Review

# Grand opening of structure-guided design for novel opioids

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**Twelve years after the publication of the first crystal structure of a G-protein-coupled receptor (GPCR), experimental crystal structures of the four opioid receptor subtypes have made their entrance into the literature in the most extraordinary way, that is, all at once. Not only do these crystal structures contribute unprecedented molecular details of opioid ligand binding and specificity, but they also represent important tools for structure-based approaches to guide the discovery of safer and more efficient opioid therapeutics. We provide here an overview of these latest breakthroughs in the structural biology of GPCRs with a focus on differences and similarities between the four opioid receptor structures, as well as their limitations, in the context of challenges for translation of this new knowledge from bench to bedside.**

## Opioids and their receptors

Opioids such as morphine, codeine, and fentanyl comprise a major class of analgesics used in the clinical management of pain. They exert their effects through activation of three major opioid receptor families [1,2], the μ-, δ-, and κ-opioid receptors (MOP, DOP, and KOP receptors, respectively, according to a IUPHAR subcommittee recommendation [3]), signaling primarily through interaction with the inhibitory G protein for adenylyl cyclase, $G_{i/o}$ [4]. These receptors were cloned in the early 1990s and belong to the superfamily of GPCRs [4]. Not only are they targets for a diverse set of small molecules, from classical morphine derivatives (also known as morphinans) to a variety of other classical and non-classical opioid ligands, but they can also be activated by peptides [5]. The latter include endogenous opioids such as endorphins, endomorphins, and morphiceptin for the MOP receptor, enkephalins for the DOP receptor, and dynorphins for the KOP receptor. The endogenous nociceptin/orphanin FQ peptide binds selectively to a fourth member of the opioid receptor family that was discovered much later [6,7] and is known as NOP or ORL-1 receptor. Despite the high sequence identity between this receptor and the other major opioid receptor subtypes {67% in the transmembrane (TM) region [8]}, the NOP receptor does not bind with the same high affinity to morphinans and other opioid ligands [6,7].

Although they are the best analgesics currently on the market, the effective medical use of opioid ligands is usually obscured by myriad undesirable side effects [4,5], including nausea, vomiting, and constipation. In addition, prolonged use of opioids often leads to the development of tolerance and/or addiction. The latter, together with a pronounced sense of euphoria, makes them among the most frequently abused drugs in the USA [9]. Although considerable resources have been devoted to the discovery of safer opioids over the past decades, the promise of more effective analgesics has gone largely unfulfilled. There are several reasons for this. First, atomic-level structural information on opioid receptors in particular, and GPCRs in general, has traditionally lagged behind because of technical difficulties in purifying and crystallizing these complex membrane protein systems. Second, similar to the other GPCRs, the biology of opioid receptors is quite complex. In fact, opioid ligands acting at these receptors can activate multiple signaling pathways, most often through either $G_{i/o}$ or arrestins. Preferential activation of a specific signaling pathway by a ligand has been termed functional selectivity, ligand-directed signaling/receptor trafficking and/or biased agonism [10,11]. Examples of this complex signaling have recently been reported in the case of all major opioid receptors [12]. For instance, it has recently been shown that opioids such as morphine and oxymorphone are effective agonists for G protein coupling but competitive antagonists or partial agonists for arrestin at the DOP and MOP receptors, respectively [13]. Similarly, 6′-guanidinonaltrindole (6′-GNTI), a potent partial agonist at the KOP receptor for G protein activation, functions as an antagonist to block arrestin recruitment [14]. By contrast, it was recently shown that the KOP receptor partial agonist 12-*epi*-salvA is an arrestin-biased ligand, and is an efficacious activator of β-arrestin-2-mediated signaling pathways [15]. Given that the most addictive opioid ligands appear to promote interactions with $G_{i/o}$ more strongly than with arrestins [13] and that, unlike analgesia, the adverse dysphoric effects induced by opioid receptor activation appear to be arrestin-dependent (at least in the case of KOP [16]), identification of effective biased opioid ligands for one protein or another represents a new direction for the development of non-addictive and/or effective analgesics. It has also been suggested that selective and specific modulation of opioid receptor signaling and function leading to analgesia without the adverse side effects depends on new therapeutic targets resulting from interaction of opioid receptors among themselves or with other GPCRs to form dimers and/or higher-order oligomers [17,18].

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The past few months have offered real breakthroughs in the opioid receptor field through the release of high-resolution crystal structures of all the existing opioid receptor subtypes. These structures correspond to Protein Data Bank (PDB) identification codes 4DKL for the MOP receptor [19], 4EJ4 for the DOP receptor [20], 4DJH for the KOP receptor [21], and 4EA3 for the NOP receptor [22]. Notably, they have provided the first high-resolution molecular insight into the selective binding of classical (e.g., morphinan derivatives) and non-classical opioid ligands to their receptors. In this review, we draw attention to differences and similarities between the different opioid receptor crystal structures, as well as other available GPCR crystal structures, and discuss their implications and limitations in the structure-based development of safer painkillers and anti-addiction medications.

## A common molecular architecture

The newly available crystal structures of mouse MOP [19], mouse DOP [20], human KOP [21], and human NOP [22] receptors at resolution of 2.8, 3.4, 2.9, and 3.0 Å, respectively, represent important milestones in our understanding of opioid receptor function. Figure 1 illustrates the overlap of the typical GPCR seven-pass TM helix folds of the four opioid receptor crystal structures. These TM helices are connected by three intracellular loops (ILs) and three extracellular loops (ELs). As expected from the high sequence identity among the TM regions of these receptors (from a minimum of 67% for the NOP receptor compared to the other opioid receptors to a maximum of 76% between the MOP and DOP receptors [8]; Figure 2), these structures share a very similar structural fold, even in the less conserved loop regions. This is especially evident in the characteristic β-hairpin structure of the EL2, which is interestingly also present in the crystal structure of the chemokine receptor CXCR4 [23], another GPCR that binds peptides.

As shown in Figure 1, the largest difference between the opioid receptor crystal structures is found in the extracellular half of TM1, which appears to be much straighter in the KOP structure compared to those of the DOP, MOP, and NOP receptors. Notably, the KOP receptor TM1 also differs from the equivalent portion of the CXCR4 crystal structure [23]. However, different conformations of TM1 have been observed even within the same crystal, as seen in the case of the turkey β1-adrenergic receptor, suggesting that different crystallization conditions or crystal contacts might be responsible for these structural deviations [24]. Particularly different is the EL3 link between TM6 and TM7 (Figure 1), which exhibits low sequence conservation (Figure 2) and high temperature factors in all opioid receptor structures. Notably, this loop has not been resolved in the KOP receptor.

## Crystal contacts or physiological interfaces?

As for the majority of GPCR crystal structures available, the four opioid receptor structures correspond to engineered receptor forms in which the T4 lysozyme (or T4L

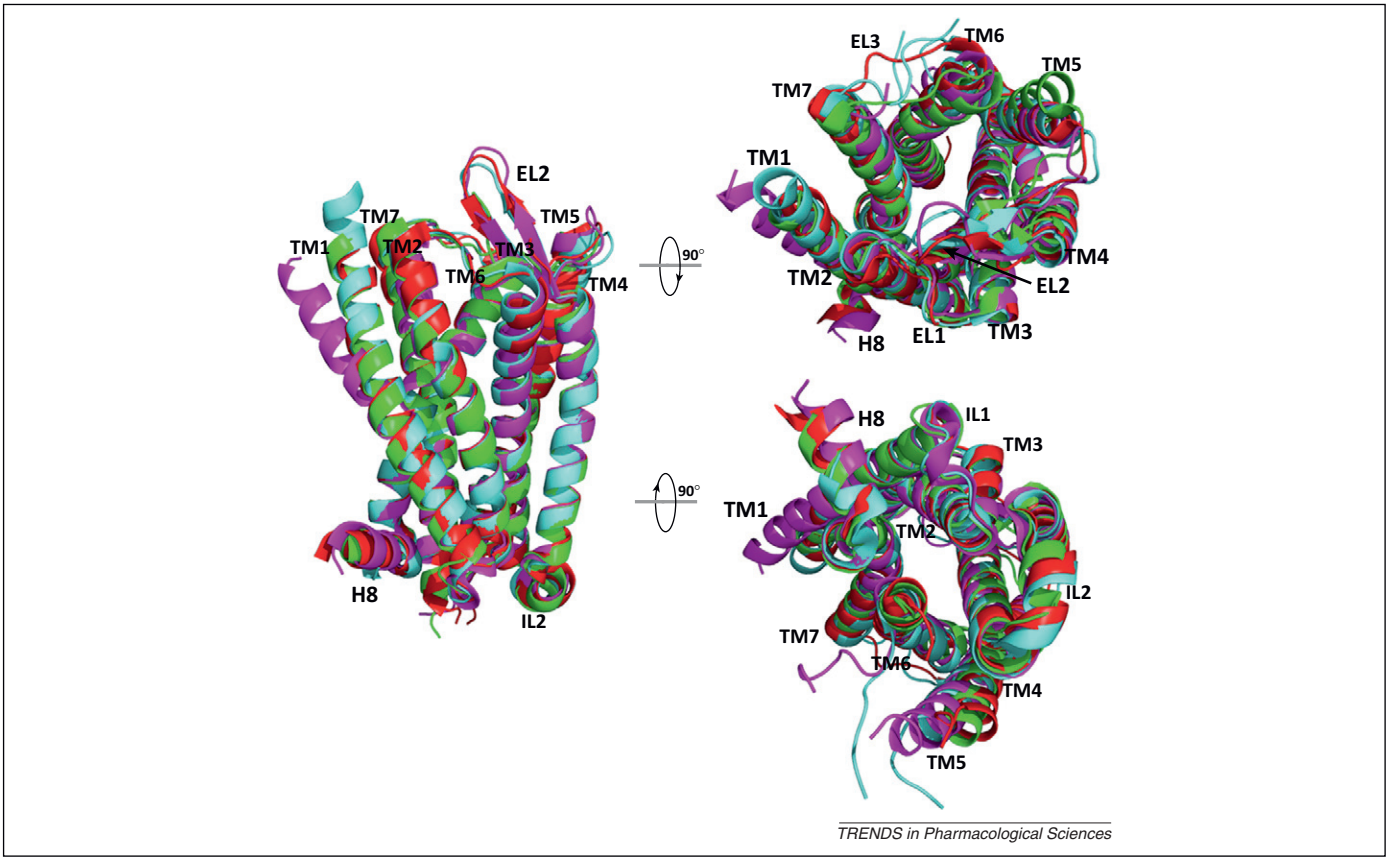


**Figure 1**. Vertical (left), extracellular (top right), and intracellular (bottom right) views of the overlap of the four opioid receptor crystal structures that are currently available. The μ-, δ-, κ-, and nociceptin opioid receptor crystal structures corresponding to Protein Data Bank (PDB) identification codes 4DKL, 4EJ4, 4DJH, and 4EA3 are shown in red, cyan, magenta, and green, respectively.

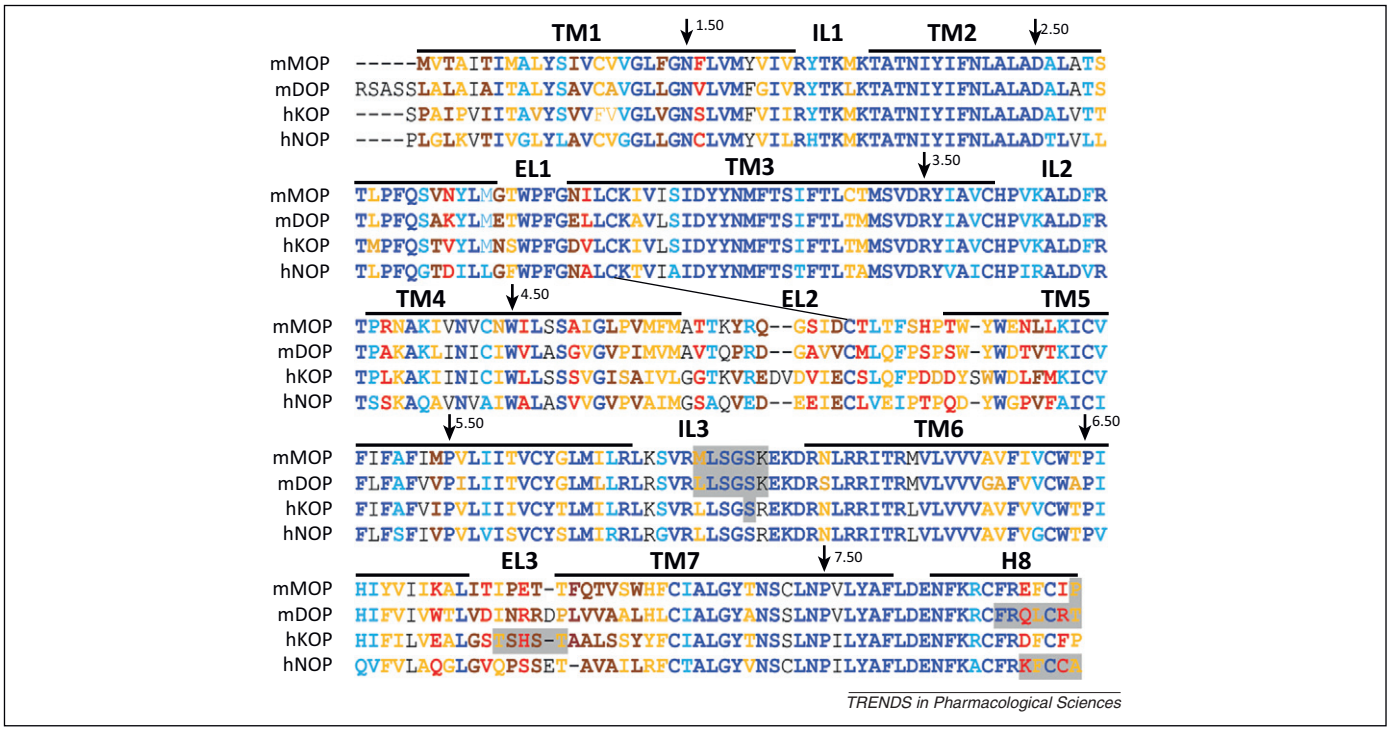


**Figure 2**. Sequence alignment of the four crystallized opioid receptors. The transmembrane (TM) helices and loop regions are indicated and labeled accordingly. Small arrows indicate the most conserved residue in each TM helix, which is assigned a number 50 in the Ballesteros–Weinstein numbering scheme. Identical residues in all four receptors are shown in blue. Identical resides in the μ-opioid (MOP), δ-opioid (DOP), κ-opioid (KOP) receptors but unique to the nociceptin opioid (NOP) receptor are shown in cyan. Divergent residues in all four opioid receptors are shown in red. Divergent residues in the MOP, DOP, and KOP, but not in NOP receptors are shown in brown. Unique residues to either MOP, DOP, or KOP receptors are shown in orange. Missing residues in crystal structures are highlighted in grey. N and C termini are not included in the alignment.

for MOP, DOP, and KOP receptors) or the thermostabilized apocytochrome $b_{562}$RIL (or BRIL for the NOP receptor) have been introduced to stabilize the receptor in a specific conformation that is amenable to crystallization. Specifically, T4L replaces the third intracellular loop in the MOP, DOP, or KOP receptor constructs used for crystallography, whereas BRIL replaces most of the N-terminal domain in the NOP receptor construct. In all of these receptor constructs, the poorly ordered N- and C-terminal domains have been removed.

Figure 3 illustrates the different crystal contacts involving the TM domains of the four opioid receptor structures. As evident from the location of T4L and BRIL, receptors adopt either parallel or anti-parallel orientations in the crystals. Specifically, as shown in Figure 3a,d, the DOP and NOP receptors only crystallized with anti-parallel arrangements of the receptors. By contrast, the MOP receptor (Figure 3b) exhibits exclusively parallel arrangements in its crystals, characterized by two different interfaces, a very compact one involving TM5 and TM6 with 28 residues involved in the interaction, and a less compact interface involving TM1, TM2, and H8. Notably, the latter is also present in the KOP receptor crystals (Figure 3c), which also feature an anti-parallel arrangement. The TM5/TM6 interface was also found in five independent crystal structures of the chemokine receptor CXCR4 [23]. In all of these structures, as well as in that of the MOP receptor, contacts between the T4L domains on each protomer account for a portion of the buried surface area across the interprotomer interface [19,23]. In the CXCR4 crystal structures, this portion is more extended than in the MOP receptor crystal structure. Although it is tempting to speculate that the parallel arrangements found in MOP and KOP receptors are indicative of physiologically relevant dimerization interfaces, it should be kept in mind that these are the results of crystallogenesis and thus might depend on the crystallization conditions and/or the different T4L interactions in the crystal.

The existence of opioid receptor dimers or higher-order oligomers with unique pharmacology and signaling properties has been postulated based on the results of several pharmacological and biochemical studies [17,18]. Prior to the opioid receptor crystal structures, it was suggested that TM1, TM4, and TM5 most often play a role in the interaction between opioid receptors. Indeed, a combination of computational and biochemical studies of the DOP receptor suggested the involvement of TM4 and/or TM5 at the dimerization interface, albeit with differing association propensities [25]. Several hypotheses of opioid receptor dimerization disruption have also been put forward recently. For instance, it was reported that a TAT-fused peptide composed of the MOP TM1 disrupts a possible interaction between MOP and DOP in the mouse spinal cord, with consequent augmentation of morphine analgesia and reduction of antinociceptive tolerance to morphine [26]. Recent studies have also reported that substitution of residues in the cytoplasmic carboxyl tail of the DOP receptor or the IL3 region of both MOP and DOP prevent heteromer formation [27]. Notably, the existence of endogenous MOP–DOP or DOP–KOP heteromers with unique pharmacological and signaling properties [28,29] have recently been supported by the use of antibodies that

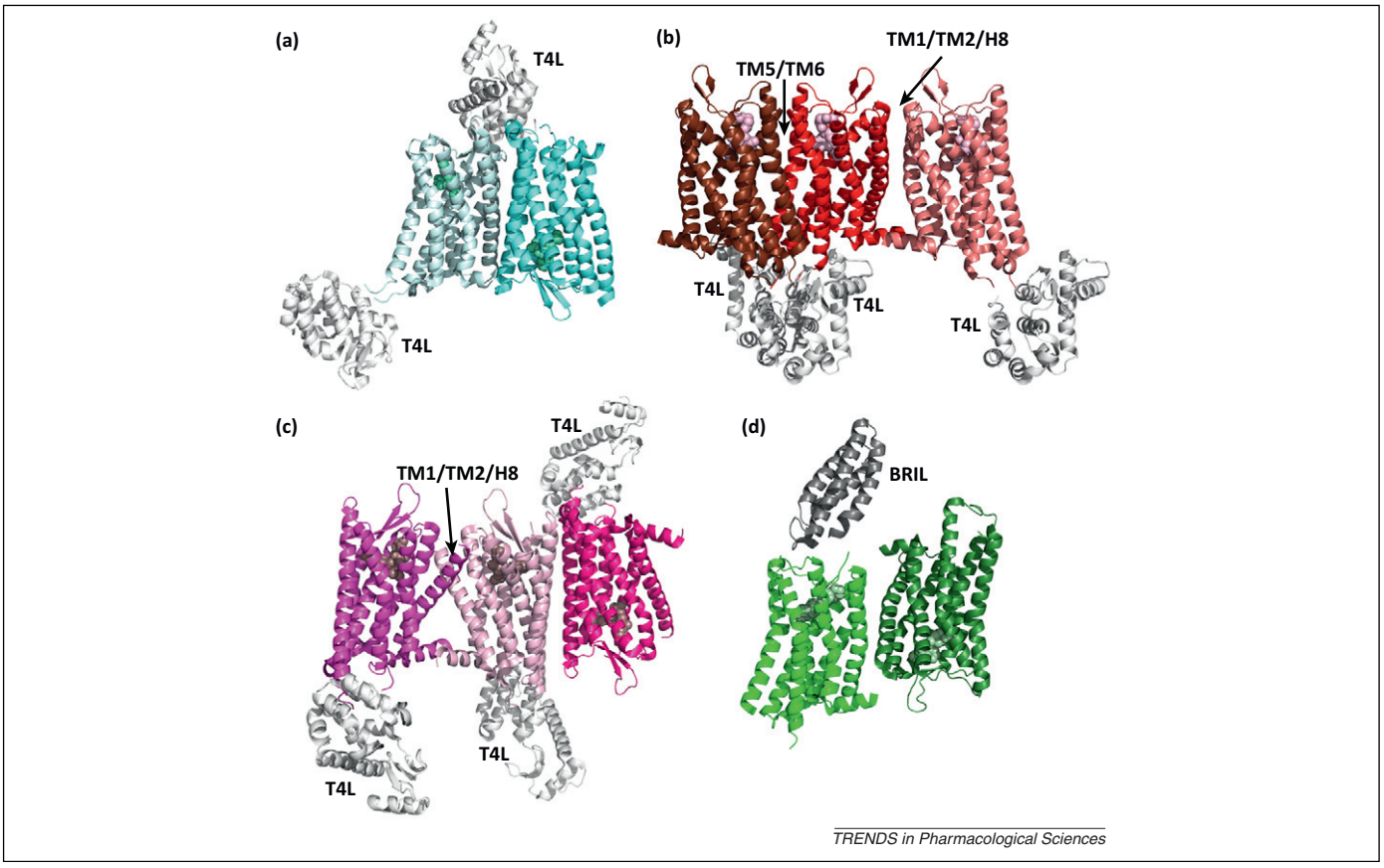


**Figure 3**. Crystal contacts involving the transmembrane (TM) domains of the four opioid receptor structures. As evident from the location of T4L (white cartoon) and BRIL (grey cartoon) in these figures, receptors adopt either parallel or anti-parallel orientations in the crystals. **(a)** Anti-parallel arrangement of the δ-opioid (DOP) receptor. **(b)** Parallel arrangement of the μ-opioid (MOP) receptor with TM5 and TM6 or TM1, TM2, and H8 at the interface. **(c)** Parallel (interface TM1, TM2, and H8) and anti-parallel arrangement of the κ-opioid (KOP) receptor. **(d)** Anti-parallel arrangement of the nociceptin opioid (NOP) receptor.

appear to recognize co-expressed receptors but not the individual proteins [30,31]. Given the different hypotheses for interfaces of opioid receptor dimerization, including those inspired by the recent receptor crystal structures, it is clear that additional studies are needed to understand how endogenous opioid receptors interact in their natural membrane environment.

## Molecular determinants of ligand recognition and selectivity

Current opioid receptor crystal structures have advanced our understanding of the way in which antagonists bind within the bundle of the receptor TM helices stabilized in an inactive conformation. Specifically, the MOP receptor has been crystallized covalently bound to the irreversible morphinan β-funaltrexamine [32] through an amino acid residue that had originally been suggested by mutagenesis studies [33]. By contrast, DOP, KOP, and NOP receptor crystal structures have been determined in the presence of non-covalent ligands, specifically naltrindole [34], James D. Thomas Tetrahydroisoquinoline carboxylic acid (JDTic) [35], and the peptide-mimetic compound 24 [36], respectively. The chemical structures of these four antagonists are illustrated in Figure 4.

The receptor-binding pockets that accommodate these opioid ligands are solvent-exposed and similar in shape. Figure 4 shows an overlay of the ligands in a representative opioid receptor crystal structure along with schema of the interaction modes for β-funaltrexamine, naltrindole, JDTic, and compound 24 in the MOP, DOP, KOP, and NOP receptor crystal structures, respectively. Residues in this figure and throughout the text are numbered according to the amino acid sequences of mouse MOP, mouse DOP, human KOP, and human NOP receptors, as well as the corresponding Ballesteros–Weinstein numbering scheme [37]. In this two-number scheme, the first indicates the TM helix number and the second one is relative to the most highly conserved residue in each TM helix indicated by the number 50. Notably, the opioid-receptor-binding pockets resemble that of CXCR4 [23], suggesting yet another commonality among GPCRs that bind both peptides and small molecules. As shown in the central panel of Figure 4, ligands occupy a common region delimited by helices TM3, TM5, TM6, and TM7 in the binding pockets of MOP, DOP, KOP, and NOP receptors. Notwithstanding the slight distortion in the binding mode of β-funaltrexamine (owing to its covalent tether to K5.39 of the MOP receptor), binding of morphinan ligands β-funaltrexamine and naltrindole is mostly confined to the TM3–TM5–TM6–TM7 section of the binding pockets of MOP and DOP receptors, respectively. By contrast, binding of ligands JDTic and compound 24 to the KOP and NOP receptors, respectively, extends to a region of the binding pocket defined by TM2, TM7, and TM3.

Figure 5 shows surface views of a representative opioid receptor crystal structure with residues colored according

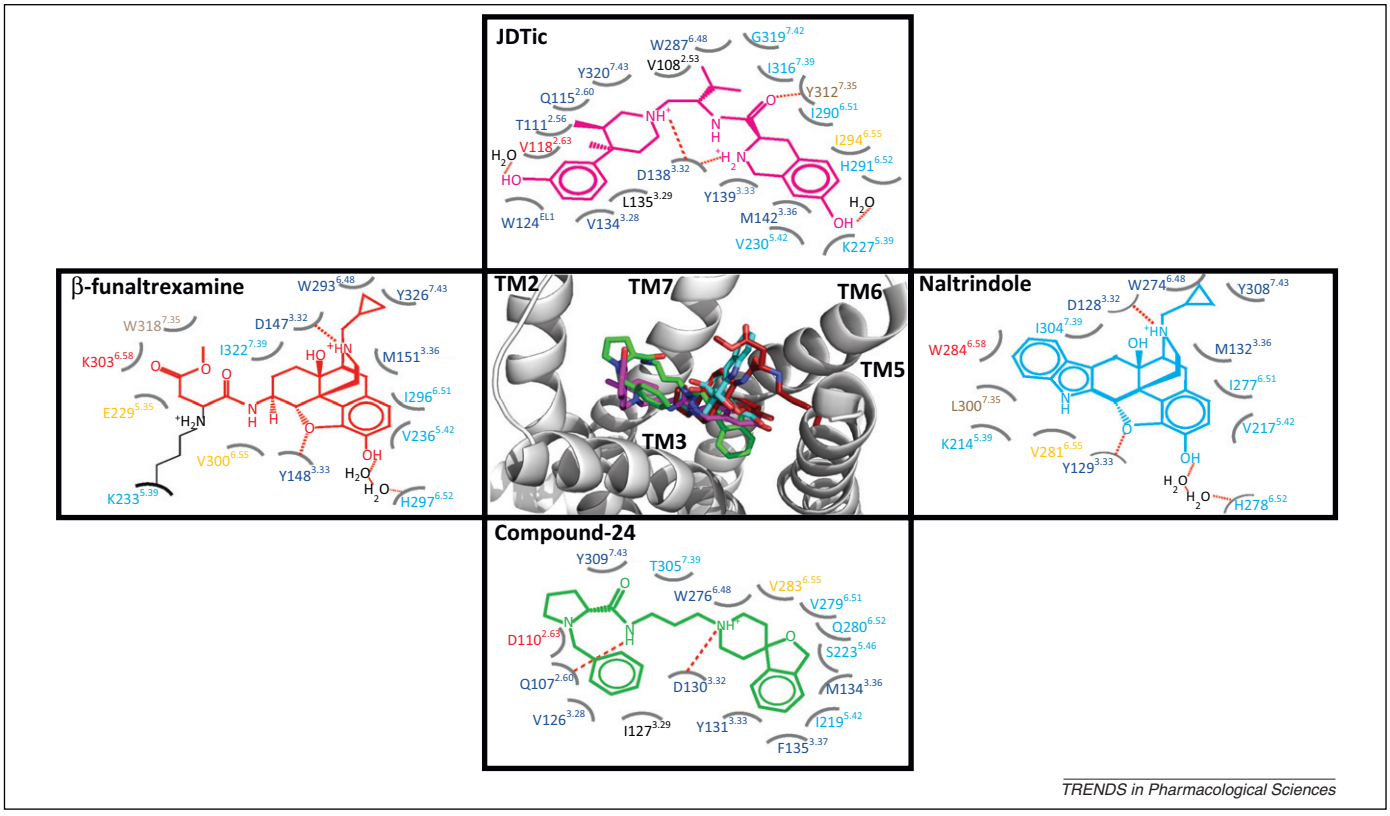


**Figure 4**. Overlay of the crystallized opioid ligands in a representative opioid receptor crystal structure along with schema of their interaction modes in each crystal structure. The central panel shows an overlay of β-funaltrexamine (red), naltrindole (cyan), James D. Thomas tetrahydroisoquinoline carboxylic acid (JDTic) (magenta), and compound 24 (green) in the MOP receptor crystal structure, which is partially shown in a grey cartoon representation. Interaction schema for β-funaltrexamine, naltrindole, JDTic, and compound 24 in the μ-opioid (MOP), δ-opioid (DOP), κ-opioid (KOP), and nociceptin opioid (NOP) receptor crystal structures are shown in the left, right, upper, and lower panels, respectively. Residues within 4 Å of any atom in the ligands are numbered according to the amino acid sequences of mouse MOP, mouse DOP, human KOP, and human NOP, as well as the corresponding Ballesteros–Weinstein numbering scheme. Identical residues in all four receptors are shown in blue. Identical resides in MOP, DOP, and KOP but unique to NOP are shown in cyan. Divergent residues in all four opioid receptors are shown in red. Divergent residues in MOP, DOP, and KOP but not NOP are shown in brown. Unique residues to either MOP, DOP, or KOP are shown in orange.

to sequence conservation. As shown by a blue surface in the middle of the TM bundle, which coincides with the bottom part of the binding pocket, this region is highly conserved, because it is the cytoplasmic side of the receptor. Although the former suggests the presence of common molecular determinants for recognition of the portion of opioid ligands that may be responsible for their efficacy (traditionally referred to as the message part of opioid ligands), the latter denotes similarities among the opioid receptors owing to their binding of the same G protein and arrestin subtypes. The fully conserved interacting residues in MOP, DOP, KOP, and NOP receptors that are within 4 Å of any atom in the ligands (Figure 4 and Table 1) are D3.32, Y3.33, M3.36, W6.48, and Y7.43. Notably, residue Y7.43 acquires a slightly different orientation in the KOP binding pocket compared to MOP, DOP, and NOP receptors, which seems to be necessary to accommodate the KOP receptor ligand JDTic. Additional interacting residues that are conserved in three of the opioid receptors are listed in Table 1. Although no crystallographic water molecules could be seen in the DOP receptor structure owing to its low resolution compared to the MOP, KOP, and NOP receptor structures, it is tempting to speculate on the presence of two conserved water molecules that could form a hydrogen bonding network between the ligands and the H6.52 residue of MOP, DOP, and KOP receptors. As reported above, this residue is a glutamine in the NOP receptor.

Figure 5 also shows that the majority of divergent residues, possibly conferring subtype specificity, are either located in the upper part of the binding pocket or in the portion of the binding pocket delimited by TM2, TM3, and TM7. The location of the majority of these residues in the upper part of the binding pocket has led to the suggestion [38] that these might form a selectivity filter [39], which happens to resemble the allosteric binding region identified in the crystal structures of muscarinic M2 and M3 receptors [40,41]. The ligand chemical moieties responsible for opiate selectivity (the so-called address portion of the ligand) interact with some of these divergent residues of the receptors shown in Figure 4. For instance, L7.35 represents a selectivity determinant for naltrindole because it corresponds to sterically incompatible residues, specifically tryptophan and tyrosine, in the MOP and KOP receptors, respectively. In the NOP receptor, there is also a leucine at this position, but it is far away (>7 Å) from the NOP receptor ligand compound 24. Additional interacting residues that may be responsible for selectivity are listed in Table 1.

## New opportunities for discovering novel opioid ligands

The newly released crystal structures suggest that although the chemical moieties responsible for opioid ligand efficacy interact similarly within the seven-TM helical bundle, two different regions of the binding pocket (the upper region mostly defined by TM5, TM6, and TM7, and

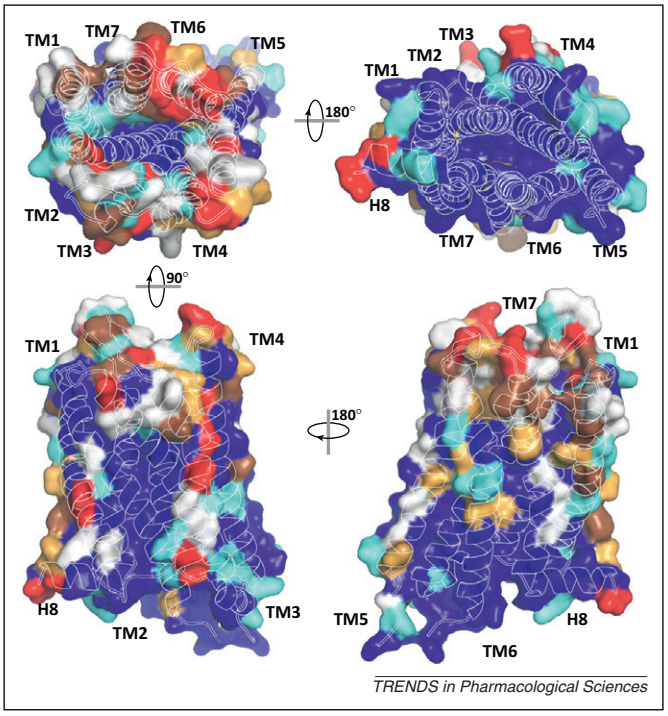


**Figure 5**. Surface views of a representative opioid receptor crystal structure with residues colored according to sequence conservation. Left and upper right panels indicate horizontal views (extracellular and cytoplasmic, respectively) of a representative opioid receptor crystal structure. Lower panels show vertical views of the same receptor. Identical residues in all four receptors are shown in blue. Identical residues in the μ-opioid (MOP), δ-opioid (DOP), κ-opioid (KOP) receptors but unique to the nociceptin opioid (NOP) receptor are shown in cyan. Divergent residues in all 4 opioid receptors are shown in red. Divergent residues in MOP, DOP, and KOP, but not in NOP receptors are shown in brown. Unique residues to either MOP, DOP, or KOP receptors are shown in orange.

**Table 1. Residues within a distance of 4 Å from ligands in the opioid receptor crystal structures[a]**

| Interacting residues | MOP | DOP | KOP | NOP |
|---|---|---|---|---|
| A/A/V/V 2.53 | – | – | + | – |
| T/T/T/T 2.56 | – | – | + | – |
| Q/Q/Q/Q 2.60 | – | – | + | + |
| N/K/V/D 2.63 | – | – | + | + |
| V/V/V/V 3.28 | – | – | + | + |
| I/L/L/I 3.29 | – | – | + | + |
| D/D/D/D 3.32 | + | + | + | + |
| Y/Y/Y/Y 3.33 | + | + | + | + |
| M/M/M/M 3.36 | + | + | + | + |
| F/F/F/F 3.37 | – | – | – | + |
| E/D/D/G 5.35 | + | – | – | – |
| K/K/K/A 5.39 | + | + | + | – |
| V/V/V/I 5.42 | + | + | + | + |
| A/A/A/S 5.46 | – | – | – | + |
| W/W/W/W 6.48 | + | + | + | + |
| I/I/I/V 6.51 | + | + | + | + |
| H/H/H/Q 6.52 | + | + | + | + |
| V/V/I/V 6.55 | + | + | + | + |
| K/W/E/Q 6.58 | + | + | – | – |
| W/L/Y/L 7.35 | + | + | + | – |
| I/I/I/T 7.39 | + | + | + | + |
| G/G/G/G 7.42 | – | – | + | – |
| Y/Y/Y/Y 7.43 | + | + | + | + |
| W/W/W124/W(EL1) | – | – | + | – |

[a]Amino acid residues in the first column refer to MOP/DOP/KOP/NOP receptors. Receptor residues are indicated with a '+' or '–' sign depending on whether or not they are within 4 Å from ligands. Identical residues in all four receptors are shown in blue. Identical residues in MOP, DOP, and KOP but unique to NOP are shown in cyan. Divergent residues in all four opioid receptors are shown in red. Divergent residues in MOP, DOP, and KOP, but not in NOP are shown in brown. Residues unique residues to either MOP, DOP, or KOP are shown in orange.

the region defined by TM2 and TM7) are involved in interactions with the chemical moieties responsible for opioid selectivity. This observation is extremely valuable for structure-based compound optimization and screening, because it offers a unique opportunity to either optimize existing opioid ligands or discover novel molecules that, by occupying different sites simultaneously, may become highly potent and/or selective for specific opioid receptor subtypes. However, it should be kept in mind that the crystal structures available may provide a limited understanding of ligand selectivity given the small chemical variability among the crystallized ligands and the current absence of agonist and peptide-bound structures of the receptors.

Another possibly novel route for developing new opioid ligands is to use dimeric interfaces as potential new targets. If the interfaces revealed by crystallography or other methods [25,26,42,43] are physiologically relevant, then it would be very helpful to use them to discover dimerization-destabilizing compounds. Even if the latter will not result in new drugs, the discovery of chemical probes that can help in understanding the potential role of oligomerization in GPCR function would be invaluable.

Finally, structural characterization of the cytoplasmic side of opioid receptors, which corresponds incidentally to the G-protein- or arrestin-binding pockets, could guide the identification of additional small molecules that can modulate opioid receptor function.

## Concluding remarks

Recent opioid receptor crystal structures provide unprecedented molecular details of opioid ligand binding and specificity. Notwithstanding the tremendous potential of current structure-guided approaches to the design of novel compounds acting at opioid receptors, there are several limitations that can still hamper full success in drug discovery. For instance, there is still much to be learned about opioid receptor structure and dynamics before we can fully understand the molecular mechanisms underlying opioid function. One problem is that the opioid receptor crystal structures available are limited to inactive forms of the receptors stabilized by high-affinity ligands during expression and crystallogenesis. More structures of different states of the receptors, ideally in complex with G protein, arrestin, or other regulatory accessory proteins such as GPCR kinases, are needed but might not be easy to obtain. Future efforts should include characterization of opioid receptor conformations with signaling and/or regulatory proteins to gain an understanding of the potential role of oligomerization and protein dynamics in receptor function.

It is well known that GPCRs can adopt multiple conformations [44,45] and opioid receptors are no exception. It is also known that while some opioid agonists (e.g., morphine [13], oxymorphone [13], 6′-GNTI [14]) can stimulate G protein signaling but not arrestin-mediated signaling,

others (e.g., 12-*epi*-salvA) are arrestin-biased ligands [15]. Structural characterization of active conformations for the G protein and the β-arrestin signaling pathways is therefore the immediate future challenge researchers working in the opioid receptor field will have to face. Not only have recent spectroscopic studies shown that ligands with different efficacies stabilize different receptor conformations [44,45], but active conformations for the G protein and the β-arrestin signaling pathways also appear to be different. Specifically, it has been suggested that β-arrestin active states require rearrangement of TM7 and H8, whereas the conformational changes associated with G protein activation mostly require movement of TM6 [46].

Although additional crystal structures are highly desirable, an understanding of how specific ligands induce or stabilize specific conformations will probably require different methods, either experimental or computational, than crystallography, because the composition of the lipid bilayer might play a key role in this understanding. Elucidation of the dynamic character of GPCRs and the way in which opioid receptors are activated by endogenous peptides and/or small-molecule ligands will then be necessary to develop more effective drugs.

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