserved elements of opioid ligand recognition while also revealing structural features associated with ligand-subtype selectivity. The binding pocket of opioid receptors can be divided into two distinct regions. Whereas the lower part of this pocket is highly conserved among opioid receptors, the upper part contains divergent residues that confer subtype selectivity. This provides a structural explanation and validation for the ‘message–address’ model of opioid receptor pharmacology^{4,5}, in which distinct ‘message’ (efficacy) and ‘address’ (selectivity) determinants are contained within a single ligand. Comparison of the address region of the δ -OR with other GPCRs reveals that this structural organization may be a more general phenomenon, extending to other GPCR families as well.

Opioid receptors have an important role in the central nervous system, regulating pain perception, hedonic homeostasis, mood and wellbeing¹. Thus, they have long been the focus of physiological and pharmacological studies, as well as being important therapeutic targets. The opioid receptors are GPCRs, and were classified based on their pharmacology and their tissue distribution into three subclasses: the μ (for morphine), the δ (for vas deferens) and the κ (for ketocyclazocine) receptors⁶. The sequence identity within the transmembrane domains (TMs) between the μ -OR and δ -OR, the μ -OR and κ -OR, and the δ -OR and κ -OR is 76%, 73% and 74%, respectively⁷. Another receptor identified by cloning, the nociceptin/orphanin FQ receptor, was classified in this family owing to a high sequence identity (67% in the TM)⁷. However, morphinans and most other classical opioid ligands have little affinity for the nociceptin receptor⁸. The μ -, δ - and κ -ORs are activated by endogenous peptides: the endorphins, enkephalins and dynorphins⁸. They are also the targets of chemically diverse small molecules with a variety of efficacy and selectivity profiles⁸. In an effort to understand better the structural basis for opioid receptor pharmacology and function, we used the *in meso* crystallization method to solve a 3.4 Å structure of a *Mus musculus* δ -OR T4 lysozyme (T4L) fusion protein (Supplementary Fig. 1) bound to naltrindole, a non-covalent δ -OR-selective morphinan antagonist⁹.

The δ -OR structure presents the typical GPCR seven-pass transmembrane helix fold (Fig. 1a), and shows marked conservation of backbone structure with other opioid receptors (Fig. 1b, c), even in

regions with low sequence conservation (Fig. 1d, e). The ligand naltrindole sits in an exposed binding pocket, similar in shape to that observed for the μ -OR and κ -OR^{2,3}. The CXCR4 receptor also has a solvent-exposed binding pocket, suggesting that this may be a feature common to some GPCRs activated by peptides. The β -hairpin in extracellular loop (ECL)2 (Fig. 1d) is observed in all three opioid receptors, despite the low sequence identity in this domain. ECL3, which is also poorly conserved among the three opioid receptors, was unresolved in the κ -OR structure and has high temperature factors in both μ -OR and δ -OR (Fig. 1e), suggesting a relatively flexible link between TM6 and TM7. Of note, the κ -OR structure shows a clear difference in the position of the extracellular half of TM1, with an outward displacement of approximately 10 Å (Fig. 1b) compared to the μ -OR and δ -OR. In this respect, the μ -OR and δ -OR resemble each other and the CXCR4 chemokine receptor more closely than the κ -OR. However, this structural difference may simply reflect differences in crystallization conditions or crystal packing influences, as is seen in the turkey β_1 -adrenergic receptor structure (PDB accession 2VT4), where two different TM1 conformations are observed in the same crystal¹⁰.

δ - and μ -ORs have been observed to form homo-oligomers in transfected cells, and functional studies suggest that they form pharmacologically distinct hetero-oligomers when they are co-expressed¹¹. It is therefore interesting that in the μ -OR crystal lattice two parallel dimeric interfaces were observed² (Supplementary Fig. 2). The most extensive interface involves TM5 and TM6 of adjacent protomers. The other interface, which is also observed in the κ -OR, involves TM1, TM2 and helix 8. In addition to this common interface, an anti-parallel interaction is also observed in the κ -OR crystal lattice. In contrast, the δ -OR crystallizes with only an anti-parallel arrangement of receptor molecules (Supplementary Fig. 2). However, inferences regarding the physiological relevance of oligomeric interfaces observed in GPCR crystal structures should be made with caution. Purified, detergent-solubilized δ -OR and μ -OR are monomeric before crystallization and the association into either parallel or antiparallel dimers occurs during crystallogenesis. The differences between the μ -OR and δ -OR dimeric interfaces probably reflect differences in the most energetically favourable interactions under the crystallography conditions and may be the consequence of one or more of the following: different crystallization conditions, a different T4L arrangement, sequence differences in the protein, and subtle differences in the structures stabilized by the different ligands.

Opioid receptors bind exceptionally well to highly diverse ligands, including morphinans, a wide variety of other small molecules, and peptides of varying length. Details of naltrindole binding to the δ -OR are presented in Fig. 2 and Supplementary Fig. 3. Despite their chemical diversity, many opioid ligands display conserved features, most notably a phenolic hydroxyl separated by six carbons from a positive charge, which mimics the amino-terminal tyrosine of all endogenous opioid peptides (Fig. 3). The morphinan ligand naltrindole used in

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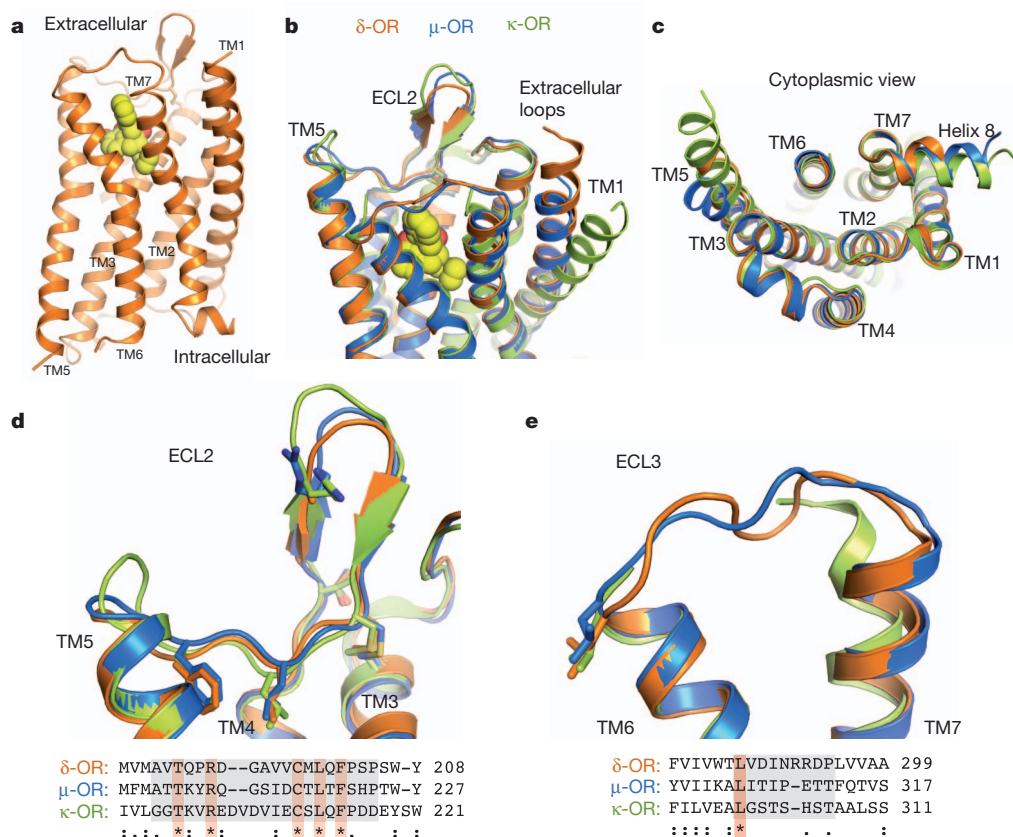


Figure 1 | Overall structure of the δ -OR. **a**, The δ -OR, orange, exhibits a typical seven-pass transmembrane architecture common to other GPCRs. **b**, **c**, This fold is highly conserved among all three classical members of the opioid receptor family. δ -OR, orange; μ -OR, blue; κ -OR, green. **d**, The opioid family possesses a conserved β -strand fold in ECL2, creating a wide, open binding pocket. Despite the structural similarity, only five residues in this region are absolutely conserved. Conserved residues are highlighted red in sequence alignment and shown as sticks. Asterisks indicate positions with complete residue conservation among opioid subtypes, colons indicate residues with strongly similar properties, and periods indicate residues with weakly similar properties. **e**, ECL3, an important selectivity determinant for ligand binding, shows modest structural variability in the μ -OR and δ -OR. In the κ -OR receptor structure this region could not be resolved owing to poor electron density. A single leucine residue is conserved in ECL3 among opioid subtypes.

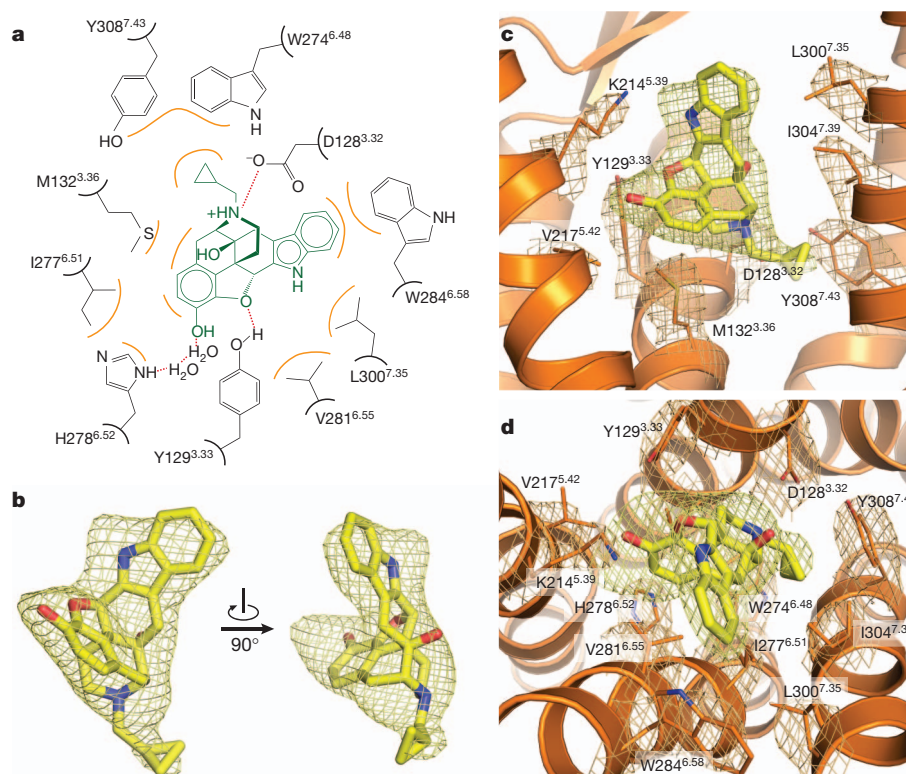


Figure 2 | Ligand-binding site of the δ -OR. **a–d**, Naltrindole binds in a deep but open binding site within the δ -OR. **a**, **b**, Selected contacts are highlighted (**a**), and a ligand $F_o - F_c$ omit map within a 2 Å radius of naltrindole is shown at

a 3 σ contour (**b**). **c**, **d**, The complete binding site is shown. 2 $F_o - F_c$ electron density maps within a 2 Å radius of binding site amino acid side chains are shown in orange at a 1.5 σ contour. The ligand omit density is shown as in **b**.

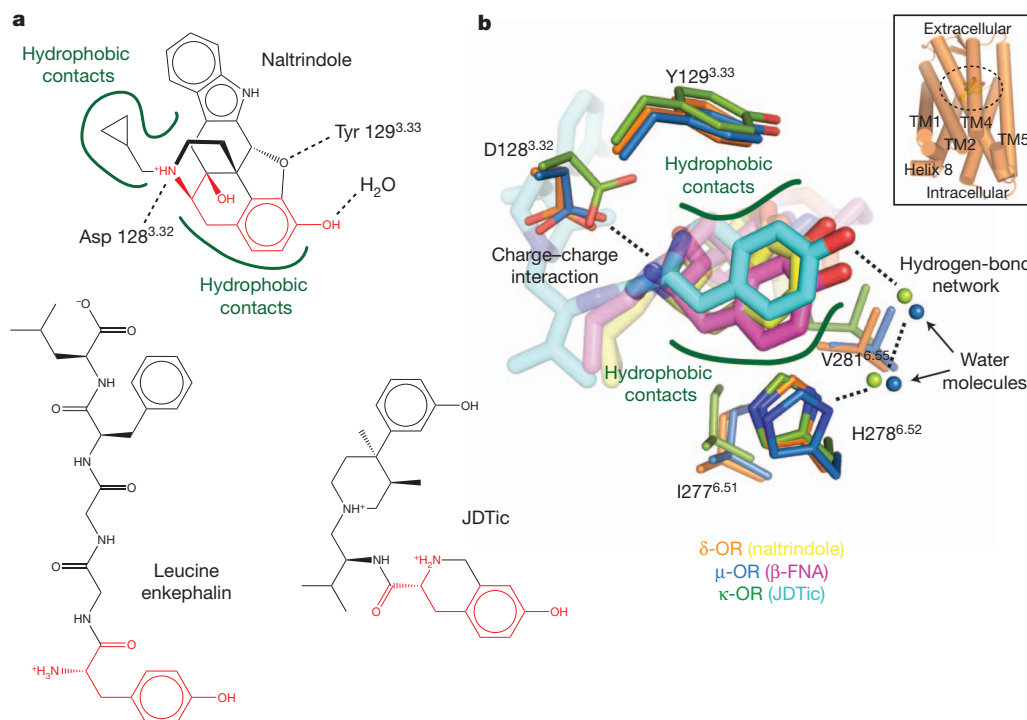


Figure 3 | A conserved opioid ligand recognition mode. **a**, Opioid receptors bind a wide variety of ligands, including morphinans like naltrindole, other small molecules such as JDtTic, and peptides like enkephalins. Most opioid ligands, including these, contain a ‘tyrosine’ pharmacophore (red) with a phenolic hydroxyl in close proximity to a positive charge. Conserved recognition features for morphinan ligands observed in the μ -OR and δ -OR are highlighted on naltrindole (top). **b**, The tyrosine pharmacophores of

naltrindole, β -FNA and JDtTic are shown in their three-dimensional context as observed in the crystal structures of the δ -OR, μ -OR and κ -OR. The inset shows the receptor orientation in the expanded view. Specific conserved interactions in all three receptors are highlighted. The δ -OR is shown in orange (naltrindole in yellow), the μ -OR in dark blue (β -FNA in pink), and the κ -OR in green (JDtTic in light blue).

crystallization of the δ -OR is non-covalent⁹, and is therefore not subject to possible distortions in its binding mode due to a covalent tether like that in the structure of the μ -OR. Indeed, the position of naltrindole in the binding pocket is shifted slightly relative to the position of the covalent morphinan ligand β -funaltrexamine (β -FNA)¹² bound to the μ -OR (Fig. 4), although all major interactions are present in both structures. As anticipated from the μ -OR structure, the leucine in the position 300^{7.35} is in contact with the indole group of naltrindole (Fig. 2). This residue is responsible for naltrindole selectivity¹³, as W318^{7.35} in μ -OR and Y312^{7.35} in κ -OR are sterically incompatible with naltrindole binding. As with the μ -OR and κ -OR, electron density near the phenolic hydroxyl of naltrindole suggests the presence of water molecules (Fig. 3b and Supplementary Fig. 4). The resolution of the δ -OR structure, however, is not sufficient to place solvent molecules with confidence. Nonetheless, this feature suggests that a two-water hydrogen-bond network linking H278^{6.52} and the ligand phenolic hydroxyl is probably a conserved feature of opioid ligand recognition, and certainly this appears to be the case in the μ -OR and κ -OR.

With the structure of the δ -OR, all classical opioid receptors have now been crystallized and solved in inactive conformations. A closely related receptor, that for the nociceptin/orphanin FQ peptide, is often classified within the opioid receptor family. However, this receptor has low or negligible affinity for most opioid alkaloids⁸, despite high sequence conservation within the TM domains. Examination of the morphinan binding site of δ -OR reveals that only a few of the critical interacting residues differ between δ -OR and the nociceptin receptor (Supplementary Fig. 5). However, mutation of certain residues in the nociceptin receptor to their δ -OR counterparts can create a high-affinity alkaloid-binding site¹⁴. These mutations change smaller amino acid side chains in the nociceptin receptor to larger residues in the corresponding positions of the δ -OR, so it is likely that the binding pocket of the nociceptin receptor is somewhat enlarged relative to

that of the δ -OR. The loss of tightly fitting hydrophobic interactions with the morphinan ring of opioid alkaloids may therefore account, at least in part, for the much higher affinity of most morphinans for the δ -OR, μ -OR and κ -OR than for the nociceptin receptor.

Opioid pharmacology has long been described in terms of the ‘message–address’ concept^{4,5}, in which the ligand can be viewed as composed of two distinct modules carrying information about efficacy (message) and selectivity (address). The structure of the δ -OR and other opioid receptors reveals this pharmacological phenomenon to be a direct consequence of opioid receptor structure. The lower portion of the binding pocket is well-conserved in both sequence and structure. In the δ -OR, this portion of the binding pocket recognizes the core morphinan group, which entails the ‘message’ of the ligand (Fig. 4a, b). In contrast, the upper binding pocket is divergent among subtypes, and is rich in selectivity determinants (Fig. 4a, b). The indole ‘address’ of naltrindole extends into this region, conferring its δ -OR selectivity (Fig. 4a, b). Similarly, the 3-hydroxyphenyl group of JDtTic extends into the address region, and would clash with δ -OR residue K108^{2.63}. This feature may account for the selectivity of JDtTic for the κ -OR and μ -OR (Fig. 4a), although it is likely that other factors contribute as well.

Development of highly subtype-selective ligands has proven to be possible for the classical opioid receptors¹⁵. However, for another GPCR family, the muscarinic acetylcholine receptors, this has proven considerably more challenging. Comparison of the message and address regions of the δ -OR with the M2 muscarinic receptor¹⁶ (Fig. 4c, d) reveals that the address region corresponds to the allosteric site of these receptors. This region is separated by a layer of tyrosines from the highly conserved orthosteric site, which matches the message region of opioid receptors. The physical separation of the two regions may therefore explain the relatively greater challenges associated with development of selective muscarinic ligands compared to opioid receptors, as well as the promising results of selective ‘dual-steric’ or

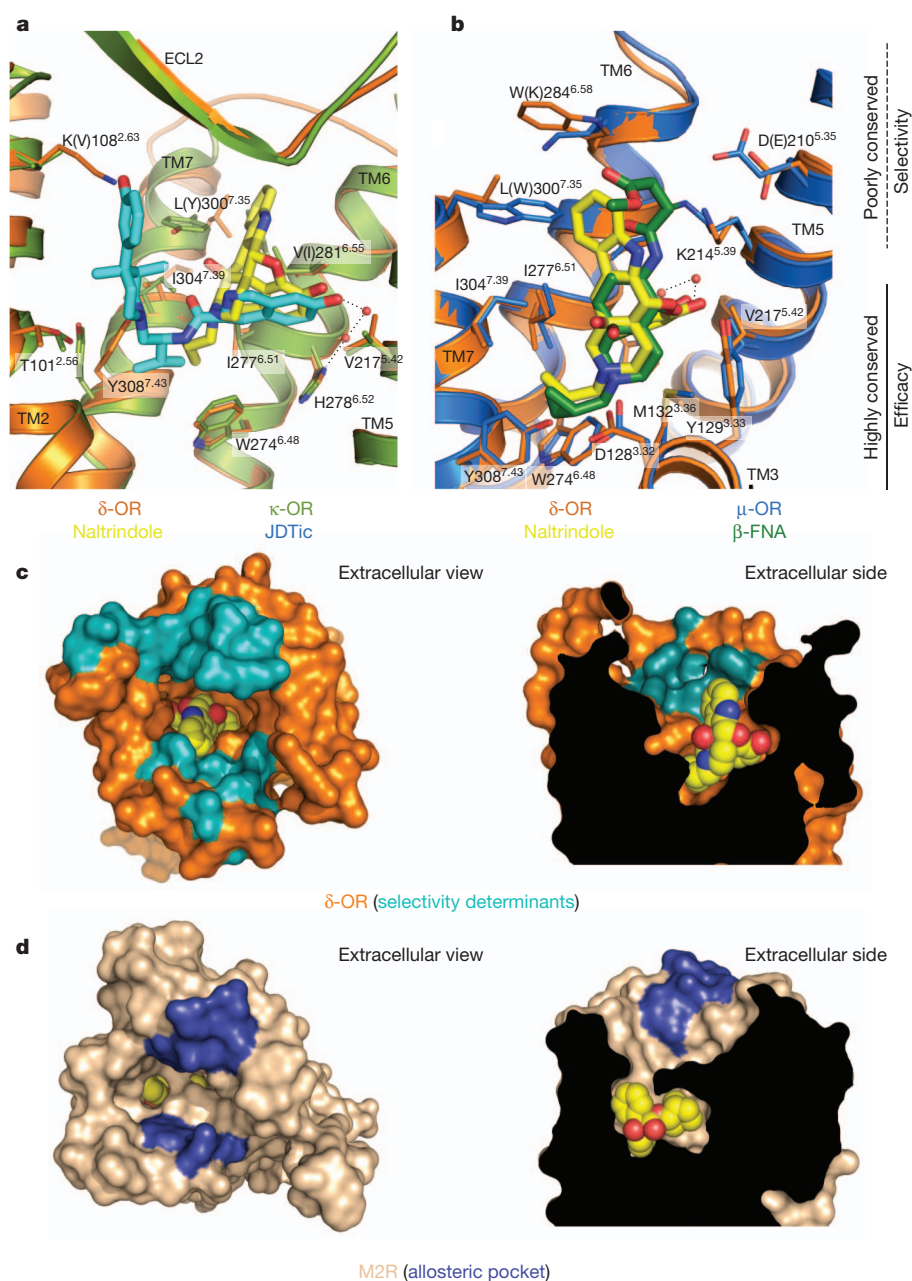


Figure 4 | The message–address hypothesis is reflected in opioid receptor structure. **a, b**, Comparison of the δ -OR with the κ -OR (**a**) and with the μ -OR (**b**) reveals high conservation in both sequence and structure in the base of the ligand-binding pocket, whereas extracellular regions are more divergent. These regions are delineated in the legend on right. The δ -OR is shown in orange (naltrindole in yellow), the κ -OR in light green (JD1c in light blue), and the μ -OR in dark blue (β -FNA is dark green). Residue numbers and labels are those for the δ -OR, with κ -OR residues in parentheses in **a** and μ -OR residues in parentheses in **b**, where sequence differs from the δ -OR. These regions interact

with ligand moieties that can target binding to a particular opioid subtype. **c**, Residues previously characterized as important for opioid-subtype selectivity^{18–20} are clustered around the upper part of the binding pocket, delineating an ‘address’ region of the receptor. The δ -OR is shown in orange (selectivity determinants in teal). **d**, This region is structurally analogous to the allosteric site in muscarinic receptors (allosteric pocket in blue), suggesting that the high selectivity of muscarinic ligands occupying this space is also a manifestation of the message–address features structurally encoded within GPCRs.

‘bitopic’ ligands that occupy both orthosteric and allosteric sites simultaneously¹⁷. The distinct message and address regions of the δ -OR and other opioid receptors then seem to be a more general feature of GPCRs, which may have implications for the development of ligands even for distantly related GPCR families.

Together with the μ -OR and κ -OR, the structure of the δ -OR completes the initial structural characterization of the opioid receptors, offering the first experimental views of the atomic details of ligand recognition and selectivity in this important GPCR family. However, such antagonist-bound structures are only the first step towards a complete understanding of opioid receptor function. Given the

importance of opioid agonists in clinical medicine, active state structures, as well as signalling complexes, will be required to fully leverage structural methods towards the development of a new generation of opioid drugs.

METHODS SUMMARY

The δ -OR–T4L fusion protein was expressed in Sf9 insect cells and purified by nickel affinity chromatography followed by Flag antibody affinity chromatography and size exclusion chromatography. It was crystallized using the lipidic mesophase technique, and diffraction data were collected at GM/CA-CAT beamline 23ID-B at the Advanced Photon Source at Argonne National Laboratory. The structure was solved by molecular replacement using merged data from 20 crystals.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.M., A.C.K. and S.G. designed experiments, performed research and analysed data. T.S.K. and F.S.T. expressed and purified receptor. W.I.W. supervised diffraction data analysis and model refinement. A.M., A.C.K., S.G. and B.K.K. prepared the manuscript. S.G. and B.K.K. supervised the research.

Author Information Coordinates and structure factors for δ -OR-T4L are deposited in the Protein Data Bank under accession code 4EJ4. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.G. (granier@stanford.edu) or B.K.K. (kobilka@stanford.edu).

METHODS

Expression and purification. We generated a *Mus musculus* δ -OR construct with features designed to enhance crystallogensis. A tobacco etch virus (TEV) protease recognition site was introduced after residue 35, and the carboxy terminus was truncated after P342. T4L 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 N terminus and an octa-histidine tag was appended to the C terminus. The mouse and human δ -OR share 94% sequence identity, with most sequence differences in the disordered N and C 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 μ M 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 h, 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 μ M naltrindole and 2 mg ml^{–1} 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, 0.5 M NaCl, 30% v/v glycerol, 2 mg ml^{–1} iodoacetamide, and 1 μ M naltrindole. This extraction reaction was mixed at 4 °C for 1 h, then centrifuged at high speed to remove cell debris. Nickel-NTA agarose was then added to the supernatant and stirred for 2 h. 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 μ M 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 μ M 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 μ M naltrindole and eluted from the anti-Flag M1 affinity resin with the same buffer containing 0.2 mg ml^{–1} Flag peptide and 2 mM EDTA. To remove flexible N and C 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 1 h 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 μ M naltrindole. After size

exclusion, naltrindole was added to a final concentration of 10 μ M. 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^{–1} using a Vivaspinn sample concentrator with a 50 kDa molecular weight cut-off (GE Healthcare). As for other GPCR-T4L fusion proteins crystallized so far, we used 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 20–55 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 h 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 μ m. Owing to radiation damage, the diffraction quality decayed during exposure. Wedges of 5–15 degrees were collected and merged from 20 crystals using HKL2000²¹. 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 Phaser²² 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 Coot²³ and refining in Phenix²⁴. To assess the quality of the final structure, we used MolProbity²⁵. As with the 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 PyMOL²⁶.

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