Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic

Aaron A. Thompson1*, Wei Liu1*, Eugene Chun1*, Vsevolod Katritch1, Huixian Wu1, Eyal Vardy2, Xi-Ping Huang2, Claudio Trapella3, Remo Guerrini3, Girolamo Calo4, Bryan L. Roth2, Vadim Cherezov1 & Raymond C. Stevens1

Members of the opioid receptor family of G-protein-coupled receptors (GPCRs) are found throughout the peripheral and central nervous system, where they have key roles in nociception and analgesia. Unlike the ‘classical’ opioid receptors, δ, κ and μ (δ-OR, κ-OR and μ-OR), which were delineated by pharmacological criteria in the 1970s and 1980s, the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP, also known as ORL-1) was discovered relatively recently by molecular cloning and characterization of an orphan GPCR3. Although it shares high sequence similarity with classical opioid GPCR subtypes (~60%), NOP has a markedly distinct pharmacology, featuring activation by the endogenous peptide N/OFQ, and unique selectivity for exogenous ligands3–5. Here we report the crystal structure of human NOP, solved in complex with the peptide mimetic antagonist compound-24 (C-24) (ref. 4), revealing atomic details of ligand–receptor recognition and selectivity. Compound-24 mimics the first four amino acids of N/OFQ (Phe1-Gly2-Gly3-Phe4), with a marked increase in potency and efficacy (Supplementary Tables 2 and 3), perhaps owing to the C-terminal NOP truncation. C-24 was selected for co-crystallization on the basis of the pronounced thermostability it imparts on the receptor (Supplementary Fig. 1), its high affinity (half-maximum inhibitory concentration (IC50) = 0.27 nM) and antagonist potency (IC50 = 0.1 nM) for NOP, and its selectivity (>1,000-fold)4. Peripherally administered C-24 is able to penetrate the central nervous system, where it antagonizes N/OFQ effects on nociception6 and produces beneficial responses in experimental models of Parkinson’s disease11. The NOP structure revealed C-24 binding deep within the orthosteric binding pocket (Fig. 1a), probably mimicking the ‘message’ domain of N/OFQ (Phe1-Gly2-Gly3-Phe4), a similar sequence to that of canonical opioid peptides (Tyr1-Gly2-Gly3-Phe4)5–7 (Supplementary Fig. 2).

Structural comparison of published GPCR crystal structures shows a modularity of the seven-transmembrane helical core, and considerable variation of the extracellular module with boundaries defined by proline-induced kinks11. NOP contains five such kinks in the seven-transmembrane core located at residue positions Pro1052-28, Pro1844-49, Pro2255-50, Pro2785-50 and Pro3165-50 (superscripts indicate residue numbers as per the Ballesteros–Weinstein nomenclature14), yielding repercussions on the shape of the ligand-binding pocket. Notably, the extracellular tip of helix V in NOP is shifted by more than 4 Å as compared with the κ-OR3 and μ-OR crystal structures (Protein Data Bank (PDB) accesses 4JH and 4DKL, respectively), thereby resulting in both a gap between helices IV and V (~12 Å between Cys of residues 184 and 215) and an expansion of the orthosteric pocket (Supplementary Fig. 5). However, compared with the chemokine receptor CXC4, the extracellular tip of helices VI and VII are tilted inwards, towards the orthosteric pocket. Unlike the κ-OR structure3, the extracellular half of helix I in NOP is pulled in towards the axis of the seven-transmembrane bundle, in a conformation that is more similar to that of the chemokine receptor (PDB accession 3ODU (ref. 15); Fig. 1b). This alternative conformation of helix I is facilitated by the presence of flexible glycine residues located at an apparent ‘hinge point’ that are conserved within the opioid receptor family: Gly651.46 and Gly681.49 in κ-OR and CXCR4. ECL2 forms an elongated domain of N/OFQ (Phe1-Gly2-Gly3-Phe4), a similar sequence to that of canonical opioid peptides (Tyr1-Gly2-Gly3-Phe4)5–7 (Supplementary Fig. 2).

Despite low sequence conservation, extracellular loops (ECLs) 1 and 2 of NOP are structurally similar to those of κ-OR and CXCR4 (Fig. 1b). Specifically, the backbone of ECL1 in NOP is nearly indistinguishable from that of κ-OR and CXCR4. ECL2 forms an elongated β-hairpin, which is tethered to the extracellular tip of helix III by a structurally conserved disulphide bond between Cys12312–13 and Cys200ECL2. This β-hairpin motif is also observed in κ-OR and CXCR4, suggesting a common structural motif of the γ-branched class A peptide-binding

1Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, USA. 2National Institute of Mental Health Psychoactive Drug Screening Program, Department of Pharmacology and Division of Chemical Biology and Medicinal Chemistry, University of North Carolina Chapel Hill Medical School, Chapel Hill, North Carolina 27599, USA. 3Department of Pharmaceutical Sciences and LTTA (Laboratorio per le Tecnologie delle Terapie Avanzate), University of Ferrara, 44121 Ferrara, Italy. 4Department of Experimental and Clinical Medicine, Section of Pharmacology and National Institute of Neuroscience, University of Ferrara, 44121 Ferrara, Italy.

*These authors contributed equally to this work.
Figure 1 | Structural overview of the NOP receptor. a, NOP (grey) is shown in ribbon representation with its ECL2 highlighted (red). The bound ligand C-24 is depicted as green spheres, and transparent disks highlight the extracellular (EC) and intracellular (IC) membrane boundaries (coloured blue and red, respectively). b, Structural superposition of NOP molecules ‘A’ and ‘B’, κ-OR (PDB accession 4DHJ)\(^\text{(1)}\) and CXCR4 (PDB accession 3ODU)\(^\text{(2)}\), coloured grey, yellow, blue and orange, respectively. Compared with κ-OR, the extracellular portion of helix I from NOP is tilted inwards towards the orthosteric pocket, in a similar conformation to CXCR4. c, Structural superposition of NOP molecules A and B and thermostabilized A\(_{\alpha 2AR}\) (PDB accession 3PWH)\(^\text{(3)}\), coloured grey, yellow and green, respectively, highlighting conformational differences between the ICLs.

Intracellular loop (ICL) 2 of NOP receptor molecule ‘B’ in the asymmetric unit forms a short \(\alpha\)-helix, which has been observed in many other GPCRs (Fig. 1c); the ICL2 is tethered to the seven-transmembrane core via a salt bridge between Arg 16\(_{\text{ICL2}}\) and in all opioid receptors and Asp 147\(_{\text{ICL3}}\) from the conserved ‘D(E)RY’ motif (Fig. 1c). Note the conformational differences between the ICL3 regions of these receptors, where NOP helices V and VI are shorter and further apart than in A\(_{\alpha 2AR}\), and the ICL3 part of ICL3 is longer than A\(_{\alpha 2AR}\) (15 residues in NOP versus 8 residues in A\(_{\alpha 2AR}\)) (Fig. 1c).

The NOP–C-24 structure highlights specific residues in the pocket that are essential for N/OFQ binding and receptor subtype selectivity. The orthosteric binding pocket of NOP is relatively large, reflecting its ability to bind large endogenous peptides. With a similar pose in both NOP molecules (root mean squared deviation (r.m.s.d.) = 0.6 Å), C-24 interacts with the ‘floor’ of the pocket through several hydrophobic and electrostatic interactions. Mutagenesis of the binding pocket of NOP defined the relative impact of specific residues on C-24 and N/OFQ binding and function (Supplementary Tables 4 and 5). The protonated nitrogen of the C-24 piperidine ring forms a crucial salt bridge with Asp 130\(_{\text{III}}\)–32 — a residue that is conserved in the opioid receptor family and all biogenic amine GPCRs. Mutations of Asp 130\(_{\text{III}}\)–32 to either alanine or asparagine abrogate N/OFQ binding, highlighting the requirement of the negative charge at this position (Fig. 2 and Supplementary Tables 4 and 5), and it has been proposed that Asp 130\(_{\text{III}}\)–32 is involved in a salt-bridge interaction with the positively charged N-terminus of N/OFQ (Supplementary Table 4). In addition to the anchoring salt bridge between Asp 130\(_{\text{III}}\)–32 and the amino moiety of C-24, the linked benzofuran/piperidine rings are buried in a hydrophobic pocket created by residues from helices III, V and VI. The benzofuran ‘head’ group is sandwiched between Met 134\(_{\text{III}}\)–36 and Tyr 131\(_{\text{III}}\)–33, in which the Met\(_{\text{III}}\)–36 side chain adopts a different, more buried rotamer as compared to κ-OR, thereby allowing a deeper penetration of the C-24 ring system. This is consistent with the modest effect of a Met 134\(_{\text{III}}\)–36Ala mutation on the potency of NOP ligands (Supplementary Tables 4 and 5). A Tyr 131\(_{\text{III}}\)–33Phe mutation had no effect on agonist binding, whereas Tyr 131\(_{\text{III}}\)–33Ala was deleterious (Supplementary Tables 4 and 5), suggesting that Tyr 131 participates in \(\pi\)-stacking interactions with Phe 1 of the peptide (Supplementary Table 4).

At the ‘tail’ end of C-24, the carbonyl group adjacent to the pyrrolidine ring is hydrogen bonded to Gln 107\(_{\text{IV}}\)–48, a residue stabilized by a hydrogen bond to Tyr 309\(_{\text{VI}}\)–45. A Gln 107\(_{\text{IV}}\)–48Ala mutation results in a 10-fold loss in C-24 binding and a more than 300-fold reduction in N/OFQ potency, and mutation of Tyr 309\(_{\text{VI}}\)–45 abolishes binding of C-24 and reduces N/OFQ potency ~7-fold (Supplementary Tables 4 and 5). Interestingly, both Gln 107\(_{\text{IV}}\) and Tyr 309\(_{\text{VI}}\) are present in the \(\alpha\)-OR structure, albeit in very different conformations (Supplementary Fig. 8).

The crystal structure of NOP in complex with C-24 afforded us a unique opportunity to determine the molecular basis for both the high-affinity binding by N/OFQ-derived peptide antagonists and their pronounced subtype selectivities (Fig. 3). Notably, we verified that the C-24 binding mode can be reliably reproduced by energy-based docking of C-24 to the NOP receptor, with an r.m.s.d. of ~0.9 Å. Moreover, docking of another piperidinamide derivative, compound-35 (C-35), closely mimics the binding of C-24, whereas docking of a less active stereoisomer compound-36 (ref. 22) yields a considerably distorted binding pose in the pyrrolidine region and a reduced binding score (not shown). C-24 has previously been proposed to mimic the N-terminal four residues of N/OFQ-related peptide antagonists [Nphe 1]N/OFQ(1-13)-NH\(_2\) (in which Nphe denotes N-benzylglycine) (Supplementary Table 4) and UFP-101 (ref. 24). Automated docking of the four N-terminal residues of UFP-101 results in a conformation of the Nphe 1-Gly 2-Gly 3-Phe 4 tetrapeptide in which the Nphe 1 and Phe 4 rings of the peptide make the same hydrophobic interactions as the aromatic rings of C-24, and the N-terminal amino group forms a salt bridge with Asp 130\(_{\text{III}}\)–32, thus supporting the proposed similarity in the binding poses between small molecules and peptide analogues (Fig. 3c).
by the highly acidic tip of ECL2 and helices II and VII, with all six basic residues of the peptide forming ionic interactions with acidic side chains of NOP (Fig. 3c–e).

Interactions of the address domain of N/OFQ(1–13) with helices II (residues 107–113)\(^{22}\) and VII (residues 296–302)\(^{19}\) were previously demonstrated by photocrosslinking, a finding consistent with our mutagenesis data showing the crucial importance of Asp 110 2.63 in
demonstrated by photocrosslinking, a finding consistent with our mutagenesis data showing the crucial importance of Asp 110 2.63 in the binding mode for this classical opioid receptor peptide. Instead, a comparison of the NOP and \(k\)-OR structures shows that several of the NOP-specific side-chain changes, including two of the substitutions mentioned earlier (Ala\(^{6.39}\), Lys and Glu\(^{6.52}\)His), are involved in a large-scale reshaping of the binding pocket and an alternative coordination of water molecules (Fig. 4). Located closer to the ligand-binding pocket entrance, Lys 223 5.35 and Glu 297 6.58 (Fig. 4a). Replacement of Lys\(^{5.39}\) to alanine in NOP precludes these stabilizing ionic interactions and is accompanied by an outwards shift of the extracellular half of helix V in the NOP crystal structure, and an inwards shift of helix VI. Opioid receptor subtype alteration of the large Lys\(^{5.39}\) side chain and the accompanying shifts of the \(\alpha\)-helices reshape the entrance to the pocket, and this probably affects the binding of address domains of peptides and synthetic ligands.

The \(k\)-OR structure reveals a cluster of water molecules that is coordinated by two of the classical opioid receptor-specific residues involved in binding pocket remodelling (Fig. 4b) — His 291 6.52 and the (nor-BNI)\(^{29}\). The crystal structures of NOP and \(k\)-OR show that the side chains of these three residues are pointing towards the interior of the binding pocket (Fig. 4 and Supplementary Fig. 8). In NOP, Gln 280 6.52 and Thr 305 7.39 are involved in C-24 interactions, and all three of the cognate residues at these positions are involved in \(k\)-OR interactions with the selective antagonist JD Tic and with the modelled nor-BNI antagonists\(^{5}\). Notably, although most of the modified side chains are polar, none form direct hydrogen bonding interactions to the ligands tested, so that the selectivity profiles cannot be explained by simple polar-to-hydrophobic (or vice versa) changes of ligand contacts. Instead, a comparison of the NOP and \(k\)-OR structures shows that several of the NOP-specific side-chain changes, including two of the substitutions mentioned earlier (Ala\(^{6.39}\), Lys and Glu\(^{6.52}\)His), are involved in a large-scale reshaping of the binding pocket and an alternative coordination of water molecules (Fig. 4).

Located closer to the ligand-binding pocket entrance, Lys 223 5.35 and Glu 297 6.58 (Fig. 4a). Replacement of Lys\(^{5.39}\) to alanine in NOP precludes these stabilizing ionic interactions and is accompanied by an outwards shift of the extracellular half of helix V in the NOP crystal structure, and an inwards shift of helix VI. Opioid receptor subtype alteration of the large Lys\(^{5.39}\) side chain and the accompanying shifts of the \(\alpha\)-helices reshape the entrance to the pocket, and this probably affects the binding of address domains of peptides and synthetic ligands.

The \(k\)-OR structure reveals a cluster of water molecules that is coordinated by two of the classical opioid receptor-specific residues involved in binding pocket remodelling (Fig. 4b) — His 291 6.52 and the...
Figure 3 | Molecular docking in the orthosteric-binding pocket. a–e. The docking of C-24 (a), its analogue C-35 (b) and peptide antagonist UFP-101 (c–e) in the NOP. The crystallographic pose of C-24 is green in all panels, and the docked molecules (C-24, C-35 and UFP-101) are coloured yellow. The Nphe 1-Gly 2-Gly 3-Phe 4 tetrapeptide portion of the docked UFP-101 is depicted as sticks, and the ‘address’ domain (residues 5–17) of this peptide is represented as a cartoon. A ‘sliced’ side-view of the pocket is shown in c, and a view from the extracellular surface is shown in d. e. The electrostatic surface potentials of the UFP-101 peptide, coloured blue to red, corresponding to positive and negative surface potentials (+3 to −3 kT e−1), respectively. ECL2 is coloured red, and the acidic Asp and Glu residues from the ECL2 β-hairpin are depicted as red sticks.

Figure 4 | Conformational differences in the ligand-binding pocket between NOP–C-24 and κ-OR–JDTic. a, ‘Sliced’ surface representation of NOP, highlighting the deep binding pocket bound with C-24 (coloured green) and JDTic (coloured magenta) from the superimposed κ-OR structure. b, c, Different views of NOP (coloured grey with green C-24) superimposed with the κ-OR structure (PDB accession 4DJH; coloured blue with magenta JDTic). Hydrogen bonding interactions are depicted as dashed yellow and black lines for NOP and κ-OR, respectively. The water molecules from the κ-OR structure are depicted as cyan spheres. Residue labels are coloured black and blue for NOP and κ-OR, respectively. The conformational shifts observed between helices V and VI that result in different binding pocket architectures are highlighted in b. The alternative rotamer of Met5.36 in the pocket (134 in NOP and 142 in κ-OR), which affects the orientation of the head group of the ligand, is highlighted in c.
METHODS SUMMARY

BRIL–NOP was expressed in Spodoptera frugiperda (Sf9) insect cells. Ligand-binding assays were performed as described in Methods. Sf9 membranes were solubilized using 0.5% (w/v) n-dodecyl-β-D-maltopyranoside and 0.1% (w/v) cholesteryl hemisuccinate, and purified by immobilized metal ion affinity chromatography. Receptor crystallization was performed by the lipidic cubic phase (LCP) method. The protein–LCP mixture contained 40% (w/v) concentrated receptor solution, 54% (w/w) monoolein and 6% (w/w) cholesterol. Crystals were grown in 40 ml protein-laden LCP bolus overlaid by 0.8 μl of precipitant solution (25–30% (v/v) PEG400, 100–200 mM potassium sodium tartrate tetrahydrate, 100 mM Bis-Tris propane, pH 6.4) at 20 °C. Crystals were collected directly from the LCP matrix and flash frozen in liquid nitrogen. X-ray diffraction data were collected at 100 K on the 23ID-B/D beamline (GM/CA-CAT) of the Advanced Photon Source at the Argonne National Laboratory using a 10-μm collimated minibeam. Diffraction data from 23 crystals were merged for the final data set. Data collection, processing, structure solution and refinement are described in Methods. Flexible docking of small molecules and peptides was performed with the ICM molecular modelling package (Molsoft LLC).

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

Received 22 February 2011; accepted 30 March 2012.

1. Mollerneau, C. et al. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett. 341, 33–38 (1994).
2. Meunier, J. C. et al. Isolation and structure of the endogenous agonist of opioid receptor-like-1 (ORL1) receptor. Nature 377, 532–535 (1995).
3. Reinscheid, R. K. et al. Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. Science 270, 792–794 (1995).
4. Goto, Y. et al. Identification of a novel spiroepiperidino opioid receptor-like 1 antagonist class by a focused library approach featuring 3D-pharmacophore similarity. J. Med. Chem. 49, 847–849 (2006).
5. Wu, H. et al. Structure of the human kappa opioid receptor in complex with JDTic. Nature advance online publication doi:10.1038/nature10939 (21 March 2012).
6. Manglik, A. et al. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. Nature advance online publication doi:10.1038/nature10954 (21 March 2012).
7. Lambert, D. G. The nociceptin/orphanin FQ receptor: a target with broad functional expression and localization. Trends Pharmacol. Sci. 25, 366–428 (1995).
8. Bes, B. & Meunier, J. C. Identification of a hexapeptide binding region in the nociceptin/orphanin FQ receptor (ORL1) receptor by photo-affinity labelling with Ac-Arg-Bpa-Tyr-Arg-Trp-Ang-NH2. Biochem. Biophys. Res. Commun. 310, 992–1001 (2003).
9. Mouledous, L. et al. Mutations in the α3 and β3 subunits of the human γ-aminobutyric acid A receptor alter ligand binding and receptor function. Mol. Pharmacol. 53, 772–777 (1998).

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

Acknowledgements This work was supported by PSI: Biology grant U54 GM094618 for studies on structure and function. R.W. is supported by NIH Roadmap grant GM073197 for technology development and R01 DA017204, R01 DA27170, and the NIH PMHS Psychoactive Drug Screening Program (X-P.H., E.V. and B.L.R.) and the Michael Hooker Chair of Pharmacology (B.L.R.), University of Ferrara (FAR grant to G.C.), Italian Ministry of University (FIRB Futuro in Ricerca 2010 grant to C.T.). We thank J. Francis for suggesting the idea to pursue the NOP receptor; J. Velasquez for help on molecular biology; T. Trinh, K. Allin and M. Chu for help on baculovirus expression; A. Walker and E. Abola for assistance with manuscript preparation; J. Smith, R. Fischetti and N. Sanishvili for assistance in development and use of the minibeam and beamtime at GM/CA-CAT beamline 23-ID at the Advanced Photon Source, which is supported by the National Cancer Institute grant Y1-CO-1020 and National Institutes of General Medical Sciences grant Y1-GM-1104.

Author Contributions A.A.T. optimized the constructs, purified and crytalized the receptor in LCP, optimized crystallization conditions, grew crystals for data collection, collected the data and refined the structure, and prepared the manuscript. W.L. assisted with LCP experiments, performed fluorescence recovery after photobleaching assays, collected diffraction data, and assisted with preparing the manuscript. E.C. and J.V. performed the construct optimization, prepared the receptor in LCP with X-ray data, assisted with preparing the manuscript. V.K. performed the receptor docking and prepared the manuscript. H.W. assisted with membrane preparations, provided advice on crystallization strategies, and assisted with preparing the manuscript. E.V. and P.H. performed ligand-binding and site-directed mutagenesis studies. C.T., R.G. and G.C. suggested the use of and synthesized numerous ligands for crystalization and pharmacological studies, and assisted with preparing the manuscript. B.L.R. supervised the pharmacology and mutagenesis studies and prepared the manuscript. V.C. assisted with the crystalization in LCP, collected diffraction data, prepared the manuscript. R.C.S. was responsible for the overall project strategy and management and wrote the manuscript.

Author Information The coordinates and the structure factors have been deposited in the Protein Data Bank under accession code 4EA3. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. 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 R.C.S. (stevens@scripps.edu).
METHODS
Cloning, expression and purification. NOP contains a ∼50-amino-acid extracellular domain at its N terminus, with a relatively high content of leucine and proline residues (26% and 14%, respectively) and three putative N-linked glycosylation sites. Despite high thermostability in the presence of select small molecule compounds, numerous attempts at crystallizing the receptor with an intact N terminus were unsuccessful. Although deletion of the C terminus (NOP-ΔC; 31-amino-acid deletion) resulted in increased expression, any truncation of the N terminus decreased the expression levels. However, replacement of the N terminus with several soluble fusion proteins restored the expression to levels that were comparable with constructs containing a full N terminus. Fusion with the most thermostabilized molecule compounds, numerous attempts at crystallizing the receptor with an intact glycosylation sites. Despite high thermo stability in the presence of select small ligands and within an r.m.s.d. of 0.5 Å from the antagonist C-24 in the structure. HEK293T cells. Mutations (Q107A, D110A, D130A, Y131A, M134A, I219A, Q280A and Y309A) were cloned from pFastBac into pCDNA3.1 and expressed in HEK293T cells. (concentration that leads to an 80% maximum response). Results were analysed using GraphPad Prism.

Crystalization. Protein samples of BRIL-AN-NOP-ΔC (concentrated to 40 mg m−1) in complex with C-24 were reconstituted into the lipidic cubic phase (LCP) by mixing with molten lipid using a mechanical syringe mixer24. The protein–LCP mixture contained 40% (w/w) protein solution, 54% (w/w) monolein (Sigma) and 6% (w/w) cholesterol (AvantiPolar Lipids). Crystalization trials were performed in 96-well glass sandwich plates24, 1 mM ATP, 25 mM imidazole, 25 mM HEPES, pH 7.5, and 10% (v/v) glycerol, exchanged into a buffer containing 500 mM NaCl, 20 mM KCl, 50 mM HEPES, pH 7.5, and 0.1% (w/v) dodecyl-β-D-maltopyranoside (DDM; Anatrace). The pure receptor was exchanged into a buffer containing 500 mM NaCl, 20 mM KCl, 50 mM HEPES, pH 7.5, and 35% (v/v) glycerol, flash frozen with liquid nitrogen, and stored at −80 °C until further use. Purified membranes were thawed and incubated with 25 mM C-24 (1-benzyl-N-((3-[[2-(hydroxyimino)methyl]-1H]-imidazol-1-yl)propyl)-pyridoline-2-carboxamide) (synthesized by C. Trapella and R. Guerrini), 500 mM NaCl, 20 mM KCl, 50 mM HEPES, pH 7.5, and 5% (v/v) glycerol, and incubated at 4 °C for 1 h. Iodoacetamide (Sigma) was then added to the membranes at a final concentration of 1 mg·ml−1 for another 15 min before solubilization with 0.5% (w/v) n-dodecyl-β-D-maltopyranoside (DDM; Anatrace), and 0.1% (w/v) cholesteryl hemisuccinate (CHS; Anatrace) for 3 h at 4 °C. The supernatant was isolated by centrifugation at 160,000g for 45 min, supplemented with 25 mM imidazole, pH 7.5, and incubated with TALON metal ion affinity chromatography resin (Clontech) overnight at 4 °C. Typically, 0.75 ml of resin (slurry) per 11 of original culture volume was used. After binding, the resin was washed with 15 column volumes of wash buffer 1 (500 mM NaCl, 20 mM KCl, 10 mM MgCl2, 50 mM HEPES, pH 7.5, 5% (v/v) glycerol, 1 mM ATP, 25 mM imidazole, 25 mM C-24, 0.05% (w/v) DDM and 0.01% (w/v) CHS); and 5 column volumes of wash buffer 2 (same as wash buffer 1, but without ATP and MgCl2), before protein elution with elution buffer (500 mM NaCl, 20 mM KCl, 50 mM HEPES, pH 7.5, 10% (v/v) glycerol, 250 mM imidazole, 25 mM C-24, 0.025% (w/v) DDM and 0.005% (w/v) CHS). Purified receptor was exchanged into a buffer containing 500 mM NaCl, 20 mM KCl, 5% (v/v) glycerol, 50 mM HEPES, pH 7.5, and 25 mM C-24 using a PD midiTrap G-25 column (GE Healthcare). BRIL-AN-NOP-ΔC was then supplemented with C-24 to a final concentration of 100 μM, and concentrated from ∼4.0 mg·ml−1 to 30 mg·ml−1 with a 100-kDa molecular mass cut-off Vivaspin concentrator (GE Healthcare). Regulator of G-protein signaling 2 (RGS2).

Docking of high-affinity NOP specific ligands was performed using an all-atom flexible receptor docking algorithm in ICM-Pro (MolSoft LLC) molecular modelling package as described previously48. Internal coordinate (torsion) movement were allowed in the side chains of the binding pocket, defined as residues within 10 Å distance of C-24 in the crystal structure. Other side chains and the backbone of the protein were kept as in the crystal structure. An initial conformation of the molecule ligand was generated by Cartesian optimization of the ligand model in Merck Molecular Force Field. Docking was performed by placing the ligand in a random position within 5 Å from the entrance to the binding pocket and global conformational energy optimization of the complex. To facilitate side-chain rotamer switches in flexible NOP receptor models, the first 10 steps of the Monte Carlo procedure used ‘soft’ van der Waals potentials and high Monte Carlo temperature, followed by another 10 steps with ‘exact’ van der Waals potentials and gradually decreasing temperature. A harmonic ‘distance restraint’ was applied between the protonated amine (of piperidine group in the small ligand or Nphe 1 in the UFP-101 peptide) and the carbonyl of the Asp 130 22 side chain in the initial 10 steps. The restraint was removed in the final 100 steps of the docking procedure. With UFP-101, the first six residues Nphe 1-Gly 2-Gly 3-Phe 4-Thr 5-Gly 6 were considered fully flexible, whereas the peptide backbone was fixed in an ideal α-helical conformation for the rest of the peptide (Ala 7–Arg 8-Lys 9-Ser 10-Ala 11-Arg 12-Lys 13–Arg 14-Lys 15-Asn 16-Gln 17). At least 10 independent runs of the docking procedure were performed for each NOP-ligand. The docking results were considered ‘consistent’ when at least 80% of the individual runs resulted in conformations clustered within an r.m.s.d. of <1 Å to the overall best energy pose for small molecule ligands and within an r.m.s.d. of <2 Å for the UFP-101 peptide. All calculations were performed on a 12-core Linux workstation.

©2012 Macmillan Publishers Limited. All rights reserved
32. Caffrey, M. & Cherezov, V. Crystallizing membrane proteins using lipidic mesophases. *Nature Protocols* **4**, 706–731 (2009).

33. Cherezov, V., Peddi, A., Muthusubramaniam, L., Zheng, Y. F. & Caffrey, M. A robotic system for crystallizing membrane and soluble proteins in lipidic mesophases. *Acta Crystallogr. D* **60**, 1795–1807 (2004).

34. Otwonowski Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326 (1997).

35. McCoy, A. J. et al. Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674 (2007).

36. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D* **66**, 486–501 (2010).

37. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D* **66**, 213–221 (2010).

38. The PyMOL Molecular Graphics System. v.1.4.1 (2011).

39. Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl Acad. Sci. USA* **98**, 10037–10041 (2001).

40. Totrov, M. & Abagyan, R. Flexible protein-ligand docking by global energy optimization in internal coordinates. *Proteins* **29** (suppl.), 213–220 (1997).

41. Katritch, V. et al. Analysis of full and partial agonists binding to β2-adrenergic receptor suggests a role of transmembrane helix V in agonist-specific conformational changes. *J. Mol. Recognit.* **22**, 307–318 (2009).