

Peer Review File

Manuscript Title: Structural basis of odorant recognition by a human odorant receptor

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

To date, no tridimensional structure of a mammalian odorant receptor has been reported. In this work, the authors solved the first structure of a human odorant receptor, OR51E2, at a high resolution (3.1Å). OR51E2 belongs to the Class I group of odorant receptors, which show some specific sequence motifs when compared to the more numerous Class II receptors, and are also more tuned to a specific group of ligands, the aliphatic odorants. The results raise important insights into the structural mechanisms of an odorant receptor activation by its ligand and should be of broad interest to the scientific community.

In order to trap the receptor in the G protein interacting conformation, the authors generated a construct containing OR51E2 fused at its C-terminal region to the mini Gs protein. A complex containing OR51E2-mini Gs bound to its ligand propionate, the nanobody Nb35 (to stabilize the GPCR/G protein complex) and the Gb1g2 subunit was purified and analyzed using cryo-EM.

The structure shows propionate bound to a small pocket located deep in the receptor and the residues that are required for the interaction. Functional cell assays with mutant forms of OR51E2 were also performed, to verify whether these residues are indeed involved in the receptor activation by the ligand. These experiments also showed that the size of the pocket determines the specificity of the receptor towards fatty acids that have short chain lengths.

Due to the strategy used, the authors were unable to determine the structure of the inactive receptor (without ligand). Instead, they modeled an inactive form. Based on the comparison of the active and inactive forms, a mechanism of activation was proposed.

These structures were also compared to those of B2-AR2, one Class A GPCR with a similar ligand binding pocket. The comparison is interesting, and reveals both, the similarities, and differences between the modes of activation of the two receptors.

The article is well written and organized, I only have the following comments:

The term 'olfactory receptor' is sometimes used as a general term to refer to all possible types of olfactory receptors, including for example TAARs and pheromone receptors. To avoid confusion, I recommend that the authors use the term 'odorant receptors' to refer to the canonical receptors.

Extended data figure 2 c- why are there two bands for OR51E2-miniGs?

Line 216- 'By contrast, the highly conserved CWxP6.50 motif in the connector region of Class A GPCRs is absent in ORs. Instead ORs contain the previously described FYGx6.50 motif in the connector region'. This is an interesting finding. What does this difference in conserved residues represent to the workings of the connector region? In other words, is connection between the binding pocket to the G protein interaction helix particularly different in ORs when compared to non-olfactory Class A GPCRs?

Line 543- Please provide the sequence used of OR51E2 (accession number).

Why was Gg2 used in the G protein complex instead of Gg13, which is the Gg subunit abundantly expressed in the mature olfactory neurons?

Line 155- and figure 3. Propionate and other short fatty acids show millimolar potency in the functional assays. This range of odorant concentrations represents low affinities, even for odorant receptors. Does OR51E2 respond to additional ligands with higher affinity? Is there some explanation for this?

What do exactly frequencies numbers represent in extended figure 6? Percentage of receptors? Please include in the figure legend.

Figure 4A- please depict the relevant TMs

Figure 5g- would help to indicate also other relevant TMs. FYG interactions are not clearly depicted, what are the TMs involved?

Other ligands have been described for OR51E2, for example, why does b-ionone work as agonist while a-ionone works as an antagonist? Could this be explained by the solved structure?

How would other ligands that are very different and larger, such as 19-hydroxyandrostenedione activate the receptor?

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5985834/>

Referee #2 (Remarks to the Author):

The molecular basis of odor recognition in mammals remains elusive since the discovery of the olfactory receptor (OR) gene family by Buck and Axel in 1991. Seminal papers by Ruta and colleagues recently revealed the structure of insect odorant receptors and the mechanisms of odor recognition, however these principles cannot be extrapolated to the mammalian ORs, due to fundamental structural and functional differences between insect and mammalian odorant receptors. Thus, the elucidation of the human OR51E2 structure with its cognate ligand, constitutes a breakthrough. To overcome a major technical hurdle in the structural analysis of mammalian olfactory receptors, which is the poor expression properties of OR proteins in heterologous cell types, the authors used an OR that is known to be expressed outside the olfactory system. Further, this is a type I OR protein, which was recently shown to elicit lower levels of ER stress, likely facilitating expression in heterologous cells (the authors should mention this). Overcoming the bottleneck of expression levels, the authors manage to obtain cryoEM structures of OR51E2 with and without the carboxylic acid propionate and in complex with Gα proteins. Among the interesting findings of this work:

1. The binding pocket of the OR protein is very shallow, much shallower than the beta-2 AR which make account for the low affinity for odorants (especially in comparison to TAARs).
2. The binding pocket depth may account for selectivity for propionate vs longer carboxylic acids
3. Propionate makes selective contacts with residues in TM2, TM5 (van der Waals) and TM6 and ECL2 (hydrogen bonds).
4. Significant differences in the mechanisms of ligand induced Gs activation are observed between this OR and beta2-AR.

Overall, the study is carefully executed, and structural observations are complemented with mutations that confirm predictions from the structure. The article is well written and of general

interest. Congratulations to the authors for this seminal contribution to the field. This article should be accepted.

Referee #3 (Remarks to the Author):

Billesbølle et al provide structural insights to a new class of olfactory receptors. The authors have selected OR51E2 for structural studies using cryo-EM, as well as utilised molecular dynamics simulations and pharmacology assays of wild type and mutants to understand ligand activation of this receptor. To gain stable constructs for structure determination, the authors fused mini-Gas to the receptor, which impaired cAMP signalling. However, a large body of work has been done to complement the insights gained through the structural data by additional functional pharmacology data as well as molecular dynamics simulations.

CryoEM structures made it feasible to gain insights into ligand binding (interaction with propionate) and inspired to conduct experiments in regard to specificity of the receptor for activation by longer chain ligands. For this, authors used mutant receptors in cAMP assays as well as sequence conservation studies that give insights into ligand recognition by other receptors of this class.

The activation process of OR51E2 was investigated using mutant receptors in cAMP, and molecular dynamics with and without propionate ligand present (in the absence of ligand-free or G protein-free experimental structures), suggesting a general similar mechanism compared to ClassA GPCRs, with differences in the dynamics of the OR-specific FYGx motif.

In addition to aforementioned motifs, ECL3 has a particular role with low conservation amongst other ORs (determined by a low AlphaFold confidence value), and ligand binding possibly restricts flexibility of the ECL3. A dynamic, non-conserved ECL3 is actually a common phenomenon of many other GPCRs. The insights around the Q181D mutant and constitutive activity are interesting, however, the MD data presented is not fully convincing.

In general, this manuscript is written very well and clear; the rationale behind experiments is reasonable and experiments tell an excellent story. However, there are some issues in regard to the analysis/presentation of data and lack of detail, e.g. for MD simulation data and atomic model of the receptor-ligand complex (listed below). These can be (hopefully easily) resolved by addressing my comments/suggestions below. Given my expertise, I have focused on the cryo-EM data presented in this manuscript.

Data and methodology: Cryo-EM

The cryo-EM data includes both a global refinement and focussed refinement for the receptor TM region. Despite the overall good map/model fit, the model has a few issues, such as bad bonds and bad angles. These might be resolved by another round of Phenix real-space refinement after using MDFF methods such as Isolde. I would also recommend the deposition of the PDB file without hydrogens present, as the resolution does not allow for hydrogens to be visible and typically PDB files at this resolution do not contain any hydrogens. (If hydrogens are important to be displayed for receptor residues interacting with propionate, then they should only be present for these ligand

binding residues).

General points:

-Figure 3 c: It needs to be clearly stated that the models of C8 and C6 are not based on experimental data, and how these were obtained (e.g. homology modelling, refinement into EM map,...)

-Ext Fig 5a-b or Ext Data Fig 8: What do the 5 different displays show (top to bottom)? Are these plots of RMSD/distances comparing different atoms or are these replicates? Please state more clearly what is shown in the different plots and how the 5 displays were obtained.

-MD 'snapshots' e.g. mentioned in Figure legends of Figure 5, Extended Data Fig. 9: please state clearly what the rationale behind the selection of MD snapshots was? Is this 'cherry-picking' of snapshots that fit the narrative of the manuscript? It would be nicer if a more unbiased approach was used, e.g. display of ever 100th or 1000th frame/snapshot from the simulation.

-Are there any raw tables of MD data, which show interactions of residues to 'quantify' comments such as in Line 287: 'In simulations..., R262 is persistently engaged toward the ligand binding site'. There is no clear evidence for the statements around the Q181D mutant and constitutive activity.

- Alphafold predictions: in the absence of any (previous) experimental data for this receptor, how reliable is the prediction of the inactive or ligand-free receptor structure? It might help if Ext Data Fig 10 showed the confidence (pLDDT) colouring for the alphafold prediction.

-Extended data fig 4: Cryo-EM maps used to display map/model fit: Please state which map was used and which map threshold was used for the figure displays

-Figure 1 is slightly misleading; it is showing the cAMP accumulation in OR51E2, next to the structure that is not capable of maintaining this cAMP response. Would it make sense to remove cAMP graph and add it to the supplementary data?

Cryo-EM map/model issues:

-Map quality around propionate ligand is OK, given its small size, however, signal for the ligand is only present in the sharpened maps, and only very weak/less convincing in half maps or unsharpened maps (while side chains in proximity are more clearly resolved). This could be due to the transient interactions of the ligand with the binding pocket residues? Have the authors tried 3D classifications with fine angular sampling or 3D variance analysis in CryoSPARC to see whether there is signal from the ligand in slightly different positions/orientations? Or any other methods to improve local resolution of the ligand? Following structural studies, authors have conducted further experiments (MD, mutagenesis in cAMP) to validate the binding pocket/pose, which are more convincing.

-What was the rationale of the selected binding pocket of propionate in the first place/initial model? Was there any previous data that suggested this location (e.g. cAMP mutagenesis data of receptor residues), or was it discovered de novo? The methods state: "The propionate model and rotamer library were generated with the PRODRG server, docked using Coot, and refined in Phenix."

However, a bit more detail into the selection criteria for different propionate positions would be desirable.

-Q181 comes close to propionate (O beta) in the consensus model provided (with Hydrogens present), however, Ext Data Fig 5 shows a distance of Q181 to Ob of propionate of 3.3Å. Which atom distances were measured here? If there is a supplementary table that summarises these interactions (including atom-atom distances), please state here, otherwise it would be helpful for the reader if the authors created a table with H-bonds, at least for ligand-receptor bonds. Does the molecular dynamics simulation provide any insights on S258 and Q181 distances over time (as shown in Ext Fig 5 for R262)?

-Backbone of receptor residues around 189-190 not fitted properly, consider remodelling or deleting.

Referee #4 (Remarks to the Author):

Overall

The manuscript "Structural basis of odorant recognition by a human olfactory receptor" by Professor Manglik and co-authors presents novel scientific insights of great importance. The first structure of a GPCR family, or protein family in general, breaks new ground for basic research and commercial applications, tend to get very highly cited and opens up for many other studies. The same can be expected for this paper, given the great number of olfactory receptors (~400), their sensory function (which can be impacted by covid), and the increasing reports of implications in additional functions, including associations with disease etiology and targeting. This is a long-awaited structure covering a major missing family of GPCRs. I congratulate the authors for having solved a cryo-EM structure of an ORs, which is a feat given their low stability (I am certain many have tried and failed).

The conclusions on ligand recognition (e.g., occluded binding mode), activation mechanisms (e.g., ECL3 movement is sufficient for activation) etc. are all original, although some references and comparisons are missing to more recent and comprehensive studies of determinants of GPCR activation (noted below). The results should be of immediate interest to many people in the GPCR and odorant research fields, and across basic research, industry e.g., perfume industry and drug discovery targeting non-olfactory functions of ORs. The manuscript does not have any flaws which should prohibit its publication in Nature, after making a revision addressing the below comments, although a few of them are of major importance. This should lift the analysis, nomenclature and computational aspects of the paper to the same high standards as the already impressive cryo-EM structure, mutagenesis, and molecular dynamics.

Major comments

1. The authors compare the structure of OR51E2 to the two most studied prototypes for class A GPCRs, the β -adrenoceptor and Rhodopsin, which is a very helpful choice for readers. However, I would request that authors also provide an answer to the more open question of which structurally characterized non-olfactory class A GPCR has the most similar structure conformation. This could be done by RMSD calculation after superposition of previously published structures to OR51E2 (e.g., on TM1-7 and optionally including H8, and structurally conserved regions of ECL1-2, and ICL1-2) or by an all-to-all comparison of C α atoms (does not depend on a superposition, as in DOI: 10.1038/s41594-021-00675-6).

2. Although previous mechanistic structural studies were limited to TM1-7, more recent and comprehensive studies have expanded to H8 and regions of ICL1-2, and ECL1-2 have also been implicated in activation (DOI: <https://doi.org/10.7554/eLife.50279> and <https://doi.org/10.1038/s41594-021-00674-7>). These (sub)segments are structurally conserved across class A GPCRs (H8 wider) and have generic residue numbers (DOI: 10.1016/j.tips.2014.11.001) enabling their comparison on par with TM1-7 and covering more of the repacking of the GPCR upon activation. Thus, the study of activation mechanisms should not be limited to TM1-7, but investigate determinants more holistically/completely.

3. The sequence alignment in Extended Data Fig. 1 of OR51E2, rhodopsin and the β 2-adrenoceptor is identical to the structure-based alignment available from the GPCR database, GPCRdb. Given that those alignments are manually curated (based on structure superposition) and include gaps where structures show a local TM helix constriction or bulge, it would not be possible to obtain the alignment elsewhere. The authors must therefore give appropriate credit, including a reference, to the source of this alignment.

4. The authors are using a structure-based sequence alignment, but this is not compatible with the (outdated) sequence-based generic residue numbering. The mentioned sequence-based generic residue numbering scheme by Ballesteros and Weinstein from 1995 was devoid of structural templates and therefore assumed that TM1-7 are perfect α -helices that does not contain any gaps – in structural and sequence alignment. The structural era proved this wrong revealing local helix bulges and constrictions, which if occurring in one of two superposed structures, represent a gap of one residue – in the structural and sequence alignment. Failure to annotate those gaps cause a detrimental offset of all following residues, such that the ‘generic’ numbers no longer index equivalent/corresponding residues.

In 2015, GPCRdb, Ray Stevens and Heptares published a structure-based numbering scheme that corrects for this and extends to helix 8 and structurally conserved segments of the first two extracellular and intracellular loops, respectively (<https://doi.org/10.1016/j.tips.2014.11.001>). Beyond the added segments, the main point of that scheme is that helix bulges or constrictions in some receptors no longer offsets the numbering relative to normal alpha-helices, such that numbers stay generic/equivalent. Hence, the publication of the structure-based GPCRdb generic residue

numbering scheme corrects and references all of the sequence-based numbering schemes for classes A (Ballesteros-Weinstein), B1 (Wootten) and C (Pin). This scheme has now been used by numerous structure studies, including the recent Nature paper extending the structure-based generic residue numbering to class D GPCRs (DOI: 10.1038/s41586-020-2994-1). To reflect the structure-based sequence alignment used, ensure that residue numbers are indeed generic/equivalent, and avoid future misinterpretation by the many studies to build on this paper – the authors should use the consistent structure-based generic residue numbering.

5. On page 6, authors write “We tested the selectivity of fatty acid odorants of various chain lengths at OR51E2 to understand how structural features in the receptor lead to odorant specificity.” However, as only one receptor is being assayed, what is being evaluated is not the selectivity of odorants, but of the receptor for different odorants. This is stated correctly later on the same page in “We speculated that the selectivity of OR51E2 for short chain fatty acids ...”.

6. The authors demonstrated that the selectivity of OR51E2 for short chain fatty acids arises from the restricted volume of the occluded binding pocket (31 Å³) by mutagenesis and analogs testing. To complete the scientific rationale, and supports its importance, authors should provide information about which other olfactory receptor (wildtype) sequences that have smaller amino acids at the mutated positions and whether some of these are known to be able to recognize longer-chain fatty acids.

7. The authors “aimed to understand how propionate binding to OR51E2 stabilizes these regions in an active conformation.”. Their reference for determinants and mechanisms for class A GPCR activation is their own paper from 2017 (reference 34), but this covers only a very limited set of receptors. Two more recent and much more comprehensive studies have since been published in 2019 (DOI: 10.7554/eLife.50279) and 2021 (<https://doi.org/10.1038/s41594-021-00674-7>). The study from 2021 was able to revise and expand class A GPCR activation determinants by using novel structure comparison tools enabling comparison across all available structure across the inactive and active states, a residue contact frequency metrics, and validation of class-generalty by sequence conservation. For example, the mentioned PIF motif is actually PIW in the majority of class A GPCRs and the so-called rotamer switch W6x48 does not undergo sidechain rotation shift but helix backbone rotation (as part of a larger rotation of TM6) upon activation. Altogether, these updates add up to be substantial and therefore necessitate the use of an updated reference for the presented comparison of OR51E2 activation determinants to non-olfactory class A GPCRs.

Furthermore, the mentioned outward movement of cytosolic TM6 is not seen in all GPCR classes, which can instead have unwinding (B1) or little movement (C). In classes where TM6 makes a large outward movement this is also accompanied by a rotational movement (not yet mentioned in the manuscript), which should also be described as it makes a large mechanistic contribution.

8. Results include the finding that “OR51E2 simulated with propionate remains in a conformation similar to the cryo-EM structure.”, which seems contradictory to the Discussion: “We propose the

following model for OR51E2 activation (Fig. 5g). In the unbound state, the extracellular segment of TM6 is dynamic. Upon binding of propionate, TM6 rotates inward towards the 7TM domain ...". Specifically, shouldn't the model of OR51E2 activation have been reflected by opposite movements in MD after removing the G protein?

Furthermore, it is highly unusual for the receptor conformation to remain similar after removing the G protein. Did the authors observe an inward movement and rotation of TM6? If not, the authors should either perform longer MD simulations or refer to previously published studies where the receptor remains in an active-like conformation after removal of the G protein?

9. The authors compare their OR51E2 structure with an AlphaFold2 model, which is "in an intermediate or inactive conformation characterized by outward displacement of R262.59 and ECL3, a G protein coupling domain in the inactive conformation, and TM6 more inwardly posed compared to active OR51E2.". It is a known limitation of the pre-generated AlphaFold2 models that they only cover one state for each protein and, like in this case, sometimes represent an intermediate or even artificial state when the available templates that underlie the models span both states. The use of an inappropriate reference model is a problem that affects all parts of this comparison. Furthermore, the authors extended to additional olfactory receptor models: "We next examined the predicted structures of all human ORs, which revealed a largely shared topology for the extracellular region for the broader family.". It is likely that many of those models share the same problem, as they too would in most cases be based on the same type of mixed templates.

This problem has been mitigated recently with the publication and pre-generation (download-ready) AlphaFold2 models in the correct, active state (https://gpccrdb.org/structure/homology_models, <https://doi.org/10.1093/nar/gkac1013>). These models were generated using AlphaFold2-MultiState, which allows AlphaFold2 to model the correct state by only using templates in the appropriate state. Download-ready models are available of OR51E2 and in total 23 homologous receptors belonging to the O51 subfamily of ORs. To ensure correct conclusions, the authors should update their comparisons to use such models, which can be downloaded or generated again using AlphaFold2-Multistate.

10. The manuscript includes the statement "We anticipate that the molecular mechanism we define here for OR51E2 is likely to extend to other Class I ORs that recognize polar, water soluble odorants with multiple hydrogen bond acceptors and donors.", but is missing data to support this statement. To support this, the authors should describe (for example in an Extended Data Table or supplementary file for download) the percent conservation among all ORs of the identified residue determinants of these molecular mechanisms. They already do so in Extended Data Fig. 6. for residues in the odorant binding pocket.

Minor comments

1. The abbreviation 7TM is used but is never defined.
2. There is a newer, more complete reference ([doi:10.1016/j.ygeno.2006.04.001](https://doi.org/10.1016/j.ygeno.2006.04.001)) than references 6-

7 for the number of human olfactory GPCRs.

3. "Although the overall conformation of OR51E2 and β 2-AR are similar (root-mean-square deviation (RMSD) of 3.2 Å), ...". RMSD values depend on the region(s) being superposed, please specify which region was used.

4. "Indeed alanine mutagenesis of OR51E2 residues in the G protein coupling region show a dramatic loss of activity for H2436.40, Y2175.58, and R1213.50 associated with poor receptor expression". The words "receptor mutants" or just "mutants" is missing after the three listed mutations, as it is the overall protein which expresses rather than single residues.

5. "A phylogenetic tree of human Class A GPCRs was made by analyzing 677 sequences." The methods section is missing key information hindering reproducibility and understand of what the results reflect. Information should be added about if these were full-length sequences or only segments which can be meaningfully aligned across receptors i.e., residue positions with generic residue numbers (representing the subsequences that can be aligned across receptors).

Furthermore, a description of how authors used the reference alignment in EDF1 to ensure that conserved sequence motives in each segment (TM1-7, H8, ICL1-2, and ECL1-2) align correctly.

6. "Extended Data Fig. 1. ... Transmembrane domains are boxed and labeled in black."

Transmembrane helices differ in length across different receptors, as evident by their structures.

This means that some residues inside the black boxes are actually not part of the TM helix, while other residues outside the boxes are actually part of a TM helix. This needs to be corrected to give correct information. For example, the boxes could be redrawn with varying width across receptors, or replaced with background coloring. Alternatively, a minimal solution would be to use the first and last helical residue of OR51E2 TMHs and define this in the legend.

7. The name of the GPCRmd database should be written with md in lower case.

8. "Olfactory receptors display significant sequence variation ...". Nature and several other journals reserve the word "significant" for statistical contexts.

Author Rebuttals to Initial Comments:

We thank the referees for their constructive comments. We provide a point-by-point response below. Revisions to the manuscript are highlighted in red text.

Reviewer Comments:

Referee #1:

Remarks to the Author:

To date, no tridimensional structure of a mammalian odorant receptor has been reported. In this work, the authors solved the first structure of a human odorant receptor, OR51E2, at a high resolution (3.1Å). OR51E2 belongs to the Class I group of odorant receptors, which show some specific sequence motifs when compared to the more numerous Class II receptors, and are also more tuned to a specific group of ligands, the aliphatic odorants. The results raise important insights into the structural mechanisms of an odorant receptor activation by its ligand and should be of broad interest to the scientific community.

In order to trap the receptor in the G protein interacting conformation, the authors generated a construct containing OR51E2 fused at its C-terminal region to the mini Gs protein. A complex containing OR51E2-mini Gs bound to its ligand propionate, the nanobody Nb35 (to stabilize the GPCR/G protein complex) and the Gb1g2 subunit was purified and analyzed using cryo-EM. The structure shows propionate bound to a small pocket located deep in the receptor and the residues that are required for the interaction. Functional cell assays with mutant forms of OR51E2 were also performed, to verify whether these residues are indeed involved in the receptor activation by the ligand. These experiments also showed that the size of the pocket determines the specificity of the receptor towards fatty acids that have short chain lengths. Due to the strategy used, the authors were unable to determine the structure of the inactive receptor (without ligand). Instead, they modeled an inactive form. Based on the comparison of the active and inactive forms, a mechanism of activation was proposed. These structures were also compared to those of B2-AR2, one Class A GPCR with a similar ligand binding pocket. The comparison is interesting, and reveals both, the similarities, and differences between the modes of activation of the two receptors. The article is well written and organized, I only have the following comments:

We thank the reviewer for putting our work into context and for the positive evaluation of its importance.

The term 'olfactory receptor' is sometimes used as a general term to refer to all possible types of olfactory receptors, including for example TAARs and pheromone receptors. To avoid confusion, I recommend that the authors use the term 'odorant receptors' to refer to the canonical receptors.

We have now changed this to “odorant receptor” throughout the manuscript.

Extended data figure 2 c- why are there two bands for OR51E2-miniGs?

We often see multiple bands for glycosylated GPCRs in biochemical preparations. Our assumption is that this reflects heterogeneous glycosylation on the N-terminus of OR51E2, which contains an NxS/T glycosylation motif. We have now added this interpretation to the figure legend.

Line 216- 'By contrast, the highly conserved CWxP6.50 motif in the connector region of Class A GPCRs is absent in ORs. Instead ORs contain the previously described FYGx6.50 motif in the connector region'. This is an interesting finding. What does this difference in conserved residues represent to the workings of the connector region? In other words, is connection between the binding pocket to the G protein interaction helix particularly different in ORs when compared to non-olfactory Class A GPCRs?

This is a very interesting distinction between the OR family compared to the broader Class A GPCR family. The importance of the CWxP^{6x50} motif for Class A GPCRs has been illuminated by structures of the same receptor in the active and inactive states. While there were initially proposals in the field that W^{6x48} in the CWxP^{6x50} motif acts as a rotamer toggle to couple agonist binding to activation, many structures, simulations, and spectroscopic studies discounted this notion. At this point, there are a number of distinct mechanisms that enable agonist binding to drive conformational changes across Class A GPCRs - one of the most common conserved mechanisms is highlighted in the manuscript (the PIF motif rearrangement).

We agree that it will be very important to understand precisely how odorant binding leads to changes in the FYGx motif. However, in the absence of an experimentally determined inactive-state structure, we are reluctant to trace a specific set of conformational changes that connect odorant binding to changes in the FYGx motif. That said, we believe that the model we proposed in Fig. 5g that is supported by data in Fig. 4, starts to outline how odorant binding is linked to changes in TM6 - this is quite distinct from other Class A GPCRs.

Line 543- Please provide the sequence used for OR51E2 (accession number).

We have now provided this accession number.

Why was Gγ2 used in the G protein complex instead of Gγ13, which is the Gγ subunit abundantly expressed in the mature olfactory neurons?

This is a good question - we used Gγ2 primarily because this isoform has been used almost exclusively for GPCR structural biology over the past decade. For example, the RCSB PDB shows almost 450 entries for Gγ2 (GNG2) and none for Gγ13 (GNG13). We agree that it will be important to address whether there are differences in OR-G protein complexes if other G protein components are used. Given the extreme technical challenges that preclude more facile structure determination of ORs, we plan to do this in future studies that will hopefully capture OR51E2, and other ORs, bound to G_{olf} and Gγ13.

Line 155- and figure 3. Propionate and other short fatty acids show millimolar potency in the functional assays. This range of odorant concentrations represents low affinities, even for odorant receptors. Does OR51E2 respond to additional ligands with higher affinity? Is there some explanation for this?

This is a good point. We should first indicate that the millimolar potency for propionate and acetate at OR51E2 in our cAMP assay is concordant with similar experiments performed by other groups using heterologous cell lines expressing either OR51E2 or the mouse ortholog Olfr78 (PMID: 23401498, 33640452). From a biochemical/biophysical perspective, the small size of propionate (only 5 heavy atoms) is also concordant with a millimolar binding potency.

The reviewer brings up a central question whether these low potencies reflect the physiologically relevant concentrations of propionate/acetate sensed by OR51E2. We first considered the olfactory role of OR51E2. Propionic and acetic acid have relatively low vapor pressures, and likely do not reach the millimolar concentrations required to activate OR51E2 in the olfactory mucosa. Studies on the mouse ortholog Olfr78 provide a potential explanation. In perhaps the most comprehensive *in vivo* study examining Olfr78 function, Soelter et al. (PMID:31919393) tested 213 odorants for activation of Olfr78-glomeruli in live mice. Out of these ligands, the most potent agonists of Olfr78 are esters of propionic or acetic acid. It is likely that the increased volatility of propionic or acetic acid esters combined with esterases in the olfactory mucosa lead to perceptible concentrations of propionate and acetate (PMID: 21123585). Based on our *in vitro* experiments, we assume these would have to reach at least high micromolar concentrations in the olfactory mucosa.

We next considered whether millimolar concentrations of propionate/acetate may be relevant in the ectopic functions of OR51E2/Olfr78 in the gut or kidney. Prior studies suggest that propionic acid can reach concentrations of 10-100 mM in the lumen of the colon (PMID: 31123355, 3297476) or 0.1-10 mM in the plasma (PMID: 23401498, 12711604, 19865172). These ranges of propionate that are concordant with our *in vitro* signaling assay. Therefore, while the true potency of propionate required to activate OR51E2/Olfr78 in the olfactory system is not precisely clear, the millimolar propionate potency we measure is likely in the physiologically relevant range for OR51E2 for its ectopic, non-olfactory functions.

What do exactly frequencies numbers represent in extended figure 6? Percentage of receptors? Please include in the figure legend.

We apologize for the lack of clarity - these numbers reflect the percentage of human ORs with the given amino acid. We have now revised the figure legend to add clarity here.

Figure 4A- please depict the relevant TMs

Thanks for catching this oversight - we have now added TM labels to this panel.

Figure 5g- would help to indicate also other relevant TMs. FYG interactions are not clearly depicted, what are the TMs involved?

We have now added TM labels to the other helices for the cartoon. We would prefer not to highlight specific interactions between the FYG motif and other residues because, while the FYGx motif is conserved across the OR family, many of the contacts we see in OR51E2 are only conserved in Class I ORs. We believe the mechanism combining movement of ECL3 coupled to FYG movement may be more broadly conserved across the OR family.

Other ligands have been described for OR51E2, for example, why does b-ionone work as agonist while a-ionone works as an antagonist? Could this be explained by the solved structure? How would other ligands that are very different and larger, such as 19-hydroxyandrostenedione activate the receptor?
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5985834/>

We agree with the reviewer that it will be highly valuable to understand how other chemically distinct ligands can interact with OR51E2, especially if they have divergent efficacy. Unfortunately, α/β -ionone and 19-hydroxyandrostenedione, are likely too large to fit in the propionate site, even with assumed flexibility of the site in the absence of propionate. We are reluctant to speculate on how ligands engage a GPCR based on incredible structural biology efforts in the GPCR field over the past 5 years, which have revealed allosteric ligand binding at many distinct sites (Fig. R1), most of which remain unpredictable prior to structure determination. A further challenge is that we are not able to obtain a response for β -ionone in our GloSensor cAMP assay. This is similar to two other recent reports, which also failed to see a response for β -ionone at either OR51E2 or the mouse ortholog Olfr78 (PMID: 23401498, 31919393).

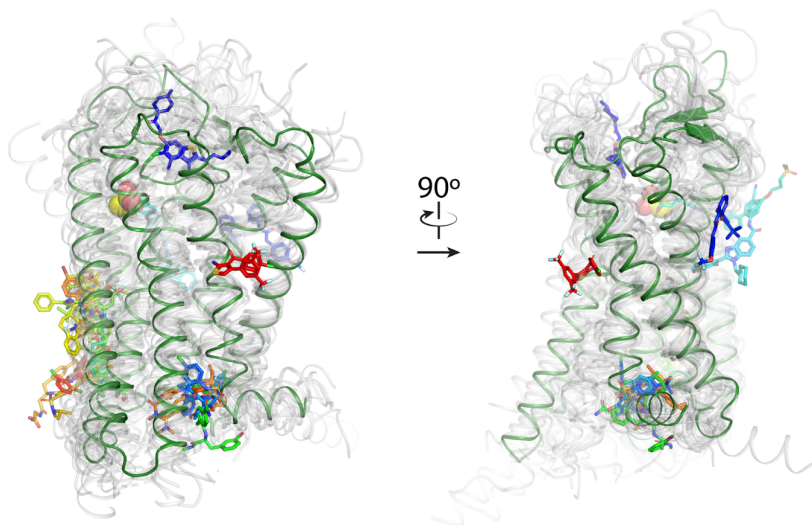


Figure R1. Allosteric ligands at Class A GPCRs. The structure of OR51E2 (green cartoon) bound to propionate (yellow spheres) is shown overlaid with 17 recent structures of other GPCRs bound to allosteric ligands. Other receptors are shown as gray transparent cartoons.

Allosteric ligands binding at distinct GPCR sites are shown in distinct colors, highlighting many distinct potential binding sites for GPCR ligands.

Referee #2:

The molecular basis of odor recognition in mammals remains elusive since the discovery of the olfactory receptor (OR) gene family by Buck and Axel in 1991. Seminal papers by Ruta and colleagues recently revealed the structure of insect odorant receptors and the mechanisms of odor recognition, however these principles cannot be extrapolated to the mammalian ORs, due to fundamental structural and functional differences between insect and mammalian odorant receptors. Thus, the elucidation of the human OR51E2 structure with its cognate ligand, constitutes a breakthrough. To overcome a major technical hurdle in the structural analysis of mammalian olfactory receptors, which is the poor expression properties of OR proteins in heterologous cell types, the authors used an OR that is known to be expressed outside the olfactory system. Further, this is a type I OR protein, which was recently shown to elicit lower levels of ER stress, likely facilitating expression in heterologous cells (the authors should mention this).

This is a good point - we now highlight that expression of Class I ORs generally leads to lower levels of ER stress by writing the following:

Additionally, Class I ORs induce decreased levels of endoplasmic reticulum stress compared to Class II ORs¹⁹, further increasing expression in heterologous cells.

Overcoming the bottleneck of expression levels, the authors manage to obtain cryoEM structures of OR51E2 with and without the carboxylic acid propionate and in complex with Galpha proteins. Among the interesting findings of this work:

- 1. The binding pocket of the OR protein is very shallow, much shallower than the beta-2 AR which make account for the low affinity for odorants (especially in comparison to TAARs).*
- 2. The binding pocket depth may account for selectivity for propionate vs longer carboxylic acids*
- 3. Propionate makes selective contacts with residues in TM2, TM5 (van der Waals) and TM6 and ECL2 (hydrogen bonds).*
- 4. Significant differences in the mechanisms of ligand induced Gs activation are observed between this OR and beta2-AR.*

Overall, the study is carefully executed, and structural observations are complemented with mutations that confirm predictions from the structure. The article is well written and of general interest. Congratulations to the authors for this seminal contribution to the field. This article should be accepted.

We thank the referee for putting this work into the broader context of the olfaction field.

Referee #3:

Remarks to the Author:

Billesbølle et al provide structural insights to a new class of olfactory receptors. The authors have selected OR51E2 for structural studies using cryo-EM, as well as utilised molecular dynamics simulations and pharmacology assays of wild type and mutants to understand ligand activation of this receptor. To gain stable constructs for structure determination, the authors fused mini-Gas to the receptor, which impaired cAMP signalling. However, a large body of work has been done to complement the insights gained through the structural data by additional functional pharmacology data as well as molecular dynamics simulations.

CryoEM structures made it feasible to gain insights into ligand binding (interaction with propionate) and inspired to conduct experiments in regard to specificity of the receptor for activation by longer chain ligands. For this, authors used mutant receptors in cAMP assays as well as sequence conservation studies that give insights into ligand recognition by other receptors of this class.

The activation process of OR51E2 was investigated using mutant receptors in cAMP, and molecular dynamics with and without propionate ligand present (in the absence of ligand-free or G protein-free experimental structures), suggesting a general similar mechanism compared to ClassA GPCRs, with differences in the dynamics of the OR-specific FYGx motif.

In addition to aforementioned motifs, ECL3 has a particular role with low conservation amongst other ORs (determined by a low Alphafold confidence value), and ligand binding possibly restricts flexibility of the ECL3. A dynamic, non-conserved ECL3 is actually a common phenomenon of many other GPCRs. The insights around the Q181D mutant and constitutive activity are interesting, however, the MD data presented is not fully convincing.

In general, this manuscript is written very well and clear; the rationale behind experiments is reasonable and experiments tell an excellent story. However, there are some issues in regard to the analysis/presentation of data and lack of detail, e.g. for MD simulation data and atomic model of the receptor-ligand complex (listed below). These can be (hopefully easily) resolved by addressing my comments/suggestions below. Given my expertise, I have focused on the cryo-EM data presented in this manuscript.

We thank the reviewer for the constructive feedback on how to improve our manuscript.

Data and methodology: Cryo-EM

The cryo-EM data includes both a global refinement and focussed refinement for the receptor TM region. Despite the overall good map/model fit, the model has a few issues, such as bad bonds and bad angles. These might be resolved by another round of Phenix real-space refinement after using MDFF methods such as Isolde. I would also recommend the deposition of the PDB file without hydrogens present, as the resolution does not allow for hydrogens to be visible and typically PDB files at this resolution do not contain any hydrogens. (If hydrogens are important to be displayed for receptor residues interacting with propionate, then they should only be present for these ligand binding residues).

We thank the reviewer for looking carefully at our model and for suggesting further changes. We have now worked on improving the overall model to decrease the number of poor bonds and angles, which were previously exclusively in the heterotrimeric G protein or the stabilizing nanobody. We understand the reviewer's concern regarding deposition of models with hydrogens as the resolution of our maps do not support accurate placement of hydrogen atoms - our main reason to include these hydrogens is that they are used in ISOLDE and Phenix real space refinement, and most refinement packages yield better structures with inclusion of hydrogens. These are also the hydrogen atoms used to initialize molecular dynamics simulations in this study. Based on current convention, however, our updated deposited structure omits all hydrogens.

General points:

-Figure 3 c: It needs to be clearly stated that the models of C8 and C6 are not based on experimental data, and how these were obtained (e.g. homology modelling, refinement into EM map,...)

We have now added the words "predicted binding pocket" in the figure and outlined in the figure legend that these are homology models of the mutants to avoid any confusion. We have further outlined in the methods the procedure for generating these models under the subsection titled "Generating OR51E2 mutant structural models, docking of C6 and C8 ligands and procedure for calculating the volume of the ligand binding pocket".

-Ext Fig 5a-b or Ext Data Fig 8: What do the 5 different displays show (top to bottom)? Are these plots of RMSD/distances comparing different atoms or are these replicates? Please state more clearly what is shown in the different plots and how the 5 displays were obtained.

We agree that this was not clear in the original manuscript. These 5 different displays represent five independent replicate simulations with different initial velocities. We have now added "Run 1" etc to each display to indicate that these are replicate simulations. We have also updated the figure legend to make this clear.

-MD 'snapshots' e.g. mentioned in Figure legends of Figure 5, Extended Data Fig. 9: please state clearly what the rationale behind the selection of MD snapshots was? Is this 'cherry-picking' of snapshots that fit the narrative of the manuscript? It would be nicer if a more unbiased approach was used, e.g. display of ever 100th or 1000th frame/snapshot from the simulation.

This is a very important point, and we are glad the reviewer brought this up. Indeed, we used an unbiased approach to pick representative simulation snapshots to show in these figures. We described this approach previously in the methods, but realize that the figure legends do not indicate how these snapshots were chosen. To recap our approach, we performed unsupervised clustering of the entire ensemble of simulation frames by the receptor backbone coordinate RMSD. We then chose to display a representative snapshot from clusters that together contain

>90% of the entire observed simulation ensemble. In Fig. 4i, Fig. 5b, and EDF 9a/b/c (OR51E2 No Ligand), three clusters together captured >90% of the total simulation ensemble - we picked the most representative snapshot from each of these clusters to display. For OR51E2 Q181^{45x53}D, a single cluster captured 90% of simulation snapshots - in this case, we displayed several snapshots from this single cluster.

We realize that this approach may be confusing to broad readership. In the revised manuscript, we now instead show simulation frames from each replicate after 1000 ns of simulation time (the last snapshot). This provides a consistent, unbiased approach to displaying the conformations sampled. Importantly, these images yield the same conclusion, i.e. that OR51E2 is structurally flexible in the absence of propionate and that the Q181^{45x53}D mutation stabilizes OR51E2 in an active conformation. To enable readers to examine these snapshots directly, we have included structure files for each snapshot shown in the figures.

However, for sake of thoroughness and for expert readership, we have provided our clustering approach to the Supplementary Information. Here, we provide a table that shows the percent of snapshots in each conformational cluster and three-dimensional rendering of the representative frames from each cluster. This is provided in Supplementary Table 7 and Supplementary Fig. 1.

-Are there any raw tables of MD data, which show interactions of residues to 'quantify' comments such as in Line 287: 'In simulations..., R262 is persistently engaged toward the ligand binding site'. There is no clear evidence for the statements around the Q181D mutant and constitutive activity.

We have now added three new panels to Extended Data Fig. 8 that show summary histograms of the simulations. This provides the reader a direct way to examine whether these statements are supported by the simulations. We additionally have added Supplementary Tables 1-6, which include summary statistics from MD simulations for all of the interactions called out in the manuscript.

- AlphaFold predictions: in the absence of any (previous) experimental data for this receptor, how reliable is the prediction of the inactive or ligand-free receptor structure? It might help if Ext Data Fig 10 showed the confidence (pLDDT) colouring for the alphafold prediction.

We have now added a panel showing the AlphaFold pLDDT for OE51E2 to Extended Data Fig. 10. We find that the AlphaFold pLDDT score is >80 for the majority of the receptor, with the exception of the distal N-terminus, ECL3, and the distal C terminus. Lower scores are found for regions that diverge from the cryo-EM structure of active OR51E2 - we interpret these as likely to be low confidence because they are conformationally flexible (an interpretation of pLDDT suggested by Jumper et al *Nature* 2021).

-Extended data fig 4: Cryo-EM maps used to display map/model fit: Please state which map was used and which map threshold was used for the figure displays

We have now explicitly indicated which maps were used to make these figures and included the map threshold.

-Figure 1 is slightly misleading; it is showing the cAMP accumulation in OR51E2, next to the structure that is not capable of maintaining this cAMP response. Would it make sense to remove cAMP graph and add it to the supplementary data?

We interpret the reviewer's concern to be that structural biology papers often show functional data for a construct that is distinct from that used for structure determination, and that the construct used for structure determination does not have the same activity that the wild-type protein. Usually this is due to mutations required to stabilize a protein for structural biology. In our case, we don't believe that our presentation is misleading. The construct for OR51E2 structure determination does not contain any mutations - it adds an amino terminal tag and a C-terminal miniG protein fusion. We explicitly indicate in the main text that fusion of the G protein decreases signaling activity, and show this data in the supplementary figures. The exact same construct without the miniG protein signals as shown in Figure 1b. To make sure there is no confusion, we have added "OR51E2" to panel b to indicate that this is data for the non-fused protein. To panel c, we now indicate "OR51E2-miniG α_s " to make it clear that this is a distinct construct from that used in panel b. We prefer to keep the signaling data in the main text as it will help readers understand the relevance of propionate to this study.

Cryo-EM map/model issues:

-Map quality around propionate ligand is OK, given its small size, however, signal for the ligand is only present in the sharpened maps, and only very weak/less convincing in half maps or unsharpened maps (while side chains in proximity are more clearly resolved). This could be due to the transient interactions of the ligand with the binding pocket residues? Have the authors tried 3D classifications with fine angular sampling or 3D variance analysis in CryoSPARC to see whether there is signal from the ligand in slightly different positions/orientations? Or any other methods to improve local resolution of the ligand? Following structural studies, authors have conducted further experiments (MD, mutagenesis in cAMP) to validate the binding pocket/pose, which are more convincing.

We agree that the density for propionate is not exceptionally strong - this could result from either flexibility of the ligand in the binding pocket, it's relatively small size, or potentially heterogeneity in ligand occupancy. Despite extensive fine angular sampling of the 7TM domain, 3D variability analysis in cryoSPARC, and 3D classification in both cryoSPARC and Relion, we are unable to identify a set of particles with improved density for propionate. Our confidence in the binding pose of propionate is indeed bolstered by the mutagenesis and simulation work. Furthermore, our experiments in Fig. 3 provide further evidence that our binding pose for propionate is likely correct (with all of the limitations that come with a moderate 3.1 Å resolution structure).

-What was the rationale of the selected binding pocket of propionate in the first place/initial model? Was there any previous data that suggested this location (e.g. cAMP mutagenesis data

of receptor residues), or was it discovered de novo? The methods state: “The propionate model and rotamer library were generated with the PRODRG server, docked using Coot, and refined in Phenix.”. However, a bit more detail into the selection criteria for different propionate positions would be desirable.

Our main reason for focusing in this region for a propionate binding site was severalfold. First, this was among the only pockets large enough in the OR51E2 structure that could accommodate propionate. Second, while the binding pocket of propionate is distinct from other Class A GPCRs, it is generally in a similar region. Third, there is good prior evidence that there is greater diversity among ORs in the region that lines the propionate binding pocket. Finally, a previous study from our group found that mutation of R^{6x59} in OR51E1 makes the receptor unresponsive to a cognate carboxylic acid (PMID: 30599066). With these constraints, we felt fairly confident that the propionate binding site would be near R262^{6x59}, with a likely model being that R262^{6x59} coordinates the carboxylic acid of propionate. We have now included more detail in the methods indicating how we initially placed propionate in the binding pocket.

-Q181 comes close to propionate (O beta) in the consensus model provided (with Hydrogens present), however, Ext Data Fig 5 shows a distance of Q181 to Ob of propionate of 3.3Å. Which atom distances were measured here? If there is a supplementary table that summarises these interactions (including atom-atom distances), please state here, otherwise it would be helpful for the reader if the authors created a table with H-bonds, at least for ligand-receptor bonds. Does the molecular dynamics simulation provide any insights on S258 and Q181 distances over time (as shown in Ext Fig 5 for R262)?

This is a very helpful point. The distances shown in Extended Data Fig. 5c were calculated as the minimum distance between heavy atoms (excluding hydrogens) in the amino acid and the specified atoms of propionate. The time trajectories for interactions between propionate and S258^{6x55} and Q181^{45x53} are not particularly informative - they show minimal deviation from the cryo-EM structure model. We have updated the figure legend to indicate how these distances were calculated and now provide a set of Supplementary Tables 1-6 that show summary statistics of the simulations.

-Backbone of receptor residues around 189-190 not fitted properly, consider remodelling or deleting.

We have now revised this region of the model to better fit the density and satisfy local geometry.

Referee #4:

Remarks to the Author:

Overall

The manuscript "Structural basis of odorant recognition by a human olfactory receptor" by Professor Manglik and co-authors presents novel scientific insights of great importance. The first structure of a GPCR family, or protein family in general, breaks new ground for basic research and commercial applications, tend to get very highly cited and opens up for many other studies. The same can be expected for this paper, given the great number of olfactory receptors (~400), their sensory function(which can be impacted by covid), and the increasing reports of implications in additional functions, including associations with disease etiology and targeting. This is a long-awaited structure covering a major missing family of GPCRs. I congratulate the authors for having solved a cryo-EM structure of an ORs, which is a feat given their low stability (I am certain many have tried and failed).

We thank the reviewer for their very positive comments about our work.

The conclusions on ligand recognition (e.g., occluded binding mode), activation mechanisms (e.g., ECL3 movement is sufficient for activation) etc. are all original, although some references and comparisons are missing to more recent and comprehensive studies of determinants of GPCR activation (noted below). The results should be of immediate interest to many people in the GPCR and odorant research fields, and across basic research, industry e.g., perfume industry and drug discovery targeting non-olfactory functions of ORs. The manuscript does not have any flaws which should prohibit its publication in Nature, after making a revision addressing the below comments, although a few of them are of major importance. This should lift the analysis, nomenclature and computational aspects of the paper to the same high standards as the already impressive cryo-EM structure, mutagenesis, and molecular dynamics.

We thank the reviewer for such an impressively constructive approach to peer review - we agree that all of these suggestions will improve our manuscript significantly!

Major comments

1. The authors compare the structure of OR51E2 to the two most studied prototypes for class A GPCRs, the b-adrenoceptor and Rhodopsin, which is a very helpful choice for readers. However, I would request that authors also provide an answer to the more open question of which structurally characterized non-olfactory class A GPCR has the most similar structure conformation. This could be done by RMSD calculation after superposition of previously published structures to OR51E2 (e.g., on TM1-7 and optionally including H8, and structurally conserved regions of ECL1-2, and ICL1-2) or by an all-to-all comparison of Cα atoms (does not depend on a superposition, as in DOI: 10.1038/s41594-021-00675-6).

Based on this request, we compared all representative active non-olfactory class A receptors with a resolution better than 3.5 Å (both X-ray and cryo-EM) that are currently annotated on the GPCRdb. This included a total of 71 structures to compare to OR51E2. We aligned all Cα atoms of these receptors with the Cα atoms of OR51E2 using Cealign. RMSD values for all Cα atoms

ranged between 2.7–5.5 Å, with 5HT1D (PDB: 7E32) being the most similar structurally related structure to OR51E2. By contrast, the β 2AR and rhodopsin RMSD values were 3.1 Å and 3.4 Å, respectively. This table is now included as Supplementary Table 8.

2. Although previous mechanistic structural studies were limited to TM1-7, more recent and comprehensive studies have expanded to H8 and regions of ICL1-2, and ECL1-2 have also been implicated in activation (DOI:<https://doi.org/10.7554/eLife.50279> and <https://doi.org/10.1038/s41594-021-00674-7>). These (sub)segments are structurally conserved across class A GPCRs (H8 wider) and have generic residue numbers (DOI: [10.1016/j.tips.2014.11.001](https://doi.org/10.1016/j.tips.2014.11.001)) enabling their comparison on par with TM1-7 and covering more of the repacking of the GPCR upon activation. Thus, the study of activation mechanisms should not be limited to TM1-7, but investigate determinants more holistically/completely.

We thank the reviewer for pointing out this broader view of GPCR activation mechanisms. Since we do not have an experimentally determined inactive structure of OR51E2 (or any other OR), our goal in this manuscript has been to try to glean the first insights that we can by comparing to other well understood Class A GPCRs - we mostly focused on very well known motifs across the GPCR family. We believe that our manuscript does highlight some of the regions indicated by the reviewer (e.g. the section titled “Structural dynamics of ECL3 in OR function” and our analysis of ECL3 movements in Figure 5). We now cite these reviews in the manuscript to highlight the more holistic perspective on GPCR activation.

3. The sequence alignment in Extended Data Fig. 1 of OR51E2, rhodopsin and the β 2-adrenoceptor is identical to the structure-based alignment available from the GPCR database, GPCRdb. Given that those alignments are manually curated (based on structure superposition) and include gaps where structures show a local TM helix constriction or bulge, it would not be possible to obtain the alignment elsewhere. The authors must therefore give appropriate credit, including a reference, to the source of this alignment.

We apologize for this oversight. We previously proposed an alignment of ORs to Class A GPCRs in de March *et al Protein Sci* 2015 (PMID: 26044705), which we used to drive our alignment. This alignment was further supported by the structure of OR51E2 and a related OR structure. We subsequently contacted Dr. David Gloriam, who for many years has led the GPCRdb to suggest the addition of OR51 family members to the GPCRdb. We are fortunate that Dr. Gloriam and colleagues recently added OR51E2 and other OR51 members to the GPCRdb, after verifying that our alignment scheme in de March *et al Protein Sci* 2015 is consistent with their standards for structure-based alignment. We now cite the latest iteration of GPCRdb to highlight the important work of the GPCRdb team.

4. The authors are using a structure-based sequence alignment, but this is not compatible with the (outdated) sequence-based generic residue numbering. The mentioned sequence-based generic residue numbering scheme by Ballesteros and Weinstein from 1995 was devoid of structural templates and therefore assumed that TM1-7 are perfect α -helices that does not contain any gaps – in structural and sequence alignment. The structural era proved this wrong

revealing local helix bulges and constrictions, which if occurring in one of two superposed structures, represent a gap of one residue – in the structural and sequence alignment. Failure to annotate those gaps cause a detrimental offset of all following residues, such that the ‘generic’ numbers no longer index equivalent/corresponding residues.

In 2015, GPCRdb, Ray Stevens and Heptares published a structure-based numbering scheme that corrects for this and extends to helix 8 and structurally conserved segments of the first two extracellular and intracellular loops, respectively (<https://doi.org/10.1016/j.tips.2014.11.001>). Beyond the added segments, the main point of that scheme is that helix bulges or constrictions in some receptors no longer offsets the numbering relative to normal alpha-helices, such that numbers stay generic/equivalent. Hence, the publication of the structure-based GPCRdb generic residue numbering scheme corrects and references all of the sequence-based numbering schemes for classes A (Ballesteros-Weinstein), B1 (Wootten) and C (Pin). This scheme has now been used by numerous structure studies, including the recent Nature paper extending the structure-based generic residue numbering to class D GPCRs (DOI: 10.1038/s41586-020-2994-1). To reflect the structure-based sequence alignment used, ensure that residue numbers are indeed generic/equivalent, and avoid future misinterpretation by the many studies to build on this paper – the authors should use the consistent structure-based generic residue numbering.

We thank the reviewer for bringing this up. We have now changed the numbering to reflect this newer scheme and cite the paper indicated.

5. On page 6, authors write “We tested the selectivity of fatty acid odorants of various chain lengths at OR51E2 to understand how structural features in the receptor lead to odorant specificity.” However, as only one receptor is being assayed, what is being evaluated is not the selectivity of odorants, but of the receptor for different odorants. This is stated correctly later on the same page in “We speculated that the selectivity of OR51E2 for short chain fatty acids ...”.

Thanks for bringing this up - we have now fixed this.

6. The authors demonstrated that the selectivity of OR51E2 for short chain fatty acids arises from the restricted volume of the occluded binding pocket (31 Å³) by mutagenesis and analogs testing. To complete the scientific rationale, and supports its importance, authors should provide information about which other olfactory receptor (wildtype) sequences that have smaller amino acids at the mutated positions and whether some of these are known to be able to recognize longer-chain fatty acids.

As shown in Extended Data Fig. 6d, no other human Class I ORs possess an alanine or glycine at positions 4x57 and 4x60. Most receptors that contain the next smallest amino acid (valine) at these positions (V@4x57: 52E8, 52E4, 52E2, 52E5, 51A2, 51A4, 51L1, 51Q1, 51B5; V@4.60: 51F2, 51B2) have not been deorphanized. OR51L1, however, harbors valine at 4x57. While this receptor has not been fully characterized, our previous deorphanization work indicated that it responds to hexanoic acid, a longer chain fatty acid (PMID: 19261596). Finally, the closest

ortholog of OR51E2 is OR51E1, an OR that responds to longer chain fatty acids. However, OR51E1 retains the same amino acid as OR51E2 at position 4x60 (L) and has a minor substitution at position 4x57 (M instead of F). Therefore the ability of OR51E1 to accommodate larger chain fatty acids likely arises from other changes in the binding pocket, but the precise mechanism remains to be determined. We now cite prior literature that these other Class I ORs have substitutions in the binding pocket and can recognize longer chain fatty acids.

7. The authors “aimed to understand how propionate binding to OR51E2 stabilizes these regions in an active conformation.”. Their reference for determinants and mechanisms for class A GPCR activation is their own paper from 2017 (reference 34), but this covers only a very limited set of receptors. Two more recent and much more comprehensive studies have since been published in 2019 (DOI: 10.7554/eLife.50279) and 2021 (<https://doi.org/10.1038/s41594-021-00674-7>). The study from 2021 was able to revise and expand class A GPCR activation determinants by using novel structure comparison tools enabling comparison across all available structure across the inactive and active states, a residue contact frequency metrics, and validation of class-generalty by sequence conservation. For example, the mentioned PIF motif is actually PIW in the majority of class A GPCRs and the so-called rotamer switch W6x48 does not undergo sidechain rotation shift but helix backbone rotation (as part of a larger rotation of TM6) upon activation. Altogether, these updates add up to be substantial and therefore necessitate the use of an updated reference for the presented comparison of OR51E2 activation determinants to non-olfactory class A GPCRs.

As indicated for comment #2 above, we agree that these two references provide a more comprehensive analysis - we cite both in the revised manuscript.

Furthermore, the mentioned outward movement of cytosolic TM6 is not seen in all GPCR classes, which can instead have unwinding (B1) or little movement (C). In classes where TM6 makes a large outward movement this is also accompanied by a rotational movement (not yet mentioned in the manuscript), which should also be described as it makes a large mechanistic contribution.

We now clarify with the following revised text:

A central hallmark of **Class A** GPCR activation is an outward displacement **and rotation** of TM6 in the cytoplasmic side of the receptor, which is accompanied by more subtle movement of the other TM helices^{36–38}.

8. Results include the finding that “OR51E2 simulated with propionate remains in a conformation similar to the cryo-EM structure.”, which seems contradictory to the Discussion: “We propose the following model for OR51E2 activation (Fig. 5g). In the unbound state, the extracellular segment of TM6 is dynamic. Upon binding of propionate, TM6 rotates inward towards the 7TM domain ...”. Specifically, shouldn’t the model of OR51E2 activation have been reflected by opposite movements in MD after removing the G protein?

Our presentation of the simulation data likely led to a lack of clarity, which is highlighted by the reviewer here. To recap, our conclusion in the discussion is based on comparing simulations of OR51E2 either in the presence or absence of propionate. In both cases, we simulated OR51E2 in the absence of the G protein. We find that the overall conformation of OR51E2 simulated in the presence of propionate, but with the G protein removed, is relatively similar to the cryo-EM structure (both at the level of propionate binding site shown in Fig. 2f, EDF 5, Fig. 5c, EDF 8c and at the level of the connector region as shown in Fig. 4j,k and EDF 8a,b). As noted below, we do not see deactivation of OR51E2 in these simulations, likely because these simulations have timescales that are too short to see this transition. This conformational similarity to the cryo-EM structure was not explicitly shown in the previous version of the manuscript, but is visualized in the revised version of Extended Data Fig. 9.

By comparison, our simulations of OR51E2 without propionate (and also without G protein) showed significantly higher flexibility in the ligand binding region (Fig. 5c and EDF 8c) and in the connector region (Fig. 4i,j,k and EDF 8a,b). This is again highlighted more clearly in Extended Data Fig. 9. Similar to our simulations of OR51E2-propionate without G protein, our simulations of OR51E2-no ligand without G protein also do not deactivate.

We used the difference in dynamics between propionate-bound and no-ligand simulations to understand the effect of propionate binding on OR51E2 - our discussion/model is based on this analysis. We think that the revised version of Extended Data Fig. 9 makes more explicit the simulations that we have conducted that led to our conclusions, and will hopefully clear up any confusion.

Furthermore, it is highly unusual for the receptor conformation to remain similar after removing the G protein. Did the authors observe an inward movement and rotation of TM6? If not, the authors should either perform longer MD simulations or refer to previously published studies where the receptor remains in an active-like conformation after removal of the G protein?

In our simulations of OR51E2 bound to propionate, but with the G protein removed, we do not observe receptor deactivation. If prior simulations of the β 2AR are informative (PMID: 22031696), we do not expect to see full deactivation of the receptor in 1 μ s simulation time. Long timescale simulations of the β 2AR previously done by Ron Dror, DE Shaw and colleagues suggest that the probability of observing deactivation varies depending on the random seed used for system setup. Indeed, only 39 out of 81 simulations led to deactivation, which occurred between 2-30 μ s of simulation time. We now explicitly state this in the results section and cite the prior work by Dror and colleagues. Importantly, our goal in this work was to use simulations to inform us of the narrower conformational ensemble around the EM structure with and without the propionate agonist.

9. The authors compare their OR51E2 structure with an AlphaFold2 model, which is “in an intermediate or inactive conformation characterized by outward displacement of R262.59 and ECL3, a G protein coupling domain in the inactive conformation, and TM6 more inwardly posed compared to active OR51E2.”. It is a known limitation of the pre-generated AlphaFold2 models

that they only cover one state for each protein and, like in this case, sometimes represent an intermediate or even artificial state when the available templates that underlie the models span both states. The use of an inappropriate reference model is problem that affects all parts of this comparison. Furthermore, the authors extended to additional olfactory receptor models: “We next examined the predicted structures of all human ORs, which revealed a largely shared topology for the extracellular region for the broader family.”. It is likely that many of those models share the same problem, as they too would in most cases be based on the same type of mixed templates.

This problem has been mitigated recently with the publication and pre-generation (download-ready) AlphaFold2 models in the correct, active state (https://gpccrdb.org/structure/homology_models, <https://doi.org/10.1093/nar/gkac1013>). These models were generated using AlphaFold2-MultiState, which allows AlphaFold2 to model the correct state by only using templates in the appropriate state. Download-ready models are available of OR51E2 and in total 23 homologous receptors belonging to the O51 subfamily of ORs. To ensure correct conclusions, the authors should update their comparisons to use such models, which can be downloaded or generated again using AlphaFold2-Multistate.

We thank the reviewer for bringing up our use of AlphaFold. To recap, our main reason for using AlphaFold in this manuscript is to assess the diversity of human OR sequences in the extracellular region. We feel comfortable assessing the meaning of pLDDT scores from AlphaFold2 as assessed by Jumper et al *Nature* 2021 (PMID: 34293799) because this has been validated across the proteome. Based on this understanding of pLDDT scores, we suggest that odorant receptor ECL3 is predicted to be more disordered compared to other extracellular regions. It is important to note that the utility of the pLDDT metric is not clear for implementations that alter the AlphaFold pipeline.

While AlphaFold-Multistate is useful for obtaining alternative GPCR conformations in the membrane spanning helices of a GPCR, it suffers from low structural accuracy in extracellular loops. The central premise of the AlphaFold-Multistate approach (along with multiple other similar AlphaFold “hacks”) is to deprecate the depth of the multiple-sequence alignment that Alphafold uses for defining residue contacts. The combination of shallower alignments, combined with a template structure certainly can provide an alternative conformation of a GPCR. However, the shallow multiple-sequence alignment often leads to poor prediction of the extracellular loops compared to structure predictions by normal AlphaFold2. Relevant to this manuscript, the active model of OR51E2 on GPCRdb right now (as predicted by AlphaFold-Multistate) shows an incorrect prediction for the extracellular region as compared to the prediction from regular AlphaFold2 (Fig. R2). More quantitatively, the RMSD (using C α atoms) of OR51E2 prediction by AlphaFold-Multistate to the cryo-EM experimental structure is 5.6 Å using the full sequence of the receptor; the RMSD of the AlphaFold2 prediction compared to the cryo-EM structure is 2.4 Å. When comparing just the extracellular regions (N-terminus, ECL1-3), the RMSD between AlphaFold-Multistate to the cryo-EM experimental structure is 12.2 Å. By contrast, the RMSD between AlphaFold2 and the cryo-EM structure is only 2.0 Å. We

therefore believe that the analysis using output from normal AlphaFold2 is most useful for our analysis of the extracellular region.

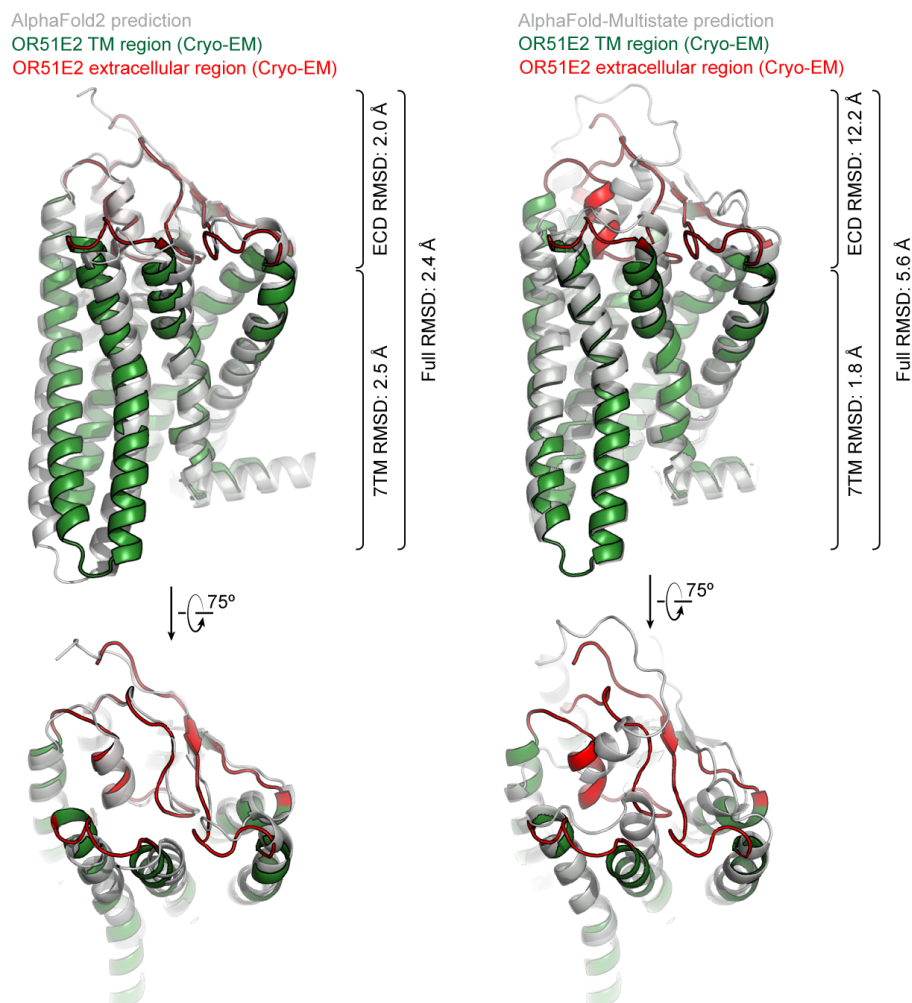


Figure R2. The cryo-EM structure of OR51E2 is overlaid with either the prediction from AlphaFold2 (left) or from AlphaFold-Multistate (right) obtained from GPCRdb. RMSD calculations for OR51E2 Cα atoms were performed for either the full receptor sequences, just the extracellular domain (ECD, depicted in red) or receptor regions excluding the extracellular domain (7TM, shown in green). Notably, the RMSD for the ECD region is significantly worse for AlphaFold-Multistate, primarily because of incorrect folding of ECL2.

10. The manuscript includes the statement “We anticipate that the molecular mechanism we define here for OR51E2 is likely to extend to other Class I ORs that recognize polar, water soluble odorants with multiple hydrogen bond acceptors and donors.”, but is missing data to support this statement. To support this, they authors should describe (for example in an Extended Data Table or supplementary file for download) the percent conservation among all ORs of the identified residue determinants of these molecular mechanisms. They already do so in Extended Data Fig. 6. for residues in the odorant binding pocket.

We have now provided a table that shows percent conservation among all human Class I and Class II ORs at the positions that we analyze in the manuscript in the G protein-coupling and connector regions. This updated table is shown in Extended Data Fig. 7f,g.

Minor comments

1. The abbreviation 7TM is used but is never defined.

We have now defined 7TM as “seven transmembrane” on the first occurrence.

2. There is a newer, more complete reference (doi:10.1016/j.ygeno.2006.04.001) than references 6-7 for the number of human olfactory GPCRs.

We have now included this reference in the text.

3. “Although the overall conformation of OR51E2 and β 2-AR are similar (root-mean-square deviation (RMSD) of 3.2 Å), ...”. RMSD values depend on the region(s) being superposed, please specify which region was used.

Thank you for pointing this out. This was calculated using C α atoms over all resolved regions of both receptors, including the transmembrane helices and the intracellular/extracellular loops. We have now added text to explicitly state this.

4. “Indeed alanine mutagenesis of OR51E2 residues in the G protein coupling region show a dramatic loss of activity for H2436.40, Y2175.58, and R1213.50 associated with poor receptor expression”. The words “receptor mutants” or just “mutants” is missing after the three listed mutations, as it is the overall protein which expresses rather than single residues.

Thanks for pointing this out, it is now corrected.

5. “A phylogenetic tree of human Class A GPCRs was made by analyzing 677 sequences.” The methods section is missing key information hindering reproducibility and understand of what the results reflect. Information should be added about if these were full-length sequences or only segments which can be meaningfully aligned across receptors i.e., residue positions with generic residue numbers (representing the subsequences that can be aligned across receptors). Furthermore, a description of how authors used the reference alignment in EDF1 to ensure that conserved sequence motives in each segment (TM1-7, H8, ICL1-2, and ECL1-2) align correctly.

We now describe the motif and or single residues conserved in all class A GPCRs or specific to non-olfactory class A GPCRs and ORs that were used to align receptors sequences.

6. “Extended Data Fig. 1. ... Transmembrane domains are boxed and labeled in black.” Transmembrane helices differ in length across different receptors, as evident by their structures. This means that some residues inside the black boxes are actually not part of the TM helix,

while other residues outside the boxes are actually part of a TM helix. This needs to be corrected to give correct information. For example, the boxes could be redrawn with varying width across receptors, or replaced with background coloring. Alternatively, a minimal solution would be to use the first and last helical residue of OR51E2 TMHs and define this in the legend.

Extended Data Fig. 1 has now been modified to show the transmembrane domains for each individual receptor.

7. The name of the GPCRmd database should be written with md in lower case.

We have now fixed this.

8. "Olfactory receptors display significant sequence variation ...". Nature and several other journals reserve the word "significant" for statistical contexts.

We have now removed use of "significant" in this and other contexts. For this specific case, we now use "substantial" instead of "significant".

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors answered satisfactorily all of my questions. I have no additional comments.

Referee #3 (Remarks to the Author):

The authors have addressed my comments appropriately and I therefore recommend the manuscript for publication. I have only added a few typo/formatting issues below:

Typos/formatting:

- Ext Dat Fig. 6: 'propionate-contacting residues contacting'
- Order in Supplementary Information file: Suppl. Table 7, Suppl. Fig 1, Suppl. Table 8 (Suppl Figure 1 should probably be listed before/after tables).
- Figure 5f: figure legend states 'human odorant receptor' whereas label inside figure 5f states 'AlphaFold – human olfactory receptors'
- Extended data Fig 3: 'Gold-standard Fourier Shell Correlation (GS-FSC)' (not 'Coefficient')
- Methods: RMSD, line 882 "stableFor this"
- adding a reference for cryosparc (e.g. in methods section or Extended data Fig 3) would be appropriate (reference for Relion was added).

Referee #4 (Remarks to the Author):

I am pleased to have read the revised manuscript "Structural basis of odorant recognition by a human odorant receptor" by Billesbølle et al. and have no further comments. My review comments have been satisfactory resolved through manuscript updates and/or by explanations in the responses in the rebuttal. This has made the revised paper sufficiently precise and clear, also on the nomenclature and computational aspects. Undoubtedly, it will gain much appreciation and attention by the readers of Nature and across the GPCR and odorant research fields.

Best regards,
David Gloriam

Author Rebuttals to First Revision:

Referees' comments:

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We thank the reviewer for their helpful and constructive review.

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- Methods: RMSD, line 882 "stableFor this"
- adding a reference for cryosparc (e.g. in methods section or Extended data Fig 3) would be appropriate (reference for Relion was added).

We have now fixed these issues and thank the reviewer for their careful attention to detail.

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David Gloriam

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