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Molecular control of δ-opioid receptor signalling

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Opioids represent widely prescribed and abused medications, although their signal transduction mechanisms are not well understood. Here we present the 1.8 Å high-resolution crystal structure of the human δ-opioid receptor (δ-OR), revealing the presence and fundamental role of a sodium ion in mediating allosteric control of receptor functional selectivity and constitutive activity. The distinctive δ-OR sodium ion site architecture is centrally located in a polar interaction network in the seven-transmembrane bundle core, with the sodium ion stabilizing a reduced agonist affinity state, and thereby modulating signal transduction. Site-directed mutagenesis and functional studies reveal that changing the allosteric sodium site residue Asn 131 to an alanine or a valine augments constitutive β-arrestin-mediated signalling. Asp95Ala, Asn310Ala and Asn314Ala mutations transform classical δ-opioid antagonists such as naltrindole into potent β-arrestin-biased agonists. The data establish the molecular basis for allosteric sodium ion control in opioid signalling, revealing that sodium-coordinating residues act as 'efficacy switches' at a prototypic G-protein-coupled receptor.

The three classical opioid receptors (μ, κ and δ-OR) and the related nociceptin/orphanin FQ peptide receptor (NOP) are G-protein-coupled receptors (GPCRs) essential for regulating nociception, mood and awareness[1]. These opioid GPCRs are activated by endogenous peptides (endorphins, enkephalins, dynorphins, nociceptin/orphanin FQ), natural alkaloids (opiates), and an expanding number of small molecule agonists through interactions with the orthosteric site located in the extracellular portion of the seven-transmembrane (7TM) bundle. Despite the progress made in understanding GPCR activation[2], the underlying molecular mechanisms and structural features responsible for many processes including signal transduction, allosteric modulation, functional selectivity and constitutive activity remain elusive[3,4].

Insights from 1.8 Å resolution δ-OR structure

Pioneering studies initiated in 1973 on opioid receptors revealed that physiological concentrations of sodium alter opiate ligand binding and signalling, albeit by unknown mechanisms[5,6]. To address the molecular basis for the striking allosteric effect of sodium on opioid receptor function, we crystallized the human δ-OR (residues 36–338) with an amino-terminal b_{562}RIL (BRIL) fusion protein (BRIL–δOR(ΔN/ΔC)) and determined the crystal structure in complex with the subtype-selective ligand naltrindole[7] at 1.8 Å resolution (Fig. 1, Extended Data Table 1 and Methods). Importantly, the high-resolution BRIL–δOR(ΔN/ΔC)–naltrindole structure contains the wild-type protein sequence, including an intact intracellular loop 3 (ICL3), providing the opportunity to study an opioid receptor that closely resembles a near native conformational state.

The 1.8 Å structure of the human δ-OR is similar to the 3.4 Å *Mus musculus* δ-OR structure fused to T4 lysozyme[8] at the ICL3 site (root mean squared deviation of 0.91 Å over all structurally characterized Cα atoms) with the distinction that the atomic details of regions crucial for receptor activity are revealed. These include: (1) a fully resolved ICL3 adopting a 'closed' inactive state conformation (Fig. 2); (2) a detailed molecular characterization of the orthosteric site with water-mediated ligand–receptor interactions (Extended Data Fig. 1); (3) a distinct conformation of the human third extracellular loop (ECL3) (Extended Data Fig. 2); and, importantly, (4) a high-resolution characterization of the allosteric sodium site, water molecules and a comprehensive network of hydrogen-bond interactions inside the 7TM core (Fig. 1 and Extended Data Figs 3, 4).

All ICL3 residues are well resolved in the BRIL–δOR(ΔN/ΔC)–naltrindole structure. The side-chain guanidinium group of Arg $257^{6.31}$ (superscripts indicate residue numbering using the Ballesteros–Weinstein nomenclature[9]) appears to have a key role in stabilizing ICL3 by forming an extensive hydrogen-bonding network with the main-chain carbonyls of Leu $240^{5.67}$, Arg 244^{ICL3} and Val 243^{ICL3}, and a salt bridge with the carboxylate group of Asp $253^{6.27}$ (Fig. 2a). The Leu 246^{ICL3} and Val 243^{ICL3} side chains insert back in the helical bundle and form a hydrophobic cluster with Val $150^{3.54}$, Leu $240^{5.67}$ and Leu $256^{6.30}$ (Fig. 2b). The loop also interacts with helix III via a water-mediated hydrogen-bond network between the main-chain carbonyl groups of Leu 246^{ICL3} and Val $150^{3.54}$, and the side chain of Arg $239^{5.66}$ (Fig. 2d). These atomic details suggest a stable 'closed' conformation of ICL3 in the inactive δ-OR, which tethers the intracellular ends of helices V and VI. Although it contrasts with the more 'exposed' ICL3 conformations in the thermostabilized A_{2A} adenosine receptor (A_{2A}AR; Protein Data Bank (PDB) accession 3PWH)[10] and rhodopsin (PDB 3CAP)[11] (Fig. 2c), the ICL3 in δ-OR is similar to that observed in the lower resolution NOP structure (PDB 4EA3)[12] (Fig. 2b). A high sequence conservation of ICL3 in all four opioid receptors, which signal primarily via $G\alpha_{i/o}$-proteins, suggests that ICL3 can adopt a similar 'closed' conformation in inactive states of all opioid receptor subtypes. The closed conformation of ICL3 may have a role in stabilizing the inactive state in opioid receptors, and thus compensate for the lack of a stabilizing 'ionic lock' in these receptors, which have a hydrophobic Leu$^{6.30}$ instead of the usual Glu$^{6.30}$ side chain that is required for an ionic lock.

In the orthosteric pocket, the BRIL–δOR(ΔN/ΔC)–naltrindole structure reveals an extensive network of water-mediated interactions with the morphinan group of naltrindole, including interactions with residues

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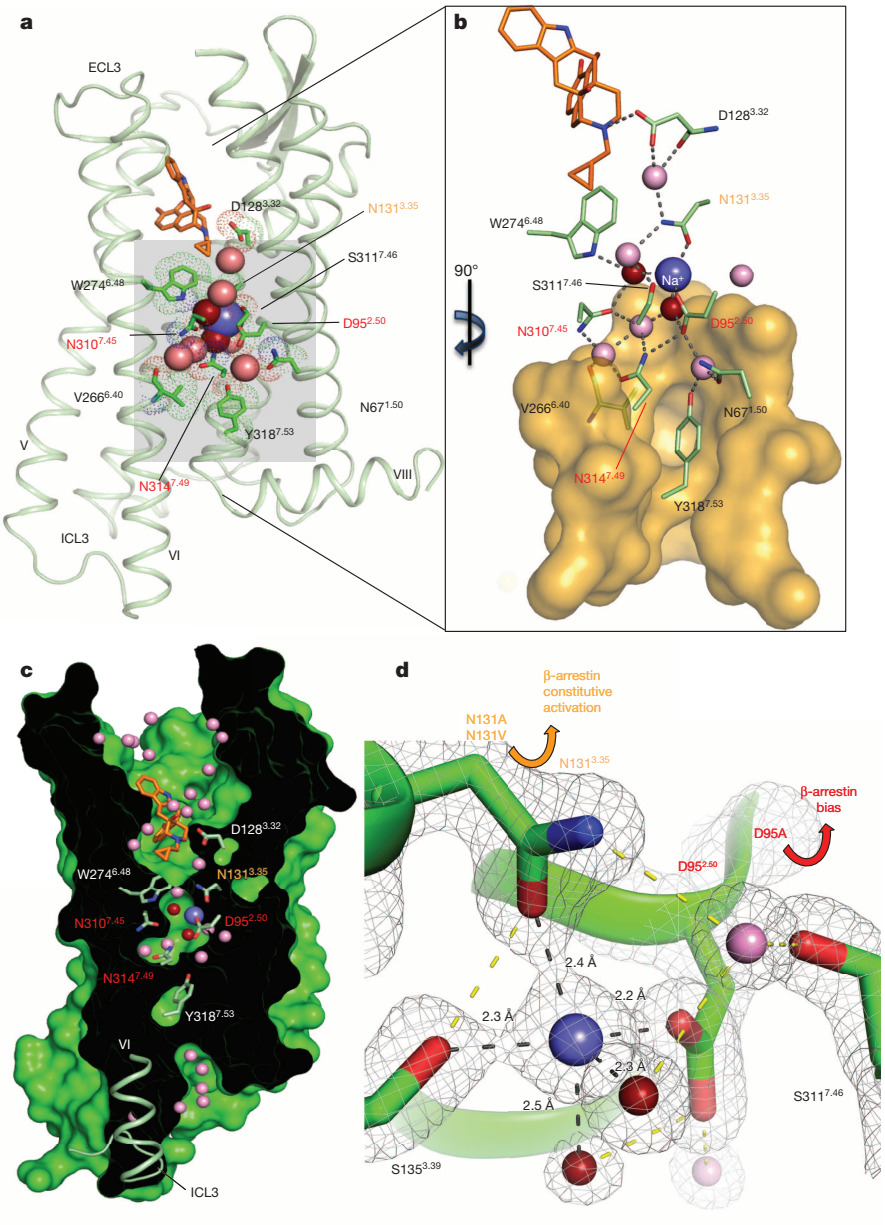

Figure 1 | Interactions in the 7TM core of BRIL–δOR(ΔN/ΔC)–naltrindole. **a**, BRIL–δOR(ΔN/ΔC)–naltrindole structure (light green, BRIL fusion omitted) and residues around the allosteric sodium site (green sticks). Sodium is shown as a blue sphere; red and pink spheres are waters in the first and second coordination shells, respectively. Naltrindole is shown as orange sticks. **b**, Hydrogen bonds (grey dotted lines) and hydrophobic residues (orange surface, located below the allosteric site). **c**, 'Sliced' surface representation of BRIL–δOR(ΔN/ΔC)–naltrindole showing the continuous pathway connectivity between orthosteric and allosteric sites. **d**, $2mF_o - DF_c$ electron density map (grey mesh) contoured at 2σ around residues, waters and sodium in the allosteric site. Hydrogen bonds are shown as black (first sodium ion coordination shell) and yellow (other hydrogen bonds) dotted lines. Arrows indicate increased β-arrestin constitutive activity of 'efficacy switch' mutants Asn131$^{3.35}$Ala and Asn131$^{3.35}$Val (yellow) and β-arrestin-biased activation in response to naltrindole in the Asp95$^{2.50}$Ala mutant (red).

in helix V and ECL2 (Extended Data Fig. 1a). For the ECL3 region, a key selectivity determinant for peptide binding to classical opioid receptors[13], we observe that the side chain of Arg 291^{ECL3} constrains a distinct loop conformation between helices VI and VII through hydrogen-bonding networks with the main-chain carbonyl groups of Val 287^{ECL3} and Trp 284$^{6.58}$, positioning the latter for a π−π interaction with naltrindole (Extended Data Fig. 2). This conformation of ECL3 is quite different from the one observed for the lower resolution *M. musculus* δ-OR structure[8], which has an asparagine side chain instead of the Asp 290^{ECL3} seen in the human δ-OR. These high-resolution details of the binding pocket and ligand interactions in the human δ-OR orthosteric site provide an excellent framework for designing new δ-OR ligands[14] and allosteric modulators[15] with improved selectivity and functional profiles.

Unique features of the δ-OR sodium site

Evidence for the presence of a sodium ion in the allosteric site is similar to that observed in the high-resolution A_{2A}AR structure (PDB 4EIY)[16], including: (1) electron density showing coordination of the proposed sodium position by five oxygen atoms; (2) short distances observed between the ion and coordinating oxygens (~2.4 Å); and (3) calculations of ion valence (Supplementary Table 1). The cavity harbouring the allosteric sodium is formed by the side chains of 16 residues, 15 of which are highly conserved in class A GPCRs (Fig. 1 and Extended

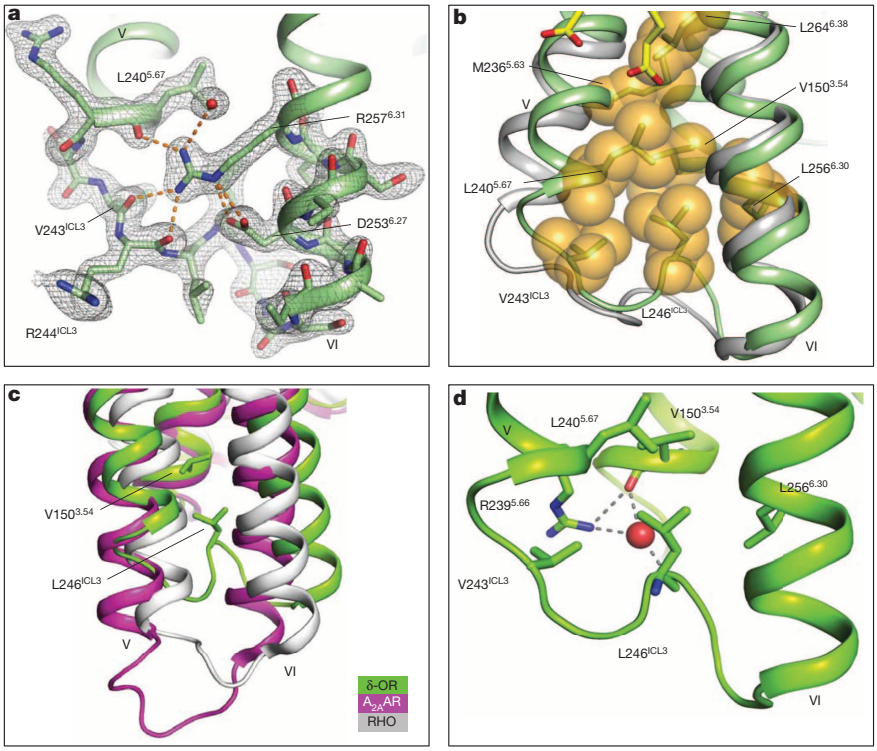

Figure 2 | Structure of the human δ-OR ICL3. **a**, $2mF_o - DF_c$ electron density map (grey mesh) of BRIL–δOR(ΔN/ΔC)–naltrindole ICL3 contoured at 1σ. Polar and ionic interactions of Arg 257$^{6.31}$ are shown by orange dashed lines. **b**, ICL3 loop comparison between BRIL–δOR(ΔN/ΔC)–naltrindole (green) and NOP[12] (light grey) structures. Hydrophobic residues in the ICL3 hydrophobic cluster are shown as orange spheres and oleic acid (OLA) molecules are represented by yellow sticks. **c**, ICL3 comparison of BRIL–δOR(ΔN/ΔC)–naltrindole (green) with A_{2A}AR (magenta; PDB 3PWH) and rhodopsin (RHO, grey; PDB 3CAP). **d**, Details of the hydrogen bonds between Arg 239$^{5.66}$, Leu 246^{ICL3}, Val 150$^{3.54}$ and water molecule (red sphere) are shown by grey dashed lines.

Data Fig. 3). Notably, the structure of BRIL–δOR(ΔN/ΔC)–naltrindole revealed that in addition to the highly conserved Asp 95$^{2.50}$ and Ser 135$^{3.39}$ side chains[16], the sodium ion is directly coordinated by a non-conserved Asn 131$^{3.35}$ side chain. Whereas Asn$^{3.35}$ is conserved among opioid receptors (Extended Data Fig. 3b), the majority (~70%) of class A GPCRs have a hydrophobic residue in this position, and in the high-resolution A_{2A}AR structure the side chain of Leu 87$^{3.35}$ is pointing towards the lipidic membrane[16]. By contrast, in the BRIL–δOR(ΔN/ΔC)–naltrindole structure the Asn 131$^{3.35}$ side chain points into the sodium pocket, placing its oxygen (OD1) and nitrogen (ND2) atoms between the ion and the orthosteric pocket (Fig. 1). These exact atom positions are occupied by two water molecules in the allosteric sodium site of the A_{2A}AR structure (Extended Data Fig. 3a). In addition to the key role of the Asn 131$^{3.35}$ side-chain OD1 atom in sodium coordination, the ND2 atom is hydrogen bonded to both side-chain OD1 and main-chain carbonyl atoms of Asp 128$^{3.32}$ via a water molecule (Fig. 1); the latter residue occupies a central position deep in the orthosteric site and establishes a salt bridge with the nitrogen group of naltrindole. These interactions between the sodium ion, Asn 131$^{3.35}$ and Asp 128$^{3.32}$ establish an apparent axis of connectivity between orthosteric and allosteric regions on the receptor characterized in the inactive state. Altogether, the allosteric sodium of δ-OR is coordinated by five oxygen atoms, from Asp 95$^{2.50}$, Ser 135$^{3.39}$ and Asn 131$^{3.35}$ side chains and two structurally conserved water molecules, which comprises the first coordination shell for the sodium ion (Fig. 1 and Extended Data Figs 3, 4).

The second coordination shell of the sodium ion in the allosteric site is formed by the side chains of three residues (Trp 274$^{6.48}$, Asn 310$^{7.45}$ and Asn 314$^{7.49}$) and two additional water molecules in contact with waters in the first shell (Fig. 1 and Extended Data Figs 3, 4). These conserved residues of the sodium pocket belong to two of the most well-known class A functional motifs: CW$^{6.48}$xP in helix VI and N$^{7.49}$PxxY in helix VII (x denotes any residue) (Fig. 1a), which have a critical role in GPCR activation processes[17]. As a whole, the cluster comprising the sodium ion and eight water molecules mediates extensive intrahelical hydrogen-bond networks between helices I, II, III, VI and VII in the core of the receptor, when it is stabilized in an inactive state conformation. By contrast, the activated agonist-bound structure of A_{2A}AR reveals a sodium site that is collapsed by an inward movement of helix VII[18], suggesting that rearrangements in this conserved sodium pocket have a key role in the activation of class A GPCRs[2].

Functional characterization of δ-OR

To correlate the structural data obtained using BRIL–δOR(ΔN/ΔC) with the wild-type δ-OR, we performed radioligand binding assays with opioid agonists and antagonists with wild-type δ-OR and BRIL–δOR(ΔN/ΔC) constructs expressed in HEK293 and *Sf*9 (*Spodoptera frugiperda*) cells, respectively. We found that the BRIL–δOR(ΔN/ΔC) and wild-type δ-OR displayed similar ligand-binding affinities (Extended Data Table 2) and, when both were expressed in HEK293 cells, they displayed similar functional coupling to $G\alpha_i$-mediated signalling (Extended Data Fig. 5a, b). Consistent with classical studies performed on opioid receptors *in situ*[5,19,20], physiological concentrations of NaCl (140 mM) reduced the affinity of the δ-OR peptide agonist DADLE ([D-Ala2, D-Leu5] enkephalin), which is structurally related to endogenous peptide agonists, at both wild-type δ-OR expressed in HEK293 cells and BRIL–δOR(ΔN/ΔC) expressed in *Sf*9 cells, while having minimal effects on antagonist-binding affinity (Extended Data Table 2). To confirm the specificity of the sodium site, we also examined the effects of other monovalent cations on the binding of ^{3}H-DADLE in saturation binding assays. Among several monovalent cations tested, only sodium at physiological concentrations reduced ^{3}H-DADLE binding (Extended Data Fig. 6).

Sodium modulates δ-OR ligand binding

Although the phenomenon of allosteric modulation of GPCR ligand affinity by sodium ions has been previously described for a number of class A GPCRs (for example, opioid, adrenergic, adenosine and dopamine receptors)[21–24], the nature of this allosteric effect, as well as sodium's affinity for the allosteric site is unknown. To quantify sodium's affinity at its allosteric binding site and to clarify the nature of the apparent negative cooperativity with respect to the peptide

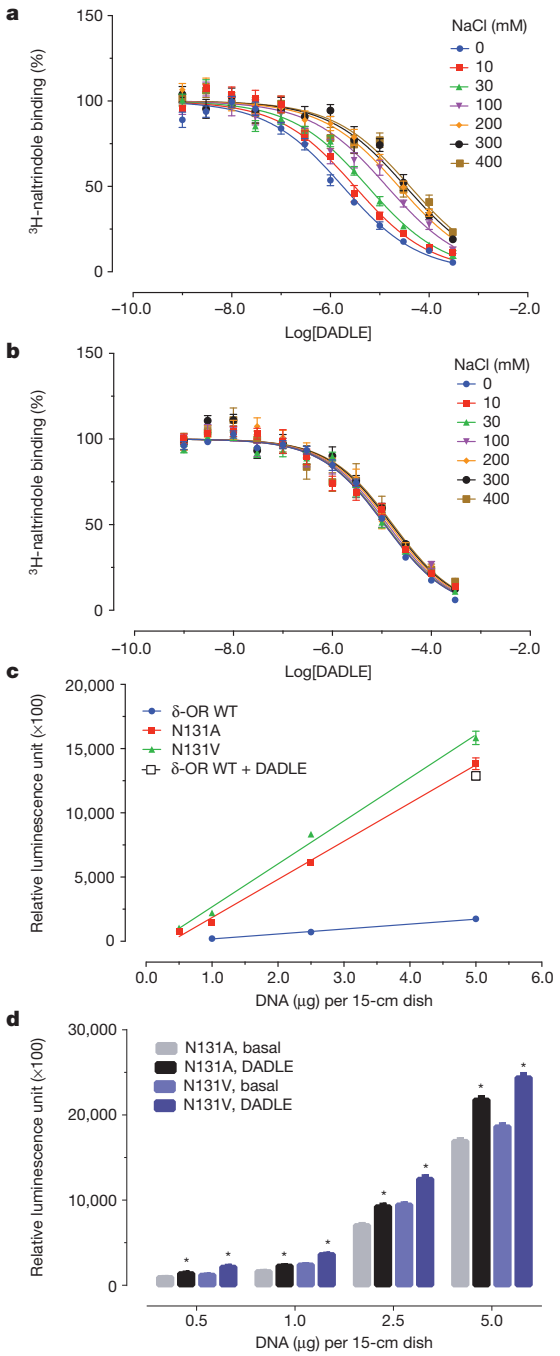

Figure 3 | Effect of sodium site mutations on sodium allosterism and β-arrestin constitutive activity. **a**, **b**, The effects of graded doses of sodium on DADLE affinity were measured at wild-type (**a**) and D95A mutant (**b**). Results were analysed using the allosteric model summarized in Table 1. **c**, The basal activity of Asn131$^{3.35}$Ala and Asn131$^{3.35}$Val is compared to wild type (WT) over a range of DNA dosages and their activity exceeded the one achieved with a saturating concentration of DADLE (10 μM) at wild type (open square). Receptors were expressed at comparable levels (wild type, 226–758 fmol mg^{-1}; Asn131$^{3.35}$Ala, 77–553 fmol mg^{-1}; Asn131$^{3.35}$Val, 176–715 fmol mg^{-1}). Results represent average ± s.e.m. from a minimum of 64 replicates from a representative assay. **d**, Mutants Asn131$^{3.35}$Ala and Asn131$^{3.35}$Val responded to DADLE (10 μM) with a modest degree of stimulation (*$P < 0.01$ (*t*-test) versus no drug addition (basal)). Low-level expression was used allowing the detection of activation by a saturating concentration of DADLE.

agonist DADLE, we performed a series of radioligand binding assays in varying NaCl concentrations and then analysed the results using a standard allosteric model[25]. Our studies revealed that sodium had essentially the same affinity and produced a similar degree of negative cooperativity at both wild-type δ-OR expressed in HEK293 cells and the BRIL–δOR(ΔN/ΔC) expressed in *Sf*9 cells, although the negative cooperativity with DADLE was slightly decreased in the crystallized construct (Fig. 3a, Extended Data Fig. 5c, d and Table 1). These findings confirm that the crystallized construct maintains essentially the same capacity for sodium-dependent allosteric regulation as the wild-type δ-OR. Moreover, by revealing the relatively high sodium affinity to δ-OR ($K_B = 13.3$ mM), our studies demonstrate that at physiological sodium concentrations (140 mM) the sodium site is likely to be saturated.

Asn 131 regulates β-arrestin bias

Given the intimate relationships between residues coordinating allosteric sodium and motifs implicated in GPCR activation (for example, Asn 314$^{7.49}$ and Tyr 318$^{7.53}$ of the NP$^{7.50}$xxY motif; Fig. 1)[4], we predicted that mutating selected sodium site residues could modulate δ-OR functionality. Accordingly, we performed functional studies on selected sodium site mutants (Figs 3 and 4, Table 1, Supplementary Table 2, Extended Data Table 3 and Extended Data Fig. 7) and discovered that mutating the sodium-anchoring δ-OR residue Asn 131$^{3.35}$ into alanine or valine considerably enhanced constitutive activity for the β-arrestin pathway (Fig. 3c, d and Extended Data Fig. 7). Notably, the β-arrestin constitutive activity of Asn131$^{3.35}$Ala or Asn131$^{3.35}$Val mutants exceeded the activation levels of wild-type δ-OR achieved with a saturating concentration of the agonist DADLE (Fig. 3c), whereas $G\alpha_i$ protein basal activity remained unaffected (Extended Data Fig. 7). The Asn 131$^{3.35}$ mutants increased receptor β-arrestin constitutive activity as well as DADLE-binding affinity, as compared with wild-type δ-OR, albeit DADLE efficacy for the β-arrestin pathway was greatly reduced (Supplementary Table 2 and Extended Data Table 3). Importantly, although the Asn131$^{3.35}$Ala mutation abolished the 'sodium effect', the receptor carrying the Asn131$^{3.35}$Val mutation retained sodium ion binding, although with lower affinity compared to wild-type receptor (Table 1).

The key differences in sodium ion binding affinity between the Asn 131$^{3.35}$ mutants provided us with a model system to further clarify the role of sodium on canonical $G\alpha_i$-protein-mediated signalling. Notably, the Asn131$^{3.35}$Ala mutant was inactive whereas the Asn131$^{3.35}$Val mutant, which partially retains the 'sodium effect', maintained $G\alpha_i$ activity, although with reduced agonist potency compared with the wild-type δ-OR (Fig. 4a and Supplementary Table 2). These data indicate that a complete disruption of the interactions between Asn 131$^{3.35}$ and the sodium ion can induce high levels of constitutive activity at non-canonical β-arrestin signalling, while simultaneously abolishing canonical G-protein signalling, essentially inducing an 'efficacy switch' from the $G\alpha_i$ protein pathway to a β-arrestin pathway. These results reveal that the non-conserved residue Asn 131$^{3.35}$ has an essential role in controlling both δ-OR functional selectivity and constitutive activity, probably through its structural role in coordinating the allosteric

Table 1 | Allosteric parameters for sodium at BRIL–δOR(ΔN/ΔC) and wild-type δ-OR

	Na^+ $pK_B \pm$ s.e.m.	Na^+ K_B (mM)	$p\alpha \pm$ s.e.m.	α	Hill coefficient
δ-OR WT (HEK293)	1.88 ± 0.10	13.3	1.93 ± 0.34	0.012	0.56 ± 0.16
D95A (HEK293)	NAE	NAE	NAE	NAE	NAE
D95N (HEK293)	NAE	NAE	NAE	NAE	NAE
N131A (HEK293)	NAE	NAE	NAE	NAE	NAE
N131V (HEK293)	1.11 ± 0.15	77	1.61 ± 0.86	0.025	0.61 ± 0.02
BRIL–δOR(ΔN/ΔC) (*Sf*9)	1.79 ± 0.11	15.9	0.86 ± 0.05	0.138	0.79 ± 0.02

Radioligand binding assays (Fig. 3a, b and Extended Data Fig. 5c, d) were analysed by the allosteric model (see ref. 25 for details). Here α defines the effect of allosteric ligand (in this case, sodium) on orthosteric ligand (in this case, DADLE); an $\alpha > 1$ indicates a positive effect thereby increasing binding affinity, whereas an $\alpha < 1$ indicates a negative effect thereby reducing binding affinity. For the Asp95$^{2.50}$Ala, Asp95$^{2.50}$Asn and Asn131$^{3.35}$Ala mutations, no allosteric effect of sodium was observed (see Fig. 3b for representative data with the Asp95$^{2.50}$Ala mutant).
NAE, no allosteric effect of sodium observed; WT, wild type.

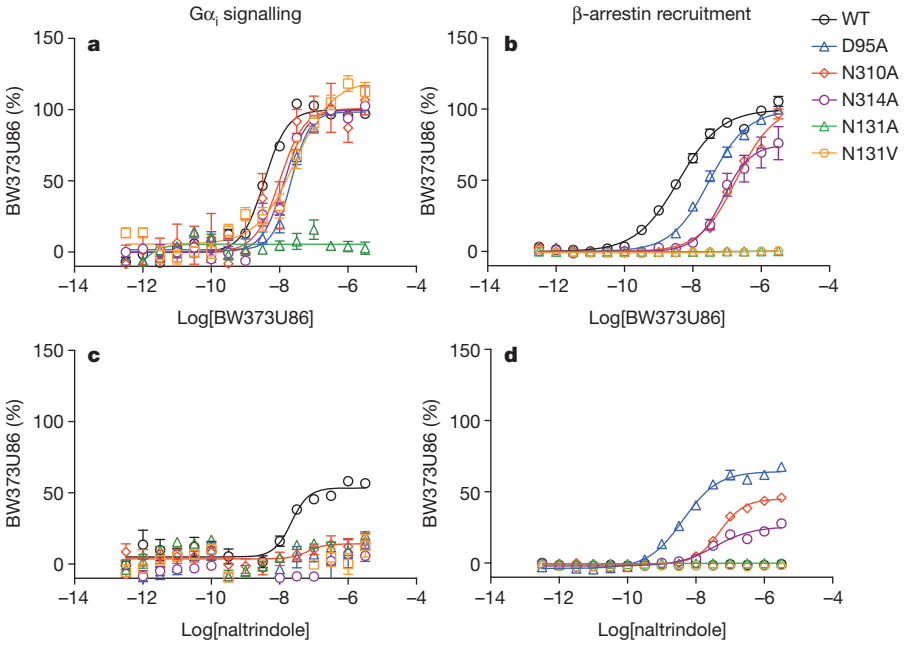

Figure 4 | Sodium-coordinating residues form an efficacy switch regulating biased signalling. Mutation of the sodium-anchoring residues Asp95$^{2.50}$, Asn310$^{7.45}$ and Asn314$^{7.49}$ promotes efficacy switching of the cyclopentene-containing antagonist naltrindole into a potent β-arrestin-biased agonist. **a**–**d**, Normalized concentration-responses of δ-OR-mediated $G\alpha_i$ signalling induced by BW373U86 (**a**) and naltrindole (**c**), and δ-OR-mediated β-arrestin recruitment with BW373U86 (**b**) and naltrindole (**d**) were quantified as in Methods. Results represent average ± s.e.m. of four independent experiments each in quadruplicate and are presented as percentage of activation by BW373U86. Receptors were all transfected with 15 μg DNA revealing weak partial agonist activity of naltrindole at $G\alpha_i$ signalling as described previously[29,30].

sodium. We also compared basal $G\alpha_i$ protein activity for the sodium site δ-OR mutants (Extended Data Fig. 7) and found that the mutants have constitutive $G\alpha_i$ activity similar to the wild-type δ-OR, with a minor increase at Asn314$^{7.49}$Ala mutant and decrease at Asn131$^{3.35}$Ala and Asn131$^{3.35}$ Val mutants. The strong β-arrestin-biased activity observed with Asn131$^{3.35}$Ala and Asn131$^{3.35}$Val could contribute to this small reduction by displacing $G\alpha_i$ protein.

Sodium-dependent opioid pharmacology

We next examined Asp 95$^{2.50}$, another key sodium site residue, and found that mutation of this residue into either alanine or asparagine abolished the 'sodium effect' in radioligand binding assays (Fig. 3b and Table 1). The small molecule agonist BW373U86 has been previously described as a selective orthosteric δ-OR agonist for which binding to the receptor is minimally affected by sodium ions[26]. Consequently, BW373U86 displays a low-to-moderate reduction in G-protein and β-arrestin signalling at sodium-anchored-residue mutants compared to wild type (Fig. 4a, b). On the other hand, the peptide agonist DADLE has a diminished potency for β-arrestin recruitment at the δ-OR mutant Asp95$^{2.50}$Ala and is inactive at both the Asn310$^{7.45}$Ala and the Asn314$^{7.49}$Ala mutants, while showing low-to-moderate reduction in G-protein-agonist efficacy (Supplementary Table 2). Importantly, we discovered that classical δ-OR ligands containing a cyclopentene functional group (naltrindole, naltriben and 7-benzylidenenaltrexone (BNTX)), which display no apparent agonist activity at the β-arrestin pathway with the wild-type δ-OR, gained potent β-arrestin-biased agonist activity at the Asp95$^{2.50}$Ala sodium site mutant (Fig. 4d and Supplementary Table 2). A similar transformative effect is observed when the sodium site residues Asn 310$^{7.45}$and Asn 314$^{7.49}$ were mutated to alanine (Fig. 4d and Supplementary Table 2). The conversion of these antagonists/weak partial agonists into β-arrestin-biased agonists by mutations in the allosteric sodium site, together with the effects of Asn 131$^{3.35}$ mutants described above, uncovered what we characterize as 'efficacy switches' within δ-OR. These efficacy switches are apparently distinct from those previously reported at which only G-protein signalling was enhanced[27,28].

The allosteric sodium-binding pocket described here in atomic detail is potentially an attractive drug discovery target. Although the binding of small molecule allosteric modulators in this highly conserved site is unlikely to have desired subtype selectivity, extension of selective orthosteric ligands into the sodium cavity may lead to bitopic compounds with new pharmacological properties, for example, inverse agonism or strong functional bias. The detailed crystal structure may also help to identify the binding site and shed light on the mode of action for the other types of positive allosteric modulator (PAM) compounds, such as those recently reported in ref. 15.

Our results reveal a profound and essential role for allosteric sodium-anchoring residues at specifying GPCR signal transduction and pharmacology. Mutation of sodium-anchoring residues within the allosteric site selectively modulates not only agonist binding, but also markedly changes GPCR functional activity by augmenting β-arrestin constitutive activity and introducing new patterns of biased signalling. In particular, these findings highlight the unexpectedly essential role for sodium-coordinating amino acids as efficacy switches for GPCR signalling.

METHODS SUMMARY

The BRIL–δOR(ΔN/ΔC) construct has its first 35 amino-terminal residues (ΔN 35) replaced with the thermostabilized apocytochrome b_{562} RIL (M7W, H102I, R106L; BRIL) and 34 carboxy-terminal residues (ΔC 34) deleted and was expressed in *S. frugiperda* (*Sf9*) insect cells for structural and radioactive ligand-binding experiments. *Sf9* membranes were solubilized in 0.75% (w/v) *n*-dodecyl-*β*-D-maltopyranoside and 0.15% (w/v) cholesteryl hemisuccinate in the presence of 25 μM naltrindole, and purified by immobilized metal ion affinity chromatography. Receptor crystallization was performed using the lipidic cubic phase (LCP) method. The protein–LCP mixture contained 40% (w/w) protein solution, 54% (w/w) monoolein (Sigma) and 6% (w/w) cholesterol. Crystallization trials were performed using 40 nl protein-laden LCP overlaid with 0.8 μl precipitant solution (31–34% (v/v) PEG 400, 0.095–0.12 M K/Na tartrate, 5% (v/v) ethylene glycol, 100 mM MES buffer, pH 6.1–6.2, and 1 mM naltrindole) at 20 °C. Crystallographic data were collected on the 23ID-D beamline (GM/CA CAT) of the Advanced Photon Source at the Argonne National Laboratory using a 20-μm collimated minibeam. Data sets from 47 different crystals were merged for the final data set (Supplementary Table 1). cAMP assays were performed in HEK293T cells co-transfected with

human wild-type δ-OR or various mutants along with a split-luciferase-based cAMP biosensor (GloSensor; Promega). δ-OR β-arrestin-recruitment assays were performed using the Tango assay as described in Methods. ^{3}H-naltrindole-binding assays were performed using *Sf*9 membranes expressing the crystallized construct BRIL–δOR(ΔN/ΔC) or HEK293 T-cell membrane preparations transiently expressing wild-type or mutant δ-OR receptors.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions G.F. designed, optimized and purified δ-OR receptor constructs for structural studies, crystallized the receptor in LCP, collected and processed diffraction data, determined the structure, analysed the data and wrote the paper. P.M.G. performed mutagenesis and signalling studies, analysed the data and wrote the paper. X.-P.H. performed ligand binding and signalling studies, analysed the data and wrote the paper. V.K. analysed the data and wrote the paper. A.A.T. designed and cloned initial δ-OR constructs. V.C. analysed the data and wrote the paper. B.L.R. supervised the pharmacology and mutagenesis studies, analysed the data and wrote the paper. R.C.S. was responsible for the overall project strategy and management, analysed the data and wrote the paper.

Author Information The coordinates and the structure factors have been deposited in the Protein Data Bank under accession code 4N6H. 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 the paper. Correspondence and requests for materials should be addressed to R.C.S. (stevens@scripps.edu) or B.L.R. (bryan_roth@med.unc.edu).