Molecular mechanism of the wake-promoting agent TAK-925

The OX₂ orexin receptor (OX₂R) is a highly expressed G protein-coupled receptor (GPCR) in the brain that regulates wakefulness and circadian rhythms in humans. Antagonism of OX₂R is a proven therapeutic strategy for insomnia drugs, and agonism of OX₂R is a potentially powerful approach for narcolepsy type 1, which is characterized by the death of orexinergic neurons. Until recently, agonism of OX₂R had been considered ‘undruggable.’ We harness cryo-electron microscopy of OX₂R-G protein complexes to determine how the first clinically tested OX₂R agonist TAK-925 can activate OX₂R in a highly selective manner. Two structures of TAK-925-bound OX₂R with either a Gq mimetic or Gi reveal that TAK-925 binds at the same site occupied by antagonists, yet interacts with the transmembrane helices to trigger activating microswitches. Our structural and mutagenesis data show that TAK-925’s selectivity is mediated by subtle differences between OX₁ and OX₂ receptor subtypes at the orthosteric pocket. Finally, differences in the polarity of interactions at the G protein binding interfaces help to rationalize OX₂R’s coupling selectivity for Gq signaling. The mechanisms of TAK-925’s binding, activation, and selectivity presented herein will aid in understanding the efficacy of small molecule OX₂R agonists for narcolepsy and other circadian disorders.
Orexin signaling in the brain is the primary mechanism connecting diurnal circadian rhythms to arousal and wakefulness. Orexin A and B are neuropeptides (33 and 28 amino acids respectively, also known as hypocretins) derived from the preproorexin precursor and produced by a small set of dedicated neurons in the lateral hypothalamus, which stimulate release of neurotransmitters in diverse brain regions to promote wakefulness. Orexin release is controlled by the circadian clock, and levels of orexin neuropeptides in mammalian cerebrospinal fluid follow a 24-hour cycle, peaking during the wake period. The orexin peptides act by binding to the GPCRs OX1R and OX2R on target neurons to activate cellular signaling by G proteins and arrestins, particularly stimulating Gq/11-mediated release of calcium from the endoplasmic reticulum.

The human disorder narcolepsy type 1 is characterized by a deficiency of orexin signaling, resulting in a pentad of symptoms including excessive daytime sleepiness, disturbed nighttime sleep, hypnagogic/hypnopompic hallucinations, sleep paralysis, and cataplexy, a sudden loss of muscle tone triggered by strong emotions. Narcolepsy type 1 affects approximately 100,000 adults in the U.S., and up to 3 million patients may be affected by the disorder worldwide. While the etiology of this disorder varies, the proximal cause of narcolepsy type 1 is the death of orexin neurons. The resulting loss of orexin expression in the narcoleptic brain has been validated in human clinical studies, and the causal relationship between orexin deficiency and the narcolepsy phenotype has been validated using preproorexin antagonist (DORA) suvorexant (Belsomra) for insomnia in 2014. Numerous small molecule chemotypes have been developed as DORAs or OX2R-selective antagonists, however the discovery of small molecule OX2R agonists has lagged far behind, with very few potent lead compounds reported in the primary literature or patents. A breakthrough in this area was recently achieved with the small molecule TAK-925, which displays low-nanomolar potency for OX2R activation and >5000-fold selectivity for OX2R over OX1R. In animal studies, subcutaneous delivery of TAK-925 during the sleep period results in a dose-dependent increase in wakefulness and reduction in sleep time. TAK-925 is currently being explored as an orexin agonist in adults in the U.S., and up to 3 million patients may be affected by the disorder worldwide. While the etiology of this disorder varies, the proximal cause of narcolepsy type 1 is the death of orexin neurons.

Experiments in canine and mouse models also established that orexin control of circadian rhythms and wakefulness occurs predominantly through the actions of OX2R, while signaling through OX1R is critical for stimulation of reward pathways. These discoveries paved the way for small molecule drug discovery efforts targeting the orexin receptors in sleep/wake disorders, culminating in FDA approval of the dual orexin receptor antagonist (DORA) suvorexant (Belsomra) for insomnia in 2014. Numerous small molecule chemotypes have been developed as DORAs or OX2R-selective antagonists, however the discovery of small molecule OX2R agonists has lagged far behind, with very few potent lead compounds reported in the primary literature or patents. A breakthrough in this area was recently achieved with the small molecule TAK-925, which displays low-nanomolar potency for OX2R activation and >5000-fold selectivity for OX2R over OX1R. In animal studies, subcutaneous delivery of TAK-925 during the sleep period results in a dose-dependent increase in wakefulness and reduction in sleep time. TAK-925 is currently being explored as an orexin agonist in humans with narcolepsy, with multiple trials ongoing.

Insights into the molecular basis for orexin receptor activation and inhibition have come from structural studies of OX2R and OX1R bound to different ligands. The first structure of OX2R with suvorexant revealed that the drug binds in a narrow membrane-embedded pocket analogous to the classic orthosteric site of adrenaline and beta-blocker binding in the β2-adrenergic receptor. Structures of OX2R and OX1R with other antagonists showed that these compounds invariably bind to the same suvorexant site. However, mutagenesis studies of the orexin receptors and other neuropeptide-activated GPCRs showed that interactions of the peptide agonists with the solvent-exposed extracellular loops (notably ECL2) are required to trigger full activation, possibly underlying the difficulty in identifying small molecule agonist drug candidates. A recent cryo-EM study showed how a small molecule could mimic orexin B (OXB) to stabilize an active conformation of OX2R bound to a G protein.

In this work, we use cryo-EM of different OX2R-G protein complexes and associated pharmacological studies to understand the molecular mechanism of the drug candidate TAK-925. These structural and functional data reveal how TAK-925 activates OX2R with high potency and subtype selectivity, and with a preference for signaling through Gq.

Results

Structure determination of TAK-925-bound OX2R coupled to Gq and G11. To capture active structures of OX2R bound to TAK-925, we purified complexes of the receptor together with heterotrimeric G proteins. Using recombinant protein from S9 insect cells, we elucidated the cryo-EM structure of OX2R coupled to a mini-Gαq/β/γ-G heterotrimer (referred to as OX2R-mGαqβγ in this manuscript) in lauryl maltose neopentyl glycol (LMNG) micelles at 3.3 Å resolution (Supplementary Figs. 1a, b, and 2), serving as a model for the Gq signaling complex leading to calcium release. In parallel, we solved the cryo-EM structure of OX2R coupled to a DNGαq11/Gq/11 heterotrimer (referred to as OX2R-Gq11) in digitonin at 3.2 Å resolution (Supplementary Figs. 1c, d, and 3), representing a Gq signaling complex. Both cryo-EM efforts took advantage of scFv16 binding, requiring generation of a modified mini-Gα16,β12-Gq β pocket residues with TAK-925 bound, compared to the OX2R-OxB complexes, and we similarly showed that Gln1343.32 was previously observed in the OX2R-Compound 1 cryo-EM envelope, atomic model, and density for TAK-925 in OX2R-mGαqβγ are shown in Fig. 1a–c, while the analogous data for OX2R-Gq11 are in Fig. 1d–f. Differences between OX2R models in these two structures are greatest at the G protein interface, although the models of the agonist-bound receptor are generally in close agreement, with a root mean square deviation (rmsd) of 0.7 Å. Both cryo-EM maps have well-defined sidechain density in the transmembrane region (Supplementary Fig. 4), and allowed placement and refinement of TAK-925 (Fig. 1c, f). The stability of the modeled ligand conformation, particular regarding the saturated rings, was validated by quantum chemistry calculations (Supplementary Fig. 5).

Binding of TAK-925 to OX2R. The molecular interactions between TAK-925 and OX2R are highly similar in the mGαqβγ and G11 complexes (Fig. 2a), which solidifies the interpretation of the details of these interfaces. The cryo-EM density for the ligand binding pockets in the OX2R-mGαqβγ and OX2R-Gq11 complexes are shown in Supplementary Fig. 6a and b, respectively. TAK-925 adopts a compact, U-shaped conformation, contacting OX2R residues on TM2, TM3, TM5, TM6 and TM7 and burying 460 Å² of solvent-accessible surface area. The methyl carbamate and sulfonamide ‘arms’ of TAK-925 extend toward polar residues on either side of the orthosteric pocket, the latter group engaging in a hydrogen bond with Gln134.3.32, while the phenyl-cyclohexane ‘tail’ projects deeper into the transmembrane region contacting Val1138.3.36, Phe227.5.42, and Ile320.5.51. TAK-925 and suvorexant share an overlapping binding site in OX2R despite having opposing ligand efficacy (Fig. 2b). The diarylsulfonamide agonist Compound 1, reported in a previous cryo-EM structure, shares elements of the TAK-925 pocket, but also extends further toward the extracellular loops of the receptor. Modulation of the position of Gln134.3.32 was previously observed in the OX2R-Compound 1 and OX2R-OxB complexes, and we similarly find that Gln134.3.32 undergoes the largest conformational switch of the orthosteric pocket residues with TAK-925 bound, compared to the suvorexant-bound inactive state (Fig. 2c). Despite their very different chemotypes, TAK-925 and Compound 1 both have sulfonamides at similar positions in the active OX2R complexes (Fig. 2b), which interact with Gln134.3.32 and promote a shifted position of TM3 (Fig. 2c). The overall structure of active OX2R with TAK-925 is similar to active OX2R with Compound 1, with
hydrogen bond to Asn3245.55. This polar residue was also previously demonstrated to be essential for antagonist binding and inhibition of OX1R in structural19,20 and mutagenesis27 studies. The residue Thr1112.61 interacts with the ligand in the OX1R-mGsqiN structure such that the sidechain oxygen is 3.8 Å from TAK-925’s sulfonamide nitrogen. While the structure does not indicate a hydrogen bond between these groups, we found that mutation of this residue to alanine led to significantly reduced potency of the agonist (Fig. 2d, top panel, EC50 2.1 μM). This loss of activity could be due to solvent-mediated effects, as discussed below. Finally, we carried out β-arrestin recruitment assays for a panel of OX1R mutants, and found that the residues important for TAK-925 activation of this pathway agree with the interactions that TAK-925 makes with the active OX1R orthosteric pocket in the cryo-EM structures (Supplementary Fig. 8, Supplementary Tables 3 and 4). Notably, large reductions in β-arrestin recruitment by TAK-925 were measured for residues Gln1343.32 and Asp3246.55 as well as Tyr3179.48 at the base of the orthosteric pocket, both of which undergo changes from the inactive to active conformations.

From ligand structure-activity relationship (SAR) studies, we found that critical determinants of TAK-925’s potency include the stereochemistry of substituents on the cyclohexane and piperidine rings, which enforces TAK-925’s stable U-shaped conformation seen in our structures (Fig. 1c, f, Supplementary Fig. 5). Furthermore, the sulfonamide of the ligand is essential for potency, and this group forms a tight polar interaction with the important residue Gln1343.32 in the active state (Fig. 2a, c). The SAR around the phenyl ring is narrow and replacement with aliphatic groups results in a reduction in potency, which is consistent with the phenyl-cyclohexyl tail of TAK-925 projecting into the deep hydrophobic pocket lined by nonpolar residues from TMs 3, 5, and 6.

Selectivity of TAK-925 for OX1R over OX2R

We have previously shown that TAK-925 has >5000 fold selectivity for OX1R over OX2R in calcium mobilization assays. Our complex structures with TAK-925 show that, as with the DORA suvorexant19,20, the agonist contact site is highly conserved in OX1R, with only two substitutions: Thr1112.61→Ser and Thr1353.33→Ala (Fig. 3a). It is possible that selectivity is enforced locally by these small differences in the respective binding pockets, however selectivity could also be the result of long-range allosteric differences. To distinguish these possibilities, we carried out subtype swap experiments similar to what we previously performed for OX1R- and OX2R-selective antagonists.20

When we mutated the two divergent binding pocket residues in OX1R to the corresponding amino acid in OX2R and carried out IP accumulation assays, we observed saturable activation by TAK-925 (EC50 = 300 nM versus >100 μM non-saturating for wild-type OX1R). The two single mutants showed a partial increase (left-ward shift) in potency, indicating that both positions contribute towards selectivity (Fig. 3b). Conversely, mutation of these two positions in OX2R to the corresponding amino acids in OX1R leads to a reduction (right-ward shift) in potency, and the double mutant results in EC50 3.6 μM versus 7.5 nM for wild-type OX1R (Fig. 3c). We also found that when OX1R Thr1112.61 is mutated to alanine (instead of serine), receptor activation by TAK-925 is diminished (Fig. 2d top panel, EC50 2.1 μM). Collectively, these data indicate that the high selectivity of TAK-925 for OX2R is largely caused by differences in the deep orthosteric pockets of the two receptor subtypes, rather than in the more divergent extracellular loops. If the active OX2R adopted a different conformation at the deep orthosteric pocket where TAK-925 binds, it would not be possible to rescue signaling with
the S103T/A127T double mutant. Likewise, this result indicates that the allosteric pathway of activation for OX1R can be turned on by agonist binding at this site, and rules out the possibility that TAK-925 has minimal potency at OX1R due to differences in the allosteric transmission mechanism.

**TAK-925 activation of OX2R**

How does TAK-925 stabilize the active conformation of OX2R? Compared to the inactive state, binding of TAK-925 causes a rotation in TM3 at the orthosteric pocket with sidechains moving towards TM2, including the shift of Gln1343.32 described above. The ‘pull’ on TM3 occurs together with an opposing downward ‘push’ on Tyr3176.48 induced by the deeper projection (relative to suvorexant) of TAK-925’s phenyl-cyclohexyl tail against TMs 3 and 6 (Fig. 4a). This conformational change is disfavored by suvorexant due to the position of its benzoxazole, which packs against TM3 and prevents its rotation. A downward shift in the aromatic residue at position 6.48 (Ballesteros–Weinstein numbering) is observed in several active GPCRs, and has been suggested to initialize the hallmark outward rotation of TM6 during GPCR activation. The signal is then further propagated down the receptor, where the ‘PIF switch’ (PVF in OX2R) adopts an active conformation (Fig. 4b), and the vacancy left by TM6’s outward movement is filled by the inward movement of TM7 toward TM3. In particular, the microswitch residue Tyr3647.53 moves ~4 Å inward (Fig. 4c) and sits between residues Leu1453.43, Ile1483.46, and Arg1523.50, while another microswitch residue Leu3066.37 moves ~5 Å outward as a result of the repositioning of Arg1523.50. In parallel with the TM3-TM6-TM7 repacking, the ionic interaction between Asp1513.49 and Arg1523.50 is broken, and the cytosolic side of the receptor opens to accommodate the G protein α5 helix (Fig. 4d). These latter conformational changes are also seen in other GPCRs.

**OX2R-G protein interaction**

A major signaling cascade activated by the orexin neuropeptides in neurons is Gq-mediated calcium release. We were able to characterize TAK-925-bound samples of active OX2R with both mGsqiN (a Gq mimetic) and Gi1, and are thus able to compare the interfaces between these complexes. The cryo-EM density for the receptor-G protein interface in the OX2R-mGsqiN and OX2R-Gi1 complexes are shown in Fig. 2b and c.

![Fig. 2 Binding of TAK-925 to OX2R](https://example.com/fig2.png)

**Figure 2** Binding of TAK-925 to OX2R. a Overlay of contact residues (sticks with purple carbons for mGsqiN-coupled OX2R and sticks with blue carbons for Gi1-coupled OX2R) within 4 Å of TAK-925 (yellow carbons) when superimposing OX2R- mGsqiN and OX2R-Gi1. The OX2R backbone (silver) is from OX2R-mGsqiN. The hydrogen bond from Gln1343.32 to TAK-925’s sulfonamide is not shown because this residue is behind the ligand from this viewpoint (same as in Fig. 2c). b Overlay of TAK-925 (sticks with yellow carbons), Compound 1 (sticks with orange carbons) and suvorexant (sticks with cyan carbons) when superimposing the OX2R polypeptides from the OX2R-mGsqiN complex (this work), the OX2R-mini-Gi1 complex (PDB 7L1V) and the antagonist-bound inactive conformation (PDB 450V). c Overlay of contact residues within 4 Å of TAK-925 when superimposing OX2R-mGsqiN and OX2R-Gi1 (this work, magenta), OX2R-mini-Gi1/Compound 1 (PDB 7L1V, orange) and the suvorexant-bound inactive conformation of OX2R (PDB 450V, cyan). TAK-925 is shown as transparent spheres. d Stimulation of Gq by OX2R wild type (WT) and mutants when bound to TAK-925 (top) and orexin B (bottom). Each data point represents an average from n ≥ 3 independent experiments (each performed in duplicate), where n is shown in Supplementary Table 2. Error bars are ±SD. Data were normalized to the WT Emax and fitted to the three-parameter model ‘log(agonist) vs response’ in GraphPad Prism 9. Source data are provided as a Source Data file.
Supplementary Fig. 6c and d, respectively. A majority of the contacts occur between OX2R and the C-terminal α5 helix of each G protein (Fig. 5a, b), as seen in previous activated receptor complexes25,33. The OX2R-mGsqiN interface has features similar to previously characterized GPCR-Gq/11 complexes34–37. In particular, the end of the C-terminal α5 helix forms a ‘hook’ that packs against the TM7-Helix8 junction, and the sidechain of Tyr235H5.23 from this hook extends across the center of the interface to form a hydrogen bond with ICL2 of the receptor (Ser164ICL2 in OX2R, Figs. 4d and 5a). A similar interaction has been observed in other GPCR-Gq complexes36,37, and Tyr235H5.23 is not conserved in Gi.

The overall conformation of the OX2R-Gi1 interface is similar to that of OX2R-mGsqiN (rmsd for receptor and Gα subunits together equals 1.3 Å). However, the OX2R-mGsqiN interface features more extensive contacts between OX2R and the mGsqiN, resulting in burial of more surface area (1039 Å2 versus 814 Å2).

Notably, the interaction of OX2R with the Gi1 α5 helix is predominantly mediated by hydrophobic contacts, and Gi1 makes only two polar contacts to the receptor’s polypeptide backbone (Fig. 5b). In contrast, active OX2R makes 7 specific polar interactions with the mGsqiN α5 helix, including from Ser164ICL2 to Tyr253H5.23 and from Thr3026.33 to the mGsqiN C-terminus (Fig. 5a, Supplementary Figs. 9 and 10). The backbone amide of Phe3718.50 also hydrogen-bonds to Asn244 H5.24 of mGsqiN (common Gα numbering system38) (Fig. 5a), and this OX2R residue undergoes a downward shift during activation concomitant with the upward movement of Tyr3647.53 (Fig. 4c).

To confirm whether OX2R can signal effectively through both G protein complexes, we carried out Gq and Gi signaling assays in HEK293 cells transfected with OX2R and G proteins. Both orexin B and TAK-925 showed poor ability to stimulate Gi1 as measured by reduction in cAMP levels after forskolin treatment (Fig. 5d). In contrast, both orexin B and TAK-925 induced robust Gq-mediated IP accumulation with EC50 values consistent with previous studies (8.2 nM and 8.1 nM, respectively) (Fig. 5c). These results show that TAK-925 is similar to OxB in selectively activating Gq signaling in cells.
Discussion

TAK-925 can fully activate OX2R despite its low molecular weight and non-peptidic structure. Earlier mutagenesis studies showed that residues on the ECL2 β-hairpin of OX2R are important for activation by OxB23, and we previously found that an α-helix preceding TM1 is involved in orexin binding20, which helps to position the neuropeptide C-terminal region to interact with the membrane-embedded site including N324 6.55. These findings made it difficult to imagine how a drug-like small molecule could recapitulate the interactions needed to activate OX2R. Our present structures of active OX2R with TAK-925, along with the recent structure with Compound 124, explain how a low-MW drug can activate the receptor (Fig. 5d) without fully mimicking the orexin neuropeptide. Indeed, TAK-925 binds in the same deep membrane-embedded site as the inhibitor suvorexant (Fig. 2a, b), but is able to engage in concerted interactions with the receptor that stabilize the active conformation (Figs. 2c and 4a). The deep burial of TAK-925 at the membrane-embedded site also facilitates high affinity and potency, which the orexin neuropeptide can only achieve through multivalent contacts analogous to Class B GPCR/neuropeptide interactions39. The mechanism of TAK-925 activation of OX2R sets a precedent that small molecule drug-like full agonists are possible even for the most challenging peptide-activated GPCR targets.

Subtype selectivity remains a major challenge in GPCR drug development, both for agonists and antagonists40,41. TAK-925 has a high degree of OX2R selectivity, and this profile may be important to avoid OX1R activation of brain reward pathways associated with addiction13, while maintaining desirable sleep/wake effects. A typical strategy for achieving GPCR ligand selectivity is to exploit subpockets of the orthosteric site that are not conserved between subtypes, or to focus on more highly divergent allosteric sites42. Our structures of TAK-925-bound active OX2R and functional data (Fig. 3) show that subtype selectivity can be achieved even when the binding pockets are extremely highly conserved (19 out of 21 residues within 5 Å of TAK-925 are identical, with only Thr→Ser and Thr→Ala substitutions). How then does TAK-925 bind so much weaker to OX1R? As with OX2R-selective antagonists such as EMPA21, the contact sphere surrounding the ligand does not have to be very different between subtypes. Instead, we propose that small cavities resulting from the two smaller amino acids in a potential

Fig. 4 Propagated changes in OX2R activation. a Conformational changes of the side chains of key residues at the orthosteric site when bound to agonist, comparing the active conformation (purple sticks, transparent gray cartoon) and the inactive conformation (cyan sticks and transparent cyan cartoon). TAK-925 is shown as yellow sticks and suvorexant is in cyan sticks. b Overlay of PIF transmission switch in the active conformation of OX2R (purple sticks and transparent spheres) and the inactive conformation of OX2R (PDB 4S0V, cyan sticks and transparent spheres). c Rewiring of micro switches on the intracellular side of OX2R when bound to agonist, comparing the active conformation (purple sticks, gray cartoon) and the inactive conformation (cyan sticks). TAK-925 is shown as yellow sticks. d Conformational changes of DRY motif when coupled to G protein, comparing the active conformation (gray cartoon and purple stick) and the inactive conformation (cyan sticks). The HS helices of Gα and Gβ are shown as orange and blue cartoon, respectively. Hydrogen bonds are shown as dotted lines.
OX₂R/TAK-925 complex lead to subtly diminished steric complementarity and less favorable desolvation upon surface burial\(^{13}\), with a large effect on binding free energy. These cavities are filled in the OX₂R S103^{2-61}/T127^{3-33} double swap mutant, allowing for activation of this receptor by TAK-925.

Other selective small molecule OX₂R agonists such as Compound 1\(^{14}\) and the structurally similar YNT-185\(^{16}\) may also rely on the subtle differences between orthosteric pockets that are important for TAK-925, however the structure of Compound 1-bound OX₂R shows that this type of diarylsulfonamide also makes contacts with the more divergent extracellular loops. Intriguingly, a recent medicinal chemistry effort\(^{44}\) succeeded in making a potent dual orexin receptor agonist by modifying YNT-185, which is predicted to interact with the biphenyl moiety that is expected to interact with the extracellular loops (given the structural analogy with Compound 1). This finding implies that OX₂R-selective arylsulfonamide agonists may derive selectivity from interactions that are far from the TAK-925 interface. Determining the importance of different contacts for selectivity of this other agonist chemotype will require additional functional studies of mutant receptors. In the case of the antagonist EMPA, a previous structural and computational study\(^{22}\) found that binding of a trapped ordered water molecule in a cavity formed at A1273.33 of the putative OX₁R complex is a major determinant of OX₂R selectivity for this ligand. A similar phenomenon may occur to make TAK-925’s interaction with OX₁R unfavorable, and will require computational approaches to elucidate. Our recent demonstration\(^{45}\) of converting a dual selective antagonist (suvorexant) into an OX₂R-selective agonist by filling in this cavity with an aliphatic group suggests that modifying TAK-925 may be a viable strategy for creating OX₂R-selective agonists.

Few GPCRs have been rigorously characterized for their ability to activate multiple G protein classes, such as \(\beta\_2\)-adrenergic receptor coupling to \(G_\alpha\) and \(G_\beta\)\(^{16}\). Several studies of OX₂R using cAMP sensors or other engineered reporter assays have indicated that OX₂R can also stimulate \(G_\beta\) and \(G_\alpha\) signaling, although with reduced orexin potency\(^{47,48}\). From these studies, one may ask whether OX₂R has any preference for activating a particular signaling cascade in cells or in vivo. Our structural data indicates that OX₂R distinguishes between \(G_\beta\) and \(G_\alpha\), both in the extent of interaction and in the number of specific polar contacts (Fig. 5a, b, Supplementary Figs. 9 and 10). Meanwhile, our functional data shows that OX₂R cannot substantially activate \(G_\alpha\) in HEK293 cells (Fig. 5d), however the caveat remains that we did not measure this activity in transfected neurons. These results bolster observations that orexins function mainly by stimulating calcium release through activating \(G_\alpha\)\(^{32,49}\), and suggest that G protein promiscuity plays a limited role in orexin signaling.

Several examples have recently been described of activated GPCR structures in complex with multiple G proteins (or G protein mimetics), which can provide insights into G protein selectivity. In most cases, the G protein known to couple most efficiently with the receptor has a larger buried surface area at the interface relative to subordinate G protein transducers\(^{37,38}\), which
holds true for OX2R in this study (see above). The multivalent interactions between the receptor and the G protein are divergent, and selectivity is largely dependent on the unique features of each GPCR. In the GCGR-Gi complex, ICL2 of the receptor engages more closely with Gi compared to Gq, and this interaction was demonstrated to be important for preferred Gi coupling.60 On the other hand, the GCGR-Gq complex features unique contacts between ICL1 and Gi and between ICL3 and Gαq.11 In the CCK1R-Gq complex, ICL3 interacts with Gαq and was shown to be important for Gi coupling potency.37 We do not observe a similar ICL3-G protein interaction in either of our OX2R-G protein complexes (Fig. 5a, b), and the tip of ICL3 is disordered in both structures, however the caveat remains that we have used a Gαi mimetic (mGαqi) rather than wild-type Gαqi protein in our study. In another example of multiple CCK1R-G protein structures, a different conformation was observed for the G protein α5 helix between mGαqi and Gαqi complexes, which may reflect the potential for structural differences or dynamics of this key divergent G protein epitope to confer selectivity.63 In our two OX2R-G protein structures, we observe highly similar conformations of the α5 helix (including the C-terminal ‘hook’) for the mGαqi and Gαqi complexes (Fig. 5a, b, rsmd of 1.3 Å as described above). On the other hand, we find that our OX2R-Gq1 interface is dominated by hydrophobic contacts, and the OX2R-Gαqi1 interface is a mixture of polar and hydrophobic interactions (Fig. 5a, b, Supplementary Figs. 9 and 10). A similar differentiation of interface properties is seen in comparing the preferential GCGR-Gq complex to the GCGR-Gαqi complex.30 Intriguingly, several of the hydrogen bonds between OX2R and the mGαqi α5 helix are conserved in CCK1R-Gq structures:36,37 Y235H5.23–S164ICL2 and N231H5.19–A1553.53 from OX2R–mGαqiN interface is dominated by hydrophobic contacts, and the OX2R–Gαqi interface is a mixture of polar and hydrophobic interactions (Fig. 5a, b, Supplementary Figs. 9 and 10). A similar differentiation of interface properties is seen in comparing the preferential GCGR-Gq complex to the GCGR-Gαqi complex.30

Cloning and expression of the human OX2R-Gαqi-scFv16 complex. The coding sequence of wild type human OX2R (residues 2–444) was synthesized and sub-cloned into pFastBac with an N-terminal FLAG tag followed by a fragment of βAR N-terminal 1–244 before OX2R, and a C-terminal 2xMBP-His tag after OX2R. A TEV cleavage site was inserted between OX2R and the MBP tag. The prolactin precursor sequence was used to increase signal peptide to increase protein expression. A deletion-negative bovine Gαqi construct called DNGαqi was generated by site-directed mutagenesis to incorporate mutations G203A31 and A326S32 to decrease the affinity of nucleotide binding and increase the stability of the Gαqi complex. All three G protein complex components, human Gαqi, human Gβ1i and human Gγ2i were cloned into pFastBac individually. For OX2R-Gαqi, scFv16 was cloned into pFastBac with a GP67 signal peptide at the N-terminal and a TEV cleavage-His tag at the C-terminus. Baculoviruses for OX2R, DNGαqi, His-tagged Gβ1i and Gγ2i, and scFv16 were co-expressed in Sf9 cells. Cell cultures were grown to a density of 3.5 × 10^6 cells/ml and infected with all five baculoviruses at a ratio of 1:1:1:1:1. 48 h after infection, cells were harvested and stored at −80 °C for further use.

Cloning, expression, and purification of scFv16. For OX2R-mGαqi-scFv16, scFv16 was expressed and purified separately. A synthesized DNA fragment encoding scFv1655 was cloned into pFastBac, with a melittin signal sequence at the N-terminus and a 10His tag at the C-terminus. The resulting construct was expressed in Sf9 cells. scFv16 was purified as previously described53. In brief, secreted scFv16 in the cell culture media was separated from Sf9 cells by centrifugation, and 10 mM Tris buffer pH 8.0 was added to balance pH. Then 1 mM NiSO₄ and 5 mM CaCl₂ were added to chelate with a GP67 signal peptide at the N-terminal and a TEV cleavage-His tag at the C-terminus. Baculoviruses for OX2R, DNGαqi, His-tagged Gβ1i and Gγ2i, and scFv16 were co-expressed in Sf9 cells. Cell cultures were grown to a density of 3.5 × 10^6 cells/ml and infected with all five baculoviruses at a ratio of 1:1:1:1:1. 48 h after infection, cells were harvested and stored at −80 °C for further use.

Purification of the human OX2R-Gαqi-scFv16 complex. Cell pellets from 6L culture of the expressed OX2R-Gαqi complex were resuspended and lysed for 30 min at 4 °C in a hypotonic buffer consisting of 10 mM HEPES pH 7.4, 160 μg/ml benzamidine, 100 μg/ml leupeptin, 1 mM MgCl₂, 0.1 mM TCEP, Apyrase (0.5 μM/μl), Lysed cells were spun down and resuspended using a dounce tissue grinder (Wheaton) in buffer consisting of 50 mM HEPES pH 7.4, 150 mM NaCl, 160 μg/ml benzamidine, 100 μg/ml leupeptin, 1 mM MgCl₂, 1% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace), 0.1% Na Cholate, 0.1% cholesteryl hemi-succinate (CHS, Steraloids), 10% glycerol, 0.5 mM TCEP, Apyrase (15 μU/μl) and 10 μM CaCl₂. Solution was incubated for 15 min at 4 °C, followed by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatant was incubated with M1 anti-Flag affinity beads (Sigma) in batch-binding mode overnight at 4 °C in the presence of 2 mM CaCl₂. After binding, the M1 beads were spun down and washed with 10 column volumes of buffer consisting of 50 mM HEPES pH 7.4, 150 mM NaCl, 160 μg/ml benzamidine, 100 μg/ml leupeptin, 1 mM MgCl₂, 1% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace), 0.1% Na Cholate, 0.1% cholesteryl hemi-succinate (CHS, Steraloids), 10% glycerol, 0.5 mM TCEP, Apyrase (15 μU/μl) and 10 μM CaCl₂. Solution was incubated for 15 min at 4 °C, followed by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatant was incubated with M1 anti-Flag affinity beads (Sigma) in batch-binding mode overnight at 4 °C in the presence of 2 mM CaCl₂. After binding, the M1 beads were spun down and washed with 10 column volumes of buffer consisting of 50 mM HEPES pH 7.4, 150 mM NaCl, 0.05% LMNG, 0.005% Na Cholate, 0.005% CHS, 5% glycerol, 1 mM MgCl₂, 2 mM CaCl₂, 0.5 mM TCEP and 10 μM TAK-925. The complex was eluted from M1 beads with buffer consisting of 50 mM HEPES pH 7.4, 150 mM NaCl, 0.05% LMNG, 0.005% Na Cholate, 0.005% CHS, 5% glycerol, 0.5 mM TCEP, 10 μM TAK-925, 200 μg/ml FLAG and 5 mM EDTA. The eluted OX2R-Gαqi-scFv16 complex was mixed at a ratio 1:1 and concentrated using a 100,000 × g for 30 min on ice. Finally, the mixture was concentrated and applied onto Superose 6 Increase 10/300 size exclusion column (GE Healthcare). The total yield of the
complex was ~3 mg and the peak corresponding to the OX,R-mGinqu-scFv16 complex was collected, concentrated and used in the following cryo-EM experiments (Supplementary Fig. 1a).

**Purification of the human OX2R-Gi1-scFv16 complex.** Cell pellets from 10L culture were thawed at room temperature and suspended in 20 mM HEPES pH 7.2, 50 mM NaCl, 2% Brij35, at 100 × g for 5 min. The supernatant was then centrifuged at 100,000 × g for 30 min. The pellets were mixed on an orbital shaker for 30 min. The plates were read on a Microbeta Trilux plate reader (Perkin Elmer) the following day. IP assays were performed using anti-Gαq (Perkin Elmer) in 3% of the total lysate of each construct. Western blot analysis was performed using anti-Gαq (Perkin Elmer) as a control for the expression of Gαq. The protein was resolved on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with 5% BSA and probed with antibodies specific for Gαq (Perkin Elmer). The bands were visualized using an ECL detection system (GE Healthcare) and quantified using ImageJ software.

**Cryo-EM data collection for the OX2R-Gi1-scFv16 complex.** Prior to freezing grids, the OX2R-mGinqu-scFv16 complex was concentrated to 9.5 mg/ml. The cryo-EM grids were prepared by applying 3.5 µl of this sample to glow-discharged Quantifoil R1/2/1.3 300-mesh Au holey carbon grids (Quantifoil Micro Tools GmbH, Germany), blotted for 4.5 s under 100% humidity at 4 °C and plunge frozen in liquid ethane cooled by liquid nitrogen using a Mark IV Vitrobot. SerialEM software61 was used for automated data collection. A Titan Krios microscope (FEI) operated at 300 kV with a K3 direct electron detector (Gatan) at a temperature (~80 K) was used to collect the final images. The Ramachandran plot, 97.6% and 2.4% of residues were in favored and allowed regions, respectively. Data collection and refinement are included in Supplementary Table 1. The cryo-EM density map has been deposited in the Electron Microscopy Data Bank under accession code EMD-25389. Atomic coordinates have been deposited in the PDB under accession code 7SQO. Structural figures were made using Pymol (Version 2.0 Schrödinger, LLC) and UCSF Chimera.

**Quantum chemistry calculation.** Northwest Computational Chemistry Package (NWChem) 6.8.165 was used to perform all electronic structure calculations. All geometries were optimized at the BLYP/6-31G* level of the Density-functional theory (DFT) in gas phase first and then with the solvation model based on density (SMD) in aqueous solution. Population analysis was performed in each case to confirm that each structure was a local minimum on the potential energy surface and to compute thermodynamic quantities. The standard Eckart projection algorithm, as implemented in NWChem, was applied to project out the translations and rotations of the nuclear hessian. Based on the frequencies obtained from the projected hessian, the zero point energy for the molecular system was calculated.

**Inositol phosphate accumulation assay for Gαq signaling.** The IP assay was adapted from a prior precedent65. HEK293 cells were maintained in DMEM-high glucose (Millipore Sigma) supplemented with 5% fetal bovine serum (Corning) and penicillin-streptomycin (Millipore Sigma). Receptor constructs used were the full-length wild-type (or mutant) human OX2R and OX2R sequenced human OR (PDB: 6DDF) as well as structures of mini-Gαq2, GOαs (PDB: 7SQO) and scFv16 (PDB: 6VMS) were used as initial models for model rebuilding. A polyalaline model was made from the amino acid sequence of each construct. The cryo-EM density map has been deposited in the Electron Microscopy Data Bank under accession code EMD-25389. Atomic coordinates have been deposited in the PDB under accession code 7SQO. Structural figures were made using Pymol (Version 2.0 Schrödinger, LLC) and UCSF Chimera.
normalized to OX2R WT Eₘ₅₀, and dose responses were fitted to the three-parameter model ‘log(agonist) vs response’ in GraphPad Prism 9 (GraphPad Software). The Kᵢ and binding affinities were calculated. Data was normalized to OX2R WT (with TAK-925 or orexin B) using one-way ANOVA followed by Dunnett’s test. All pharmacological parameters are displayed in Supplementary Table 2.

β-arrestin PathHunter assay. β-Arrestin recruitment activity was measured by using the PathHunter system (DiscoveRX) according to the manufacturer’s instruction. Receptor constructs used were the full-length wild-type (or mutant) human OX2R cloned into the pCMV-ProLink (DiscoveRX) vector. Cells stably expressing Eα β-arrestin2 (DiscoveRX) were plated on 384 well white plates (Thermo Fisher) at 4000 cells/well in HamF12 (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (Corning) and penicillin-streptomycin (Wako Pure Chemical Industries) and incubated overnight at 37 °C under 5% CO₂. Cells were transfected with plasmids encoding wild type or each mutant ProLink-tagged human OX2R using FuGENE HD (Promega). The following day, the media was replaced with HBSS (GIBCO) containing 20 mM HEPES (GIBCO) and 0.1% BSA (Wako Pure Chemical Industries), and treated with TAK-925 for 2 h at 37 °C. Detection reagent was added and incubated for 1 h at room temperature. Luminescence signal was detected using an EnVision plate reader (PerkinElmer). β-arrestin assays on different constructs were performed in two or three independent experiments (each done in quadruplicate). Dose response curves (shown in Supplementary Fig. 8) were fitted to the three-parameter model ‘log(agonist) vs response’ in GraphPad Prism to determine IC₅₀ values. Fitted IC₅₀ values were analyzed for statistical significance compared to OX2R WT (with TAK-925 or orexin B) using one-way ANOVA followed by Dunnett’s test.

Inhibition of forskolin-stimulated cAMP Production.

To measure G, signaling HEK293 cells were transfected with pcGloSensor™–22F cAMP Plasmid (Promega) and CMV expression plasmid encoding human OX2R. To enhance the signal for the cAMP assay, we co-transfected a G, expression plasmid (CMV-driven) in which the full-length human Gα₁, subunit sequence was cloned into pcDNA3. The following day, cells were transfected with cAMP assay buffer, HBSS with 20 mM HEPES pH 7.4 (Millipore Sigma). Cells were suspended in assay buffer containing 0.5 mg/ml Luciferin (Gold Biotechnology) and plated on 96 well tissue culture-treated white plates with opaque bottoms (Thermo Fisher). The cells were incubated at 37 °C for 90 min. Forskolin (Millipore Sigma) diluted in assay buffer was added to 100 μM final concentration. Luminescence reading was read at room temperature repeatedly in a CLARIOstar microplate reader (BMG Labtech) until the signal stopped increasing. Agonist diluted in assay buffer was added from a 7X stock and the plates were read repeatedly until the signal was stable. Assays for either TAK-925 or orexin B were performed in 3 independent experiments (each done in duplicate). cAMP luminescence data were plotted as a fraction of the maximal signal with forskolin, and dose responses were analyzed by one-way ANOVA (and Nonparametric or Mixed) and plotted as a log(agonist) vs response column with scattered points using GraphPad Prism 9 (GraphPad Software).

Data availability

The structural data generated in this study have been deposited in the Protein Data Bank (PDB) under accession codes 7QOQ and 7QOR, and cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-25389 and EMD-25399. The pharmacological data generated in this study are compiled in the Source Data file provided with this paper. Source data are provided with this paper.

Received: 28 February 2022; Accepted: 5 May 2022; Published online: 25 May 2022

References

1. Li, J., Hu, Z. & Lecea, L. De The hypocretins/orexins: integrators of multiple physiological functions. Br. J. Pharmacol. 171, 332–350 (2014).
2. Zeitzer, J. M., Nishino, S. & Mignot, E. The neurobiology of hypocretin (orexins), narcolepsy and related therapeutic interventions. Trends Pharmac. Sci. 27, 568–574 (2006).
3. Marston, O. J. et al. Circadian and dark-pulse activation of orexin/hypocretin neurons. Mol. Brain 1, 19–19 (2008).
4. Scammell, T. E. & Winrow, C. J. Orexin receptors: pharmacology and therapeutic opportunities. Annu. Rev. Pharmacol. Toxicol. 51, 243–266 (2011).
5. Bassetti, C. L. A. et al. Narcolepsy — clinical spectrum, aetopathophysiology, diagnosis and treatment. Nat. Rev. Neurol. 15, 519–539 (2019).
6. Silber, M. H., Krahn, L. E., Olson, E. J. & Pankratz, V. S. The epidemiology of narcolepsy in olmsdet county, minnesota: a population-based study. Sleep 25, 197–202 (2002).
7. Peyron, C. et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat. Med. 6, 991–997 (2000).
8. Mignot, E. et al. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hyposomnias. Arch. Neurol. Chic. 59, 1553–1562 (2002).
9. Chemelli, R. M. et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98, 457–451 (1999).
10. Hara, J. et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron 30, 345–354 (2001).
11. Lin, L. et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98, 365–376 (1999).
12. Willie, J. T. et al. Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. Neuron 38, 715–720 (2003).
13. Baiem, C. et al. Orexin/hypocretin role in reward: implications for opioid and other addictions. Brit J. Pharm. 172, 334–348 (2015).
14. Coleman, P. J., Gotter, A. L., Herring, W. J., Winrow, C. J. & Renger, J. J. The discovery of suvorexant, the first orexin receptor drug for insomnia. Annu. Rev. Pharmacol. Toxicol. 59, 509–535 (2019).
15. Lebod, T. P., Bonaventure, P. & Shireman, R. T. Selective orexin receptor antagonists. Bioorg. Medicinal Chem. Lett. 23, 4761–4769 (2013).
16. Nagahara, T. et al. Design and synthesis of non-peptide, selective orexin receptor 2 antagonists. J. Med. Chem. 58, 7931–7937 (2015).
17. Fujimoto, T. et al. Discovery of TAK-925 as a potent, selective, and brain-penetrant orexin 2 receptor antagonist. ACS Med. Chem. Lett. 13, 457–462 (2022).
18. Yukiakie, H. et al. TAK-925, an orexin 2 receptor-selective agonist, shows robust wake-promoting effects in mice. Pharm. Biochem Be 187, 1277294 (2019).
19. Yin, J., Mobarec, J. C., Kolb, P. & Rosenbaum, D. M. Crystal structure of the human OX2 orexin receptors. Nat. Struct. Mol. Biol. 23, 293–299 (2016).
21. Suno, R. et al. Crystal structures of human orexin 2 receptor bound to the subtype-selective antagonist EMPA. Structure 26, 7–19.e5 (2018).

22. Rappas, M. et al. Comparison of orexin 1 and orexin 2 ligand binding modes using X-ray crystallography and computational analysis. J. Med. Chem. 63, 1528–1543 (2020).

23. Malherbe, P. et al. Mapping the binding pocket of dual antagonist almorexant to human orexin 1 and orexin 2 receptors: comparison with the selective OX1 antagonist SB-6204 and the selective OX2 antagonist N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonfyl)-amino]-N-pyridin-3-ylmethyl-acetamide (EMPA). Mol. Pharmacol. 78, 81–93 (2010).

24. Hong, C. et al. Structures of active-state orexin receptor 2 rationalize peptide and small-molecule agonist recognition and receptor activation. Nat. Commun. 12, 815 (2021).

25. Koehl, A. et al. Structure of the µ-opioid receptor-Gi protein complex. Nature 558, 547–552 (2018).

26. Németh, R. et al. Mini-G proteins: novel tools for studyingGPCRs in their active conformation. Plos One 12, e0175642 (2017).

27. Tran, D.-T. et al. Chimeric, mutant orexin receptors show key interactions between orexin receptors, peptides and antagonists. Eur. J. Pharm. 667, 120–128 (2011).

28. Ballesteros, J. A. & Weinstein, H. [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neuropsyco. 25, 366–428 (1995).

29. Zhou, Q. et al. Common activation mechanism of class A GPCRs. Elife 8, e03279 (2019).

30. Huang, W. et al. Structural insights into µ-opioid receptor activation. Nature 524, 315–321 (2015).

31. Latorraca, N. R., Venkatakrishnan, A. J. & Dror, R. O. GPCR dynamics: structure in motion. Chem. Rev. 117, 139–155 (2017).

32. Maeda, S., Qu, Q., Robertson, M. J., Skiniotis, G. & Kobilka, B. K. Structures of β-arrestin1 and β-arrestin2 in complex with a GPCR-activating G-protein. Structure 134, 552–557 (2019).

33. Kim, K. et al. Structure of a hallucinogen-activated 5-HT2A serotonin receptor. Cell 182, 1574–1588.e19 (2020).

34. Mobbs, J. I. et al. Structures of the human cholecystokinin 1 (CCK1) receptor bound to Gs and Gq mimetic proteins provide insight into mechanisms of G protein selectivity. Plos Biol. 19, e3001295 (2021).

35. Liu, Q. et al. Ligand recognition and G-protein coupling selectivity of cholecystokinin A receptor. Nat. Chem. Biol. 17, 1238–1244 (2021).

36. Flock, T. et al. Universal allosteric mechanism for Ga activation by GPCRs. Nature 524, 173–179 (2015).

37. Liang, Y.-L. et al. Phase-plate cryo-EM structure of a class B GPCR-G-protein complex. Nature 546, 118–123 (2017).

38. Zhang, A., Neumeyer, J. L. & Baldessarini, R. J. Recent progress in development of dopamine receptor subtype-selective agents: potential therapeutics for neurological and psychiatric disorders. Chem. Rev. 107, 274–302 (2007).

39. Ye, N., Neumeyer, J. L., Baldessarini, R. J., Zhen, X. & Zhang, A. Update 1 of: Recent progress in development of dopamine receptor subtype-selective agents: potential therapeutics for neurological and psychiatric disorders. Chem. Rev. 113, PR123–PR178 (2013).

40. Conn, P. J., Christopoulos, A. & Lindsey, C. W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat. Rev. Drug Discov. 8, 41–54 (2009).

41. Biela, A. et al. Dissecting the hydrophobic effect on the molecular level: the role of water, enthalpy, and entropy in lipid bilayer to thermolysin. Angew. Chem. Int. Ed. 52, 1822–1828 (2013).

42. Zhang, D. et al. Discovery of arylsulfonamides as dual orexin receptor agonists. J. Med. Chem. 64, 8806–8825 (2021).

43. Hellmann, I. et al. Structure–based discovery of a subtype-selective orexin 1 receptor antagonist. Proc Natl Acad. Sci. USA 117, 18059–18067 (2020).

44. Marenich, A. V., Cramer, C. J. & Truhlar, D. G. Universal solvation model of Coot. J. Comput Chem. 331 (2014).

45. Rasmussen, S. G. F. et al. Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477, 549–555 (2011).

46. Maeda, S., Qu, Q., Robertson, M. J., Skiniotis, G. & Kobilka, B. K. Structures of the M1 and M2 muscarinic acetylcholine receptor-G-protein complexes. Science 364, 552–557 (2019).

47. Kim, K. et al. Structure of a hallucinogen-activated 5-HT2A serotonin receptor. Cell 182, 1574–1588.e19 (2020).

48. Shibayama, N. et al. Structure of the human orexin 2 receptor-Gs protein complex determined using X-ray crystallography. J. Biol. Chem. 273, 21752–21758 (1998).

49. Zhu, Y. et al. Structure of a D2 dopamine receptor-G-protein complex in a lipid membrane. Nature 584, 125–129 (2020).

50. Qiao, A. et al. Structural basis of Gs and Gi recognition by the human glucagon receptor. Science 367, 1346–1352 (2020).

51. Lee, E., Faussig, R. & Gilman, A. G. The G226A mutant of Gα alpha highlights the requirement for dissociation of G protein subunits. J. Biol. Chem. 267, 1212–1218 (1992).

52. Posner, B. A., Mizon, M. B., Wall, M. A., Sprang, S. R. & Gilman, A. G. The A3265 mutant of Gαi1 as an approximation of the receptor-bound state. J. Biol. Chem. 273, 21752–21758 (1998).

53. Yin, J. et al. Structure of a D2 dopamine receptor-G-protein complex in a lipid membrane. Nature 584, 125–129 (2020).

54. Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. Elife 7, 163 (2018).

55. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Meth. 14, 331–332 (2017).

56. Zhang, K. GetCf: Real-time CTF determination and correction. J. Struct. Biol. 193, 1–12 (2016).

57. Rohou, A. & Grigorieff, N. CTFIND4: Fast and accurate defocus estimation from electron micrographs. J. Struct. Biol. 192, 216–221 (2015).

58. Jung, A. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).

59. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

60. Broughton, K. The Buccaneer software for automated model building. 1. Tracing protein chains. Acta Crystallogr D. Biol. Crystallogr 62, 1002–1001 (2006).

61. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr D. Biol. Crystallogr 66, 486–501 (2010).

62. Liebschner, D. et al. Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. Acta Crystallogr D. Biol. Crystallogr 75, 861–877 (2019).

63. Valiev, M. et al. NWChem: A comprehensive and scalable open-source solution for large scale molecular simulations. Comput Phys. Commun. 181, 1477–1489 (2010).

64. Marenich, A. V., Cramer, C. J. & Truhlar, D. G. Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions. J. Phys. Chem. B 113, 6378–6396 (2009).

65. Mathiesen, J. M. & Ramirez, M. T. The metabotropic glutamate receptor 4 is internalized and desensitized upon protein kinase C activation. Br. J. Pharmacol. 148, 279–290 (2006).

66. Hepler, J. R. et al. Functional importance of the amino terminus of Gαq (+). J. Biol. Chem. 271, 496–504 (1996).

67. Kumar, B. A., Kumar, P., Soni, C. & Yadav, P. N. Chapter 2 GloSensor assay for discovery of GPCR-selective ligands. Methods Cell Biol. 142, 27–50 (2017).
Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30601-3.

Correspondence and requests for materials should be addressed to Mack Flinspach, Jef K. De Brabander or Daniel M. Rosenbaum.

Peer review information Nature Communications thanks Yanan Zhang and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022