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Behavioral Neuroscience of Orexin/ Hypocretin

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Andrew J. Lawrence • Luis de Lecea
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Behavioral Neuroscience of Orexin/Hypocretin

 Springer

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Preface

Twenty years have passed since the discovery of the Hypocretins/Orexins [1, 2], as we have witnessed an amazing set of discoveries demonstrating key roles for these transmitters in arousal and beyond. This volume entitled *Behavioral Neuroscience of Orexin/Hypocretin* in the series *Current Topics in Behavioral Neurosciences* focuses on the most recent advances on this neurotransmitter system and brings together scientists from around the world who provide a timely discussion of how this peptide regulates different aspects of behavior. We have attempted to give readers a broad taste of the current knowledge base surrounding the evolutionary conservation of orexin/hypocretin systems as demonstrated in zebrafish (see [3]), orexin/hypocretin receptor structure (see [4]), orexin/hypocretin signaling (see [5]), and the role of orexin/hypocretin in numerous physiological and behavioral processes. For example, the critical role of orexin/hypocretin in arousal is canvassed (see [6]) as well as the evidence for the involvement of orexin/hypocretin systems in Alzheimer's disease (see [7]). From a systems physiology perspective we cover the role of orexin/hypocretin in energy balance (see [8]), plus how orexin/hypocretin modulates cardiovascular and respiratory function (see [9]). From a clinical perspective this issue details the development of OX2 receptor antagonists as sleep aids (see [10]).

Over the past decade or so there has been intense interest regarding how orexin/hypocretin regulates both natural and aberrant reward-seeking. Included in this issue are chapters on orexin/hypocretin and addiction (see [11]), plastic changes associated with drug abuse (see [12]), and orexin/hypocretin and alcohol use/abuse (see [13]). Related to this facet of orexin/hypocretin neurobiology we also address how this system impacts stress reactivity (see [14]).

The discovery of the hypocretins/orexins has led to many important advances in the neurosciences: identifying a molecular marker to untangle the cellular composition of the lateral hypothalamus, providing a mechanism for sleep/wake regulation, a diagnostic tool for narcolepsy, a first-in-class chemical entity approved for the treatment of primary insomnia, and the first implementation of optogenetics in vivo. Overall this issue captures many of these discoveries. We acknowledge that

this is a fast-moving field and therefore cannot encompass every relevant topic; however our intention was to appeal to a broad cross section of basic scientists and clinicians.

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The Human Orexin/Hypocretin Receptor Crystal Structures

Jie Yin and Daniel M. Rosenbaum

Abstract The human orexin/hypocretin receptors (hOX₁R and hOX₂R) are G protein-coupled receptors (GPCRs) that mediate the diverse functions of the orexin/hypocretin neuropeptides. Orexins/hypocretins produced by neurons in the lateral hypothalamus stimulate their cognate GPCRs in multiple regions of the central nervous system to control sleep and arousal, circadian rhythms, metabolism, reward pathways, and other behaviors. Dysfunction of orexin/hypocretin signaling is associated with human disease, and the receptors are active targets in a number of therapeutic areas. To better understand the molecular mechanism of the orexin/hypocretin neuropeptides, high-resolution three-dimensional structures of hOX₁R and hOX₂R are critical. We have solved high-resolution crystal structures of both human orexin/hypocretin receptors bound to high-affinity antagonists. These atomic structures have elucidated how different small molecule antagonists bind with high potency and selectivity, and have also provided clues as to how the native ligands may associate with their receptors. The orexin/hypocretin receptor coordinates, now available to the broader academic and drug discovery community, will facilitate rational design of new therapeutics that modulate orexin/hypocretin signaling in humans.

Keywords Antagonist • Crystal structure • GPCR • High-resolution • Hypocretin • Orexin

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1 Goals Behind Solving Structures of hOX₁R and hOX₂R

The motivation for structural studies of the orexin/hypocretin receptors is twofold. First, we would like to understand at the atomic level how the native ligands (orexin-A and orexin-B) and synthetic antagonists bind to the receptors, and how ligand binding stabilizes distinct receptor conformations. This goal is fundamentally a problem of understanding GPCR allostery for the specific case of the orexin/hypocretin receptors. Second, by studying receptor–ligand interactions, we hope to create knowledge and tools that translate into the design of more potent and selective modulators of orexin/hypocretin signaling.

hOX₁R and hOX₂R exhibit different physiological functions and pharmacology [1]. The two receptor subtypes are expressed differentially in various CNS regions [2], and pharmacological and genetic studies have uncovered differences in their behavioral functions. The hOX₂R is the more evolutionarily ancient of the two subtypes [3], and plays a more significant role in controlling circadian rhythms, sleep, and arousal [4, 5]. Thus modulation of hOX₂R has become an attractive therapeutic strategy for sleep and wake disorders such as insomnia (e.g., the FDA-approved drug suvorexant) and narcolepsy (for potential small molecule orexin mimetics). The hOX₁R functions in modulating reward [6, 7], nociception [8], and stress [9], and inhibition of this subtype has developed into an active therapeutic area for disorders such as addiction [10].

2 Challenges for Solving High-Resolution Crystal Structures of GPCRs

The key to determining high-resolution structures of the hOX₁R and hOX₂R was the ability to obtain diffraction-quality crystals. A decade ago, ligand-activated GPCRs were thought to be largely intractable targets for structure determination. However crystallization of GPCRs has recently become possible due to a number of breakthrough technologies that were developed for GPCRs and other integral membrane proteins. First, methods of expression and purification from recombinant systems such as *Spodoptera frugiperda* (Sf9 insect cells) [11], *Pichia pastoris* (yeast) [12, 13], and mammalian cells (such as HEK293) [14] have allowed labs to purify milligram quantities of many GPCRs. Second, protein engineering methods

including crystallizable domain chimeras [15], thermostabilizing mutations [16], and antibodies [12, 17] and nanobodies [18, 19] have made purified GPCRs more stable and amenable to forming three-dimensional crystals. Third, detergents such as the neopentyl glycols [20] were developed which stabilize GPCRs during solubilization, purification, and reconstitution. Finally, lipid-mediated crystallization techniques such as lipidic cubic phase (LCP) [21] and bicelles [22] have facilitated GPCR crystal formation by promoting lateral crystal contacts between the receptors' transmembrane (TM) regions.

Despite these advances, GPCRs are still highly challenging targets for X-ray crystallography. To highlight this point, the only previous successful crystallography efforts for GPCRs in the β branch of the rhodopsin family were for thermostabilized mutants of the rat neurotensin receptor (rNTR1) bound to neurotensin agonist peptides [23–25]. In order to crystallize hOX₁R and hOX₂R, we solubilized the protein out of Sf9 cell membranes in lauryl maltose neopentyl glycol (LMNG), and purified milligram quantities of the receptors to homogeneity. Further, we created chimeras with domains such as T4L that had previously facilitated GPCR crystallization [15]. After using these methods, we were still not able to obtain diffraction-quality crystals. To finally overcome this barrier, we had to identify and develop a new fusion domain – PGS (*Pyrococcus abyssi* glycogen synthase) – that yielded high-resolution diffracting crystals for both hOX₁R [26] and hOX₂R [27] when fused at the third intracellular loop (ICL3). The ICL3 fusion strategy is successful for GPCR crystallization because it removes an inherently flexible region of the receptor (ICL3), which may hinder crystal contact formation, and adds a stable folded structure in its place that can successfully mediate lattice contacts [15]. While other crystallizable domains have been developed as fusion protein partners [28], the new PGS domain proved indispensable for our efforts to crystallize both orexin receptor subtypes.

3 Comparison of hOX₁R and hOX₂R to Other GPCRs

The crystal structures that we obtained of the orexin/hypocretin receptors each have high-affinity antagonists bound (see Sect. 5). The extracellular surfaces of hOX₁R and hOX₂R broadly resemble those of other peptide-binding GPCRs. Figure 1 shows identical views of four different GPCRs activated by peptide hormones that have been structurally characterized: hOX₂R [27], rat neurotensin receptor rNTR1 [25], opioid receptor μ OR [29], and the chemokine receptor CXCR4 [30]. Along with other related examples [31, 32], these structures support the idea that all peptide-activated GPCRs in the rhodopsin family (Class A) contain a short β -hairpin (two antiparallel β -strands) in the second extracellular loop (ECL2) situated above the orthosteric pocket. In the “partially active” conformation shown for rNTR1, the agonist peptide Neurotensin₈₋₁₃ packs against the β -hairpin motif. This motif may be a general platform for GPCR-peptide interaction due to the large flat surface area it presents, although other peptide-bound structures are needed to

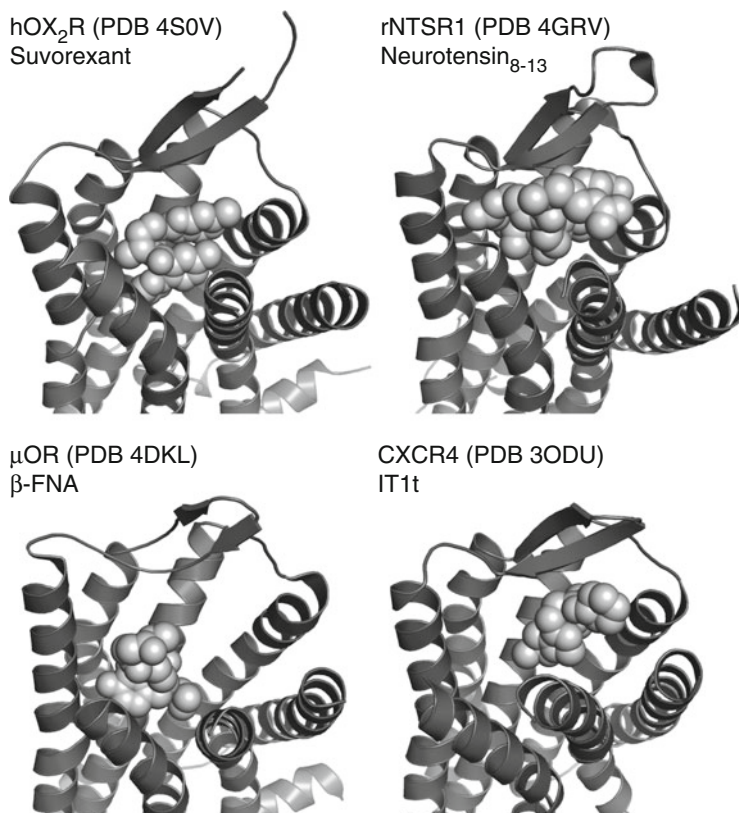


Fig. 1 Crystal structures of different peptide-activated GPCRs. Receptors are depicted as dark gray cartoons, and antagonist ligands are depicted as light gray spheres

confirm this prediction. The sites of several of the most deleterious reported mutations for orexin/hypocretin affinity and potency are on this β -hairpin [33]. In contrast, in the secretin family of GPCRs (Class B), high-affinity peptide recognition requires a large folded N-terminal extracellular domain [34].

As illustrated in Fig. 1, small molecule antagonists for hOX₂R, μ OR, and CXCR4 are each positioned in the orthosteric pocket that is created by the TM α -helices embedded within the membrane. In fact, the position of the drug suvorexant (used for co-crystallization of both hOX₁R and hOX₂R) is very similar to the binding site for β -blocker inverse agonists such as carazolol in the β_2 adrenergic receptor (β_2 AR), the most extensively studied model system for ligand-activated GPCRs (Fig. 2a). Structures of β_2 AR with antagonists [15], agonists [18, 35], and G proteins [19] led to a model in which agonist-induced inward movements of the TM α -helices at the orthosteric binding pocket initiate conformational changes through the transmembrane 7TM bundle. These changes ultimately lead to outward movement of TMs 5 and 6 at the intracellular surface, which is necessary for the engagement of G proteins. Based on this model, also recapitulated in other members

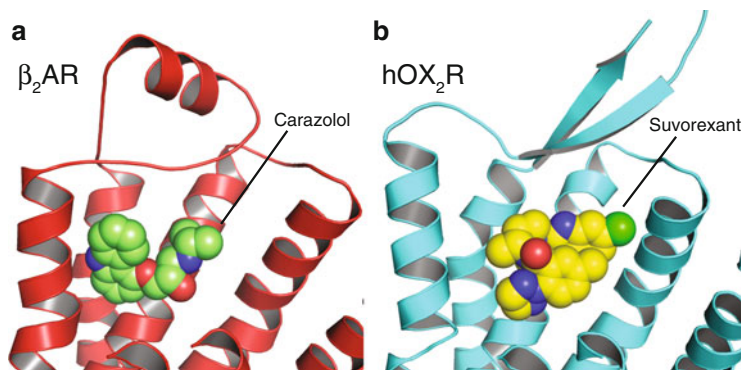


Fig. 2 Analogous positions of antagonist binding in the orthosteric pockets of different GPCRs. (a) Carazolol inverse agonist (spheres with green carbons) bound to the β_2 adrenergic receptor (red cartoon). (b) Suvorexant antagonist (spheres with yellow carbons) bound to hOX₂R (cyan cartoon)

of the GPCR superfamily [36], binding of antagonists such as suvorexant (Fig. 2b) are hypothesized to stabilize the inactive conformation by preventing inward movement of the TM α -helices at the orthosteric site, as well as competing for binding surfaces with the native orexin/hypocretin ligands. Comparison of the published structures of inactive [23] and partially active [25] conformations of rNTSR1 showed that a peptide agonist can promote an outward shift of TM6 at the intracellular surface, through binding of a surface that overlaps with suvorexant's binding site in the orexin receptors.

4 Overall Structures of hOX₁R and hOX₂R

The global structures of hOX₁R and hOX₂R are shown in Fig. 3. To date, we have reported the structure of hOX₁R bound to the dual orexin receptor antagonist (DORA) suvorexant at 2.75 Å resolution (Fig. 3a); the structure of hOX₁R bound to the type 1-selective antagonist SB-674042 at 2.8 Å resolution (Fig. 3b); and the structure of hOX₂R bound to suvorexant at 2.5 Å resolution (Fig. 3c) [27, 37]. The root mean squared deviation (rmsd) between superimposed hOX₁R and hOX₂R is 0.4 Å over 282 C α 's, indicating that these two receptors (with 64% sequence identity) are very similar in three-dimensional structure (Fig. 3d). Beyond this similarity, the rmsd between the hOX₂R structure (Fig. 3c) and the inactive-state structure of the β_2 AR (with 23% sequence identity) is only 2.2 Å, highlighting the strong structural conservation within the GPCR superfamily. The major difference between the crystal structures lies in the extracellular region containing the ECL2 and N-terminus. The hOX₁R has a short α -helix preceding TM1, which packs against the ECL2 (Fig. 2a, b). We did not observe such a motif in our hOX₂R structure (Fig. 3c); however, this α -helix may exist but have too much flexibility to

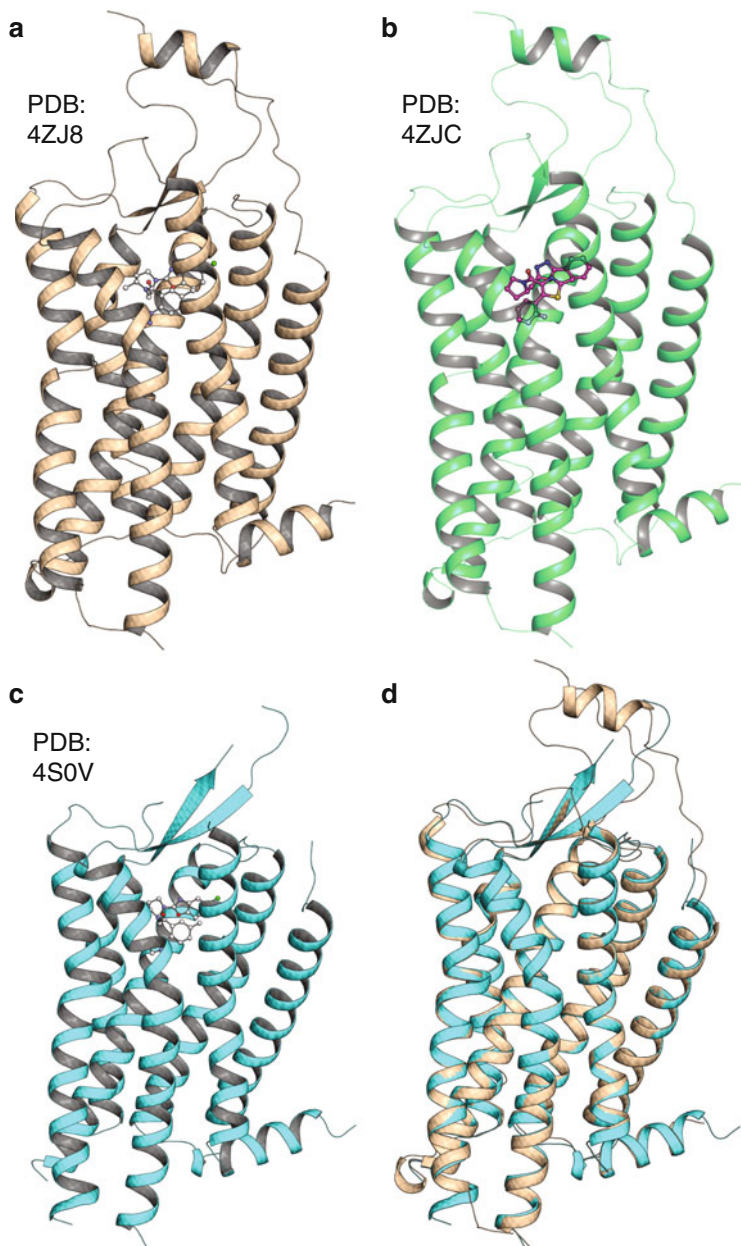


Fig. 3 Structures of orexin receptors bound to small molecules. **(a)** hOX₁R (wheat cartoon) bound to suvorexant (balls and sticks with gray carbons). **(b)** hOX₁R (green cartoon) bound to SB-674042 (balls and sticks with magenta carbons). **(c)** hOX₂R (cyan cartoon) bound to suvorexant (balls and sticks with gray carbons). **(d)** Superposition of hOX₁R and hOX₂R

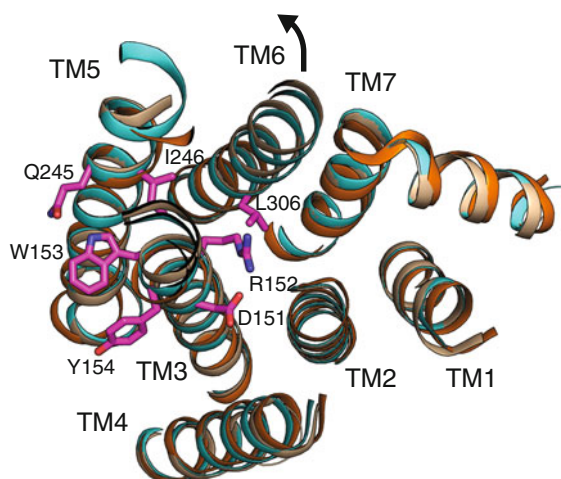


Fig. 4 Structures of inactive-state GPCRs at the intracellular surface of the membrane. Superposition of suvorexant-bound hOX₁R (wheat cartoon), suvorexant-bound hOX₂R (cyan cartoon), and tiotropium-bound M3 muscarinic acetylcholine receptor (orange cartoon, PDB 4DAJ), viewed from intracellular side. Intracellular loops are removed for clarity. The DRWY residues on TM3 and interacting residues on TMs 5 and 6 are shown as magenta sticks. *Arrow* indicates outward movement of TM6 during activation

visualize in the crystal's electron density. We believe that this N-terminal α -helix is directly involved in orexin/hypocretin recruitment and receptor activation (see Sect. 6).

Like all GPCRs, hOX₁R and hOX₂R translate agonist binding into functional responses through receptor-mediated activation of intracellular heterotrimeric G proteins, principally G_{q/11} and G_{i/o} for the orexin/hypocretin receptors [38]. Based on previous structural and biophysical studies of other GPCRs [18, 35, 36], the binding of agonists should stimulate outward movement of the TM α -helices at the cytoplasmic surface to facilitate binding of G proteins [19]. In the inactive state of the β_2 AR, TM5 and TM6 pack against TM3 bearing the conserved DRY motif at the intracellular surface, blocking epitopes involved in G protein binding. The DRY motif is conserved throughout Class A GPCRs, and these residues are important for maintaining a stable inactive-state conformation with low basal activity [39, 40]. For hOX₁R and hOX₂R, the DRY motif is changed to DRWY, and these residues are tightly packed against residues from TM5 and TM6 (Fig. 4), analogous to the antagonist-bound M3 acetylcholine receptor (another G_q-coupled GPCR). Therefore we can conclude that the antagonist-bound crystal structures of hOX₁R and hOX₂R represent inactive conformations [26, 27].

5 Binding of Small Molecule Antagonists

Orexin receptor antagonists are prospective therapeutics for a number of different human diseases, as detailed elsewhere in this volume. So far, the only such molecule to be approved by the FDA is suvorexant (Belsomra) [41] for treatment of insomnia. An important goal of characterizing the structures of the receptors is to understand the precise mechanisms by which antagonists bind and prevent activation. The solvent-exposed orthosteric binding sites where small molecule antagonists bind to hOX₁R and hOX₂R are well ordered in the crystal structures, along with the bound small molecules. Figure 5 shows the ligand binding poses and detailed interactions with the receptors for all three crystal structures we have reported: hOX₁R with suvorexant (Fig. 5a), hOX₁R with SB-674042 (Fig. 5b), and hOX₂R with suvorexant (Fig. 5c) [26, 27]. The binding sites include contributions from the extracellular ends of all TMs except TM1, as well as from the ECL2. Several of the amino acids that make the greatest contact with the ligands (in terms of buried surface area) have been previously characterized in mutagenesis studies as contributing greatly to antagonist affinity, adding further functional support to our structural data [42–45].

The two ligands that we have so far co-crystallized with the orexin receptors both adopt a compact horseshoe-like bound conformation, in which two aromatic groups, separated by a spacer group, engage in intramolecular aromatic stacking interactions (Fig. 5). For suvorexant analogs, a related 3D conformation of the isolated ligand in solution was previously reported, and suggested to be relevant to the receptor-binding conformation [46]. Our structures support the idea that these molecules and related antagonists prepay some of the entropic cost of ligand binding by constraining their 3D conformations through intramolecular packing. Indeed, a large number of the small molecule orexin/hypocretin receptor antagonists discovered by different laboratories have the same basic form, in which two aromatic moieties are separated and presented by a small ring scaffold [47]. We predict that many of these molecules will bind in a similar mode as we have elucidated in our crystal structures.

In conjunction with solving the hOX₁R structures, we carried out several different computational analyses to better understand how subtype-selective ligands such as SB-674042 discriminate between hOX₁R and hOX₂R. These studies demonstrated how the very highly conserved orthosteric binding pockets, which have only two subtle differences in amino acid composition (Fig. 4d), create small differences in pocket volume and shape that can be exploited to achieve selectivity toward either subtype [26]. The hOX₁R-selective SB-674042 occupies slightly more space in the orthosteric pocket and clashes with the two larger divergent residues (Thr111 and Thr135) in the resulting slightly smaller hOX₂R pocket. In contrast, docking and simulation of the hOX₂R-selective antagonist 2-SORA-DMP indicated that better shape complementarity and van der Waals contacts with hOX₂R lead to greater affinity compared to hOX₁R with its larger orthosteric pocket. While our structural observations and calculations are consistent with the

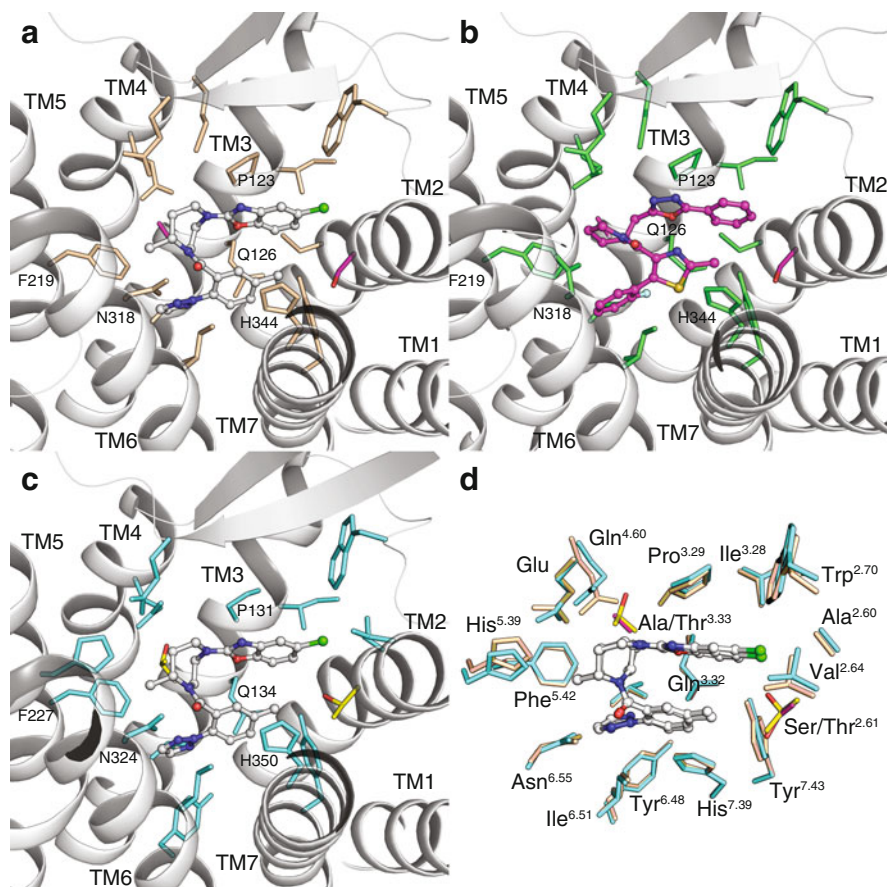


Fig. 5 Binding pockets and antagonist interactions with the orexin/hypocretin receptors. (a) Contact residues within 4 Å between suvorexant (balls and sticks with gray carbons) and hOX₁R (gray cartoon with wheat sidechains). (b) Contact residues within 4 Å between SB-674042 (balls and sticks with magenta carbons) and hOX₁R (gray cartoon with green sidechains). (c) Contact residues within 4 Å between suvorexant (balls and sticks with gray carbons) and hOX₂R (gray cartoon with cyan sidechains). (d) Superposition of binding pocket residues of suvorexant-bound hOX₁R (wheat sticks) and suvorexant-bound hOX₂R (cyan sticks). Labels use Ballesteros-Weinstein numbering in superscript. The two divergent binding site residues are displayed as magenta sticks for hOX₁R and yellow sticks for hOX₂R

subtype selectivity of these antagonists, one caveat is that the crystallographic coordinates represent saturated complexes with high ligand occupancy and do not inform kinetic mechanisms that influence binding selectivity. Intriguingly, the antagonist-bound orexin receptor structures revealed a “lid” over the binding pocket formed by multiple salt bridges, leaving only a constricted solvent channel to the orthosteric site. This feature implies that the receptor’s extracellular surface must breathe in order to allow access for antagonists, which may influence both

association and dissociation. The precise contributions of binding pocket residues and the extracellular structure to ligand binding kinetics can now be probed with pharmacological studies of mutant receptors guided by the high-resolution structures. Another important factor that is not captured by our structures is ligand and binding pocket desolvation that occurs during complex formation [48]. In future studies, interactions of water molecules with the receptor, ligand, and bound complex can be simulated using our crystallographic coordinates as a framework, to achieve a more complete understanding of the large differences in subtype affinity displayed by selective orexin receptor antagonists.

6 Clues for Orexin/Hypocretin Interaction with hOX₁R and hOX₂R

We currently lack a clear understanding of how the orexin/hypocretin neuropeptides bind and activate their cognate GPCRs. One intriguing clue from the hOX₁R structure was the ordered N-terminal region prior to TM1, containing a short amphipathic α -helix that is positioned over the orthosteric binding pocket (Fig. 6a). This region is conserved in all known vertebrate orexin/hypocretin receptor sequences, from fish and amphibians to humans [49] (Fig. 6b). Given that the NMR structures of orexin-A and orexin-B also revealed amphipathic α -helices [50, 51], we hypothesized that the structured N-terminal region is involved in binding and recruitment of the neuropeptides through interactions of α -helices. Using a combination of binding and receptor activation assays, we showed that this element is essential for potent orexin-A activation for both hOX₁R and hOX₂R. We also found that mutation of a polar residue in the orthosteric binding pocket (hOX₁R N318 or hOX₂R N324) severely diminished orexin-A potency [26]. Previous site-directed mutagenesis experiments demonstrated that residues in the ECL2 β -hairpin (e.g., hOX₁R D203A or hOX₂R D211A) are critical for orexin-A potency [33]. Putting these findings together, we propose that orexin-A binds to hOX₁R or hOX₂R through a polytopic interface involving all three of these receptor sites (Fig. 6a). In this context, the published result that 17 or more amino acids in the orexin/hypocretin neuropeptides are required to reach low-nanomolar potency [52] can be easily rationalized. A detailed picture of how this interface forms and influences receptor conformation will await structures of the neuropeptide-bound GPCRs. It is worth noting that juxtamembrane N-terminal regions are involved in binding peptide agonists for a number of other rhodopsin family GPCRs, including formyl peptide receptor [53], cholecystokinin (CCK) receptor [54], and the tachykinin receptors [55]. The N-terminal region also plays a key role in chemokine receptors, providing an extended epitope that interacts with the folded globular domain of the chemokine hormone [56, 57].

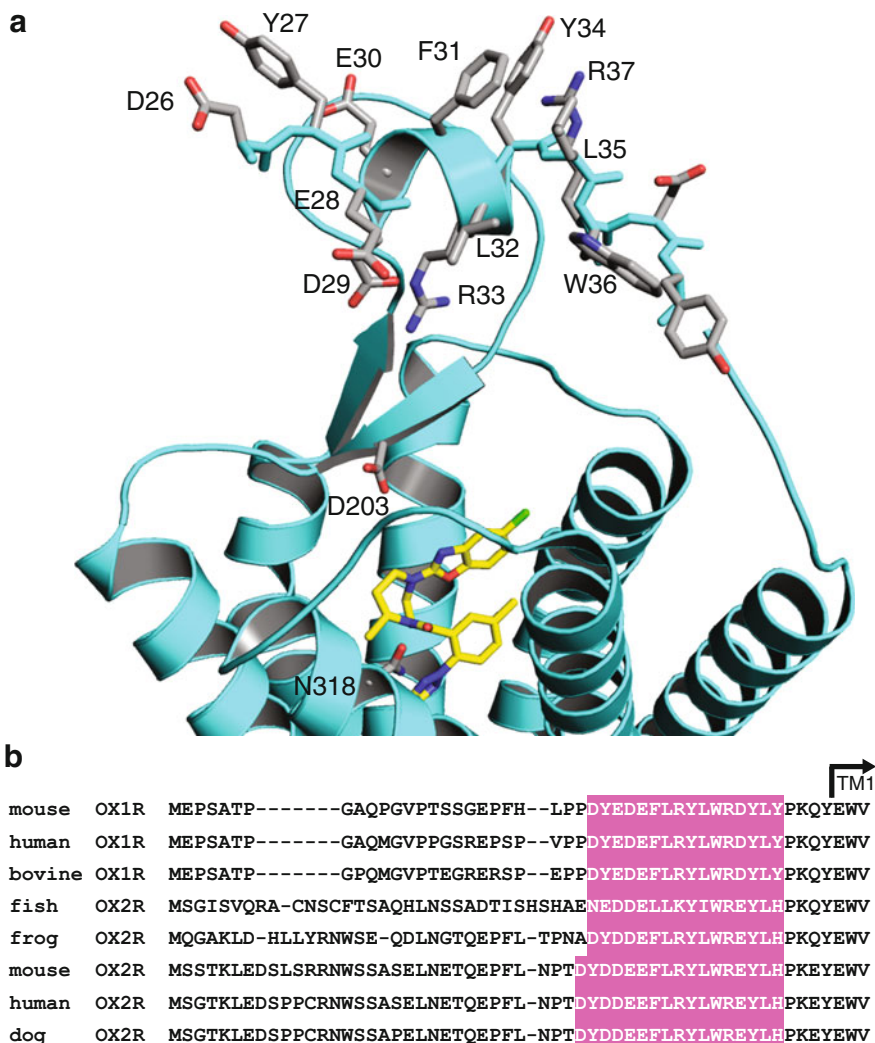


Fig. 6 Structure of the N-terminal extracellular region of hOX₁R. **(a)** The amphipathic α -helix at the N-terminus preceding TM1 in hOX₁R structure (cyan cartoon, sidechains with gray carbons). **(b)** Sequence alignment of orexin receptor N-termini, including *Mus musculus* (mouse), *Homo sapiens* (human), *Bos Taurus* (bovine), *Danio rerio* (fish), *Xenopus laevis* (frog), and *Canis lupus* (dog) sequences (adapted from Yin et al. [26])

7 Conclusions and Future Prospects

The crystal structures of the human orexin/hypocretin receptors have provided an atomic-level framework for understanding binding and subtype selectivity of small molecule antagonists, including the clinically used suvorexant and the hOX₁R-

selective SB-674042 [26, 27]. In addition, the structures have revealed a previously unknown role for the receptor N-terminal region in recruitment of the orexin/hypocretin neuropeptides [26]. We anticipate that our publicly deposited coordinates (PDB accession codes 4SOV, 4ZJ8, 4ZJC) and future co-crystal structures using our reported constructs and protocols will aid the design and optimization of orexin/hypocretin receptor antagonists with improved affinity and subtype selectivity.

To fully elucidate the mechanism for orexin hormone activation of the orexin receptors, we must obtain structures bound to agonists, as well as complexes with G proteins or G protein-mimetic antibodies. Crystallographic and biophysical studies of β_2 AR other ligand-activated GPCRs have shown that extracellular agonist binding and intracellular conformational changes leading to signaling are weakly coupled [35, 58]. Therefore structures of the receptors bound to the orexin neuropeptides alone (akin to Egloff et al. [23]) may not reveal the propagated conformational changes through the membrane that ultimately result in signal propagation. In studies of active β_2 AR [18], M3 muscarinic acetylcholine receptor [36], and μ opioid receptor [59], this problem was overcome by selecting nanobodies (small single-chain antibody domains derived from llamas) that stabilize the active conformation by binding at the G protein coupling site [60]. Discovery of active state-stabilizing nanobodies for the orexin receptors may similarly enable structure determination of an active neuropeptide-bound GPCR, illuminating how the peptide agonist allosterically promotes the active conformation. Further, the structures of the orexin receptors in different conformations will allow design of biophysical experiments to measure the dynamic changes between states, for example, by fluorescence [61] and NMR spectroscopy [62]. Finally, the biochemical precedents for homogeneous purification and crystallization of the orexin receptors may facilitate attempts to co-crystallize these GPCRs with G proteins, such as the G_q heterotrimer. While this goal is highly challenging (there is only one GPCR-G protein complex structure solved to date, for β_2 AR) [19], only a complex with a G protein will ultimately explain how orexin neuropeptides stimulate G protein signaling. Importantly, these developments would pave the way for structure-guided design of small molecule activators of orexin signaling, which have so far been extremely challenging to isolate. The coming years promise to be an exciting time for biophysical characterization and manipulation of orexin/hypocretin signaling.

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Orexin/Hypocretin Signaling

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Abstract Orexin/hypocretin peptide (orexin-A and orexin-B) signaling is believed to take place via the two G-protein-coupled receptors (GPCRs), named OX₁ and OX₂ orexin receptors, as described in the previous chapters. Signaling of orexin peptides has been investigated in diverse endogenously orexin receptor-expressing cells – mainly neurons but also other types of cells – and in recombinant cells expressing the receptors in a heterologous manner. Findings in the different systems are partially convergent but also indicate cellular background-specific signaling. The general picture suggests an inherently high degree of diversity in orexin receptor signaling.

In the current chapter, I present orexin signaling on the cellular and molecular levels. Discussion of the connection to (potential) physiological orexin responses is only brief since these are in focus of other chapters in this book. The same goes for the post-synaptic signaling mechanisms, which are dealt with in Burdakov: Postsynaptic actions of orexin. The current chapter is organized according to the tissue type, starting from the central nervous system. Finally, receptor signaling pathways are discussed across tissues, cell types, and even species.

Keywords Adenylyl cyclase • Calcium • Diacylglycerol lipase • Endocannabinoid • G_i • G_q • G_s • Heterotrimeric G-protein • Hypocretin • K⁺ channel • Na⁺/Ca²⁺-exchanger • Non-selective cation channel • Orexin • OX₁ receptor • OX₂ receptor • Phospholipase A₂ • Phospholipase C • Phospholipase D • Protein kinase C

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