Narcolepsy type 1 (NT1) is a chronic neurological disorder that impairs the brain’s ability to control sleep-wake cycles. Current therapies are limited to the management of symptoms with modest effectiveness and substantial adverse effects. Agonists of the orexin receptor 2 (OX2R) have shown promise as novel therapeutics that directly target the pathophysiology of the disease. However, identification of drug-like OX2R agonists has proven difficult. Here we report cryo-electron microscopy structures of active-state OX2R bound to an endogenous peptide agonist and a small-molecule agonist. The extended carboxy-terminal segment of the peptide reaches into the core of OX2R to stabilize an active conformation, while the small-molecule agonist binds deep inside the orthosteric pocket, making similar key interactions. Comparison with antagonist-bound OX2R suggests a molecular mechanism that rationalizes both receptor activation and inhibition. Our results enable structure-based discovery of therapeutic orexin agonists for the treatment of NT1 and other hypersomnia disorders.
Orexin A (OXa) and orexin B (OXb), also termed hypocretin-1 and hypocretin-2, respectively, are excitatory neuropeptides produced in the hypothalamus that control sleep/wake behavior by promoting and maintaining wakefulness, and suppressing rapid eye movement (REM) sleep1–3. Loss of orexin-producing neurons is the cause of narcolepsy type 1 (NT1), also termed classical narcolepsy or narcolepsy with cataplexy4–6, a lifelong neurological disorder that affects 1 in 2000 to 1 in 4000 people7–9. Patients suffer from excessive daytime sleepiness and abnormal REM sleep-related symptoms, such as hallucinations, sleep paralysis, and cataplexy. Available therapies are limited to managing symptoms with modest effectiveness and moderate to severe adverse effects8–10.

OXa and OXb signal through the two closely related receptors, orexin receptor type 1 and type 2 (OX1R and OX2R, respectively), expressed widely across the central nervous system1. OX1R and OX2R are class A G-protein-coupled receptors (GPCRs) of the β-branch that signal predominantly through heterotrimeric Gq/11, leading to increased cytosolic Ca2+ levels10. Inhibition of both OX1R and OX2R with antagonists provides an effective treatment of insomnia with two drugs recently reaching the market11–14. Conversely, intracerbroventricular administration of OXa promotes wakefulness and suppresses REM sleep in mice15 and, more recently, the discovery of non-peptide orexin agonists have demonstrated potential for the treatment of NT1 and other hypersomnia disorders14–18, by targeting the orexin system via selective activation of OX1R. Despite progress with two OX2R-selective agonists in early clinical trials19, identification of efficacious oral small-molecule agonists with drug-like properties remains challenging.

Recently reported structures of antagonist-bound OX1R and OX2R20–23 have greatly advanced our understanding of the molecular basis of antagonist recognition and subtype selectivity. Yet, their utility for dissecting the molecular mechanism of activation and for driving the discovery of small-molecule agonists has remained limited. In this work, we determined single-particle cryo-electron microscopy (cryo-EM) structures of OXa- and OXb-protein complexes bound to OXb and the small-molecule agonist agonist 3′-N-(3-(2-((2H1,2,3-triazol-2-yl)benzamido)ethyl)phenyl)sulamoyl)-4′-methoxy-N,N-dimethyl-[1,1′-biphenyl]-3-carboxamide (compound 1; Fig. 1a) to further our understanding of orexin signaling and to enable structure-based drug discovery of novel therapeutics for the treatment of NT1. Our results shed light on the molecular details of peptide and small-molecule agonist recognition, reveal the global and local conformational changes associated with receptor activation, and suggest a molecular mechanism for activation by peptide and small-molecule agonists.

Results

Structure determination. We reconstituted an OX2R-G-protein complex from purified subunits, using an engineered variant of human OX2R with a truncated carboxy-terminus and a shortened intracellular loop (ICL) 3. This construct enabled high-level heterologous expression and purification of homogeneous material, while maintaining wild-type-like affinity for both the natural peptide agonists and the small-molecule agonist compound 1 (Supplementary Fig. 1). We used a Ga-subunit based on a previously described chimeric minimal Ga-construct with altered receptor specificity to allow productive interaction with Gq-coupled GPCRs. We included residues of the αN helix of Ga41 to obtain a Ga-construct we refer to as mini-Gaα. The altered amino-terminus allowed the use of previously described antibody fragment scFv16 that stabilizes the nucleotide-free receptor-G-protein complex25,26. To obtain a structure of OX2R activated by an endogenous peptide agonist, we purified the receptor in the presence of OXb and assembled a complex with mini-Gαq, Gβ1γ2, and scFv16. Samples were vitrified on electron microscopy grids and the structure was determined by single-particle cryo-EM to a nominal resolution of 3.2 Å (Supplementary Table 1 and Supplementary Fig. 2). The resulting density map was of high quality and allowed modeling of sidechains for most of the amino acids of the complex (Supplementary Fig. 3). Residues N20–M28 at the carboxy-terminus of OXb were well-resolved, while only poor or no density for the remainder of the peptide was observed. We obtained improved density maps at an overall resolution of 3.0 Å for the structure with compound 1 (Fig. 1a) by including a synthetic nanobody, Sb51, for additional conformational stabilization and to aid alignment of particle projections (Supplementary Table 1, and Supplementary Figs. 4 and 5). Sb51 binds to the extracellular surface of OX2R contacting residues in extracellular loop (ECL) 2, and part of ECL3 without directly interacting with compound 1 or significantly altering the structure of the small-molecule binding site, suggesting that the compound 1–receptor interactions are likely unaffected by the presence of the nanobody. Sb51 was omitted, however, for the complex with OXb as it was likely to clash with the much larger peptide agonist or indirectly affect the receptor–peptide interface.

Overall structures of active-state OX2R. The structures of OX2R bound to OXb and compound 1 are very similar, with root mean square deviations of 0.77 and 0.54 Å, when comparing equivalent α-carbons of OX2R and of the entire receptor-G-protein complexes, respectively (Supplementary Fig. 6). The main structural differences are in ECL2 and ECL3 of OX2R, regions that contribute to the epitope of Sb51. The structures of the nucleotide-free complexes and the relative orientation of the individual subunits closely resemble previously reported structures of GPCRs bound to G proteins Gαi, Gαo, Gαs or Gα11 (refs. 25,27–30), and are consistent with what has been referred to as a canonical-state complex31 (Fig. 1c, d). Comparison of the OXb-bound structure with the structures of inactive-state OX2R reveals the conformational changes the receptor undergoes upon agonist binding and activation (Fig. 2a–c). The observed reorganization of transmembrane (TM) helices 5–7 at the cytosolic interface are consistent with the key conformational changes associated with the conserved mechanism of GPCR activation32,33, and result in the intracellular half of TM6 swinging outward to allow insertion of the α5 helix at the carboxy-terminus of the Ga-subunit deep into the core of OX2R. Furthermore, the arrangements of several structural motifs associated with GPCR activation suggest that the receptor has been visualized in a fully activated state (Fig. 2d). They include the microswitches R152S, S157N (Ballesteros-Weinstein numbering in superscript34), and Y364F of the D3.49P3.50Y3.51 motif and the N7.49P7.50X7.53 motif, respectively, as well as the hydrophobic core triad IY3.40P5.50S5.44 (also termed connector region).

The rearrangements on the extracellular side are more subtle and lead to the contraction of the solvent-accessible central cavity of the receptor (Fig. 1c). Of note is that in the structures of antagonist-bound OX2R, TM2 is partially unwound and kinked toward TM7 facilitated by conserved P1099–39, while in active-state OX2R, the helical twist is tightened, causing an inward displacement of residues on either side of the kink. Moreover, enabled by sidechain rearrangements in the hydrophobic core of OX2R, TM3 undergoes a minor rotation and moves along the helical axis toward the extracellular side. In addition, the entire helix is slightly shifted toward the center of the helical bundle.

Peptide binding in the receptor core. The 28 amino acid neuropeptide OXb is a potent orexin agonist with modest selectivity for OX2R (Fig. 1b)3. Our cryo-EM map showed strong density
only for the carboxy-terminal portion of OxB (N20–M28). This segment adopts an extended conformation and reaches far into the core of OX2R, contacting all TM helices except TM1, as well as residues in ECL2 and ECL3 through an extensive interface of hydrophobic and polar interactions (Fig. 3a–c and Supplementary Table 2). This extended conformation was unanticipated since in solution both OxA and OxB fold into two \( \alpha \)-helices oriented approximately perpendicular to each other and molecular docking of OxA and OxB to models of both receptors suggested plausible binding poses with \( \alpha \)-helical carboxy-terminal portions. In contrast, the natural peptide agonists of the endothelin ET\( _B \) receptor and neurotensin NTS1 receptor, both GPCRs of the same subfamily as OX2R, are disordered in isolation and only become conformationally restricted upon binding to their cognate receptors. Similar polar interactions at the base of the binding pocket between an agonist peptide and residues at positions 3.32 and 7.43 have been observed for the \( \mu \)- and \( \delta \)-opioid receptors. In the ET\( _B \) receptor, Q1813.32 forms hydrogen bonds with the backbones of ET-1 and ET-3 in much the same way as observed in the OX2R–OxB complex. The pocket widens toward the extracellular side, exposing residues N20–A23 of OxB to the surrounding milieu. Several polar interactions with the mainchain and sidechains of OxB form the interface with OX2R in this region. The sequence of the carboxy-terminal segment of OxB differs from that of OxA only at the terminal residue (L33 in OxA, M28 in OxB; Supplementary Fig. 3a). It is therefore likely that the observed receptor–peptide interactions made in this region are conserved across the two endogenous agonists.

Receptor–peptide interactions at the extracellular surface. Our cryo-EM map contains two less well-defined density features.
protruding into the extracellular space that are absent in the map of the complex with compound 1 (Supplementary Fig. 8); one is continuous with the amino-terminal end of TM1 and likely accounts for a short \( \alpha \)-helix observed in structures of antagonist-bound OX2R and OX2R\(^{21-23} \), albeit in varying orientations, indicating flexibility in the absence of a peptide agonist. In proximity is the second feature that connects to the stronger density describing the carboxy-terminal segment of OXB. Its shape and location are consistent with an \( \alpha \)-helical amino-terminal portion of the peptide. While we excluded both structural elements from our final model of the OX2R–G-protein complex, we hypothesized that the amino-terminal segment of OXB forms a tripartite interaction with the amino-terminal \( \alpha \)-helix and ECL2 of OX2R. The involvement of these parts of the receptor in peptide-mediated activation has been demonstrated by mutagenesis\(^{21,45} \). Guided by the cryo-EM map, we constructed a model of OX2R bound to full-length OXB comprising residues of the amino-terminal \( \alpha \)-helix and a complete ECL2 (Supplementary Fig. 8). To probe the stability of the receptor–peptide interface in the extracellular region, this model was subjected to all-atom molecular dynamics (MD) simulations (Fig. 4). We performed four independent 1000-ns MD simulations for a combined total of 4 \( \mu \)s of simulation time. The helical structures of the amino-terminal portion of OXB and the amino-terminal \( \alpha \)-helix of OX2R, as well as the \( \beta \)-sheet in ECL2 were found to be stable throughout each microsecond-scale MD trajectory. Some flexibility was observed for the distal region of ECL2, where in two trajectories formation of a short \( \alpha \)-helix was observed. However, the OX2R–OXB interface remained intact throughout all simulations with the peptide in contact with both ECL2 and the amino-terminal \( \alpha \)-helix of OX2R. Together, the cryo-EM map and the MD simulations suggest that these extracellular regions of OX2R engage in agonist peptide binding.

Small-molecule agonist recognition. We have recently discovered diarylsulfonamide compound 1, a potent and selective OX2R agonist (Fig. 1a, b). With a local resolution of 3 Å, the cryo-EM map was of excellent quality in the OX2R core, featuring well-defined density for a single molecule of compound 1 in the orthosteric pocket (Fig. 3d, e and Supplementary Fig. 5). The ligand is kinked around a central sulfonamide, positioning the two flanking phenyl rings approximately perpendicular to one another. This core interacts extensively with OX2R; the sulfonamide forms a bidentate hydrogen bond interaction with Q1343.32, while the adjacent phenyl rings contact a lipophilic patch formed by P1313.29, M1914.64, T1112.61, and V1142.64. The two ends of the compound project into distinct regions. The amide-linked phenyltriazole inserts into the hydrophobic bottom of the pocket that accommodates carboxy-terminal M28 of OXB in peptide-bound OX2R. It makes hydrophobic contacts to C107 2.57, T1353.33, V1383.36, I3206.51, and N3246.55 and binds through \( \pi \)-\( \pi \) interactions to F2275.42. The amide carbonyl of the linker forms a hydrogen bond with H3507.39, whose sidechain also contacts the terminal 1,2,3-triazole moiety and the distal phenyl ring of the core, thereby stabilizing the kinked conformation of compound 1. The other end of the agonist extends toward the extracellular side, a region occupied by I25 of OXB in the OX2R–OXB complex. However, facilitated by conserved P1092.59, the last two turns of TM2 are shifted outward in compound 1-bound OX2R, enlarging the subpocket between TM2 and TM7 to accommodate the substituted biphenyl fragment of the ligand. In addition, Y3437.23 assumes a different sidechain rotamer to interact with the terminal N,N-dimethylbenzamidine (Fig. 3f). The observed ligand conformation and receptor–ligand interactions are unrelated to those proposed for a similar non-peptide OX2R agonist based on molecular docking into models derived from a structure of antagonist-bound OX2R\(^{16,46} \), underscoring the need for experimental structures to guide drug design.

Comparison with antagonist binding. Superposition of our structures of agonist-bound OX2R with those of the inactive receptor bound to the antagonists suvorexant\(^{20} \), HTL6641 (ref. 23), or EMPA\(^{22} \) reveals that all three antagonists occupy the bottom-most region of the central cavity, overlapping with the last three residues of OXB and the portion of compound 1 containing the sulfonamide and the amide-linked phenyltriazole. Compound 1 and all three antagonists place similar chemical groups in three distinct locations (Fig. 5a–c): (i) an aromatic ring is anchored at the bottom between the sidechains of F2275.42 and I3206.51. (ii) An amide carbonyl is placed in the center of the pocket, interacting with H3507.39. Notably, this contact is water-mediated in the case of all three antagonists\(^{20,22,23} \), whereas in active-state OX2R, owing to conformational changes in TM7, H3507.39 sits lower and more central, allowing direct interaction
with the amide linker in compound 1. (iii) A second aromatic moiety is positioned further up overlapping with the location of the sidechain of L26 of OxB. Although for both compound 1 and all three antagonists, these groups interact with the lipophilic patch around P131.32, the phenyl ring of the compound 1 diaryl sulfonamide core is located further toward the extracellular side. This is noteworthy as it leaves space for Q134.32 to adopt an extended conformation and to make the observed bidentate interaction with the sulfonamide of the agonist. Similarly, the position of L26 of OxB allows Q134.32 to extend and form a hydrogen bond with the OxB backbone. It is the position and sidechain rotamer of this residue that constitutes the most substantial difference in the binding sites of inactive-state and active-state OX1R (Fig. 5d, e and Supplementary Fig. 9). Our cryo-EM maps unambiguously show that in both structures of agonist-bound OX1R, the sidechain of Q134.32 is extended and projects upward, i.e., toward the extracellular side (Supplementary Figs. 5f and 5f). In addition, this configuration of Q134.32 and its interaction with OxB remained stable in our microsecond-scale MD simulations (Supplementary Fig. 10). In contrast, this sidechain is positioned further down and points downward toward the cytoplasm, when OX1R is bound to any of the antagonists. A similar arrangement of the equivalent residue Q126.32 is observed in all available structures of OX2R captured in an inactive state (Supplementary Fig. 11) 21,22. High-resolution structures of both receptors reveal that this sidechain conformation is stabilized by a hydrogen bond to a tyrosine at position 7.43, and through interactions with a network of water molecules and polar sidechains, which in OX1R also includes a sodium ion 22.

**Discussion**

The cryo-EM structures of active-state OX2R bound to OxB and compound 1 reveal the molecular details of peptide and small-molecule agonist binding. Both OxB and compound 1 are selective orexin agonists with one and four orders of magnitude higher potency at OX2R compared to OX1R, respectively (Fig. 1b). With active-state structures of only one of the two receptors and only with selective agonists at hand, additional structural and functional data will be required to obtain a detailed understanding of the molecular details underlying receptor subtype selectivity. However, the selectivity of OxB and that of compound 1 likely have distinct molecular bases. The sequence of the last nine amino acids of OxB only differs from that of non-selective OxA at the terminal residue (Met in OxB and Leu in OxA). Therefore, subtype selectivity likely arises from interactions of the amino-terminal portions of the two peptides, which differ both in sequence and structure 35,36, with residues at the extracellular surface of the receptors, where the sequences of OX1R and OX2R diverge. In contrast, the binding site of compound 1 is entirely located in the highly conserved core of the receptor, where OX2R only differs from OX1R at positions 2.61 (T111.261 in OX2R; S103.261 in OX1R) and 3.33 (T135.333 in OX2R; A127.333 in OX1R). The resulting differences in shape and electrostatics of the small-molecule agonist binding sites of the two receptors are likely subtle, making it challenging to understand the basis for receptor subtype selectivity, in particular in the absence of structures of active-state OX1R. Since compound 1 does not make any direct polar contacts with T111.261 or T135.333, it is tempting to speculate that water-mediated interactions and/or rearrangements of water networks in the binding pocket have a key role in the observed selectivity for OX1R akin to what has recently been proposed for the OX2R-selective antagonist EMPA based on high-resolution crystal structures of EMPA-bound OX1R and OX2R 23. Additional structures of active-state OX1R and OX2R bound to subtype-specific small-molecule agonists—preferably at resolutions at which water molecules can be resolved—will be required to elucidate the molecular bases of small-molecule agonist selectivity.

The structures presented in this study suggest that interactions in the core of the receptor are sufficient to elicit the conformational changes in OX2R required for receptor activation. OxB
induces these rearrangements through an extended carboxy-terminal segment inserted deep into the orthosteric pocket. This is in agreement with previous observations that OxA truncated from its amino-terminus down to a segment that is highly conserved between the two endogenous neuropeptides contains the residues critical for functional potency on OX1R. Our experimental and computational results also demonstrate the presence of contacts between the amino-terminal portion of OxB and extracellular regions of the receptor consistent with a model proposed based on mutagenesis studies of OX1R, in which the peptide agonist binds to the receptor through a polytopic interface. In line with the weaker density observed in the cryo-EM map, our MD simulations reveal increased flexibility of the receptor–peptide interface in the extracellular region compared to that in the OX2R core. The amino-terminal portion of OxB, while not directly involved in activation, may have a role in initial binding of the peptide and may function as “address” component of a classic “message-address” system, in which the peptide’s carboxy-terminal segment carries the “message”. This concept has also been proposed for peptide agonist binding to other receptors, including opioid, nociceptin, and, endothelin receptors.

Based on the key structural differences between agonist- and antagonist-bound OX2R, we propose a common mechanism of activation by the endogenous peptide agonists, as well as the small-molecule agonist compound 1 (Fig. 6). This mechanism also rationalizes how antagonists of the three classes discussed above keep the receptor in an inactive conformation. GPCRs are dynamic proteins that transition between fully inactive, fully active, and intermediate state conformational ensembles even in the absence of a ligand. Binding of an agonist or antagonist shifts this dynamic equilibrium toward an active or inactive conformation by stabilizing the respective arrangement of key structural elements. The outward swing of TM6, essential for the formation of the receptor–G-protein complex, is coupled to the upward movement of TM3 through rearrangements in the connector region. Our structures reveal that both OxB and compound 1 stabilize the upward displacement of TM3 in two ways: (i) direct stabilization through hydrogen bond interaction...
with the upward-shifted and extended sidechain of Q134$^{3.32}$ and (ii) indirect stabilization by filling the space vacated by the relocated Q134$^{3.32}$. For OxB, this is accomplished by part of the sidechain and the carboxy-terminal amide group of M28, while in the case of compound 1 it is the terminal 1,2,3-triazole group that is inserted. The upward movement of TM3 is accompanied by an inward shift of TM2. Consequently, C107$^{2.56}$ moves adjacent to the 1,2,3-triazole of compound 1 and the carboxy-terminal amide group of OxB, respectively, effectively forming a wedge that prevents TM3 from sliding down into the position observed in inactive-state OX2R and Q134$^{3.32}$ from adopting the downward-facing sidechain rotamer (Fig. 5f). In contrast, the antagonists act in the opposite way, stabilizing inactive-state OX2R by placing a bulky substituent in a position that would clash with the upward-shifted and extended Q134$^{3.32}$, and by leaving room for the downward-facing rotamer of this sidechain found in inactive-state OX2R. Whether the mechanism proposed here extends to other classes of orexin agonists remains to be elucidated, but it is consistent with all available structures of inactive-state OX1R and OX2R determined with antagonists of diverse chemotypes.

Fig. 5 Comparison of agonist and antagonist binding. Superpositions of OX2R in inactive (gray) and active (green and purple) conformations. a-c Comparison of the binding mode of compound 1 (yellow) and those of the antagonists EMPA (blue; PDB ID 5WQC), suvorexant (orange, 4SOV), and HTL6641 (dark green; 6TPN), respectively. Water molecules mediating interactions with the sidechain of H350$^{7.39}$ in the antagonist-bound complexes are rendered as red spheres. d-f Conformational changes (white arrows) in TM2, TM3, Q134$^{3.32}$, and C107$^{2.56}$ upon activation by OxB (cyan) and compound 1 are highlighted by white arrows.
such a central role of the sidechain at position 3.32 facilitating the 
interactions with Pd/C catalyst to reveal an aniline intermediate, 
ingested by transfection of mammalian cells in suspension using the 
and complexed with mini-Gsqi,Gi1 to the amino-terminus of mini-Gs/q71, a chimeric 
synthetic nanobody Sb51 were included. In parallel, the receptor was deglycosylated using 500 
the presence of 50 μM small-molecule agonist as described above and biotinylated using BirA biotin-
by SEC using a Superdex 200 Increase column, which was cooled in liquid nitrogen, and stored at 
the amino-termini were fused to the carboxy-terminus of MBP, carrying an amino-
to carry out the first step in commercially available reagents. An EDC/HOAt-mediated amide bond 
the resulting aryl bromide was subjected to a Pd-catalyzed Suzuki 
and a deca-histidine tag at its amino and carboxy termini, respectively, was 
the synthesis of compound 1. Compound 1 was accomplished in four steps from commercially available reagents. An EDC/HOAt-mediated amide bond 
activation provides critical information for structure-based drug 
the base. The resulting aryl bromide was subjected to a Pd-catalyzed Suzuki 
resulting from the biotinylated Avi-tag on a neutravidin-coated WAVEchip 4PCP (Creoptix). 
the sidechain at position 3.32 facilitating the 
the base. The resulting aryl bromide was subjected to a Pd-catalyzed Suzuki 
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the base. The resulting aryl bromide was subjected to a Pd-catalyzed Suzuki 
resulting from the biotinylated Avi-tag on a neutravidin-coated WAVEchip 4PCP (Creoptix).
eV was used during data collection. Fully automated data collection was carried out using Latitude in Gatan Imaging Suite (Gatan Inc.) with a nominal defocus range set from −0.8 to −2.5 µm. Image Shift was used with nine exposure groups per stage shift to improve the throughput of data collection. A total of 38,810 and 17,956 movies were collected for the samples with OxB and compound 1, respectively. For data collection is summarized in Supplementary Table 1, and Supplementary Figs. 2 and 4.

Cryo-EM data processing. The cryoSPARC Live application of cryoSPARC v. 2 (Structura Biotechnology) was used to streamline the movie processing, CTF estimation, particle picking, and 2D classification. Preprocessing involved anisotropic motion correction60 and local CTF estimation. The data were curated by keeping only data with better than 6 Å determined by CTF fit resolution. Particle picking started with blob picking −150 Å in diameter. Once a small set of particles was extracted and 2D classifications were obtained, the good 2D classes were used as template picking on the entire dataset. From the ~14 million (OxB) and ~7.5 million (compound 1) particles initially picked, ~2.9 million and ~1.9 million particles were kept from the good 2D classes, respectively. Then ab initio 3D reconstruction was carried out in cryoSPARC v. 2 (ref. 61) and one out of three classes was giving the reconstruction of the density from ~0.8 million and ~1.1 million particles for OxB and compound 1, respectively. Homogeneous refinement coupled with non-uniform and global CTF refinements gave the final 3D reconstructions at 3.2 Å (OxB) and 3.0 Å (compound 1) resolution, respectively (0.143 gold-standard FSC with correction of masking effects62). Local resolution estimates, shown in Supplementary Figs. 2 and 4, were determined in cryoSPARC for data processing, see Supplementary Table 1, and Supplementary Figs. 2 and 4.

Model building and refinement. Starting models for OX2R, mini-Gsqi, Gqα15, and scFv16 were based on Protein Data Bank (PDB) entries 4SOV, 5G53, 3SN6, and 6DDE, respectively. For an initial model of nanobody SB51, a homology model based on PDB entry 3K1K was constructed using Maestro (Schrödinger). For the complex with compound 1, its five subunits were placed into the density map by rigid-body fitting. The fit of the intracellular half of TM6, Helix8, and ECL2 of OX2R, as well as the α5 helix of mini-Gsqi was manually adjusted in Coot63. Missing residues of the αN helix of mini-Gsqi were manually fitted and sequences adjusted where required. The resulting initial model was further refined in iterative rounds of manual modifications in Coot and real-space refinement using the Phenix software package64. Coordinates and restraints for compound 1 were generated using Grade (Global Phasing Ltd), and the ligand was manually fitted into the density using real-space refinement in Coot and further refined using Phenix. The refined model for the complex with compound 1 was used as starting point for the refinement of the structure with OxB, using a similar iterative model building and refinement strategy. Model quality was assessed using Molprobity65 as implemented in Phenix. Map and model statistics are detailed in Supplementary Table 1. Even though present in the OX2R construct no or poor density was observed for residues M1 to L48 (complex with OxB) or M1 to E54 (complex with compound 1) preceding the five subunits. Disruptive residues in C22 of C22 and 290 of IC54, and residues beyond A378 in Helix8. Due to the absence of SB51, the complex with OxB additionally lacked density for residues P198 to L206 of ECL2. These regions were therefore left unmodeled. For the same reason, the linker replacing the Gαa helical domain and flanking residue stretches R61 to H64 and T206 to G208 of mini-Gsqi (Gαa/nanobody) residues M1 to N4 and E63 to L71 at the termini of Gqα2, and most residues of the interdomain linker of scFv16 were not included in the final model. In addition, several predominantly surface-exposed sidechains throughout the complex were truncated to their β-carbon atoms due to lack of density. Molecular representations were generated using PyMol (Schrödinger) and Chimera66.

Radioligand-binding assay. Cell membranes from a Chinese hamster ovary (CHO) cell line stably expressing wild-type OX2R and from transiently transfected Expi293 cells (Thermo Fisher) overexpressing engineered OX2R were incubated at room temperature for 6 h with [125I]-OxA (PerkinElmer) in assay buffer (25 mM HEPES-NaOH pH 7.4, 2.5 mM CaCl2, 1.25 mM MgCl2, and 0.175% (w/v) CHAPS) in a total volume of 200 µl. Unbound ligand was removed by rapid filtration through GF/C glass filters and 3 x 3 ml washes with 50 mM Tris-HCl pH 7.4, 200 mM NaCl, and 0.2% (v/v) CHAPS. Bound radioactivity was measured through liquid scintillation using Ecolume Liquid Scintillation Cocktail (MP Biomedicals) and detected using a Tri-Carb liquid scintillation counter. Saturation measurements were carried out by incubating membranes (20 µg total protein/well) with a range of concentrations of [125I]-OxA (0.002–8 nM). For competition studies, membranes (20 µg total protein/well) were incubated with a range of concentrations of compound 1 and OxB (100 µM−0.02 nM and 1 µM−0.0002 nM, respectively) and with [125I]-OxA at 0.005 nM. Data were analyzed using GraphPad Prism (GraphPad Software, Inc.).

Insoluble monophosphate accumulation assay. Agonist-dependent insoluble monophosphate (IP1) accumulation was measured using the IP-One Gq kit (Cisbio), a cell-based homogeneous time-resolved fluorescence assay, according to the manufacturer’s instructions. Briefly, cryo-preserved CHO cells stably expressing human OX2R and human OX2R were thawed, and resuspended in IMDM medium ( Gibco) at densities of 2.0 x 10^5 cells/ml and 4.0 x 10^5 cells/ml, respectively. Cells were seeded into 384-well plates (50 µl per well) and incubated at 37°C in 5% CO2 for 20–24 h. In a separate plate, a dilution series of each agonist was prepared in Stimulation Buffer (Cisbio). The culture medium was removed and cells were incubated with 14 µl of agonist-containing Stimulation Buffer at 37°C. After 1–2 h, 6 µl of Lysis and Detection Buffer (Cisbio) containing IP1-d2 and IP1 Tb cryptate antibody conjugates were added, and the reaction was incubated at room temperature protected from light. After 1 h, fluorescence was measured on an EnVision fluorescence plate reader (PerkinElmer) with the excitation wavelength set to 320 nm, and emission monitored at 620 nm (donor) and 665 nm (acceptor). Data were analyzed using GraphPad Prism.

Molecular dynamics simulations. A model of OX2R bound to full-length OxB was built in Coot63 based on the cryo-EM structure of the OX2R-G-protein complex bound to the carboxy-terminal portion of OxB, using the weak density in the extracellular region as a guide. This model was comprised of residues F39–W251 and R292–A378. The disordered amino-terminal region and IC13 were excluded due to missing density and available templates, while residues L39–L48 of the amino-terminal α-helix of OX2R were included. This OX2R-OxB complex was prepared for MD simulations using Maestro (Schrödinger). Atomic coordinates from the model were imported and refined using the Protein Preparation Wizard with default parameters and settings. The protonation states were determined and set for all titratable residues at pH 7.4. Hydrogens were added to the system and then minimized. The OX2R-OxB complex model was aligned to the Orientation of Proteins in Membranes database structure of PDB 5WQC (http://opm.phar.umich.edu/), inserted into a POPC bilayer and solvated using the System Builder Wizard in Maestro. Finally, water molecules (using the TIP3P water model) and ions were added, using the default parameters provided in the Maestro interface. MD simulations were performed using Desmond66 as included in the Schrödinger 2020-1 release, using OPLS3e force field for all atoms. Simulations were carried out in the NPT ensemble at 300 K and 1 bar. The default Desmond protocol was followed for relaxation. Restraints of 5 kcal mol^-1 Å^-2 were applied to the heavy atoms of those residues that are in direct contact with the extracellular interface with OX2R to ensure that the receptor remained in an active conformation for the duration of the simulation. Individual simulations were run to a final production length of 1000 ns, with 2000 frames recorded from each independent replicate run for analysis. A total of four independent classical MD trajectories were run for an aggregate of 4 µs of simulation time.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The cryo-EM density maps for the OxB-bound and the compound 1-bound OX2R-G-protein complexes have been deposited in the Electron Microscopy Data Bank under access codes EMD-23118 and EMD-23119, and their coordinates are available from the Protein Data Bank under accession numbers 7IIIU and 7IIIY, respectively. All other data related to this study are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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Author contributions
C.H. prepared cryo-EM grids, collected and processed cryo-EM data, determined and refined the structures, and prepared figures. N.J.B. optimized and carried out purification of OX2R, and reconstituted and purified OX2R-G-protein complexes. B.Z. performed radioligand-binding assays and, together with D.L.H., characterized the OX2R-mini-Gαq interaction. S.T. purified OX2R and G1β1γ2, and characterized compounds. L.X. carried out molecular dynamics simulations with input from S.A.H. and prepared figures. J.M.S. and A. T.P. optimized and carried out mammalian and insect cell expression. C.M. performed IP1 accumulation assays. M.J.R., M.T.R., S.J.S., V.L.R., and J.C.K. developed structure–activity relationship data for the compound 1 chemical series. M.J.B. designed and synthesized compound 1. M.T.R. prioritized compounds for structure determination. K.A.A. and L.X. performed in silico analysis. J.A.O. optimized IP1 accumulation assays and characterized S651. T.P.M. contributed to the characterization of engineered OX2R and provided insight into orexin biology. C.S. supervised cryo-EM facility. A.B. supervised protein expression and purification. S.M.S. and K.H. were responsible for overall project strategy. K.H. orchestrated the project, optimized and characterized engineered OX2R and mini-Gαq constructs, optimized expression and purification for all components of the OX2R-G-protein complexes, determined, refined, and analyzed the structures, prepared figures, and wrote the manuscript. All authors commented on the manuscript.

Competing interests
The authors declare the following competing interests: all authors are current or past employees of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA.

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