Activation of the GLP-1 receptor by a non-peptidic agonist

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Class B G protein-coupled receptors are major targets for treatment of chronic disease, including diabetes and obesity[1]. Structures of active receptors revealed that peptide agonists engage deep within the receptor core leading to an outward movement of extracellular loop (ECL) 3 and tops of transmembrane helices (TMs) 6 and 7, an inward movement of TM1, reorganisation of ECL2 and outward movement of the intracellular side of TM6 allowing G protein interaction and activation[2-6]. Here we have solved the structure of a novel non-peptide agonist, TT-OAD2, bound to the GLP-1 receptor. This

identified an unpredicted non-peptide agonist binding pocket where reorganisation of TM6/ECL3/TM7 manifested independently of direct ligand interaction within the deep transmembrane domain pocket. TT-OAD2 exhibits biased agonism, and kinetics of G protein activation and signalling that are distinct from peptide agonists. Within the structure, TT-OAD2 protruded beyond the receptor core to interact with the lipid/detergent providing an explanation for the distinct activation kinetics that likely contributes to the clinical efficacy of this compound series. This work alters our understanding of the events that drive class B receptor activation.

Class B peptide GPCRs regulate the control of glucose and energy homeostasis, bone turnover, and cardiovascular development and tone[1]. Multiple peptide agonists are clinically approved for disorders of energy and bone metabolism[1], however, attempts to develop non-peptide, orally available analogues have yielded only limited success. Understanding the structural basis of class B GPCR activation is crucial to rational development of peptidic and non-peptidic drugs. Recent structural determination of full-length, active class B receptors bound to peptide agonists[2-6] confirmed that the N-terminus of the peptide ligands, required for receptor activation, binds deep within the 7 TM bundle. This is associated with an outward movement of the top of TM6 and TM7 (and interconnecting extracellular loop (ECL) 3) and a large kink in the centre of TM6 that opens up the intracellular face of the receptor to allow G protein coupling[2-4,7-10]. In parallel, a conformational reorganisation of ECL2 and an inward movement of TM1 facilitates peptide interaction and receptor activation.

The GLP-1R receptor (GLP-1R) is an established therapeutic target for type 2 diabetes and obesity[11]. Despite their clinical success, GLP-1R peptide drugs are suboptimal due to their route of administration and side-effect profiles, most notably nausea and vomiting that reduce patient compliance[11]. For many years oral GLP-1R agonists have been pursued, with recent studies reporting promising clinical trial data for oral semaglutide, a new formulation of the approved peptide semaglutide[12,13]. However, it induced greater severity of nausea and gastrointestinal side effects than those observed with injectable GLP-1 mimetics[13]. Future development of non-peptide drugs could offer more traditional small molecule absorption characteristics that may assure better long-term patient compliance with potential for reduced gastrointestinal liability, especially for patients who are co-administering with additional medications.

A number of non-peptidic GLP-1R agonists have been identified[14]. One class form covalent interactions with C347$^{6.36}$ and are predicted to allosterically disrupt polar networks at the base of the receptor, promoting activation[15], while other small molecule compounds bind to unknown sites at the receptor extracellular face[14,16,17]. However, it is assumed that these molecules may need to mimic key interactions of the peptide N-terminus deep within the TM core to initiate receptor activation, as is seen for short stabilised 11mer peptides, that occupy an overlapping site to full-length peptides[18].

The current study investigated TT-OAD2 (Figure 1A), a non-peptidic compound reported in the patent literature and part of the chemical series that contains the vTv Therapeutics investigational drug candidate, TTP273. TTP273, an orally administered GLP-1R agonist, successfully completed phase IIa efficacy trials for type 2 diabetes (ClinicalTrials.gov Identifier: NCT02653599) where it met its primary endpoint, reducing glycated haemoglobin (HbA1c) in type 2 diabetic patients, with no reported cases of nausea[19], suggesting a potential clinical advantage for compounds of this series. Little has been disclosed about the molecular properties of this compound series, however recent progression of TTP273 has been hampered by unexpected complexity in identifying optimal dosing that may be linked to a lack of understanding of its mechanism of action. Assessment of acute *in vivo* activity in humanised GLP-1R mice revealed TT-OAD2 is insulinotropic and that this effect is dependent on the GLP-1R (Figure 1B).

TT-OAD2 is a biased agonist with slow kinetics

In HEK293 cells overexpressing the GLP-1R, TT-OAD2 only partially displaced the orthosteric probes ^{125}I-Ex4(9-39) and ROX-Ex4 (Figure 1C, Extended Data Figure 1A), consistent with an allosteric mode of interaction[16]. While the GLP-1R signals to multiple cellular pathways, TT-OAD2 only activated a subset of these responses; it was a low potency partial agonist for cAMP accumulation, with only weak responses detected for iCa^{2+} mobilisation and ERK1/2 phosphorylation at very high concentrations (100 μM) (Figure 1D) and no detectable β-arrestin-1 recruitment. These data are indicative of bias towards cAMP and away from these other pathways relative to endogenous GLP-1. There is significant interest in exploiting biased agonism at GPCRs to maximise the beneficial effects of receptor activation, while minimising on-target side-effect profiles.

CRISPR engineered HEK293 cells where $G_{s/olf}$ or $G_{i/o/z}$ proteins were deleted revealed, G_s was essential for cAMP production, however, this response, for both ligands, was also dependent on the presence of $G_{i/o/z}$ proteins. (Extended Data Figure 1B). Assessment of proximal activation of G_s and G_i transducers using split luciferase NanoBit G protein sensors (Extended Data Figure 1C) determined GLP-1 decreased luminescence in a bi-phasic, concentration-dependent, manner for both G proteins with similar potencies in each phase. For TT-OAD2, the G_i sensor gave a similar decrease in luminescence to GLP-1, however, enhanced luminescence was observed for the G_s sensor, suggestive of a different mechanism of G_s activation. To probe these differences further, we employed membrane-based assays of bioluminescence resonance energy transfer (BRET) G protein sensors to assess the rate and nature of the G_s conformational change. In contrast to the rates of change in G_i conformation, which were similar for both ligands, (Extended Data Figure 1), there was marked distinction in kinetics for G_s coupling. GLP-1 promoted a rapid change in G_s protein conformation, but for TT-OAD2 this was very slow (Figure 1E). However, both agonists induced a similar plateau of the measured response (Figure 1E) that was reversed by excess GTP (Extended Data Figure 1D), indicative of a similar overall conformational rearrangement. Taken together, this suggests that slower G_s conformational transitions, required for exchange of GDP for GTP and G_s activation, would result in lower turnover of G protein and rate of cAMP production by TT-OAD2. Direct kinetic measurements of cAMP production validated this hypothesis (Figure 1F. Extended Data Figure 1E). Overall, this data revealed TT-OAD2 as a biased agonist only capable of activating a subset of pathways with limited efficacy and with distinct activation kinetics relative to peptide agonists.

TT-OAD2 has an unexpected binding mode

To understand how TT-OAD2 binds and activates the GLP-1R we determined the GLP-1R structure bound to TT-OAD2 and the transducer heterotrimeric G_s (Figure 2A). Complex formation was initiated in *Tni* insect cells by stimulation with 50 μM of TT-OAD2 then solubilised and purified (Extended Data Figure 2A). Vitrified complexes were imaged by single particle cryo-EM on a Titan Krios. Following 2D and 3D classification, the most abundant class was resolved to 3.0 Å (Extended Data Figure 2C-2F, Supplemental Data Table 1). The cryo-EM density map allowed unambiguous assignment of the TT-OAD2 binding site and pose, and clear rotamer placement for most amino acids within the receptor core and G protein (Figure 2B, Extended Data Figures 3 and 4A). The GLP-1R ECD, and the $G\alpha_s$ α-helical domain were not resolved at high resolution, consistent with their greater mobility. Rigid body

fitting of an available x-ray structure of the GLP-1R ECD domain (PDB-3C5T)[20] was performed into the density to generate a full-length model.

TT-OAD2 bound high up in the helical bundle interacting with residues within TM1, TM2, TM3, ECL1 and ECL2 (Figure 2, Extended Data Figure 4A). The majority interactions are hydrophobic in nature (Figure 2) including numerous pi-pi stacking interactions between receptor aromatic residues and phenolic regions within the ligand. Unexpectedly, TT-OAD2 adopts a "boomerang-like" orientation within the binding site with the 3,4-dichloro-benzyl ring of TT-OAD2 protruding beyond the receptor core through TMs 2 and 3, interacting with $W203^{2.73}$, and embedding in the detergent micelle, consistent with likely interactions with the lipid bilayer in a native system. $F230^{3.33}$ and $W297^{ECL2}$ interact with the 2,3-dimethyl-pyridin-4-yl-phenol region, $Y220^{ECL1}$ forms a hydrogen bond with the 2,3-dimethyl-pyridine ring and $K197^{2.67}$ forms a polar interaction with the propionic acid part of the ligand. Additional hydrophobic contacts are formed with TT-OAD2 by $Y145^{1.40}$, $L201^{2.71}$, $I196^{2.69}$, $A200^{2.70}$, $L217^{ECL1}$, $V229^{3.32}$ and $M204^{3.36}$ (Figure 2, Extended Data Figure 4A). Molecular dynamics simulations (MD) of the TT-OAD2-GLP-1R-Gs complex predicted additional transient interactions with TM1, 2, 3, ECL1, ECL2 and the ECD of the GLP-1R (Extended Data Table 1). Assessment of TT-OAD2-induced cAMP production at alanine mutants of key receptor residues within the binding site revealed either reduced potency (pEC$_{50}$), reduced Emax or both relative to the wildtype receptor (Figure 2, Supplemental Data Table 2). Application of the operational model of agonism revealed these mutations directly alter TT-OAD2 functional affinity (K_A) and/or efficacy (τ) (Supplemental Data Table 2) highlighting the importance of these residues in TT-OAD2 function.

Peptide vs non-peptide binding sites

The TT-OAD2 binding pose has very limited overlap with full-length peptides, GLP-1 and $ExP5^{3,6}$ (Figure 3, Extended Data Figure 5). Structural comparisons, combined with associated MD performed on models generated from the cryo-EM data, identified only 10/29 residues that interact with both TT-OAD2 and GLP-1. Moreover, the persistence and nature of ligand interactions formed by common residues differed (Figure 3C, Extended Data Table 1). In contrast to TT-OAD2, peptide ligands engage TMs 5-7 in addition to extensive interactions deep within the bundle in TMs 1-3 (Figure 3, Extended Data Figure 5, Extended Data Table 1).

The relatively limited overlap between the peptide and TT-OAD2 binding sites suggests this compound series may modulate peptide function in a physiological setting. To address this, we assessed the effect of TT-OAD2 on the signalling of two physiological ligands (Extended Data Figure 6). TT-OAD2 inhibited GLP-1- and oxyntomodulin-mediated cAMP, calcium, pERK1/2 and β-arrestin responses in a concentration-dependent manner (Extended Data Figure 6). This suggests that the profile of signalling observed from the GLP-1R when using TT-OAD2-like compounds as drugs may depend upon the dose administered; at high concentrations their presence would likely inhibit all endogenous peptide effects, biasing receptor responses primarily to cAMP formation mediated by the compound itself. However, at lower concentrations, some endogenous peptide signalling may still occur. Interesting TTP273 was reported to exhibit greater clinical efficacy at lower concentrations, implying that maintenance of some aspects of physiological signalling may be important for clinical efficacy.[19]

GLP-1R conformational changes and activation

At a gross level, the TT-OAD2-complexed GLP-1R helical bundle displays the key hallmarks of activated, peptide-occupied, class B GPCRs[2-6]. At the extracellular face this includes the large outward movement of TM6/ECL3/TM7, inward movements of TM1, helical extensions within TMs 2 and 3, a reordering of ECL1 and conformational transitions within ECL2 that elevates upward towards the extracellular side (Extended Data Figure 5). At the intracellular side, there is an equivalent large outward movement of TM6 away from the centre of the helical bundle, and the smaller outward movement of TM5. It is important to note that the fully-active state is driven in part by allosteric conformational changes, including those in the extracellular face, linked to G protein binding[21]. Nonetheless, all the GLP-1R structures are solved with the same G protein yet reveal conformational differences at their extracellular face, including within the extent of movement of TM6/ECL3/7 and the conformation of the ECD, TM2/ECL1 and ECL2 that are linked to the bound agonists (Figure 3A and 3B, Extended Data Figure 5B and 5C). This suggests that distinct receptor activation triggers converge to common changes at the intracellular face that allow coupling to transducers.

While the low resolution of the receptor ECD for the TT-OAD2 complex indicates extensive mobility, it occupied a distinct orientation relative to the TM core in comparison to peptide-

bound complexes, whereas both GLP-1 and ExP5 bound receptors stabilised a similar conformation[3,6] (Extended Data Figure 5A). Similarly, the short 11mer peptide HepP5 forms few interactions with the ECD[18] and also occupies a distinct orientation relative to GLP-1 and ExP5, but this conformation also differs from that stabilised by TT-OAD2 (Extended Data Figure 5C). The cryo-EM map of the TT-OAD2 bound receptor complex supports extended interactions of the ECD with ECL1 and ECL2 (Extended Data Figure 4B) and this is supported by MD that predicts interactions of $R40^{ECD}$ with $D215^{ECL1}$ and $E34^{ECD}$ with $R299^{ECL2}$ (Extended Data Table 2). This later interaction is particularly important as $R299^{ECL2}$ directly, and stably interacts with peptide ligands, but in the TT-OAD2 bound receptor, stabilises the N-terminus of the ECD in a position that may play an analogous role to the peptide in stabilising ECL2. Indeed, in our models, the position of the far N-terminal ECD helix overlapped with the location of the C-terminal region of GLP-1 and ExP5 when comparing the TT-OAD2 and peptide bound structures (Figure 3A). Thus, the ECD is likely to be important for both stabilising the TT-OAD2 binding site and facilitating receptor activation, as previously proposed for different classes of peptide ligands[22,23].

Distinctions from peptide-bound receptors observed within TM2/ECL1 and ECL2 (Figure 3B) are likely driven by direct ligand interactions by TT-OAD2 (Figure 2), while those within TM6 and 7 by direct interactions formed by peptide agonists. MD also supports a role of membrane lipid interactions in directly stabilising both these regions within the TT-OAD2 bound structure (Extended Data Figure 7). Remarkably, the helical bundle of the TT-OAD2 complexed receptor is in a more open conformation than the peptide occupied receptors, largely due to the top of TM6/ECL3, TM7 and TM1 residing 16 Å, 6 Å and 7 Å further outwards relative to the GLP-1 bound structure (measured from the Cα's of $D372^{6.62/ECL3}$, $F381^{7.37}$ and $P137^{1.32}$, respectively (Figure 3B). The orientation of TM6/ECL3/TM7 also differs between ExP5 and GLP-1 bound structures with ExP5 adopting a more open conformation[3], however, the outward positioning of ECL3 induced by TT-OAD2 is much larger (Extended Data Figure 5B). Peptide-bound structures of all solved class B GPCRs revealed direct interactions of the engaged peptide with residues within TM5, TM6, TM7 and ECL3 with the peptide volume (minimally) presumed to actively contribute to the outward conformational change in this region[2-4,8,9,24]. In the apo state of the glucagon receptor, interactions occur between ECL3 and the ECD that contribute to maintenance of receptor quiescence[7,8,25,26]. MD on the GLP-1R structures, performed following removal of either TT-OAD2 or GLP-1, predict that the GLP-1R ECD also

adopts both open and closed conformations in the apo state where it can form transient interactions with both ECL2 and ECL3 (Extended Data Figure 8)[25]. Combining this information with the GLP-1R active structures suggests that interactions, with either peptide or non-peptide agonists, are sufficient to release ECL3-ECD constraints, lowering the energy barrier for receptor activation. However, the degree of ligand interaction with TM6-ECL3-TM7 determines the extent to which the TM bundle opens and this in turn directly contributes to G protein efficacy and biased agonism, as these regions (TM6-ECL3-TM7 and TM1) have been identified as key drivers for these phenomena, particularly for the GLP-1R[3,27,28,29].

Despite the different binding modes, commonalities observed in interactions with TT-OAD2 and peptide with TMs 1-3 and stabilisation of ECL2 are sufficient to initiate conformational transitions that propagate to a similar reorganisation of the class B GPCR conserved central polar network that is linked to activation, albeit the mechanism for this differs for peptide agonists vs TT-OAD2 (Figure 4A, Supplemental Data Video 1, Extended data Figure 9). MD of the GLP-1 bound GLP-1R predicted persistent interactions between Y152$^{1.47}$, R190$^{2.60}$, Y241$^{3.44}$ and E364$^{6.53}$ and the N-terminus of GLP-1 that directly engage the central polar network (Figure 4A, Extended Data Tables, 1 and 2, Supplemental Data Video 1). In contrast, TT-OAD influences the central polar network allosterically via interactions with K197$^{2.67}$, Y145$^{1.40}$ and Y148$^{1.43}$. TT-OAD2 also promotes unique hydrogen bond networks with crucial residues in TM2 (Figure 4A, Extended Data Table 2) that result in different interaction patterns at the top of TM1 and TM2 relative to peptide-occupied receptors. These effects propagate to the polar network through transient contacts between TT-OAD2 with Y148$^{1.43}$ and Y152$^{1.47}$ that in turn interact with R190$^{2.60}$ of the central polar network (Supplementary Data Movie 2). When bound by GLP-1, the polar network is stabilised by ligand and a network of water molecules, whereas for TT-OAD2, this occurs via a distinct network of structural waters rather than by the ligand (Figure 4B, Supplementary Data Movie 1). These differences in the mechanism of conformational transitions and stabilisation of conserved polar networks are summarised in Extended data figure 9 and may contribute to the different kinetic profiles of G protein activation, as well as the full vs partial agonism for cAMP production.

Collectively, our work provides key advances in understanding class B GPCR activation and G_s protein efficacy, identifying a novel non-peptide binding site within the GLP-1R that can promote distinct efficacy and biased signalling relative to peptide ligands, and this may extend to other class B GPCRs. The demonstration that non-peptide agonists of the GLP-1R are not

required to mimic the extensive receptor contacts formed by peptides within the TM cavity to promote receptor activation will advance the pursuit of non-peptide agonists for therapeutically important class B receptors.

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Supplemental information is linked to this manuscript.

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Author Contributions

P.Z designed and performed the majority of the pharmacological studies with assistance from T.T.T; Y.L.L expressed and purified the complex; R.D performed cryo-sample preparation and imaging to acquire EM data; M.J.B and R.D processed the EM data and performed EM map calculations; M.J.B built the model and performed refinement; M.M.F performed the mutagenesis studies, L.C performed studies in the HEK293 CRISPR KO cells; G.D and C.A.R designed, performed and analysed the MD simulations; F.S.W and M.G.B provided TT-OAD2.

M.E.C, M.G.B and K.W.S designed and oversaw the in vivo studies; P.Z, Y.L.L, M.J.B, G.D, C.A.R, F.S.W, K.W.S, R.D, P.M.S and D.W performed data analysis;, P.Z, Y.L.L, M.J.B, G.D, C.A.R, F.S.W, K.W.S, A.C, L.J.M, M.W.W and R.D assisted with data interpretation, figure and manuscript preparation; P.M.S and D.W designed and supervised the project, interpreted the data and wrote the manuscript.

Data availability statement

All relevant data are available from the authors and/or included in the manuscript or Supplementary Information. Atomic coordinates and the cryo-EM density map have been deposited in the Protein Data Bank (PDB) under accession number 6ORV and EMDB entry ID 20179.

Competing interests

F.W.S., M.E.C., and K.W.S. are employees of Eli Lilly and Company.

Figures

Figure 1. Pharmacology exhibited by TT-OAD2 relative to GLP-1. A, Chemical structure of TT-OAD2. **B**, Plasma insulin induced by GLP-1 (10 μg/kg), TT-OAD2 (3 mg/kg) or GIP (Gastric inhibitory polypeptide, 25 μg/kg) in an acute IVGTT on humanized GLP-1R (hGLP-1R-KI) and GLP-1R knock out (GLP-1R KO) mice. **C,** Whole cell binding assays showing the ability of GLP-1 and TT-OAD2 to displace ^{125}I-exendin(9-39). **D,** cAMP accumulation, intracellular calcium mobilization, ERK1/2 phosphorylation and β-arrestin recruitment. **E,** Agonist induced changes in trimeric G_s conformation in cell plasma membrane preparations for GLP-1 (left) and TT-OAD2 (middle). Rates (top right) and plateau (bottom right) at saturating concentrations (1μM GLP-1, 10μM TT-OAD2) were quantified by applying a one phase association curve. **F,** Kinetics of cAMP production measured by an EPAC biosensor for GLP-1 (left) and TT-OAD2 (middle). Rates were quantified using approximate EC_{50} and Emax concentrations (1nM and 0.1μM for GLP-1, 0.1μM and 10μM for TT-OAD2) by applying a one phase association curve. For E and F, arrows refer to the timepoint ligand or vehicle was added. Parameters derived from kinetic data are represented as scatter plots with each individual experiment shown by black circles. All experiments were performed in GLP-1R expressing HEK293A cells. Panel B is mean + S.E.M from 4-5 animals per treatment, representative of 3 independent experiments. Panels C-F are means + S.E.M of 4-5 independent experiments (in duplicate or triplicate). *Statistically different using a student's paired t-test ($P<0.05$).

Figure 2. TT-OAD2:GLP-1R:Gs Cryo-EM structure reveals non-peptide binding site. Top, Orthogonal views of the TT-OAD2:GLP-1R:Gs complex cryo-EM map (left) and the structure after refinement in the cryo-EM map (right), colour coded to protein chains; GLP-1R (blue), TT-OAD2 (red), heterotrimeric Gs (α:gold, β:dark cyan, γ:purple, Nb35:salmon). **Middle,** TT-OAD2 interacts with the top of the GLP-1R bundle. Interacting residues of GLP-1R (blue) with TT-OAD2 (red). **Bottom,** TT-OAD2-mediated cAMP production on alanine mutants of key residues assessed in ChoFlpIn cells (mean + S.E.M of 4 independent experiments performed in duplicate).

Figure 3. Comparisons of GLP-1R conformations induced by GLP-1 and TT-OAD2. A and B Superimposition of the GLP-1R from PDB 5VAI (GLP-1R/G protein:orange, GLP-1:green) and the TT-OAD2 structure (GLP-1R/G protein:blue, TT-OAD2:red) reveals partial overlap of peptide and TT-OAD2 binding sites and conformational differences in the receptor. **A,** Left; full complex. Middle; close up of ECD and the top of the TM bundle. Right; Close up of the TM bundle **B,** Left; 16Å, 7Å and 6Å differences occur in the location of TM6/ECL3, TM7 and TM1, respectively. Middle; A 4Å shift in the location of the top of TM2 result in distinct conformations of ECL1. Right; the intracellular region of the GLP-1R helical bundles have similar overall backbone conformations. **C,** Comparison of the GLP-1R:TT-OAD2 and GLP-1R:GLP-1 contacts during MD simulations performed on the GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs complexes. Left; top view and Right; side view of the GLP-1R TM domain (ribbon representation – TT-OAD2 in red sticks, GLP-1 not shown). TT-OAD2 made contacts (red coloured ribbon) with ECL1 and residues located at the top of TM2 and TM3. GLP-1 was able to engage TM5, TM6, and TM7 of the receptor and side chains located deep in the bundle (blue coloured ribbon). Residues that are involved both in the GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs complexes are indicated with * and coloured according to the algebraic difference in occupancy (contact differences in % frames) between GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs. Red indicates regions more engaged by TT-OAD2 and blue more engaged by GLP-1). The ECD is not shown. Plotted data are summarised in Extended Data Table 1.

Figure 4. TT-OAD2 interactions lead to reorganisation and stabilisation of the central polar network via a distinct mechanism to GLP-1. Summaries of interactions observed in Supplemental Data Video 2 of MD simulations on TT-OAD2 and GLP-1 bound GLP-1R that predict interactions stabilizing the active conformation of the central polar network. **A.** Left; GLP-1 (brown ribbon) D^{9} (brown stick) forms an ionic interaction (red dotted lines) with $R190^{2.60}$, which is involved in key hydrogen bonds with $N240^{3.43}$ (in turn interacting with $S186^{2.56}$). At the top of TM2, $K197^{2.67}$, $D198^{2.68}$, and $Y145^{1.40}$ are stabilized in polar interactions (red dotted lines). Right; TT-OAD2 (brown stick and transparent surface) forms ionic interaction (red dotted lines) with $K197^{2.67}$ and hydrophobic contacts with $Y145^{1.40}$ and $Y148^{1.43}$ (cyan transparent surfaces) modifying the interaction network at the top of TM1. $Y148^{1.43}$ transiently interacts with $R190^{2.60}$ and partially reorients $N240^{3.43}$ and $S186^{2.56}$. TM6 and TM7 were removed for clarity. **B,** GLP-1R TM sites are occupied by structural water molecules; blue spheres indicate receptor volumes occupied by low-mobility water molecules (occupancy > 75% frames). Left; the GLP-1R:GLP-1:Gs complex stabilises the central TM polar residues through waters interacting with $Y152^{1.47}$, $T391^{7.46}$, $R190^{2.60}$, $E364^{5.53}$ (supplementary data video 1). Right; the GLP-1R:TT-OAD2:Gs complex is characterised by structural water molecules interacting with $N320^{5.50}$ and $E364^{6.53}$ (Supplemental Data Video 1).

Methods

TT-OAD2 synthesis

Several azoanthracene-based derivatives are reported as potent agonists of the glucagon-like peptide-1 receptor (WO10114824), and a compound from this series known as OAD2 was selected for our studies (WO14113357). OAD2, (S)-2-{[(3S,8S)-3-[4-(3,4-dichloro-benzyloxy)-phenyl]-7-((S)-1-phenyl-propyl)-2,3,6,7,8,9-hexahydro-[1,4]dioxino[2,3-g]isoquinoline-8-carbonyl]-amino}-3-[4-(2,3-dimethyl-pyridin-4-yl)-phenyl]-propionic acid, was synthesized using procedures previously described (see example 179 in WO10114824), and a dihydrochloride salt form (OAD2.2HCl) was prepared by standard methods from the free base. Therefore, TT-OAD2 is the dihydrochloride salt of OAD2 in patent WO14113357. The purity of TT-OAD2 was determined by LC/MS to be 98.62%.

Constructs

GLP-1R was modified to contain either a 2xcMyc-N-terminal epitope tag (for signalling and radioligand binding assays) or a Nanoluc tag (with a 12xGly linker – for NanoBRET binding studies) after the native signal peptide. For β-arrestin recruitment assays, a C-terminal Rluc8 was fused to the C-terminus of the receptor. For G protein conformational assays, a Nanoluc flanked by SGGGGS linkers was inserted into Gαs and $G\alpha i_2$ after G(h1ha10) in Gαs or E(HA.03) in $G\alpha i_2$ as described previously[30,31]. These were used in conjunction with an N-terminally Nluc labelled $G\gamma_2$. For G protein steady state assays G protein NanoBit split luciferase constructs were generated by fusing the LgBIT after G(h1ha10) in Gαs or E(HA.29) in $G\alpha i_2$ and the SmBIT to $G\gamma_2$. For structural studies, human GLP-1R in the pFastBac vector was modified to include an N-terminal Flag tag epitope and a C-terminal 8xhistidine tag; both tags are removable by 3C protease cleavage. These modifications did not alter the pharmacology of the receptor[3]. A dominant negative $G_{\alpha s}$ ($DNG_{\alpha s}$) construct was generated previously by site directed mutagenesis to incorporate mutations that alter nucleotide handling, stabilize the G_0 state and interactions with the βγ subunits[30].

Insect cell expression. GLP1R, human $DNG_{\alpha s}$, His6-tagged human $G_{\beta 1}$and $G_{\gamma 2}$were expressed in *Tni* insect cells (Expression systems) using baculovirus. Cell cultures were grown in ESF 921 serum-free media (Expression Systems) to a density of 4 million cells per ml and then infected with three separate baculoviruses at a ratio of 2:2:1 for GLP1R, $DNG_{\alpha s}$ and $G_{\beta 1\gamma 2}$.

Culture was harvested by centrifugation 60 h post infection and cell pellet was stored at -80 °C.

TT-OAD2:GLP-1R:Gs Complex purification. Cell pellet was thawed in 20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$ supplemented with cOmplete Protease Inhibitor Cocktail tablets (Roche). Complex formation was initiated by addition of 50 μM TT-OAD2, Nb35–His (10 μg/mL) and Apyrase (25 mU/mL, NEB) to catalyse hydrolysis of unbound GDP and allow for stabilisation of the G_0 state; the suspension was incubated for 1 h at room temperature. Membrane was solubilized by 0.5% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) supplemented with 0.3% (w/v) cholesteryl hemisuccinate (CHS, Anatrace) for 2 h at 4 °C. Insoluble material was removed by centrifugation at 30, 000g for 30 min and the solubilized complex was immobilized by batch binding to M1 anti-Flag affinity resin in the presence of 3 mM $CaCl_2$. The resin was packed into a glass column and washed with 20 column volumes of 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 1 μM OAD, 0.01% (w/v) MNG and 0.006% (w/v) CHS before bound material was eluted in buffer containing 5 mM EGTA and 0.1 mg/mL Flag peptide. The complex was then concentrated using an Amicon Ultra Centrifugal Filter (MWCO 100 kDa) and subjected to size-exclusion chromatography on a Superdex 200 Increase 10/300 column (GE Healthcare) that was pre-equilibrated with 20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1 μM OAD, 0.01% (w/v) MNG and 0.006% (w/v) CHS to separate complex from contaminants. Eluted fractions consisting of receptor and G-protein complex were pooled and concentrated. Final yield of purified complex was approximately 0.15 mg per liter of insect cell culture.

Electron microscopy

Samples (3 μL) were applied to a glow-discharged Quantifoil R1.2/1.3 CuRh 200 mesh holey carbon grid (Quantifoil GmbH, Großlöbichau, Germany) and were flash frozen in liquid ethane using the Vitrobot mark IV (Thermo Fisher Scientific, Waltham, Massachusetts, USA) set at 100% humidity and 4°C for the prep chamber. Data were collected on a Titan Krios microscope (Thermo Fisher Scientific) operated at an accelerating voltage of 300 kV with a 50 μm C2 aperture at an indicated magnification of 105K in nanoprobe EFTEM mode. Gatan K3 direct electron detector positioned post a Gatan Quantum energy filter (Gatan, Pleasanton, California, USA), operated in a zero-energy-loss mode with a slit width of 25 eV was used to acquire dose fractionated images of the GLP1R TT-OAD2 bound sample without an objective aperture. Movies were recorded in hardware-binned mode (previously called counted mode on the K2

camera) yielding a physical pixel size of 0.826 Å/pix with an exposure time of 3.715 seconds amounting to a total dose of 65.6 e-/Å^2 at a dose rate of 12.2 e-/pixel/second which was fractionated into 62 subframes. A second dataset of 1568 micrographs was also recorded using the same microscope but in "Super-resolution" mode on the K3 detector, the physical pixel size was 0.413 Å with an exposure time of 4.015 sec amounting to a total dose of 63.5 e-/Å^2 which was fractionated into 67 subframes. Defocus range was set between -0.7 to -1.5 μm. A total of 3,158 + 1,568 movies were collected in two data collection sessions.

Electron microscopy data processing

Movies were motion-corrected with UCSF MotionCor2[32] (movies collected in super-resolution mode were Fourier scaled by a factor of ×2 to match the pixel size of the larger dataset). This was followed by CTF estimation sing the GCTF software packag[33]. Particles were picked from the micrographs using the automated reference-free procedure in RELION[34,35]. Reference free 2D and 3D classification (by generating multiple *ab initio* models with no structural identity enforced) was carried out in CryoSPARC (version 2.5.0[36]. A homogeneous subset of particles was then subjected to movie refinement and Bayesian particle polishing as implemented in RELION (version 3.0). This homogeneous subset of polished particles was used in a 3D refinement in RELION and then further classified into 3D classes with alignment of Euler angles not taken into account. Particles belonging to the 3D class which yielded the best resolved map were then subjected to signal subtraction to subtract density due to the detergent micelle and the alpha domain of the G-protein. Final 3D refinement was performed in RELION (3.0) yielded a map of resolutions 3.01 Å. Local resolution estimations were performed using the ResMAP software packag[37].

Atomic model refinement

Fitting the model to the cryoEM electron density map was achieved using the MDFF routine in namd[38]. The fitted model was further refined by rounds of manual model building in coot[39] and real space refinement as implemented in the Phenix software package[40], the model restraints for the TT ligand were prepared by using the coordinates generated from Chem3D™ and the ELBOW software package[41]. The ligands were fitted after the first round of real-space refinements, manually first in coot[39], then refined using Phenix real-space refinement[42]. Ramachandran, rotamer and secondary structure restraints were applied for the first round of real-space refinement, and after manual inspection and adjustment of the model in coot further

real-space refinements were carried out with only Ramachandran and rotamer restraints applied and the model/data weight was allowed to freely refine. The density around the extracellular domain was poorly resolved (local resolution estimated at > 8 Å) and was not modelled.

Modelling Methods for MD preparation

The two missing receptor loops, namely the stalk region and ICL3 were generated using PLOP[43]; ICL3 was additionally minimized in the presence of Gα to eliminate steric clashes. Based on an analysis of the electron density of our structures, TM1 for the GLP-1 bound 5VAI structure[6] was replaced by TM1 from the P5-bound structure (PDB code 6B3J[3]) by the method of molecular superposition. The missing residues in the stalk region were reconstructed using Modeller[44] subject to the constraint that the high variability positions[45] in the GLP-1R multiple sequence alignment (E133-R134) faced outwards. The missing loops in the G protein were generated by molecular superposition, using VMD[46], of the corresponding loops in the β_2-adrenergic receptor:G protein complex[47], PDB code 3SN6 to the flank either side of the gap, since this particular x-ray structure (with 99% identity to the G protein used in this study) generally gave a lower RMSD on molecular superposition than plausible alternative G protein structures (eg. PDB 5VAI). The joining point was taken as the closest atom pairs (usually separated by ~0.2 Å) that maintained an appropriate Cα- Cα distance (3.7 – 3.9 Å) across the join; selected residues spanning the join were minimized using PLOP where additional refinement was deemed necessary. The exception to this was the loop between A249-N264, which was completed using the shorter loop from the adenosine A_{2A} receptor: G protein complex, PDB code 5G53[48]. The helical domain, between residues G47 and G207, which is not visible in the cryo-EM structure, was omitted as in earlier work.

MD methods

Four GLP-1R complexes (GLP-1R:TT-OAD2:Gs, GLP-1R:TT-OAD2, GLP-1R:GLP-1:Gs, and GLP-1R:GLP-1 - Table Methods 1) and two apo GLP-1R structures (obtained by removing both the Gs protein and the ligands - Table Methods 1) were prepared for simulation with the CHARMM36 force field[49], through use of in-house python htmd[50] and TCL (Tool Command Language) scripts. The pdb2pqr[51] and propka[52] software were used to add hydrogen atoms appropriate for a pH of 7.0; the protonation of titratable side chains was checked by visual inspection. The coordinates were superimposed on the corresponding GLP-1R coordinates from the OPM database[53] so as to orient the receptor prior to insertion[54] in a rectangular pre-built 125 Å x 116 Å 1-palmitoyl-2-oleyl-sn-glycerol-3-phosphocholine (POPC) bilayer; lipid

molecules overlapping the receptor were removed. TIP3P water molecules were added to the 125 Å x 116 Å x 195 Å simulation box using the VMD Solvate plugin 1.5 (Solvate Plugin, Version 1.5. at <http://www.ks.uiuc.edu/Research/vmd/plugins/solvate/). Overall charge neutrality was maintained by adding Na^+ and Cl^- counter ions to a final ionic concentration of 150 mM using the VMD Autoionize plugin 1.3 (Autoionize Plugin, Version 1.3. at <http://www.ks.uiuc.edu/Research/vmd/plugins/autoionize/). CGenFF force field parameters[55-57] and topology files for TT-OAD2 were retrieved from the Paramch[56] webserver. No further optimization was performed because the obtained parameters were associated to low penalty scores.

Systems equilibration and MD settings

ACEMD[58] was used for both equilibration and MD productive simulations. Isothermal-isobaric conditions (Langevin thermostat[59] with a target temperature of 300 K and damping of 1 ps^{-1} and Berendsen barostat[60] with a target pressure 1 atm) were employed to equilibrates the systems through a multi-stage procedure (integration time step of 2 fs). Initial steric clashes between lipid atoms were reduced through 3000 conjugate-gradient minimization steps, then a 2 ns MD simulation was run with a positional constraint of 1 kcal mol^{-1} $Å^{-2}$ on protein atoms and lipid phosphorus atoms. Subsequently, 20 ns of MD were performed constraining only the protein atoms. In the final equilibration stage, protein backbone alpha carbons constraints were applied for a further 60 ns.

Productive trajectories in the canonical ensemble (NVT) at 300 K (four 500 ns-long replicas for each GLP-1R complex - Table Methods 1) were computed using a thermostat damping of 0.1 ps^{-1} with an integration time step of 4 fs and the M-SHAKE algorithm[61] to constrain the bond lengths involving hydrogen atoms. The cut-off distance for electrostatic interactions was set at 9 Å, with a switching function applied beyond 7.5 Å. Long range Coulomb interactions were handled using the particle mesh Ewald summation method (PME)[62] by setting the mesh spacing to 1.0 Å. Trajectory frames were written every 100 ps of simulations.

MD Analysis

The first half (500 ns) of the MD replicas involving GLP-1R:TT-OAD2, GLP-1R:GLP1 complexes as well as the apo-GLP-1R (TT-OAD2), and apo-GLP-1R (GLP-1) systems (Table Methods 1) were considered as part of the equilibration stage and therefore not considered for analysis. Atomic contacts (atom distance < 3.5 Å) were computed using VMD[46]. Hydrogen

bonds were identified using the GetContacts analysis tool (at https://getcontacts.github.io/), with the donor-acceptor distance set to 3.3 Å and the angle set to 150°. Videos were generated using VMD[46] and avconv (at https://libav.org/avconv.html). Root mean square fluctuation (RMSF) values were computed using VM[46] after superposition of the MD trajectories frames on the alpha carbon of the TM domain (residues E138$^{1.33}$ to V404$^{7.60}$). The orientation of the N terminal helix of the extracellular domain (ECD) of GLP-1R was drawn in VMD considering a representative frame every 10 ns. In order to detect volumes within the TM domain of GLP-1R occupied by water molecules with low mobility (structural water molecules), the AquaMMapS[63] analysis was performed on 10 ns-long MD simulations of the GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs complexes (coordinates were written every 10 ps of simulation); all the alpha carbons were restrained in analogy with the approach proposed by Wall M.E *et al.*[64].

Whole cell radioligand binding assays

HEK293 cells were seeded at 30 000 cells per well in 96-well culture plates and incubated overnight in DMEM containing 5 % FBS at 37 °C, 5 % CO_2. Media was replaced with HBSS containing 25 mM HEPES and 0.1 % (w/v) BSA with 0.1 nM [^{125}I]-exendin(9–39) and increasing concentrations of unlabeled agonist. Cells were incubated overnight at 4 °C, washed three times in ice cold buffer and then solubilized in 0.1 M NaOH. Radioactivity was determined by gamma counting. Non-specific activity was defined using 1 μM exendin(9–39).

cAMP accumulation assays

Cells were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated over-night in DMEM containing 5% FBS at 37°C in 5% CO_2. cAMP detection was performed as previously described in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthin[65]. All values were converted to cAMP concentration using a cAMP standard curve performed in parallel and data was subsequently normalised to the response of 100 μM forskolin in each cell line. In one series of experiments, vehicle or increasing concentrations of TT-OAD2 was added 30 minutes prior to assay of peptide response.

cAMP kinetics studies

HEK293A cells were transfected with an Epac-cAMP sensor (CAMYEL) and hGLP-1R at optimized ratio. Ligand-mediated cAMP production was measured 48 hours post transfection.

Briefly, culture media was replaced with assay buffer (1XHBSS, 10mM Hepes, 0.1% BSA, pH 7.4). BRET signals were measured at 1 minute intervals using a PHERAstar plate reader (BMG LabTech) in the absent or present of increasing concentration of ligands. Forskolin (100 μM) was used as a positive control, and data were normalised to the Forskolin response.

β-arrestin recruitment assays

Cells were transiently transfected with GLP-1R-Rluc8 and β-arrestin1-Venus at a 1:4 ratio and seeded at a density of 30,000 cells/well into 96-well culture plates and incubated for 48 hours in DMEM containing 5 % FBS at 37°C in 5% CO_2. β-arrestin recruitment was performed as previously describe[66]. In one series of experiments, vehicle or increasing concentrations of TT-OAD2 was added 30 minutes prior to assay of peptide response.

ERK1/2 phosphorylation assays

HEK293 cells expressing stably expressing the GLP-1R were seeded at a density of 30, 000 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2. Receptor-mediated pERK1/2 was determined using the AlphaScreen ERK1/2 SureFire protocol as previously described[14]. Data were normalized to the maximal response elicited by 10% FBS determined at 6 min. In one series of experiments, vehicle or increasing concentrations of TT-OAD2 was added 30 minutes prior to assay of peptide response.

Ca^{2+} mobilisation assays

HEK293 cells stably expressing the GLP-1R were seeded at a density of 30,000 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and receptor- mediated intracellular calcium mobilisation determined as previously described[65]. Fluorescence was determined immediately after ligand addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings taken every 1.36 s for 120 s. The peak value was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP. In one series of experiments, vehicle or increasing concentrations of TT-OAD2 was added 30 minutes prior to assay of peptide response.

Generation of stable cell lines containing WT and mutant GLP-1Rs.

Mutant receptors were generated in a 2xc-Myc epitope-tagged receptor using QuikChange site-directed mutagenesis (Invitrogen) and sequences confirmed. WT and mutant receptors were

stably expressed in CHOFlpIn cells using the FlpIn Gateway technology system and selected using 600 μg/mL hygromyocin B.

NanoBRET ligand binding

HEK293A cells were transiently transfected with Nluc-hGLP-1R. 48 hours post transfection, cells were harvested and plasma membrane was extracted as described previously[31]. 1 □g per well of cell membrane was incubated with furimazine (1:1,000 dilution from stock) in assay buffer (1× HBSS, 10 mM HEPES, 0.1% (w/v) BSA, 1× P8340 protease inhibitor cocktail, 1 mM DTT and 0.1 mM PMSF, pH 7.4). RhodamineX-Ex4 (Rox-Ex4) was used as fluorescent ligand in the NanoBRET binding assay. BRET signal between Nluc-hGLP-1R and Rox-Ex4 was measured using PHERAstar (BMG LabTech) at 10 seconds interval (25 °C), a 2 minutes baseline was taken before addition of Rox-Ex4 (Kd concentration, 3.16nM, determined previously), the measurement continued for 15 minutes followed by adding increasing concentration of TT-OAD2, or unlabelled Ex4 as a control. Data were corrected for baseline and vehicle treated samples.

G protein Conformation assays

HEK293AΔS/Q/12/13 cells stably expressing the GLP-1R (tested and confirmed to be free from mycoplasma) were transfected with a 1:1:1 ratio of nanoluc–$G\alpha_s$[72]: $G\beta_1$: venus-$G\gamma_2$ 24 h before collection and preparation of cell plasma membranes. 5 □g per well of cell membrane was incubated with furimazine (1:1,000 dilution from stock) in assay buffer (1× HBSS, 10 mM HEPES, 0.1% (w/v) BSA, 1× P8340 protease inhibitor cocktail, 1 mM DTT and 0.1 mM PMSF, pH 7.4). The GLP-1R-induced BRET signal between $G\alpha_s$ and Gγ was measured at 30 °C using a PHERAstar (BMG LabTech). Baseline BRET measurements were taken for 2 min before addition of vehicle or ligand. BRET was measured at 15 s intervals for a further 7 min. All assays were performed in a final volume of 100 μl.

G protein NanoBIT assays

HEK293A WT cells stably express hGLP-1R were transiently transfected with Gα-LgBIT, Gβ1, Gγ2-SmBIT (1:5:5) 48 hours before the assays. Cells were then incubated with coelenterazine H (5 μM) for 1 hour at room temperature. Luminescence signals were measured using a Clariostar plate reader (BMG LabTech) at 30 second intervals before and after ligand addition (25 °C). Data were corrected to baseline and vehicle treated samples.

In vivo IVGTT assays

Intravenous glucose tolerance tests were performed in male human GLP1R knock-in (hGLP1R KI) and Glp1r knock-out (Glp1r KO) mice (all on C57/Bl6 background[67]). Catheters were placed in the right carotid artery and left jugular vein of animals 6-11 months of age. Approximately one week later, mice (n=4-5/group) were fasted overnight and the catheters were exteriorized as animals acclimated to test cages. Vehicle (5% DMSO, 20% captisol in NaHPO4, pH2, 1ml/kg), GLP-1(7-36)NH2 at 10 □g/kg, GIP(1-42) at 25 □g/kg, or OAD2 at 3 mg/kg was administered intravenously one minute prior to glucose load (0.5 g/kg). Blood samples were collected at -10, 0, 2, 4, 6, 10, 20, and 30 minutes to determine blood glucose concentrations via glucometer (Roche, Aviva) and plasma insulin measurement (Alpco, Cat # 80-INSMSU-E10). All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Eli Lilly and Company and the NIH Guide for the Use and Care of Laboratory Animals.

Data analysis

Pharmacological data were analysed using Prism 7 (GraphPad). Concentration response signalling data were analysed using a three-parameter logistic equation, or via operational analysis. Changes in the rate of change in BRET kinetic data were fitted to one-phase association curve. Statistical analysis was performed with either one-way analysis of variance and a Dunnetts post-test or a paired *t*-test, and significance accepted at $P < 0.05$.

Graphics

Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081).

Methods Only References

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Extended Data

Extended Data Figure 1. **Binding, transducer coupling and signalling mediated by TT-OAD2. A,** Kinetic ligand binding assay using ROX-Exendin-4 as the fluorescent probe. TT-OAD2 is only able to partially displace the probe and with slower kinetics relative to exendin-4 that shows complete displacement of the probe with rapid kinetics. **B,** cAMP accumulation studies using GLP-1 and TT-OAD2 as the agonist in WT HEK293 cells and HEK293 cells where $G_{s/olf}$ (ΔG_s) or all $G_{i/o/z}$ ($\Delta G_{i/o/z}$) have been depleted using CRISPR/Cas9. **C,** HEK293A cells transiently transfected with the GLP-1R and the NanoBit constructs for $G\alpha_s$ and $G\alpha i_2$ ($G\alpha$-Lgbit, $G\gamma_2$-Smbit). Luminescence signal was assessed over time (0-20 min) in the presence of increasing concentrations of GLP-1 and TT-OAD2. Concentration response curves are expressed as AUC (0-20min) for each concentration and normalised to the negative response observed by GLP-1 at 1μM. **D,** Agonist induced changes in trimeric G_s protein conformation. Ligand induced changes in BRET were measured in plasma membrane preparations performed in kinetic mode until kinetic equilibrium was reached for vehicle or increasing concentrations of GLP-1 (left) and TT-OAD2 (right). Addition of GTP dissociated the trimeric G protein complex stabilised by GLP-1 occupied and TT-OAD2 occupied GLP-1R. **E**, Agonist induced changes in trimeric G_{i2} protein conformation. Ligand induced changes in BRET were measured in plasma membrane preparations performed in kinetic mode until kinetic equilibrium with a saturating concentration of GLP-1 and TT-OAD2 (left). The BRET signal decreased in the presence of GTP, suggesting GTP dissociated the G_{i2} protein complex stabilised by GLP-1 occupied and TT-OAD2 occupied GLP-1R. Quantification of the plateau (middle) and the rate of ligand induced conformational change (right) for each agonist (1μM GLP-1 and 10μM TT-OAD2) was calculated by applying a one phase association curve to the kinetic data with values from each individual experiment show in black circles. **F,** Concentration response curves of production in live HEK293 cells expressing the GLP-1R and an EPAC BRET biosensor in the presence of different concentrations of GLP-1 and TT-OAD2; left, cAMP response taken 25 minutes post ligand addition, and right, AUC of the response calculated as area under the curve across the full kinetic trace for each ligand concentration (from data in Figure 2D). All data are means + S.E.M of four to six independent experiments performed in duplicate or triplicate.

Extended Data Figure 2. **Purification, cryo-EM data imaging and processing of the TT-OAD2:GLP-1R:Gs complex. A,** Representative elution profile of FLAG purified complex on Superdex 200 Increase 10/30 SEC. **B,** Representative micrograph of the TT-OAD2:GLP-1R:Gs complex. Red circles are highlighting examples of individual particles **C**, Two-dimensional class averages of the complex in MNG micelle. **D,** Cryo-EM data processing workflow. **E,** "Gold standard" Fourier shell correlation (FSC) curves, showing the overall nominal resolution at 3.0Å. **F.** 3-D histogram representation of the Euler angle distribution of all the particles used for the in the reconstruction overlaid on the density map drawn on the same coordinate axis (map is colored according to local resolution the same as **G**). **G,** Cryo-EM density map coloured according to resolution. Left, map with the GLP-1R ECD masked; right, map including the extracellular ECD domain of the GLP-1R.

Extended Data Figure 3. **The atomic resolution model of the TT-OAD2:GLP-1R:Gαs heterotrimer in the Cryo-EM density map.** EM density map and the model are shown for all seven TM helices and H8 of the receptor, the α5 helix of the $G\alpha_S$ Ras-like domain and TT-OAD2. All TMs exhibit good density, with TM6 that displays flexibility being the least well resolved region.

Extended Data Figure 4. Cryo-EM density supports ligand interactions in the TT-OAD2 binding site. A, Interacting residues predicted by LigPlot using the full-length model with ECD. **B,** The pose of TT-OAD2 and interactions with residues within TM1, TM2, TM3, ECL1 and ECL2 are supported by well-resolved density in the cryo-EM map. **C,** Density for the ECD was visible in the cryo-EM and supports extended interactions of the ECD with ECL1 and ECL2, as well as with the ligand TT-OAD2.

Extended Data Figure 5. **Comparison of the TT-OAD2:GLP-1R:Gs complex with peptide agonist bound GLP-1R structures and the inactive class B GPCR GCGR TMs. A,** Structures of agonist bound GLP-1R; from left to right: GLP-1R (orange) bound to GLP-1 peptide (green) in an active conformation, GLP-1R (pink) bound to ExP5 peptide (cyan) in an active conformation, GLP-1R (blue) bound to non-peptide TT-OAD2 (red) in an active conformation, GLP-1R (pale green) bound to 11mer peptide HepP5 (purple) in a partially active conformation. Far right: Overlay of GLP-1R agonist bound structures highlighting variations within the ECD position in the different structures. Inset, Differences in the location

of the ECD are supported by density in the cryo-EM maps; shown are the GLP-1-bound (orange) and TT-OAD2 bound (blue) GLP-1R. **B-C:** Various overlays of these structures (using the same colours) to compare conformational differences between the different structures. **B,** overlay of TT-OAD2 bound:GLP-1R Gs structure with the full-length peptide bound Gs structures and the inactive GCGR (grey) bundle reveals common conformational transitions occur in all agonist bound structures relative to the inactive GCGR, but the extent of these movements differ. A more open helical bundle is observed for the TT-OAD2 bound GLP-1R than either GLP-1 or ExP5 bound due to a distinction in the conformations of TM1, TM6, TM7 and ECL3 at the extracellular side of the receptor induced by the binding of the different ligands (left and middle). Middle; Differences in the conformation of TM2 between the inactive and peptide-agonist bound structures is also evident. Right; At the intracellular face all active structures display a similar large outward movement of TM6 and a smaller movement within TM5. **C;** Comparison of TT-OAD2 bound GLP-1R with the small peptide HepP5 bound GLP-1R structure. Left; TT-OAD2 and Hep-P5 occupy a partially overlapping binding site but promote distinct conformations of the ECD and TM bundle of the GLP-1R, Middle; HepP5 engages deeper in the helical bundle than TT-OAD2 and promotes a more closed helical bundle due to differences induced in the conformation of TM1, TM6, TM7 and ECL3. Right; Overlay of the TT-OAD2, Hep-P5 and GLP-1 bound GLP-1R TM bundles reveals HepP5 induces a similar conformation of the helical bundle to GLP-1 while TT-OAD2 induces a distinct conformation.

Extended Data Figure 6. Pharmacological responses exhibited by endogenous ligands GLP-1 and oxyntomodulin in the presence o TT-OAD2. Signalling profiles of GLP-1 and oxyntomodulin, following 30 minute preincubation of vehicle (0) or increasing concentrations of TT-OAD2. All data were performed in HEK293A cells stably expressing the GLP-1R and data are means + S.E.M of three to four independent experiments performed in duplicate.

Extended Data Figure 7. GLP-1R domains are stabilized by either ligand contacts or lipid interactions. A, Top; RMSF values ofalpha carbons computed during MD simulations of the GLP-1R:GLP-1:Gs complex (black line) and the GLP-1R:TT-OAD2:Gs complex (red line); TM helices, intracellular loops (ICLs), and extracellular loops (ECLs) positions are indicated. Bottom left; RMSF values plotted on the GLP-1R structure bound to GLP-1 (transparent ribbon); Bottom right; RMSF values plotted on the GLP-1R structure bound to TT-OAD2 (transparent stick representation). ECL1 and ECL3 were more dynamic in the GLP-1 bound

receptor than the TT-OAD2-bound structure. In contrast, ECL2 and the top end of TM5 were more mobile in the GLP-1R:TT-OAD2:Gs complex. **B,** GLP-1R contacts formed with membrane lipids during MD simulations of the GLP-1R:TT-OAD2:Gs and the GLP-1R:GLP-1:Gs systems. Two sides views of the receptor are shown (ribbon and transparent surface). When bound to TT-OAD2, ECL1, TM3, the distal end of TM6, and ECL3 are more in contact with the membrane lipids (magenta). TM1 and TM7, on the other hand, are more prone to interact with the membrane when GLP-1 is bound (green). The outward movement of ECL3 in the GLP-1R:TT-OAD2:Gs complex (stabilised by a hydrogen bond network different than GLP-1R:GLP-1:Gs -Extended Data Table 2) produces more interactions with the lipids, possibly further stabilizing the open conformation of TM6/ECL3/TM7.

Extended Data Figure 8. Dynamics of the extracellular domain (ECD) of GLP-1R. **A,** The vector (shown here as a green arrow) connecting $S49^{ECD}$ and $E34^{ECD}$ alpha carbons (ECD N-terminal helix) are shown in the box. **B,** Left; ECD N-terminal helix orientations observed during the MD simulation of the GLP-1R:GLP-1:Gs (black arrows), the GLP-1R:GLP-1 complex (obtained by removing G protein - blue arrows), and the apo-GLP-1R (obtained by removing both the Gs protein and GLP-1 - cyan arrows) are shown on the left viewed from the top and side of the bundle. The receptor is shown as a dark grey ribbon. During MD with GLP-1 bound, the N-terminal helix was oriented vertically (black and blue arrows), while in the apo-form the ECD N-terminal helix was more dynamic and experienced both open and closed conformations (this latter is analogous to the suggested ECD dynamics for the glucagon receptor). Right; The ECD N-terminal helix orientations of the GLP-1R:TT-OAD2:Gs (red arrows), the GLP-1R:TT-OAD2 complex (obtained by removing G protein orange arrows), and the apo-GLP-1R (obtained by removing both the Gs protein and TT-OAD2 - yellow arrows). The receptor is shown as a red ribbon. The distal end ($S49^{ECD}$) of the helix was more mobile than the proximal one ($E34^{ECD}$), which had an overall tendency to remain in the proximity of the TT-OAD2 binding site, driven by transient interactions with the ligand (Extended Data Table 1) and hydrogen bond with $R299^{ECL2}$ side chain (Extended Data Table 2). MD simulations are therefore suggesting a different behaviour for residue $R299^{ECL2}$, stably involved in interactions with the peptide in the GLP1-R:GLP-1:Gs complex (Extended Data Table 1), and instead interacting with $E34^{ECD}$ and other residues located at the ECL2 ($E294^{ECD}$, $D293^{ECD}$ and $N300^{ECD}$) in the GLP1-R:TT-OAD2 Gs complex (Extended Data Table 2).

Extended Data Figure 9. Proposed activation mechanism of class B GPCRs. In the inactive conformation the top of the TM domai is stabilised by interactions of the ECD with the TM6-ECL3-TM7 region (left). **Top**; Activation of class B GPCRs by peptides occurs via a 2 domain mechanism. Engagement of the peptide with the receptor ECD releases ECD constraints on the TM domain promoting outward movements of TMs 1, 6 and 7 by peptide (top left). Interaction of the peptide N-terminus in the bundle within TMs 1,2,3,5,6 and 7 promotes TM1, 6 and 7 to close in around the peptide (middle). Direct engagement of peptides with the central polar network facilitates conformational transitions required for G protein coupling and activation The active conformation of the central polar network is stabilised by a series of structural waters (top right). **Bottom**; Interaction of the non-peptide TT-OAD2 at the top of the GLP-1R TM bundle releases ECD constraints on the TM bundle resulting in movements of TM1,6 and 7 outwards. TT-OAD2 does not engage TMs 5-7 and the bundle remains open. TT-OAD allosterically promotes conformational rearrangement of the central polar network to stabilise the fully active receptor conformation that allows coupling to G protein. The central polar network is stabilised by a distinct network of structural waters relative to peptide-mediated activation (bottom right).

Extended Data Table 1. GLP-1R – TT-OAD2 and GLP-1R - GLP-1 contacts during MD simulations performed on the GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs complexes. Contacts involving the GLP-1R transmembrane (TM) domain are determined as the sum of the occupancy (reported as % of frames) of all the contacts involving each residue. Values higher than 100% indicate residues able to interact with more than one peptide side chain. A contact was considered productive if the distance between the residue and the ligand was less than 3.5 Å. Data are summarised in Figure 4C**.** TT-OAD2 mainly interacted with TM2, ECL1, and TM3. Interactions with TM1 and ECL2 were present but not persistent (with the exception of $W297^{ECL2}$). The N-terminal helix of the extracellular domain (ECD) was engaged in (many) transient interactions. GLP-1, overall, interacted with a different set of residues and was able to further involve TM5, TM6, and TM7. TT-OAD2 and GLP-1 common contact residues (indicated with *) were located at TM1, TM2, and ECL2. Ligand contacts formed via interaction with the receptor backbone rather than a side chain interaction are indicated by $^{\#}$.

Extended Data Table 2. Main GLP-1R - GLP-1R intramolecular hydrogen bonds during MD simulation. Data expressed as the occupancy (% of frames) in which the interactions were present in the GLP-1R:TT-OAD2:Gs and GLP-1R:GLP-1:Gs complexes. Differences between GLP-1R:GLP-1:Gs and GLP-1R:TT-OAD2:Gs are reported in the right column; green indicates more contacts in GLP-1 vs TT-OAD2 and red more contacts in TT-OAD2 vs GLP-1. Grey cells indicate that hydrogen bonds were not present.