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Recent Developments in the Study of Opioid Receptors

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Running Title:

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Abbreviations:

β_2-AR -	β_2-adrenergic receptor
β-FNA	β-funaltrexamine (MOPr-selective irreversible antagonist)
C-24	novel NOPr-selective antagonist; 1-benzyl-N- {3-[spiroisobenzofuran-1(3H),4'-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide
CHO	Chinese hamster ovary (cells)
DOP-r	δ-opioid receptor
ECL	extracellular loop
GPCR	G-protein-coupled receptor
ICL	intracellular loop
JDTic	KOPr-selective antagonist: (3R)-1,2,3,4-tetrahydro-7-hydroxy-N- [(1S)-1-[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl] methyl]-2-methylpropyl]-3-isoquinolinecarboxamide
KOP-r	κ-opioid receptor
MOP-r	μ-opioid receptor
NOP-r	nociceptin-orphanin FQ receptor
OP-r	opioid receptors (as a family)
SNP	single nucleotide polymorphism
TM	transmembrane

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Abstract:

It is now about forty years since Avram Goldstein proposed the use of the stereo-selectivity of opioid receptors to identify their receptors in neural membranes. This year the crystal structures of the four members of the opioid receptor family were reported, providing a structural basis for understanding of critical features affecting the actions of opiate drugs. This minireview summarizes these recent developments in our understanding of opiate receptors. Receptor function is also influenced by amino acid substitutions in the protein sequence. Among opioid receptor genes, one polymorphism is much more frequent in human populations than the many others that have been found, but the functional significance of this single nucleotide polymorphism (SNP) has been unclear. Recent studies have shed new light on how this SNP might influence opioid receptor function. In this minireview, the functional significance of the most prevalent genetic polymorphism among the opioid receptor genes is also considered.

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Introduction:

Avram Goldstein was already an established investigator when he became interested in the actions of opiate drugs in the late 1960s. An early goal was the identification and characterization of the opiate receptor (then always referred to in the singular). This required a reliable assay. Avram's strategy was to use two criteria, the well-defined stereoselectivity of the opioid receptor and the sensitivity of opiate analgesic action to antagonism by naloxone, to identify that component of total binding of the radiolabeled opiate that represented binding to the receptor (Goldstein et al, 1971). In this initial study, the fraction of opiate binding attributable to the receptor was rather small, but the same basic strategy was used later, together with opiate ligands with much higher radiochemical specific activity and a more efficient method of elimination of non-specific binding, by Lars Terenius and the Snyder and Simon groups (Pert & Snyder 1973; Terenius, 1973; Simon et al, 1973) to show the presence in the brain and gastrointestinal tract of binding proteins with high specificity for opiate drugs. This unambiguous demonstration of the binding to opioid receptors from three independent labs triggered continuing studies of the properties of these receptors, and also the search for an endogenous agent (again always discussed in the singular at this time) that was presumed to be the physiological regulator of the opiate drug receptor. This minireview summarizes recent developments in our understanding of opiate receptors following the publication this year of the crystal structures of all four members of the opioid receptors family, and recent studies evaluating the role in mu-opioid receptor function of the most prevalent genetic polymorphism among the opioid receptor genes.

Insights from structural studies of opioid receptors:

Forty years after the initial demonstration of the presence in brain of receptors for opiate drugs, crystal structures for all four members of the opioid receptor family have now been reported. Avram would be particularly pleased that one of the two responsible groups, the Kobilka group, is based in the Department of Pharmacology at Stanford University, a department that Avram established in 1955. He would also be delighted that the Nobel Prize Committee has recently recognized Dr. Kobilka and his mentor, Robert Lefkowitz, for their contributions to the elucidation of the structures and functions of all GPCR. Crystal structures for the mouse mu (MOP-r)- and delta (DOP-r)-opioid receptors were reported from the Kobilka lab (Manglik et al, 2012; Granier et al, 2012)

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while crystal structures for the human kappa (KOP-r) and nociceptin (NOP-r) receptors were reported by the Stevens lab at the Scripps Research Institute in La Jolla, CA (Wu et al, 2012; Thompson et al, 2012). The reported structures provide a number of insights into the actions of opiate drugs, and a few surprises. A comparison of the major features of the reported crystal structures of the four receptors making up the opioid receptor family is contained in Table 1.

Achieving crystallization of these G-protein coupled receptors is a major technological achievement. It required substantial molecular engineering of the receptors during which highly disordered regions of the receptor were replaced with fragments of another protein known to assist in structural stabilization; residues 2 to 161 of T4 lysozyme were inserted into the third intracellular loop of the opioid receptors to facilitate their crystallization. The highly flexible N- and C- terminus regions of the wild-type receptor proteins sequences were also truncated to aid crystallization, and a Flag-tag and a poly-His sequence with cleavage sites were inserted on the truncated N-terminus or the truncated C-terminus, respectively, to aid purification of the expressed engineered receptors. Despite this extensive engineering, each receptor when expressed in cells in culture retained the ability to bind highly selective ligands with only modest changes in affinity, and was capable of supporting agonist-induced changes in signal transduction pathways.

Antagonist ligands in the receptor complexes:

To further aid in the crystallization process, each receptor was bound to a tightly binding selective antagonist drug. The irreversible selective ligand, β-funaltrexamine (β-FNA) was bound to the mu opioid receptor (MOP-r; Manglik et al, 2012); a covalent link between β−FNA and the epsilon amino group of a lysine (K233) residue in the 5th trans-membane domain of MOP-r was identified. The delta receptor (DOP-r) was crystalized in complex with the high affinity DOP-r-selective reversible antagonist, naltrindole (Granier et al, 2012). The engineered human kappa receptor (KOP-r) was crystalized in complex with the high affinity KOP-r-selective reversible antagonist, JDTic (Wu et al, 2012), while the engineered human NOP receptor (NOP-r) was crystalized in complex with the novel high-affinity NOP-r-selective antagonist, C-24, from Banyu Pharmaceuticals (Thompson et al, 2012). The C24 structure is analogous to the first four amino acid residues of the endogenous ligand, nociceptin/orphanin FQ); C24 has a Ki of 0.3 nM for the wild-type receptor and about 2 nM for the engineered NOP-r. The use of antagonists as the co-

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crystalized ligand facilitates the formation of crystals by freezing the receptors in their relatively stable inactive conformations.

Transmembrane domains:

Each receptor has seven α-helical trans-membrane domains (7TM) that are aligned around a central ligand binding pocket as anticipated from earlier studies comparing analogous sequences in rhodopsin with the OP-r sequences, and considering the placement of the α-helical transmembrane domains of rhodopsin. There appears to be considerable similarity in the overall orientation of the 7TM helices between the four members of the opioid receptor family, although the spatial alignment of NOP-r differs in places from the more conserved orientations of the MOP-r, DOP-r and KOP-r transmembrane domains (Thompson et al, 2012). All four receptors have bends in some of the TM helices (TM2, TM4, TM5, TM6 and TM7) induced by the presence Pro residues roughly centered in each TM domain (Thompson et al, 2102). These Pro residues are highly conserved across most GPCR; their presence is emphasized in the description of the crystal structure of the β-adrenergic receptor (β_2-AR), the first GPCR to be crystalized (Cherezov et al, 2007). The bends in the TM domains contribute to the shape of the ligand binding pocket for each receptor. In contrast to the conserved TM domains, the extracellular loops (ECL) and the intracellular loops (ICL) show much extensive variation between members of the opioid receptor family. The ECL2 domains of KOP-r and NOP-r differ from those of MOP-r and DOP-r by the increased frequency of acidic amino acid residues (Asp, Glu), making the entrance to the ligand binding pocket in these receptors highly acidic (Thompson et al, 2012). This may be related to the highly basic nature of dynorphin A and nociceptin/orphanin FQ, the endogenous ligands for KOP-r and NOP-r, respectively. The overall structure of GPCR receptors is also supported by the presence of one or more conserved Cys- Cys bonds. In the opioid receptor family there is just one conserved Cys-Cys disulphide bond in a similar location in each receptor, linking the second extracellular loop (ECL2) to the intracellular end of TM3.

Ligand Binding Pocket:

There are also many similarities in the binding pockets of each opioid receptor (Fig. 1). In all cases, the binding pocket is located in the center of the receptors, deep within the hollow created by the encircling TM domain regions. The pocket appears more open to the extracellular fluid than is reported for the binding pocket of other GPCRs with small

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molecule endogenous ligands. Manglik et al (2012) suggest that the open nature of the opioid receptor ligand binding pocket is consistent with the very short dissociation half-lives of highly potent MOP-r antagonists; for example, diprenorphine, Ki 72 pM, has a dissociation half-life of 36 mins from MOP-r). In contrast the M_3 muscarinic receptor structure displays a much more restricted entry to its ligand binding site and the potent M_3 receptor antagonist, tiotropium (Ki, 40 pM), has a dissociation half-life of about 35 hrs. The amino acid residues within the opioid receptor binding pocket with which the very high affinity highly selective antagonists used in these studies interact are in part specific to the unique characteristics of these very specialized ligands. Nevertheless, several similarities across the receptor types are apparent. Conserved Asp residues [D147(3.32)*, D128(3.32), D138(3.32), D130(3.32), in MOP-r, DOP-r, KOP-r and NOP-r, respectively] are located in essentially the same location within the third TM helix of each receptor (Fig. 1). Mutation of this Asp residue in each receptor to a non-charged alternative amino acid results in loss of opioid activity. The Asp residue is thought to form a charge - charge interaction with a positively charged group in the ligands binding to each receptor. It has long been assumed than an ionic interaction between the ligand and each opioid receptor is a critical feature in the binding of opiate ligands to their receptors (Beckett & Casy, 1954). The structural basis for this is now apparent. Another common feature of the binding site is the presence of a conserved His residue in three of the four opioid receptors [H297(6.52); H278(6.52); H291(6.52), in MOP-r, DOP-r and KOP-r, respectively]. In NOP-r, this His is replaced by a Gln [Q280(5.52)]. The His residues in the three "classic' opioid receptors are thought to interact by hydrogen bonding through two associated water molecules with the tyrosine-like hydroxyl moieties of the morphinan ligands (Manglik et al, 2012). There are a number of other amino acids located in close contact with the docked antagonist molecules in these receptors (Fi g. 1, Table 1). Some of these interactions are probably specific for the unique high-affinity antagonist ligands selected for the crystallization, but many may also be important in the docking and agonist action of physiologic agonists. It should be noted that the Lys residue [K233(5.39)] covalently linked to the β-FNA in the MOP-r crystal is likely to be a special case resulting from the covalent nature of this interaction.

Receptor oligomerization:

The MOP-r and KOP-r crystals formed as parallel dimers tightly associated through TM5 and TM6, and to a lesser extent between TM1 and TM2, although in the KOP-r crystal antiparallel dimers were also observed. In contrast, DOP-r was reported to crystallize

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exclusively as antiparallel dimers (Granier et al, 2012). The antiparallel form appears unlikely in biological membranes; the authors argue that the antiparallel arrangement may reflect an energetically favorable arrangement during the crystallization process with naltrindole. It is highly unlikely that the antiparallel arrangement is a reflection of intermolecular associations that occur *in vivo* (this would require that the binding pocket of one of the receptors in the dimer faced the interior of the cell). The presence of parallel dimers in MOP-r and KOP-r crystals provides a structural basis for earlier studies reporting the homo- and hetero-dimerization of OP-r in biologic membranes (Cvejic & Devi,1997; Jordan & Devi, 1999; George et al, 2000). It should be noted that the observed dimerization in the crystals occurs during crystallization - the engineered receptors were purified as monomers - so there is no certainty that the oligomerization forms found in the crystal represent functional dimer forms present *in vivo*.

The role of membrane cholesterol in determining the preferred structures of GPCRs and in modulating opioid receptor function also requires consideration. Cholesterol was used to facilitate the crystallization of β_2AR bound to an antagonist (Cherezov at al, 2007) and when this GPCR was co-crystallized together with Gs in the presence of a β_2AR agonist (Rasmussen et al, 2011). Cherezov et al (2007) report that cholesterol mediates the parallel association of dimers in β_2AR crystals, raising the possibility that it plays a similar role in facilitating dimer formation *in vivo*. Crystallization of the four OP-r also required the presence of cholesterol (Manglik et al, 2012; Granier et al, 2012, Wu et al, 2012; Thompson et al, 2012) although the role of cholesterol as a factor determining the observed structures of these receptors is not discussed by the authors. It has long been known that modulating the cholesterol content of OP-r-expressing cell membranes can alter the binding and signal transduction properties of the receptors (Lazar & Medzihradsky, 1992; Xu et al, 2006; Gaibelet et al, 2008; Zheng et al, 2012), although the author differs in their proposed (non-mutually exclusive) mechanisms (e.g., altered membrane microviscosity; receptor partition into lipid rafts, facilitation of association with G proteins, facilitation of dimer formation, modulation of receptor palmitoylation).

Agonism at opioid receptors:

The elucidation of the crystal structure of all members of the opioid receptors family provides a strong basis for design of selective ligands for each receptor, but the antagonist-bound crystal structures shed less light on the changes in receptor structure and conformation that result in the induction of agonist effects. Like other GPCR, most

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of the observed actions of opioid receptor ligands require the activation of a G protein to trigger further down-stream events within a cell. OP-r predominantly couple with Gi or Go, to cause dissociation of the $G\alpha\beta\gamma$ complex and trigger down-stream cellular process. Recently, the Kobilka lab reported the crystallization of he β_2AR complexed with the G_s α–subunit (Rasmussen at el, 2011), another extraordinary technical achievement requiring crystallization conditions maintaining the association of an agonist bound receptor with the G protein heterotrimer. The agonist form of the β with Gs indicates that agonism requires substantial changes in the orientation of β_2AR complexed microdomains within the Gs a-subunit. To date there is no report of the crystallization of an OPr or any other Gi/o coupled GPCR in complex with the Gi/o α subunit. The Rasmussen et al (2011) study indicates a pathway towards crystallization of an agonist-form OPr crystal, but many technical challenges will need to be overcome to achieve this. It remains to be determined if Gi and/or Go activation results from a re-orientation of the C-termini of these proteins that is analogous to the agonist-activated β_2AR mediated re-orientation of the Gs α-subunit C-terminus.

Opioid receptor polymorphisms and receptor function

The primary sequence of a GPCR is a major determinant of the secondary and tertiary structure of the mature receptor. It is therefore possible that polymorphisms in an opioid receptor gene might result in the expression of receptor with a modified tertiary structure and altered functional activity. There are numerous single nucleotide polymorphisms (SNPs) in the human MOP-r gene, but most are rare and their functional significance, if any, is unknown (see review by Mague & Blendy, 2010). At this time, a polymorphism in an opioid receptor gene that alters the major conformation of the expressed receptor has not been reported. In the MOP-r gene, one SNP (rs 1799971) occurs relatively frequently in some human populations. The polymorphism is located in exon 1, where a change in adenosine (A) to guanosine (G) in nucleotide position 118 (A118G) results in a change in amino acid sequence in which Asn 40 in replaced by Asp (designated N40D). This SNP has now been studied more extensively than the other SNPs in MOP-r or any SNPs in the other OP-rs. A118G occurs with variable frequency in different human populations, with the highest reported allelic frequency of 118G being 48.5% in a Japanese population. In contrast, the 118G allelic frequency is 15.4% in European-Americans, 14% in Hispanics, 8% in Bedouins, and 5% in African Americans (Gelernter et al, 1999); other studies show approximately similar relative distributions by population

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and confirm the high expression of this SNP in Asian populations (Bond et al, 1998; Tan et al, 2009). Initial reports suggested that this SNP was associated with addictive behaviors for several drugs, but more extensive studies have not confirmed this apparent association, and the effect of the A118G polymorphism has been variously reported to be either an increase or a reduction in the risk of substance abuse. There is more consistent agreement that A118G is associated with impaired opioid signaling through MOP-r and a need for increased opiate drug doses in patients with the G variant in a variety of painful conditions (see review by Mague & Blendy, 2010).

The N40D (A118G) mutation occurs in the N-terminus extracellular domain of MOP-r, a part of the receptor that is highly disordered. Manglik et al (2012) removed this extracellular domain in their engineered receptor to facilitate its crystallization. It is therefore unlikely that this SNP alters the basic three-dimensional structure of the MOP-r protein. Early reports suggested that A118G resulted in increased signaling through MOP-r by the endogenous ligand β-endorphin (Bond et al, 1998), but more recent studies found unchanged opioid ligand binding with impaired opioid signaling in the 118G variant (Mague & Blendy, 2010; Oertel et al, 2012). Krosliak et al (2007) reported that 118G reduced the level of MOP-r protein (observed as reduced ligand binding Bmax for opioid ligands) and a lower potency of opiates as inhibitors of adenylyl cyclase in oocytes transfected with this receptor variant. In order to evaluate the function of this receptor more fully, Mague et al (2009) generated a mouse analog with nucleotide A112 of the mouse MOPr gene mutated to a G (A112G), resulting in conversion of Asn38 to Asp38 (N38D; corresponding to N40D in the human gene). The mutated mouse receptor displayed essentially unchanged ligand binding affinities for several ligands but reduced levels of receptor mRNA and protein expression were observed in most brain regions, suggesting that a reduction in the number of receptors may account for the impaired signaling. One effect of the N40D change is the loss of an N-glycosylation site on the N-terminus of the receptor protein. Huang et al (2012) have confirmed that the N38D (A112G) receptor shows reduced glycosylation in homozygous A112G mice, and that the reduced glycosylation is associated with a reduction in the stability of the modified receptor. Thus one potential explanation of the reduced level of receptor expression is a reduced stability of the less-glycosylated MOP-r protein.

Other factors also contribute to the reduced levels of receptor protein in those carrying the A118G mutation. Zhang et al (2009) have shown that a G in position 118 is associated with reduced levels of the MOP-r mRNA expression in CHO cells expressing

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transfected variant forms of the receptor mRNA, and that 118A mRNA was significantly more abundant than the 118G mRNA in human autopsy brain tissue from eight heterozygous subjects. This raises the interesting question of how a change in the gene sequence in the coding region of the gene might affect the levels of the expressed mRNA. Zhang et al (2009) discuss the possibility that 118G causes reduced mRNA stability, but did not find an allele-specific impaired mRNA stability in transfected CHO cells.

Oertel et al (2012) now offer an alternative explanation. They have shown that the A118G variant introduces a newly identified methylation site on the OPRM1 gene (the gene coding for MOP-r) at nucleotide position +117. The extent of methylation of the OPRM1 DNA at +117 and at downstream methylation sites in DNA extracted from the brains obtained post-mortem from heroin abusers (who died from an opiate overdose) and in controls, comparing methylation between the 118A and 118G alleles was dependent on whether A or G was present at position 118. Significant increases in methylation ($P < 0.05$ or greater) were found at positions +117, +145, +150, and +159 in 118G-carrying heroin-abuser subjects, but were not observed in control subjects carrying the 118G allele. The significance of the altered methylation was evaluated by comparing the levels of MOP-r mRNA expression in 118A and 118G carriers in both heroin abusers and control subjects. Heroin abusers carrying 118A exhibited significantly higher MOP-r mRNA levels in two brain regions (thalamus, S11 cortex) than 118A controls, and an increased in the level of MOP-r binding sites; in contrast, heroin abusers with 118G (either one or two copies) expressed levels of MOPr mRNA and MOPr binding that were very similar to 118G controls in both brain regions. These results indicate that the presence of the 118G allele impairs the increased expression of MOP-r mRNA that occurs when MOP-r signaling efficiency is reduced after chronic opiate drug exposure. The sites showing increased methylation with the 118G allele include two predicted binding sites for the transcription factor Sp1 in OPRM1 DNA, providing a possible explanation for the reduced ability of chronic opiate drug users with the A118G polymorphism to increase MOP-r RNA expression in response to impaired receptor signaling efficiency. The reason that increased OPRMR1 DNA methylation was observed only in heroin abuser 118G carriers but not in the 118G carrier controls is unexplained at this time. Nevertheless, it is clear that the A118G polymorphism occurring in a significant fraction of most populations modifies the regulation of MOP-r expression and the sensitivity to the actions of opiate drugs. This study points to the

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complexity of the interaction of genetic, epigenetic and environmental factors in the regulation of expression of the MOP-r gene.

Concluding thoughts:

These recent developments in opioid receptor research demonstrate that the research field in which Avram Goldstein played a prominent founding role forty years ago is still very active. Increased understanding of the three-dimensional structure of all members of the opioid receptor family will make it possible to design drugs with increased specificity for each receptor type, and to address possible allosteric regulation of receptor function. We await with interest the determination of the three-dimensional structures of the receptors when complexed with agonists and effector proteins. Improved understanding of how receptor expression and function is modified by primary sequence variations will contribute to our understanding of the differences between individuals in their responses to opiate drugs. Avram's seminal contributions to the development of this research field continue to bear fruit.

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Authorship Contribution:

The author is responsible for the entire content of this minireview.

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Footnote:

* Throughout, amino acid residues are indicated by their single letter amino acid code, followed by their numeric position in the receptor amino acid sequence. This is followed where appropriate by their location with each TM helix (in parentheses), using the Ballesteros-Weinstein numbering system, as reported by the cited authors.

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Figure Legend:

Fig. 1. Comparison of the ligand binding pockets of the four members of the opioid receptor family, all viewed from the extracellular surface.

A. The ligand binding site of the mu-opioid receptor (MOP-r) in complex with β-FNA (green) covalently bound to the receptor via K233(5.39). The red spheres indicate water molecules linking H297(6.52) to the phenolic group of β-FNA; polar contacts are indicated with red dotted lines [with D147(3.32), and Y148(3.33)] and hydrophobic interactions are in orange. Light blue mesh indicates the electron density around the receptor protein side chains (from Manglik et al, 2012, Fig. 3, panel b, with permission). **B**. The ligand binding site of the delta opioid receptor (DOP-r) in complex with naltrindole (yellow) and the protein chain in brown, showing the close proximity of D128(3.32) and Y129(3.33) to the ligand. H278(6.52) is also strategically located (from Granier et al, 2012, Fig 2, panel d, with permission). **C**. The ligand binding site of the kappa- opioid receptor (KOP-r) in complex with JDTic (yellow) and the protein chain in blue, with the polar contact of D138(3.32) (highlighted in orange) with the ligand shown as a dotted line. H291(6.52) is also located in close proximity with the ligand. In this panel, water molecules that are part of the crystal structure are shown as magenta spheres; hydrophobic surfaces are indicated in green, hydrogen bond donors in blue and hydrogen bond acceptors in red. Black indicates the protein interior (from Wu et al, 2012, Fig 2, panel a, with permission). **D**. The ligand binding pocket of the nociceptin-orphanin FQ receptor (NOP-r) in complex with the peptide mimetic agent, C-24 (green, with purple mesh) showing the proximity of D130(3.32) which forms a salt bridge (not shown here) with the ligand. The critical H residue in the other opioid receptors is replaced in NOP-r with Q280(6.52) (from Thompson et al, 2012, Fig 2, panel d, with permission).

Table 1. Comparison of Features of Reported Crystal Structures for the Four Opioid Receptors Complexed with Antagonist Drugs.

Feature	MOPr (Manglik et al, 2012)	DOPr (Granier et al, 2012)	KOPr (Wu et al, 2012)	NOPr (Thompson et al, 2012)
Receptor engineering to enable crystallization	Mouse receptor, with N & C terminus truncations; inserted N-term. Flag tag and C-term. poly-H to aid purification; lysozyme T4L residues 2 - 161 inserted in ICL3; crystalized using lipidic cubic phase technique with cholesterol	Mouse receptor, with N & C terminus truncations; inserted N-term. Flag tag and C-term. poly-H to aid purification; lysozyme T4L residues 2 - 161 inserted in ICL3; crystalized using lipidic cubic phase technique with cholesterol	Human receptor; N & C terminus truncations; inserted N-term. Flag and C-term. poly-H to aid purification; lysozyme T4L residues 2 - 161 inserted in ICL3; single point mutation I135; crystalized using lipidic cubic phase technique with cholesterol	Human receptor; replaced N terminus with a stabilized apocytochrome b-RIL fragment and a Flag sequence; truncation of C-terminus; crystalized using lipidic cubic phase technique with cholesterol
Co-crystalized ligand	**β-FNA**, MOPr-sel. irreversible antagonist	**Naltrindole**, DOPr-sel. reversible antagonist	**JDTic**, KOPr sel reversible antagonist; Ki 0.32 nM	**C-24**, NOPr sel reversible antagonist; Ki 0.27 nM
Transmembrane (TM) domains and Extra/Intra-Cell Loops (ECL/ICL) (Sequence homology data from Granier et al and Thompson et al).	7TMs with similar placement to rhodopsin, and Pro-related bends in α–helices	7TMs, 76% homol to MOPr; similar placement to rhodopsin, with Pro-related bends in α–helices	7TMs, 73% homol to MOPr; ECL2 forms a β-hairpin	7TMs, 67% homol to MOPr, similar placement to rhodopsin, with Pro-related bends in α–helices; ECL2 forms a β-hairpin; ECLs enriched in D,E residues, acidic relative to other OPrs; ICL2 forms a short α-helix
Disulphide bridge	C140 - C217, links ECL2 to end of TM3	not reported	C131 - C210, links ECL2 to end of TM3	C123(3.25) - C200(ECL2)
Opioid ligand binding pocket	"Open" binding pocket deep in cell membrane; should facilitate rapid dissociation of reversible ligands	"Open" binding pocket deep in cell membrane, similar to binding pocket in MOPr & KOPr	"Open" binding pocket deep in cell membrane, similar to binding pocket in MOPr & DOPr	Binding pocket is "relatively large", capable of binding large peptides
Critical ligand binding residues	D147(3.32) -charge -charge interaction with ligand; H297(6.52)+2H_2O - hydrogen bonding to phenolic OH and aromatic ring of morphinans; (Ligand specific: K233(5.39) - covalent link to β-FNA)	D128(3.32) - charge - charge interaction with ligand; H278(6.52) +2H_2O - hydrogen bonding to phenolic OH of naltrindole; (Probable ligand specific roles for W274(6.48), Y308(7.43), M132(3.35), I277(6.51), Y129(3.33), V281(6.55), L300(7.35z),W284(6.58)	D138(3.32) - charge- charge interaction with ligand; W287(6.48), H291(6.52) - hydro-phobic interactions with ligand. (Probable ligand specific roles for: V118(2,63), V134(3.28), L135(3.29), Y139(3.33), M142(3.36), V230(5.42), K227(5.39), I294(6.55), I290(6.51), Y312(7.35) I316(7.39), G319(7.42), V108(2.53), Q115(2.60), T111(2.56)}	D130(3.32) - charge-charge interaction with ligand; other binding pocket residues show reduced homology with KOPr or MOPr, reflecting low affinity for classic opioids; H(6.52) replaced by Q280(6.52); M134(3.36) is re-oriented relative to M142(3.36) in KOPr; A216(5.39) replaces K, T305 (7.39) replaces I in other OPrs
Oligomerization	Crystalizes as parallel dimers, tightly associated through TM5, TM6	Crystalizes as antiparallel dimer, possibly reflecting energetically favorable interactions unique to crystallization conditions	Crystalized as parallel dimers; structures of the two molecules in the dimer are similar, but not identical - for examples in ICL2.	not reported

Table Footnote:
Specific amino acids are indicated by their single letter amino acid code, with numbers indicating their position in the receptor sequence; numbers in parentheses indicate their position within the transmembrane α-helices (using the Ballesteros-Weinstein nomenclature); e.g., H297(6.52) indicates a His residue in sequence position 297, located in the 6th TM α-helix at position 52 within the helix; position 52 refers to the residue location relative to the most conserved amino acid within the helix which is arbitrarily given the locator 50 - position 52 is 2 residues towards the C-terminus from the most conserved amino acid; a position number of less than 50 indicates a location towards the N-terminus relative to the most conserved amino acid. Note the conservation across the receptors types of the positions within the α-helix structure of amino acid residue critical for ligand binding; e.g., D147(3.32); D128(3.32), D138(3.32) and D130(3.32) in MOP-r, DOP-r, KOP-r and NOP-r respectively.

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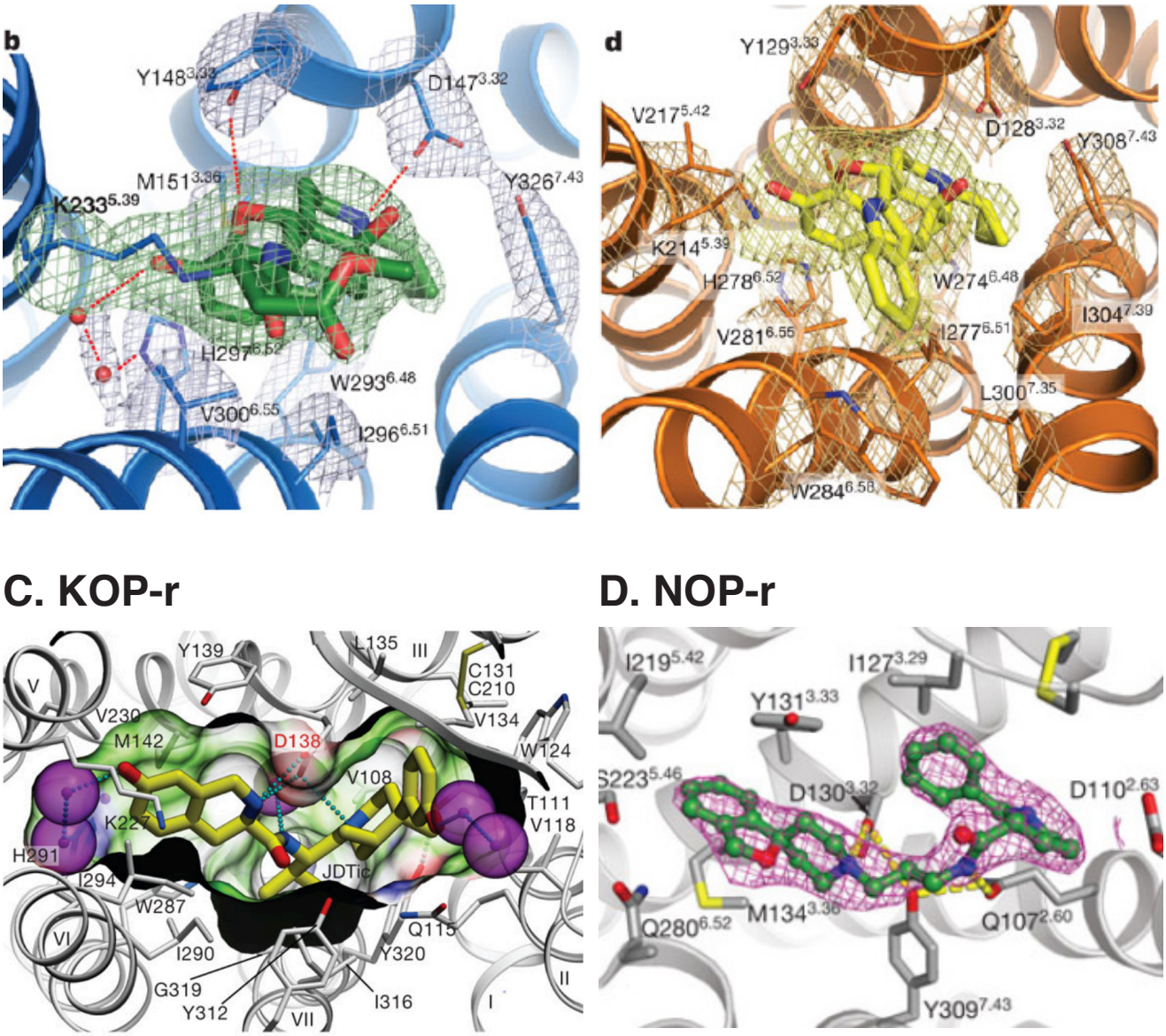

Figure 1