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Author
Manglik, Aashish

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Molecular basis of opioid action: from structures to new leads

Aashish Manglik[1,2]

[1]Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94158, USA

[2]Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, CA 94158, USA

Abstract

Since the isolation of morphine from the opium poppy over two centuries ago, the molecular basis of opioid action has remained the subject of intense inquiry. The identification of specific receptors responsible for opioid function and the discovery of many chemically diverse molecules with unique opioid-like efficacies has provided glimpses into the molecular logic of opioid action. Recent revolutions in the structural biology of transmembrane proteins have, for the first time, yielded high-resolution views into the three dimensional shapes of all four opioid receptors. These studies have begun to decode the chemical logic that enables opioids to specifically bind and activate their receptor targets. A combination of spectroscopic experiments and computational simulations have provided a view into the molecular movements of the opioid receptors, which itself gives rise to the complex opioid pharmacology observed at the cellular and behavioral levels. Further diversity in opioid receptor structure is driven by both genetic variation and receptor oligomerization. These insights have enabled computational drug discovery efforts, with some evidence of success in the design of completely novel opioids with unique efficacies. The combined progress over the past few years provides hope for new, efficacious opioids devoid of the side effects that have made them the scourge of humanity for millennia.

Keywords

Opioid; Opioid receptor; G protein-coupled receptor; analgesic; MOP; KOP; DOP

Introduction

Purification of morphine from the opium poppy over two centuries ago ushered in a new era of analgesic pharmacology(1). Morphine provided the first example that a purified natural product could recapitulate the complex pharmacology of plant matter. Though morphine enabled control over the desired effect of pain relief, it retained the critical liabilities of opium. Over the following two centuries, enormous effort focused on identification of the ideal opioid, one that provides analgesia without either the lethal side effect of respiratory suppression or the tolerance and physical dependence that contribute to addiction. Critical to this search has been synthesis of thousands of molecules with similar properties to the

Corresponding Author: Aashish.Manglik@ucsf.edu.

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original opium plant matter. While initially driven by variations on the morphine scaffold itself, subsequent efforts have identified diverse molecules with opioid-like effects, ranging from completely synthetic opioids like fentanyl to endogenous peptides like β-endorphin.

Identification of the molecular targets for opioids, initially by radioligand binding studies(2–6) and subsequently by the cloning of individual receptor genes(7–12), generated an entirely new molecular perspective on opioid pharmacology. These studies revealed the remarkable complexity of the endogenous opioidergic system, with four receptors, the μOR, δOR, κOR, and nociceptin receptor, as well as numerous endogenous opioid peptides that act either promiscuously or selectively at each subtype(13). Activation of each of these opioid receptors instigates numerous intracellular signaling pathways. Most proximally to the membrane, opioid receptors activate $G_{i/o}$ heterotrimeric G proteins. Activated opioid receptors are rapidly desensitized by the action of GPCR-regulated kinases (GRKs), which phosphorylate receptors, and β-arrestins, which internalize phosphorylated receptors. The interaction dynamics of opioid receptors with these proteins, and numerous other intracellular partners, gives rise to varied signaling outcomes(14).

This biological complexity, combined with the exceptionally diverse molecular properties of opioid receptor-binding molecules, continues to provide hope that an ideal opioid analgesic, one devoid of the lethal and addictive properties of most opioids, may exist. Critical to achieving this goal is an understanding of the molecular logic of opioid activity. A great hope of such insight would be the ability to rationally design opioids with desired specificity and efficacy. Though still at an early stage, new biophysical approaches have enabled a deep molecular analysis of opioid receptors, providing a completely new view of opioid action. This review will highlight the key insights gained from such studies, how they reconcile various aspects of opioid function and complexity, and how they offer a new approach for the discovery of novel opioids.

Molecular recognition of opioids

Over a half century prior to the discovery of opioid receptors, the structure of morphine itself was proposed(15). The ability to modify morphinan and other opioid scaffolds led to numerous compounds with varying levels of analgesic activity. Levying this broad structure-activity-relationship, Beckett and Casy proposed a common set of "rules" that govern opioid analgesic activity(16–18). Central to this concept was a common receptor binding site that recognizes diverse opioid analgesics. Key features included an anionic site to interact with the positively charged amine common to most opioids, a cavity to accommodate amino group substituents, and a flat surface to accommodate an aromatic ring (Figure 1a).

Initial efforts to determine the structures of opioid receptors focused on the inactive, "off' state. Like other G protein-coupled receptors (GPCRs), opioid receptors are incredibly dynamic proteins(19). Most structural biology methods, by contrast, require conformationally homogeneous protein samples for high resolution visualization. To generate such biochemical preparations, high affinity or covalent antagonists were used to stabilize the inactive conformation of the receptors. Combined with advances in GPCR

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biochemistry and X-ray crystallography(20), such preparations enabled the first structural insights into all four opioid receptors (Figure 1c)(21–24).

For the classic opioid receptors (μOR, δOR, κOR), these structures revealed a common logic for opioid recognition. As predicted by Beckett and Casy five decades prior, the binding sites contain an anionic aspartic acid residue that forms a salt bridge with the positively charged amino group of opioid ligands (Figure 1b). A deep cavity accommodates the aliphatic substituents on the amino group. Molecular recognition of the common phenolic group in morphinans, which had been postulated to resemble the amino-terminal tyrosine residue of endogenous opioids like β-endorphin, was more unprecedented. The phenol of co-crystallized morphinans (β-funaltrexamine for μOR and naltrindole for δOR) and a synthetic opioid (JDTic for κOR) engages an extended hydrogen bonding network between two water molecules and a conserved histidine residue in transmembrane helix 6 (TM6). A similar positioning of the phenolic tyrosine in opioid peptide ligands was initially observed for the bifunctional μOR agonist/δR antagonist DIPP-NH2 bound to the δOR(25) and subsequently in a cryo-electron microscopy structure of the μOR agonist DAMGO(26).

These structures also revealed the molecular basis for ligand selectivity at different opioid receptors. For example, naltrindole is 20-fold more selective for δOR over the μOR(27). Mutagenesis studies had demonstrated that a specific region in the opioid receptors around extracellular loop 3, more specifically W318 in the μOR, was important in naltrindole selectivity(28). The opioid crystal structures provided high resolution insight into this selectivity; naltrindole binding to the μOR is sterically precluded by the bulky W318 residue but tolerated by leucine at the same position in the δOR. More broadly, these structures revealed significant diversity in the binding pockets of the opioid receptors. Subtype-specific molecules extend a functional group into one of these subpockets, thereby increasing affinity at one opioid receptor while decreasing affinity at another.

Radioligand binding studies in the 1970s revealed a strong effect of various cations on opioid pharmacology, with sodium increasing the affinity for antagonists and divalent cations, like magnesium, increasing agonist affinity(2, 4, 29). These early studies provided evidence that opioid receptors existed in multiple conformations, with ions or guanine nucleotides allosterically influencing the conformational state of the receptor. Though residues involved in sodium binding had been identified for other GPCRs by mutagenesis studies, the structural basis for sodium modulation of GPCR function came from a high-resolution structure of the δOR(30), which revealed a sodium ion deep in the core of inactive δOR. Visualization of this site provided a structural rationale for how a single ion allosterically regulates opioid receptor conformation.

For the broader GPCR family, numerous small molecule allosteric modulators have been discovered that either potentiate or dampen signaling by endogenous hormones. As therapeutic candidates, allosteric modulators retain the spatial and temporal context of endogenous signaling, providing a unique avenue towards the design of safer drugs. Recent efforts have identified a number of small molecule allosteric modulators for the opioid receptors that potentiate the activity of endogenous endorphins and enkephalins(31–33). Optimization of allosteric modulators, however, remains challenging, in part due to a lack of

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experimental high resolution structures of opioid receptors bound to such molecules. Classic pharmacological studies and molecular dynamics simulations have begun to fill this gap(34–36). Further characterization of allosteric sites will likely enable not only ways to potentiate opioidergic signaling, but also potential avenues for exceptional opioid antagonists effective in reversing the effect of exceptionally potent opioids like carfentanil(37).

Opioid receptor activation and signal transduction

A deeper understanding of how opioids initiate cellular signaling has required structures of activated opioid receptors bound to opioid agonists. This poses several technical challenges, as agonist-bound GPCRs are highly dynamic(38) and biochemically unstable(39). Pioneering structural elucidation of active β2-adrenergic receptor required a number of new approaches in GPCR structural biology, including the identification of exceptionally potent agonists(40), the development of new detergents to stabilize transmembrane proteins(41), and single domain antibody fragments from camelids (nanobodies) that would "lock" the receptor in an active conformation(42, 43).

Initial insights into opioid receptor activation came from a high-resolution X-ray crystal structure of the μOR(44) bound to BU72, an exceptionally potent morphinan agonist with 60 pM affinity(45). Though BU72 preferentially stabilizes the active conformation of μOR, obtaining crystals required a camelid-derived single domain antibody (nanobody 39, Nb39) that binds to the intracellular domain of the activated receptor. The structure of the μOR-BU72-Nb39 complex revealed important features that drive opioid receptor activation. The largest change in the receptor upon activation is a ~10 Å outward displacement of transmembrane helix 6 (TM6), coupled with more subtle motions of TM5 and TM7 (Figure 2A). These movements, which have also been observed in other active GPCRs(39), open a large cavity in the intracellular side of the receptor which couples to heterotrimeric G proteins, GRKs and β-arrestin(46).

In contrast to this large conformational movement, the changes around the ligand-binding pocket are more subtle. Indeed, the majority of chemical contacts between the BU72 agonist and the μOR are similar to that observed for the inactive receptor bound to the morphinan irreversible antagonist β-funaltrexamine (Figure 2C). However, the larger cyclopropyl group of β-funaltrexamine displaces the ligand within the binding pocket towards the extracellular side of the receptor. Further insights into how these subtle changes drive receptor activation came from molecular dynamics simulations of the μOR, which revealed that TM3 in the receptor acts as a key conduit between the ligand binding pocket and the intracellular domain.

High sequence similarity between the μOR, δOR, and κOR may suggest a conserved activation mechanism across the opioid receptor family. The molecular pharmacology of morphinan agonists, however, suggests much more complexity and diversity in the chemical interactions that drive activation of the individual opioid receptors. Within the oripavine class of morphinan compounds, for example, diprenorphine is a potent μOR antagonist while displaying potent partial agonism at δOR and κOR(47). By contrast, buprenorphine is a partial μOR agonist with potent κOR antagonism(48). A recently determined structure of

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activated κOR bound to the morphinan agonist MP1104 and the same nanobody used for active μOR, Nb39, has revealed some of the key similarities and differences in opioid receptor activation(49). Although the global conformational changes in the intracellular domain and regions that connect the ligand-binding pocket and the intracellular domain are shared between μOR and κOR, more subtle differences in the ligand binding pockets likely drive differences in the efficacy of opioids like diprenorphine and buprenorphine at individual receptors.

A deep molecular understanding of opioid action requires not only insight into how opioids activate their receptors, but also how these activated receptors engage downstream signaling partners. Obtaining structures of opioid receptors engaged with G proteins, GRKs, and β-arrestins presents new technical hurdles, with a primary challenge being the relative biochemical instability and dynamics of such complexes(46). Rapid advances in cryo-electron microscopy (cryo-EM), however, have enabled significant advances in the structural biology of such GPCR-effector complexes(50). This technology, combined with new stabilizing antibody fragments(51), recently enabled determination of the structure of the μOR in complex with the peptide agonist DAMGO and the heterotrimeric G_i protein(26). The overall conformation of the μOR bound to G_i is similar to that when bound to Nb39, with a key difference in the conformation of intracellular loop 3 (Figure 2B). As previously observed for the β_2AR in complex with the stimulatory G protein G_s(52), active μOR induces a large conformational change in the C-terminal helix of the α-subunit of G_i. This change, coupled with a relative disordering of the helical domain of the α-subunit, leads to release of GDP, providing the key basis for how the μOR acts as a guanine exchange factor for G_i. Comparison of the structure of μOR bound to G_i with prior structures of other GPCRs in complex with G_s provides clues into the selective coupling of opioid receptors to G_i, with a primary cause being a steric incompatibility between the active μOR and the C-terminal α5 helix of G_s. These detailed insights provide a mechanistic understanding of opioid signaling. A more complete picture of opioid receptor regulation will require structures of opioid receptors in complex with GRKs and β-arrestins. Such studies have the potential to illuminate how opioids both activate their receptors and induce recruitment of signaling regulators.

Conformational dynamics and functional selectivity

Structural studies have provided high-resolution insights into the chemical recognition of opioids and the conformational changes that drive opioid receptor signaling. However these are single snapshots of a highly dynamic process involving drug binding, receptor conformational changes, and subsequent interactions with cellular signaling partners. Such complexity is critical for opioid receptor function as it likely drives many aspects of opioid pharmacology, including the ability of some opioid agonists to preferentially activate G protein signaling over β-arrestin recruitment(53). Such “biased” opioid agonists have been proposed to display increased therapeutic indices(54). The biophysical basis of their unique efficacy, however, remains poorly understood. Methods like X-ray crystallography and cryo-EM are relatively poorly positioned to provide insight into the dynamics of transmembrane proteins. Instead, approaches that directly observe the conformational ensemble of opioid receptors are required.

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Experimental methods to examine the molecular movements of transmembrane proteins rely on spectroscopic "probes" that change in some measurable physical property as the protein visits different conformational states(55). Among many such approaches, nuclear magnetic resonance spectroscopy (NMR) is uniquely positioned to identify conformations not observed by high resolution structures. In one illustrative example, NMR of dynorphin bound to the κOR revealed significant disorder in the "YGGF" region of the peptide, showing that structural dynamics are present in even the key regions of opioid peptide as they bind their receptors(56). Further NMR experiments have directly examined the opioid receptors. Solution-state NMR of ^{13}C-methyl probes chemically attached to lysine residues in the μOR(57) revealed that agonists only partially activate the receptor, which is similar to dynamics of the β2-adrenergic receptor(38, 58). Evidence for conformational states that remain unobserved by high-resolution structural methods was also demonstrated in another NMR study that labeled methionine residues within the core of the μOR(59). Spectra from these methionine residues clearly demonstrate that the G protein biased agonist TRV130(60) induces a different conformation of the μOR than the balanced agonist DAMGO. Most importantly, these NMR studies show that the μOR exists in a conformational equilibrium in solution, with agonists and antagonists stabilizing unique active or inactive states, respectively. The question of what drives partial agonist efficacy, though, remains poorly explored for opioid receptors and for GPCRs broadly. Recent biophysical studies on the β-adrenoceptors suggest that partial agonists stabilize unique GPCR conformational ensembles with a resulting unique ensemble of the GPCR-G protein complex(61–64). For opioid receptors, it remains unknown whether partial agonists induce unique receptor states, alter receptor conformational dynamics, or influence some other property of the receptor. Spectroscopic techniques like NMR are best poised to answer such questions.

Despite providing the best experimental evidence of conformational heterogeneity, current NMR studies lack atomic-level precision for the entire receptor. Computational molecular dynamics simulations provide a critical bridge between atomic-level insights and molecular motion(19). Simulations can enable direct visualization of ligand binding events, conformational changes in receptors, and activation of intracellular signaling partners. Enhanced simulation approaches have demonstrated the binding and unbinding pathways for opioid ligands and identified binding pockets for allosteric modulators(32). Simulations have also revealed key conduits of allosteric communication between the ligand binding pocket and the intracellular domains of opioid receptors(44), providing clues into how biased agonists achieve their conformational selectivity(65). Although remarkable recent progress has enabled long simulation timescales, a key limitation remains the inability to fully access the broad conformational ensemble of complex proteins like GPCRs. Future efforts combining enhanced simulation approaches and theories for analyzing dynamic systems(66) have the potential to provide an unprecedented level of insight into the experimentally observed conformational dynamics that drives opioid receptor pharmacological complexity(67).

Genetic variation in opioid receptor structure

Structural and biophysical approaches to examine opioid receptor structure have, by necessity, focused on a singular "wild-type" gene product. Genetic variation, however,

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generates significant further heterogeneity in opioid receptor structure. Two sources of such variation, including alternative splicing of opioid receptor genes and population-level polymorphisms in coding or regulatory regions, have direct consequences for opioid receptor structure and function.

The most common μOR variant substitutes Asn40 with Asp(68), which ablates one of the five potential N-linked glycosylation sites within the amino terminus. This mutation leads to decreased cell surface expression and overall receptor stability combined with more subtle effects on the binding affinity of opioid peptides and small molecules(69, 70). Increasing evidence suggests that this variant may alter pain threshold levels, sensitivity to clinically used opioids, and risk of opioid-induced respiratory depression(71–73). At a structural level, the direct consequence of this variant remains unknown due to truncations in the presumably flexible amino terminus required for crystallography.

A number of less common opioid receptor variants map to regions that have been observed by structural methods(74–76), providing a mechanistic basis to understand opioid receptor functional variance. For example, the recent cryo-EM derived structure of the μOR-G_i complex provides a structural rationale for the complete loss of signaling activity for the Arg181Cys variant of μOR(74); in this case, the Arg181 makes a direct contact with the C-terminus of the $G\alpha_i$ subunit. Other rare opioid receptor variants identified by large exome datasets map to many regions of the various opioid receptors, including the ligand binding pocket, key microswitches responsible for receptor activation, interaction interfaces between receptors and signal transducers, and posttranslational sites critical for regulatory processes(76). Many such variants have important consequences for opioid receptor signaling, but the exact mechanism remains unknown. A key future challenge will be to develop structure-guide models, similar to that for Arg181, that enable accurate prediction of how such variants will recognize different opioids and signal to different downstream partners.

Alternative mRNA splicing drives significant protein diversity within the human genome(77). Splicing within the 3’ region of the *OPRM1* gene leads to alternative carboxy-termini for both rodent and human μOR which likely has important implications for tissue-specific regulation of opioid receptors. At a structural level, the most intriguing variants are exon 11 splice variants that lead to a gene product encoding a 6TM variant of the μOR with truncation of the first transmembrane helix (TM1). The functional significance of such 6TM variants has remained controversial, likely stemming from the low probability that such a gene product would successfully fold and traffic to the cell membrane. Indeed, functional expression of the 6TM variant in model cell lines requires co-expression with the ORL1 receptor(78, 79). Intriguingly, however, the analgesic activity of the potent opioid agonist IBNtxA was in *OPRM1*$^{-/-}$ knockout mice virally transduced with a cloned 6TM μOR missing TM1(80). Although TM1 makes no direct contacts with either morphinan ligands or DAMGO in structures of μOR, this pharmacological observation is challenging to reconcile with the extensive contacts that TM1 makes with TM2 and TM7, which suggest that it is critical for proper receptor folding.

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Receptor oligomerization

Although monomeric opioid receptors are sufficient to activate heterotrimeric G proteins(81), a number of studies have proposed that opioid receptors can form higher-order oligomers. Several lines of evidence support functional interactions between individual opioid receptors and either other opioid receptors or other GPCRs(82). Co-immunoprecipitation, selective heteromer-specific antibody generation, unique pharmacological outputs, and biophysical experiments in living cells have provided support for the existence of opioid receptor dimers. Though such studies may support proximity between individual receptors, they do not directly support a physical association enabling allosteric communication from the ligand-binding pocket of one individual receptor to the binding-pocket of a neighboring receptor. Such direct evidence is critical to understanding, and eventually rationally manipulating, the unique pharmacological outcome of opioid receptors as part of putative hetero- or homodimers. Intriguingly, the crystal structure of inactive μOR displayed a large homodimeric interface between TM5 and TM6(22). Although this extensive interface was not observed in structures of the δOR or the κOR, a more limited interface including TM1 and helix 8 was observed for both μOR and κOR. The extensive TM5/TM6 interface observed for inactive μOR is incompatible with the active state, providing a potential mechanism for how dimerization may influence opioid pharmacology. However, the interfaces observed in such structures may represent artifacts from the conditions required for crystallogenesis, and alternative approaches are needed to test the existence and functional relevance of opioid receptor homo and heterodimerization. Indeed, recent molecular dynamics simulations and single molecule diffusion experiments suggest that the lifetime of opioid receptor homodimers are likely to be very transient(83). Reconciling how such transient interactions lead to unique pharmacological outcomes observed in cellular and *in vivo* systems will require significant further biochemical study, including the eventual reconstitution of opioid homo- and hetero-dimers.

Structure enabled drug discovery for opioid receptors

A great hope of the mechanistic studies outlined above is that they may enable the discovery of safer opioid analgesics. Though two centuries of medicinal chemistry efforts have yielded thousands of molecules with varying efficacies and potencies at the opioid receptors, the core molecular scaffolds on which this diversity rests is much more limited, stemming primarily from the morphinans derived from the opioid poppy and a few related synthetic scaffolds. More recent approaches to opioid discovery have leveraged high-throughput screening and defined signaling assays in model cell lines(84, 85).

Opioid receptor structures have provided an unprecedented opportunity for computational drug discovery. Computational approaches directly predict whether a given molecule will productively bind in the receptor pocket(86, 87). A number of improvements in computational chemistry over the past two decades have significantly increased the accuracy of such "docking" calculations and rapidly increasing computational power has enabled screening of ever larger libraries. Such computational drug discovery provides several advantages. First, they enable sampling of an immense breadth of biologically compatible chemical space that is significantly larger than traditional high-throughput screening and

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even the largest DNA-encoded libraries described to date(88). With the simple hypothesis that new, structurally divergent chemicals acting at the opioid receptors are likely to come with unique biological properties(86), this provides a powerful discovery platform for potentially safer opioids. Second, molecules derived from computational docking have strong hypotheses for binding interactions within the receptor pockets, providing an easier path towards optimization of affinity and receptor selectivity. Finally, docking enables targeting of new allosteric sites revealed in the structures(89, 90).

In an early example using the inactive structure of the κOR, virtual screening enabled discovery of a potent agonist for the κOR(91). A subsequent virtual screen of against the κOR similarly yielded 11 new chemical scaffolds, with one (compound 81) displaying G_i biased agonism(92). For the μOR, docking 3 million molecules against the inactive μOR yielded seven completely new molecular scaffolds targeting the receptor(93). Further optimization of one of these yielded the G_i biased agonist PZM21, which, in preliminary rodent studies, provides some separation of analgesia from important side effects like respiratory suppression. It is notable that each of these campaigns derived agonists while starting from the inactive structure of the receptors, demonstrating that docking, although highly useful as a discovery engine, is far from providing true predictions on molecular efficacy. An alternative strategy to specifically target opioids to sites of injury used computational approaches to alter the molecular properties of fentanyl, generating a molecule (NFEPP) that is selectively protonated in inflamed peripheral tissues(94). The resulting selectivity increased the overall therapeutic window of this potent analgesic. While each these successes are encouraging, a critically important goal for the future is to predictively design not only the desired selectivity and efficacy at each of the four opioid receptor subtypes, but also the molecular properties needed to advance molecules into the clinic.

Conclusion and Outlook

Rapid progress over the past decade in the structural biophysics and cellular biology of opioid receptors has yielded one of the most detailed perspectives on this important family of drug targets. While the cellular biology and circuit-level function of opioid receptors are clearly critically important for the range of opioid receptor function, even the receptor proteins themselves have a remarkable array of sequence-level diversity. Each of these opioid receptor variants may form higher order interactions with either other receptors or signaling adapters, likely giving rise to unique signaling outcomes. Even the receptor proteins themselves are incredibly conformationally dynamic, which yields a broad range of diverse signaling outcomes for agonists that target the same receptor. A critical goal remains to understand this complexity and to harness it towards the development of novel opioids devoid of their lethal side effects.

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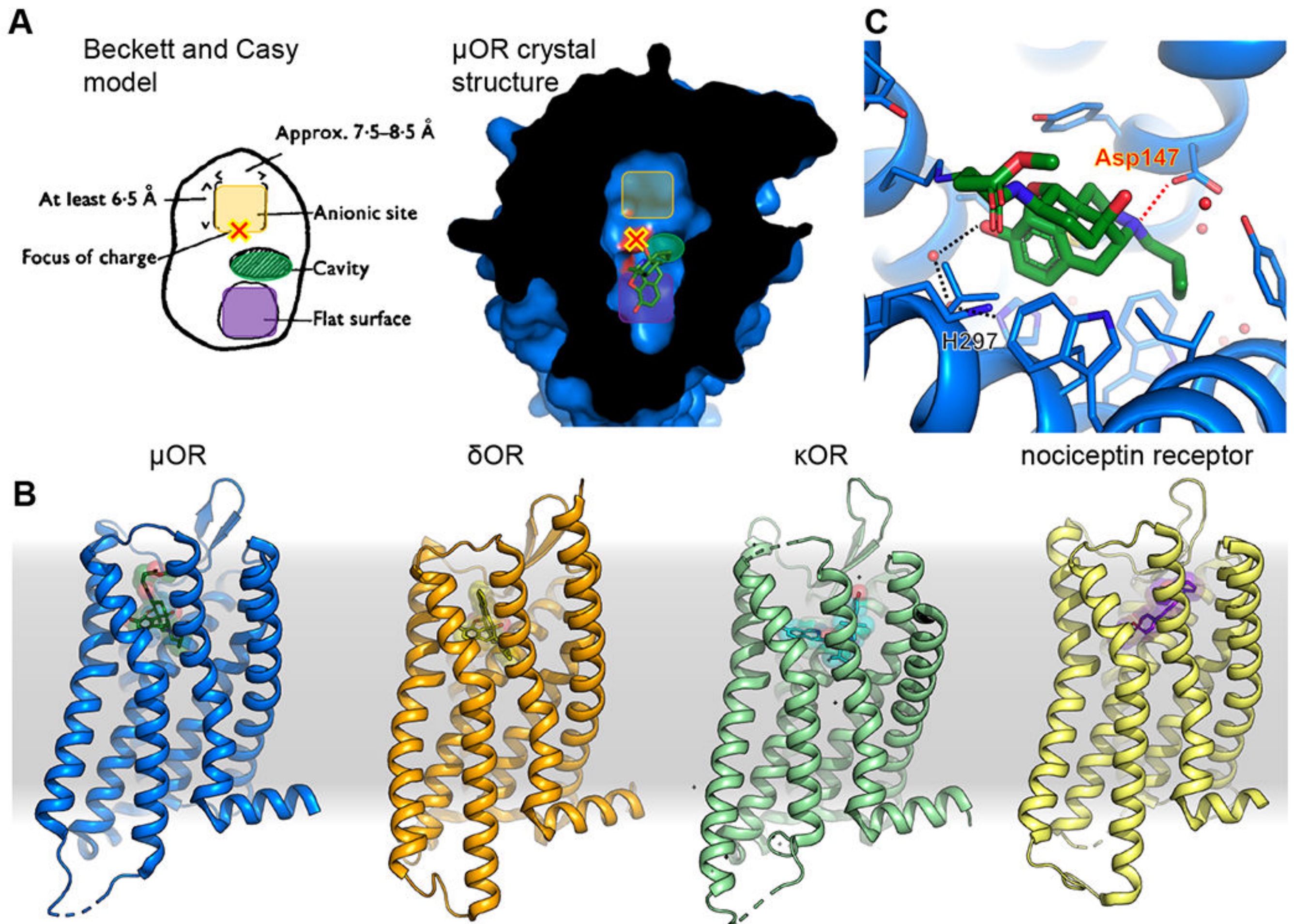

Figure 1. Molecular recognition of opioids.

A. Comparison of an opioid receptor model proposed by Beckett and Casy based on extensive structure-activity relationships of opioid-active compounds. The crystal structure of μOR revealed many features consistent with this model, including a key anionic site, a cavity to accommodate substitutions on a common amine in opioid ligands, and a flat surface to enable recognition of a phenolic moiety common to many opioids. B. Inactive-state structures of all four opioid receptors bound to antagonists. C. Close up view of the μOR binding site with the covalent antagonist β-funaltrexamine shown in green sticks. The position of the key anionic site (Asp147 in murine μOR) and a conserved histidine residue responsible for recognition of opioid phenols is shown.

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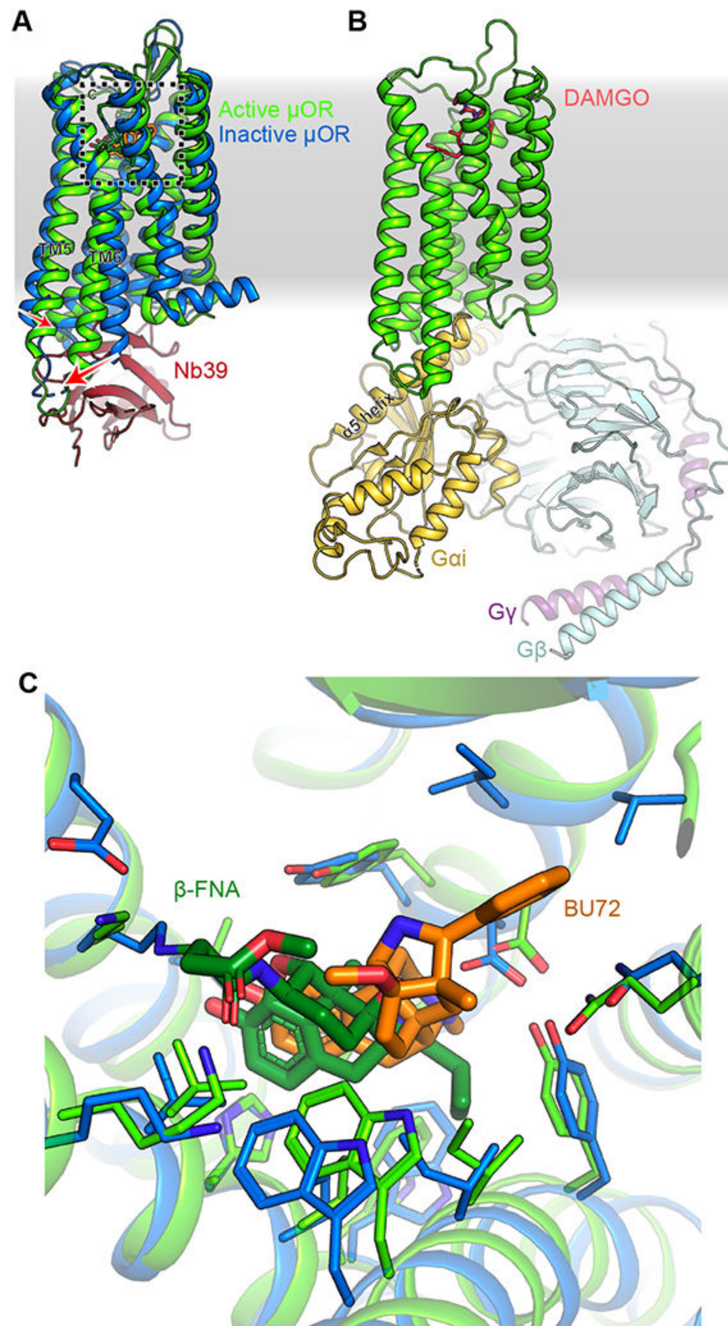

Figure 2. Opioid receptor activation.

A. Comparison of the inactive structure of μOR bound to the antagonist β-funaltrexamine and the active state bound to the agonist BU72 and a stabilizing nanobody (Nb39). Key structural changes associated with activation include and outward displacement of transmembrane helix 6 (TM6) and an inward displacement of TM5. B. Structure of μOR bound to the peptide agonist DAMGO and the heterotrimeric inhibitory G protein. The α5 helix of the G_i α subunit binds in the core of the μOR leading to subsequent release of the bound GDP. C. Close up view comparing the binding pocket of active and inactive μOR.

Many of the contacts between the receptor and agonist/antagonist are conserved. These subtle changes, however, critically drive opioid receptor activation.

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Table 1.

Biophysical approaches to understand opioid receptor function

Technique	**Enabling technologies**	**Key insight gained**	**Key limitations**
X-ray crystallography	•Protein engineering •Stabilizing antibodies •Membrane protein crystallization techniques •Microfocus diffraction beamlines	•Three dimensional structures of receptors and ligands •High resolution insights into the chemistry of ligand recognition	•Obtaining crystals is challenging •Limited information on protein dynamics •Dynamic complexes less amenable to crystallogenesis
Cryo-electron microscopy	•Direct electron detectors •New computational algorithms for single particle analysis	•Three dimensional structures of receptors and ligands •Structural heterogeneity of dynamic complexes	•High resolution structures remain challenging •Extensive optimization often required
Nuclear magnetic resonance spectroscopy	•Novel detergents to stabilize membrane proteins •High field magnets	•Structural dynamics of receptors and ligands •Directly observe transient ligand-receptor interactions	•Need three dimensional structures to provide context •Receptor preparations challenging to produce; limited to smaller protein fragments
Molecular dynamics simulations	•Dedicated hardware/software for simulation •Increasing computational power	•Structural dynamics of receptors and ligands •Atomic “microscope” detailed insights into allostery and molecular recognition	•Limited to shorter timescales (milliseconds) •Requires significant computational resources for long-timescale simulations