

Structural basis of ligand recognition and gating in mosquito IRs

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The sense of smell is a fundamental sensory ability crucial for animal survival, with the olfactory receptor genes playing a key role in the functioning of the olfactory system. Since Linda Buck and Richard Axel identified this family of genes in mammals in 1991, their function has been revealed in various species, including insects. However, it is only recently that researchers have been able to use advanced techniques to observe how olfactory receptors bind their relevant ligands at the atomic level. Beyond being of great scientific interest, this breakthrough opens up new avenues for developing molecules that target insect olfactory receptors (ORs) for various applications. Another important family of insect olfactory receptors, the ionotropic receptors (IRs), was discovered some years ago as detectors for acids, amines, moisture, and temperature. However, the structural mechanisms underlying ligand specificity and channel activation in these receptors have remained unclear. The manuscript by Li et al. presents a significant advance in the field of insect olfaction by offering valuable insights into the structural mechanisms of the *Anopheles stephensi* IR8a-IR75k complex by using cryo-EM. By resolving the ligand-free, nonanoic acid (NA)-bound, and octanoic acid (OA)-bound states, the authors reveal a dynamic ligand-binding pocket in IR75k, where ligand specificity is determined by the alkyl chain length and carboxylate geometry. These findings highlight a fascinating mechanism where ligand binding induces the closure of IR75k's bilobed ligand-binding domain, triggering conformational shifts that propagate through mechanical linkers to open the pore. This novel mechanism is critical for understanding how mosquitoes detect host odors and could inform the development of targeted insecticides and repellents. Overall, this study represents a milestone in insect olfactory research, with significant implications for vector control strategies. It provides crucial insights into odorant recognition and channel gating of the IR heterocomplex. It also offers structural resources that could support the development of innovative insecticides and repellents for mosquito control. The design of the study is rigorous, and the results are convincing. I do, however, have some concerns and suggestions that could improve the overall strength and clarity of the work. Below, please find my comments:

Major comments

1. It is well established that ionotropic receptors (IRs) often function through a combination of multiple receptors expressed together. I wonder whether only the IR8a-IR75k complex is co-expressed in a single neuron to perform its function, or if other IRs are involved in this process. Is there any evidence to support that only the IR8a-IR75k complex is expressed within a single neuron and thereby defining its functional characteristics?
2. The authors report a 2:2 ratio of the IR8a-IR75k complex in vitro for functional activity. What is the expression ratio of these receptors in vivo, within neurons? Could the authors use genetic techniques, such as reporter gene labeling, to visualize or sequence these receptors at the single-cell level in order to confirm their expression ratio in vivo? This could provide more direct evidence of how these receptors interact in the living neuron.
3. The study focuses on two ligands, nonanoic acid and octanoic acid, to investigate odorant recognition and channel gating of the IR heterocomplex. Have the authors considered testing additional acids or other stimuli to assess the full ligand response profile of the IR8a-IR75k complex. There may be other ligands that can activate the complex, which could offer further insight into its functional range.
4. At the end, the authors briefly mention comparative analyses with vertebrate ionotropic glutamate receptors to identify specific druggable sites for IR inhibition. However, this analysis feels somewhat underdeveloped. Given the detailed structural data of the *Anopheles stephensi* IR8a-IR75k complex presented in the manuscript, the authors could pursue

further work, such as an AI-driven virtual screening process, to identify molecules that can block the binding of nonanoic acid and octanoic acid to the IR8a-IR75k complex. These molecules could then be tested for their bioactivity to confirm their ability to disrupt mosquito responses to these acids, potentially leading to novel mosquito control strategies aimed at interfering with host-seeking behavior. At the same time, I realize that this might be a full topic of a follow-up study.

5. I suggest the authors add a paragraph in the discussion to compare the structural mechanisms of the IR8a-IR75k complex with the OR-Orco heterocomplex. This comparison would provide valuable insights into how these two olfactory receptor families recognize different odorant substances, highlighting the distinct mechanisms underlying their respective functions in insect olfaction.

6. In the results section the authors present highly interesting evolutionary data when comparing different anthropophilic species. It would have been very interesting to see the same comparison with some closely related non-anthropophilic species. Is the difference in host preference mirrored in more drastic changes in the critical, ligand-binding locations of the receptor? Such a comparison might even provide further insights regarding target sites for manipulation of the system.

7. In general, I find the discussion lacking. It is totally concentrated on the "druggable sites." There is so much more to discuss in this highly interesting study. Especially, I lack a deeper discussion regarding the evolution of receptors, and, in connection to point 6 above, I would like to see a specific discussion regarding the evolution of receptor specificity in connection to host-shifts. The data is at the fingertips of the authors.

Minor comments

1. I recommend the authors provide some more background on prior research regarding the function of the IR8a-IR75k complex. Specifically, what ligands are known to interact with IR8a-IR75k, and what are the associated behavioral responses? This additional context would offer compelling evidence for why the authors chose the IR8a-IR75k complex to study the structural mechanisms of the IR complex via cryo-EM.

2. Line 58, The term "olfactory receptors" should be clarified, as it does not include gustatory receptors. It may be helpful to explicitly differentiate these receptor types to avoid any confusion.

3. Line 61, The statement that mosquitoes detect key stimuli such as CO₂ and body heat should specify that this detection occurs at close distances. This is important for understanding the context of mosquito sensory behavior.

4. Line 64, It is important to note that gustatory receptors (GRs) generally detect non-volatile compounds or contact stimuli. This distinction will help clarify their role compared to olfactory receptors.

5. Line 68-69, There is a logical gap in the transition from discussing IRs to introducing ionotropic glutamate receptors (iGluRs) from *Drosophila*. The authors should explain the similarity between these gene families to make the transition clearer and more relevant to the current study.

6. Line 150, There is some confusion in this section. In the abstract, the authors mention reporting the cryo-EM structures of the *Anopheles stephensi* IR8a-IR75k complex in ligand-free (apo), nonanoic acid (NA)-bound, and octanoic acid (OA)-bound states. However, in this section, the authors state that they identified only two primary classes of particles: one representing the apo form of the complex and the other representing the OA-bound form. Could the authors clarify this discrepancy?

7. Line 190, The authors mention that "evolutionary analysis revealed strong conservation of key residues (W288, Y290, F318, R323, Y481, Y485, and E531) among anthropophilic *Anopheles* species (Extended Data Fig. 7), indicating the conservation of the ligand recognition mechanism." If the conservation of the ligand recognition mechanism is being emphasized, it would be beneficial to validate this by testing it in these *Anopheles* species. If such testing is not conducted, I suggest rephrasing this statement to avoid implying direct functional conservation without supporting evidence.

In conclusion, I find this manuscript highly interesting to a wide audience. My main comments concern the evolutionary perspective both in the bioinformatics analysis and in the discussion. I suggest the authors take a careful look at this aspects.

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

Pan et al. provide the first high-resolution single particle CryoEM structures of an insect chemosensory receptor complex from the iGluR-like family of variant invertebrate called the Ionotropic Receptors (IRs). On the surface, this is an important achievement: in the 16 years since these receptors were first identified in *Drosophila melanogaster* in 2009, there have been many genetic studies in *Drosophila* and mosquito to document the importance of these receptors for insect behavior; and there have been a few studies that attempted to understand the mechanism by which these ligand-gated ion channels interact with odorants and activate neurons, largely 2-electrode voltage recordings in *Xenopus* oocytes.

Pan et al. took the appropriate next steps to carry out single particle CryoEM analysis to gain structural insights into IR function. They selected two *Anopheles* IRs with previously known ligands (Ir75k/Ir8a) to attempt to understand the apo unbound form of this presumed dimer of dimers and how it changes when it binds a chemical ligand, in this case either nonanoic acid (NA) or octanoic acid (OA).

The experimental results have a number of serious gaps of evidence that do not justify the strong conclusions and interpretation that the authors reach. Until these gaps are addressed with data, the current results are of limited interest only to specialists in the field.

Major issues:

1. The structures for the ligand bound forms do not include the transmembrane domains. This means that we are left with structures of only the extracellular ligand-binding domains bound to NA or OA. While it is interesting to see how these carboxylic acids interact with Ir75k, the structures do not and cannot provide any insights into how ligand binding impacts gating of these ion channels if the structure does not include any data from the transmembrane domains and the ion-conducting pore. Everything in Figure 4 is therefore purely speculative and needs to be removed or significantly toned down.
2. The mutagenesis of the binding site produces severe impacts on ion conductance, but the authors do not adequately consider the alternative hypothesis that the mutations impact trafficking or protein expression. The photomicrographs in Supplementary Figures 4 and 6 show bright staining in likely intracellular intramembrane compartments but this technique would not resolve the amount of surface expression of the IRs. One would need an extracellular epitope and antibody. Furthermore single-channel recordings would provide much stronger resolution than the whole cell patch recordings provided here. So the interpretations about ligand binding being mediated by these residues is based on incomplete data.
3. Figure 5 and associated text is entirely speculative and adds little to the paper, especially not having the full structure of the dimer of dimers, it feels very premature to speculate about the 'drugability' of these proteins. I would recommend deleting Figure 5 and associated text.
4. The paper lacks key experimental details and it is therefore difficult for others to reproduce these experiments (see below) and the flow of the paper could be improved as it jumps around between different topics

Minor points in order of occurrence:

1. Abstract – the authors should frame the problem in context of what is known about vertebrate iGluRs and why this family is different and interesting and worthy of study.
2. Line 25: most readers will not know that "Ionotropic Receptor" is a specific term for a class of iGluR-like proteins – this should be stated clearly
3. Line 33: the C-lobe is never defined here or later in the paper.
4. Line 39: given the incomplete evidence of mechanism, it is premature to claim that these structures will be useful to prevent malaria.
5. Line 46: delete "hematophagous" – technical jargon and not needed here
6. Line 49: delete the medical terms – the symptoms (itching, redness, swelling) are sufficient to convey the point
7. Line 55: suggest to use "chemosensory receptor" as an overall term for insect ORs, IRs, and GRs because "olfactory receptor" is not precise given the broad ligand tuning of these three multi-gene families
8. Line 56: Anopheles mosquitoes have olfactory neurons in the maxillary palp, proboscis in addition to the antenna
9. Line 57-58: the authors introduce only the concept of the antenna but the maxillary palp expresses the GRs that respond to CO₂
10. Line 65-66: the structural biology of Ors and GRs is still at an early stage and neither the stoichiometry or mechanisms are at a point of consensus. Particularly GRs are likely to function as heteromultimers, this just has not yet been explored. So this part of the introduction should be more general.
11. Line 68-69: this does not seem relevant - the insect iGluRs are highly related to the vertebrate iGluRs for which there are structures. The authors should emphasize here that the insect IRs are highly divergent from iGluRs and that there has been no structural insight (until this paper). I would delete references 35-36 and focus on the unknowns of the IRs rather than the known of the iGluRs
12. Line 75-76: this is not true - no one knows the mechanism of ligand recognition of IRs - all that is known is rather indirect information that they are activated by these ligands, but most of these data are indirect from in vivo measurements in mutants compared to wild type.
13. Line 78-79: reference 37 does not use any mosquito receptors and is a pure characterization of IRs in *Drosophila*. The reference should be changed or the text aligned to the data in the reference
14. Line 86: Please state that this is the major malaria vector in India, not in Africa, where *Anopheles gambiae* is the main vector
15. Line 90-92: this overstates what applications emerge from this paper – recommend to remove or significantly soften the claims
16. Line 97: mosquito genomes are heterogenous within a species. Was there any effort to determine if this is the major haplotype in this strain? What tissue was used to make the cDNA library – what method was used to make it? There are no experimental details anywhere that I could find.
17. Line 102-103: overstates the results – there is no TMD structure
18. Line 108-109: this statement does not follow directly from the data. Ir8a is thought to be a lactic acid receptor – have the authors considered the possibility that the IRs are flexible in contributing as structural elements in one complex and ligand-binding elements in another complex?
19. Line 111: typo – appears to refer to a single transmembrane domain but as the authors know this is a multi-TMD protein family
20. Line 127-128: there is a problem with the flow of the logic – data from the functional assay are mentioned here, but the assay is not introduced until Line 141
21. Line 130-131: overinterpreted – no evidence that steric hindrance is responsible
22. Line 133: these experiments do not provide evidence of correct trafficking – they only confirm that the proteins are expressed – most staining is intracellular.
23. Line 146: important caveat to mention is that these earlier studies used expression in *Xenopus* oocytes not the HEK293 cells used here. There should be some consideration of confounds of the two different heterologous expression systems.
24. Line 155-157: this is the key gap in the paper. Many of the strong conclusions that the authors reach are not evaluable without this information
25. Line 164-165: conclusions overstated given weak data

26. Line 188: the photomicrographs do not show "proper membrane localization" – they show mostly intracellular staining. Nothing can be interpreted from these images to speak to cell surface expression.
27. Line 197-198: greatly overstates the conclusions relative to the available data
28. Line 209: please define C-lobe
29. Line 213-223: without a complete structure of the ligand-bound form of this channel, it is not possible to draw these strong conclusions about gating. This whole passage needs to be deleted or substantially toned down.
30. Line 256-302: I would delete all of this and the associate figure. It is pure speculation and detracts from the paper. There is also no consideration of how these theoretical disruptors of IR function will be deployed in the real world to actual mosquitoes.
31. Line 313: what was the source tissue of the RNA? How was the library made?
32. Line 323: Please define "P/S"
33. Line 348: please provide the missing technical details of how these mutant plasmids were generated. Was the entire ORF sequenced to confirm that only the desired mutations were present?
34. Line 358: "identical conditions" relative to what?
35. Line 378: what was the supplier for NA and OA, the catalogue number, the purity etc?
36. Line 381-383: what was the supplier for the antibody, the catalogue number, the dilution, etc?
37. Line 384: what was the protocol for ECL – no information provided
38. Line 600: 72 references is a lot of references for a brief paper, consider curating them to the most essential
39. Line 680-681: this is the source of the concern that these mutants are just poor expressers or folders, rather than it being LBD mutations.
40. Figure 4: without any data of the TMD in the ligand bound state, everything here is highly speculative and not backed up by experimental data.
41. Figure 5: suggest to delete – entirely speculative

Review prepared by Leslie VossHall

Referee #4

(Remarks to the Author)

Authors present a highly significant structural and functional investigation of the ionotropic receptor (IR) complex IR8a-IR75k from the primary malaria vector, *Anopheles stephensi*. Using single-particle cryo-electron microscopy (cryo-EM), the authors determine the first high-resolution structures of this complex in ligand-free (apo), nonanoic acid (NA)-bound, and octanoic acid (OA)-bound states.¹ Structures reveal that the functional receptor is a (IR8a)-(IR75k) (2:2) heterotetramer, where two IR8a co-receptor subunits form a central scaffold for two ligand-binding IR75k "tuning" subunits.

Authors suggest a "molecular lever" model for channel activation, wherein ligand binding induces a large-scale rotational closure of the IR75k ligand-binding domain (LBD). This conformational change is then propagated via mechanical linkers to the transmembrane domain (TMD), leading to pore opening. This study reveals a detailed molecular basis for the receptor's specificity for medium-chain carboxylic acids.

This is an important study that provides the first atomic-resolution structure of a mosquito ionotropic receptor complex. The work is novel and of importance to the fields of vector biology, insect neurobiology, and structural biology.

Major comments:

A major weakness of this study is the lack of direct biochemical validation for the reported 3D architecture and its proposed subunit interfaces. The model is currently presented without the support of orthogonal experimental evidence. To validate the structural model, the authors must perform additional assays. For instance, cysteine cross-linking experiments to confirm the proximity of specific residues at the proposed interfaces in the functional receptor complex. Such validation is considered a minimal requirement in the field. Authors may see the seminal structural papers on the related iGluR receptors.

Another weakness of this manuscript is that the TMD structure could not be resolved in either the NA- or OA-bound states. Thus, the entire model of pore dilation is an inference based on superimposing the observed LBD movements onto the static, closed-pore TMD structure from the apo state. The causal link between LBD closure and TMD opening is hypothesized, not structurally demonstrated. This limitation must be more prominently acknowledged in the text, and the model should be presented as a well-supported hypothesis rather than a definitive conclusion. Authors may add some additional experiments to strengthen these claims, e.g., by MD simulations.

The authors report the half-maximal effective concentration (EC₅₀) for nonanoic acid (NA) as ~17 μM and for octanoic acid (OA) as ~46 μM. The manuscript would be significantly strengthened by providing a direct measure of binding affinity (K_d), which is distinct from functional potency (EC₅₀). This would allow to deconvolve the energetic contributions of ligand binding from the mechanics of channel gating.

Minor Comments:

The authors mention that NA-bound and OA-bound structures are at resolutions of 2.36 Å and 2.55 Å. It gives the impression that this is the resolution for the entire structure. However, in reality, it's just the ECD domains. This must be explicitly made clear.

A particularly important observation that deserves greater emphasis in the discussion is the unique, non-swapped architecture of the IR8a-IR75k complex. In vertebrate iGluRs, the subunit partners swap between the ATD and LBD layers, creating a complex "dimer of dimers" arrangement that is crucial for receptor assembly and allosteric modulation. The structures presented here reveal a simpler, non-swapped arrangement. This likely represents a significant evolutionary divergence. The authors should comment more explicitly on the functional implications of this architectural difference. Does it allow for greater promiscuity in co-receptor partnering? Does it streamline the receptor for a more direct ligand-gating response, bypassing the allosteric control layer provided by the NTD interactions in iGluRs? Highlighting these key differences would further underscore the novelty of the IR structure and likely its adaptation from a synaptic to a chemosensory role.

The manuscript includes a discussion on several potential "druggable sites" that could be targeted to disrupt mosquito olfaction. While identifying such sites is a valuable outcome of structural work, the claims made in this section are currently without any experimental support and must be considered highly speculative. The authors have not presented any data such as compound screening, binding assays, or functional inhibition assays to validate any of these proposed targets. Therefore, this section should be significantly toned down to more accurately reflect the speculative nature of these ideas.

For the benefit of readers working in the membrane protein biology field, a detailed purification protocol highlighting the approximate detergent concentration at the various steps of purification, and protein concentration would be useful. For example, what was the approximate protein and detergent concentration in the sample applied to the EM grids?

The paragraph on page 11 (lines 247-254) is a summary of the activation mechanism that has already been detailed. It disrupts the narrative flow and may be removed.

On page 13, line 274, the text refers to "Fig. 7," but the manuscript only contains five figures. This must be corrected; the reference is likely to a panel within Figure 5.

The term "Ca atoms" is used throughout the manuscript (e.g., lines 117, 168, 171). The correct notation for alpha-carbons is "C α atoms" and should be corrected.

The manuscript contains several minor typographical and grammatical errors. These must be checked very carefully and corrected.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have made substantial efforts to address the comments raised by all reviewers, and I am now happy to recommend the manuscript for publication. I have only one minor suggestion for the authors to consider. The findings presented in Extended Data Figs. 1d–e, concerning the functional validation and gene expression analysis of IR8a–IR75k in *Anopheles* mosquitoes, are currently described in the Introduction. As these data represent experimental results from the present study, I suggest that they be moved to and briefly described in the Results section rather than in the Introduction.

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

The authors have produced a revision that addresses all of the concerns I raised in the initial review. Most importantly they have addressed the gap that the original submission did not have the full structure of the ligand-bound IR, only the extracellular soluble domain. After what sounds like absolutely heroic work, the authors now come back with the full structure, which makes the paper complete and compelling. I also appreciate that the authors conducted a number of other important experiments requested by the reviewers, specifically exploring the ligand selectivity in more depth, and firming up the heterologous expression of the mutants. The second most important thing the authors have done is to write a proper methods section that now explains all necessary experimental details. The third most important thing the authors have done

is a substantial rewrite to improve the flow of the manuscript and to remove the highly speculative aspects that several reviewers noted. Overall this paper is an order of magnitude or more improved and I am pleased to offer my strong and unqualified support for publication.

Review prepared by Leslie Vosshall

Referee #4

(Remarks to the Author)

The authors have successfully addressed the key concerns raised in the revised manuscript. I congratulate the authors for delivering a high-quality and impactful study that not only provides a structural blueprint for how mosquitoes detect human host odors but also insights into divergence from vertebrate iGluRs.

The determination of the full-length open-state structure, supported by molecular dynamics simulations and rigorous surface-localization controls, provides a solid foundation for their conclusions.

In my opinion, the authors should include the MST results in the supplementary information; omitting binding affinity metrics leaves a minor experimental gap, as they rely entirely on functional EC50 values from heterologous cells to infer ligand sensitivity.

Minor comments:

Figure 1d: Please clarify whether rotameric flexibility was accounted for during minimum pore radius calculations?

Figure 2b: provide specific Hill slope values, which are omitted from the figure legend.

A detailed proofreading of the revised manuscript text reveals several typographical errors, character corruptions, subject-verb agreement mismatches, and grammatical inconsistencies. Below is a categorized list of these errors along with their corresponding locations in the text:

The manuscript still needs a thorough proofreading to weed out some remaining typos and grammatical errors. Some are listed below:

"Ca atoms" is used instead of "C_α" atoms at multiple places in the text. Please fix. Maybe it's getting corrupted during the PDF conversion.

Line 177: "aligned Co atoms" contains a typo

Line 178: Use "asymmetric" instead of "asymmetry" ?

Figure 1 Legend: Typo "tunning" receptor IR75k

Line 673: Use "principal" instead of "principle" ?

Line 720: Use "confirmed" instead of "conformed" ?

Line 777: Use "does" instead of "does " ?

Line 827: Use "NA-bound" instead of "NA-boud" ?

Janesh Kumar

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Point-to-point responses

Referee #1 and Referee #2:

We are truly grateful for the reviewer's thorough and generous evaluation of our work. We greatly appreciate their recognition of the significance of our structural findings on the *Anopheles stephensi* IR8a-IR75k complex. Their insightful and constructive comments have been invaluable in guiding us to further strengthen the depth, clarity, and broader impact of our manuscript. We have carefully considered each point raised and have addressed them in detail below.

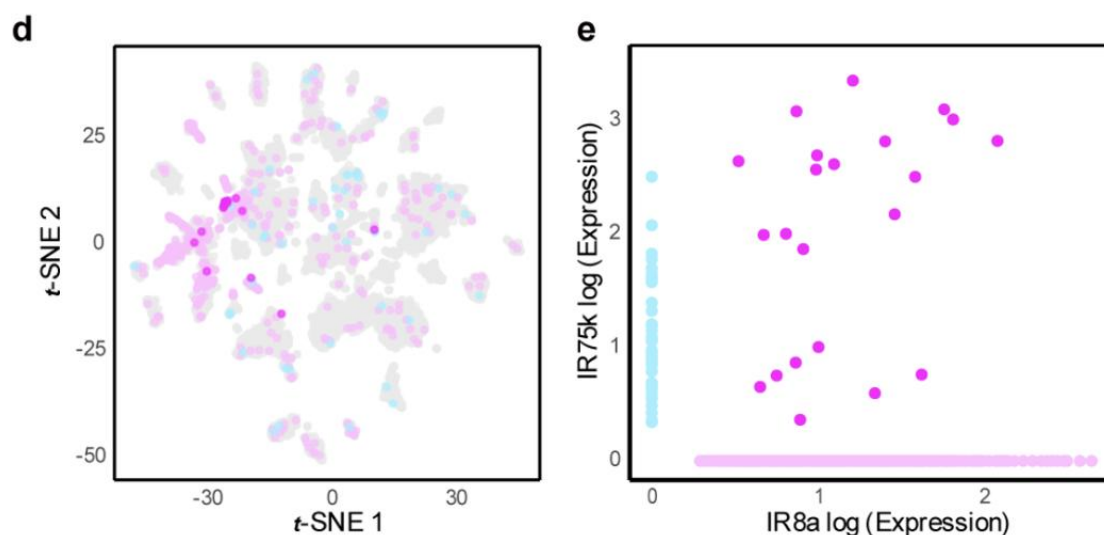
Major concerns:

1. It is well established that ionotropic receptors (IRs) often function through a combination of multiple receptors expressed together. I wonder whether only the IR8a-IR75k complex is co-expressed in a single neuron to perform its function, or if other IRs are involved in this process. Is there any evidence to support that only the IR8a-IR75k complex is expressed within a single neuron and thereby defining its functional characteristics?

We thank the reviewer for raising this important question regarding the co-expression profile of IRs in the olfactory neurons under study. We agree that insect ionotropic receptors can function in combinatorial receptor complexes and that neuronal IR expression patterns may include multiple IR transcripts. To address this issue, we re-examined our single-nucleus RNA-seq dataset, specifically focusing on the neuronal population co-expressing IR75k and IR8a. Within this population, we found that no other IR was universally co-expressed (present in 100% of the nuclei).

Importantly, our biochemical, structural, and electrophysiological data demonstrate that IR8a and IR75k are sufficient to assemble into a functional ligand-gated ion channel responsive to medium-chain carboxylic acids.

In response to the reviewer's comment, we have revised the Introduction to include this expression context and have added Extended Data Fig. 1d and 1e to visually present the co-expression data. We believe these additions further strengthen the physiological relevance of our structural findings.



Extended Data Figs. 1d-e | Function validation and gene expression analysis of IR8a-IR75k from *Anopheles* mosquitoes.

d, t-SNE plot of nuclei, embedding with each nucleus assigned to one of four categories based on the log-normalized co-expression of IR8a and IR75k: no expression of either gene (gray), IR8a only (light pink), IR75k only (light blue), and co-expression of both IR8a and IR75k (purple). **e**, Bivariate scatter plot of log-normalized expression for IR8a (x-axis) and IR75k (y-axis). Each point represents an individual nucleus expressing at least one of the two genes (log-normalized expression > 0), colored as in **d**.

2. The authors report a 2:2 ratio of the IR8a-IR75k complex in vitro for functional activity. What is the expression ratio of these receptors in vivo, within neurons? Could the authors use genetic techniques, such as reporter gene labeling, to visualize or sequence these receptors at the single-cell level in order to confirm their expression ratio in vivo? This could provide more direct evidence of how

these receptors interact in the living neuron.

We thank the reviewer for raising this important question regarding the stoichiometry of IR8a and IR75k *in vivo* versus *in vitro*.

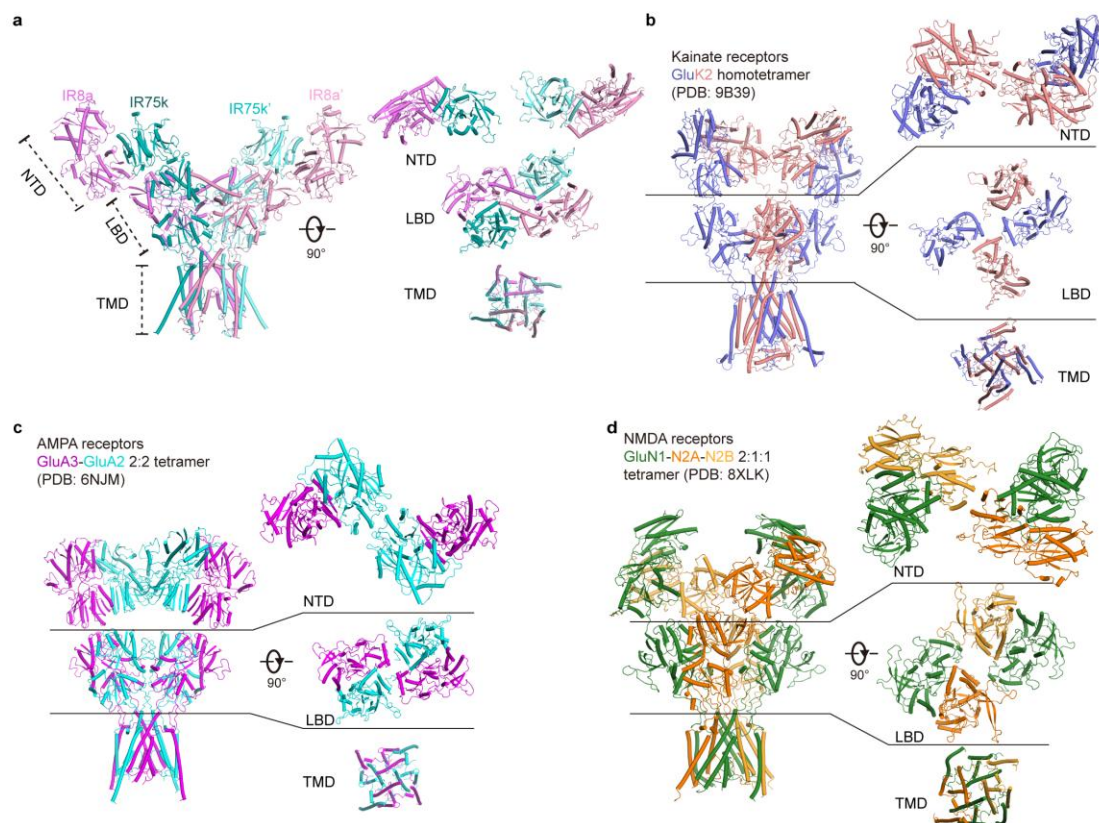
It is important to note that mRNA levels do not necessarily dictate protein assembly stoichiometry, which is shaped by post-transcriptional regulation, trafficking, and subunit dynamics. Nevertheless, our single-nucleus RNA-seq data confirm that IR8a and IR75k are co-expressed within the same neuronal population, supporting their cellular co-localization (Extended Data Fig. 1).

Turning to the assembly stoichiometry, the 2:2 ratio reported in our study is derived from cryo-EM structural analysis of the assembled complex and reflects its functional quaternary organization. Regarding the assembly specificity, our biochemical data argue against alternative stoichiometries. We co-expressed tagged IR8a and IR75k and purified the complex via tandem affinity purification. Had heterogeneous assemblies (e.g., 1:3 or 3:1) existed, they would have been detected as distinct subpopulations during cryo-EM 3D classification; instead, we observed a single, homogeneous 2:2 population. This is further supported by the conserved tetrameric architecture of the iGluR family, which requires four subunits to form a functional pore domain (Extended Data Fig. 3).

We fully agree that direct determination of receptor stoichiometry *in vivo* would be highly valuable. However, such an approach would require technically demanding protein-level quantification—such as endogenous epitope tagging combined with super-resolution imaging or quantitative proteomics of antennal tissue. To our knowledge, endogenous IR protein levels in mosquito antennae are relatively low, and these methods are not yet readily applicable to IRs in insect tissues.

In summary, our structural and biochemical data support a 2:2 assembly as the

functional unit of the IR8a-IR75k receptor, while our transcriptomic data confirms their co-expression *in vivo*. We hope this clarification addresses the reviewer's concern.

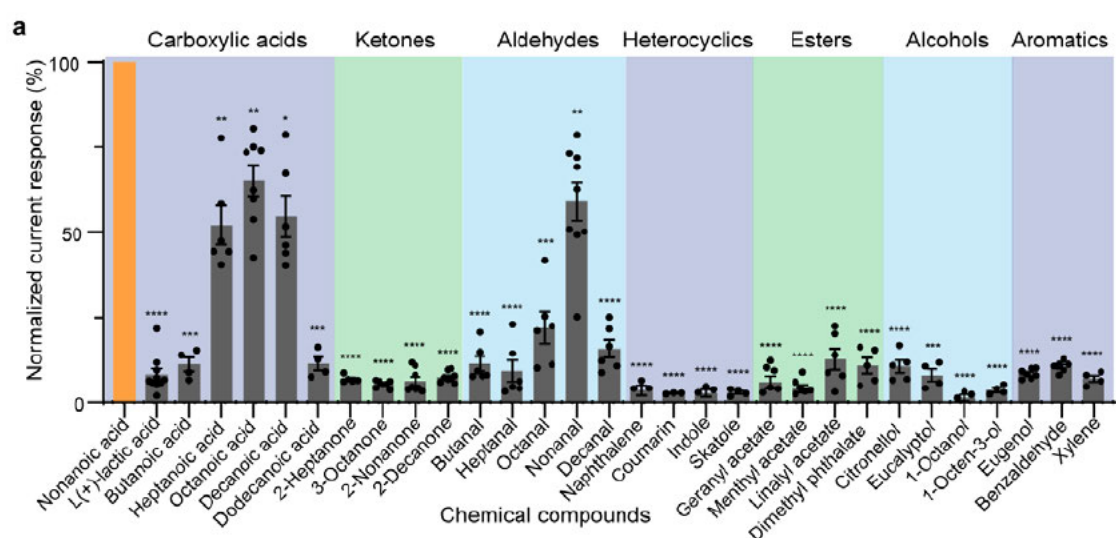


Extended Data Fig. 3| Overall architecture of IR8a-IR75k heterotetramer is different from that of canonical vertebrate iGluRs. a-d, Side and top views of IR8a-IR75k heterotetramer, GluK2 homotetramer (PDB: 9B39), GluA3-A2 2:2 heterotetramer (PDB: 6NJM) and GluN1-N2A-N2B 2:1:1 heterotetramer (PDB: 8XLK). To make it clear, the top views of the four complexes are divided into NTD, LBD and TMD layers. All the structures were prepared in PyMOL if not otherwise indicated.

3. The study focuses on two ligands, nonanoic acid and octanoic acid, to investigate odorant recognition and channel gating of the IR heterocomplex. Have the authors considered testing additional acids or other stimuli to assess the full ligand response profile of the IR8a-IR75k complex. There may be other ligands that can activate the complex, which could offer further insight into its

functional range.

We thank the reviewer for the insightful comment. We fully agree that expanding the ligand panel is essential for comprehensively defining the functional profile of this heteromeric receptor. In response, we have tested a broader range of chemical compounds, including ketones, aldehydes, heterocyclics, esters, alcohols, and aromatic compounds. The results of these additional assays have been incorporated into the revised manuscript as updated Fig. 2a.



Adapted from Fig. 2 | Functional characterization and cryo-EM structure determination of NA-bound full-length open state of IR8a-IR75k.

a, Normalized responses of IR8a-IR75k complex to a panel of chemical compounds. The recombinant IR8a-IR75k complex was expressed in HEK293T cells, and its functional response to various chemical agonists was measured. The compounds are categorized into structural groups. All ligands were applied at 300 μ M. Brown-Forsythe tests and Welch's ANOVA were employed for multiple comparisons between NA and other compounds (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Bars represent mean $\pm$ s.e.m ($n=4-10$, n is the number of experimental cells from which recordings were conducted).

4. At the end, the authors briefly mention comparative analyses with vertebrate ionotropic glutamate receptors to identify specific druggable sites for IR inhibition. However, this analysis feels somewhat underdeveloped. Given the detailed structural data of the *Anopheles stephensi* IR8a-IR75k complex presented in the manuscript, the authors could pursue further work, such as an AI-driven virtual screening process, to identify molecules that can block the binding of nonanoic acid and octanoic acid to the IR8a-IR75k complex. These molecules could then be tested for their bioactivity to confirm their ability to disrupt mosquito responses to these acids, potentially leading to novel mosquito control strategies aimed at interfering with host-seeking behavior. At the same time, I realize that this might be a full topic of a follow-up study.

5. I suggest the authors add a paragraph in the discussion to compare the structural mechanisms of the IR8a-IR75k complex with the OR-Orco heterocomplex. This comparison would provide valuable insights into how these two olfactory receptor families recognize different odorant substances, highlighting the distinct mechanisms underlying their respective functions in insect olfaction.

We sincerely thank the reviewer for these two insightful suggestions. Regarding the druggable site identification and virtual screening, we fully agree that this represents a valuable and logical next step based on our structural data of the *Anopheles stephensi* IR8a-IR75k complex. We believe that a rigorous druggability assessment, especially one involving AI-driven virtual screening followed by experimental bioactivity validation, deserves a dedicated investigation rather than a brief add-on to the current manuscript. Therefore, in this revision, we have removed the preliminary druggable sites prediction. We have kept all relevant structural data, and we are actively pursuing this virtual screening and compound testing as a standalone follow-up study, which we plan to present in the near future.

Regarding the comparison between the IR8a-IR75k complex and the OR-Orco heterocomplex, we greatly appreciate this suggestion. In the revised manuscript, we have added a new paragraph in the Discussion section that systematically compares the structural mechanisms of these two olfactory receptor families, with an emphasis on their distinct ligand recognition modes, gating mechanisms, and functional implications in insect olfaction. We have also incorporated a schematic illustration in the same figure to visually highlight these key differences. We believe this addition significantly strengthens the broader impact of our work by placing the IR complex within the larger context of insect chemosensation. Please refer to Fig. 5.

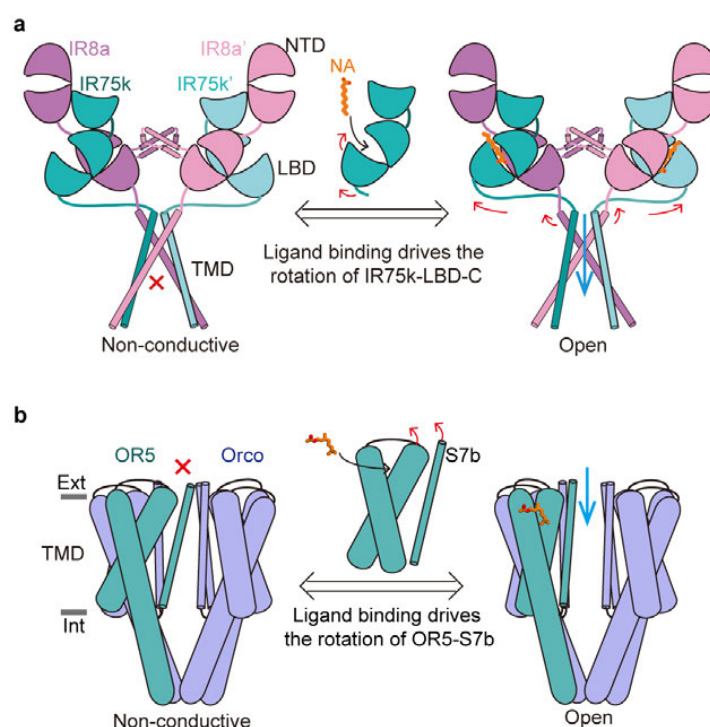


Fig. 5 | Different activation mechanisms of IRs and ORs.

a, Model of ligand induced channel activation of IRs. The subunits are represented with the corresponding colors as in previous figures. Extensive inter-subunit contacts between the extracellular domains of IR8a and IR75k form a largely rigid scaffold, except for the C-lobe of the IR75k-LBD. Upon ligand binding, the IR75k LBD undergoes closure with C-lobe's rotation,

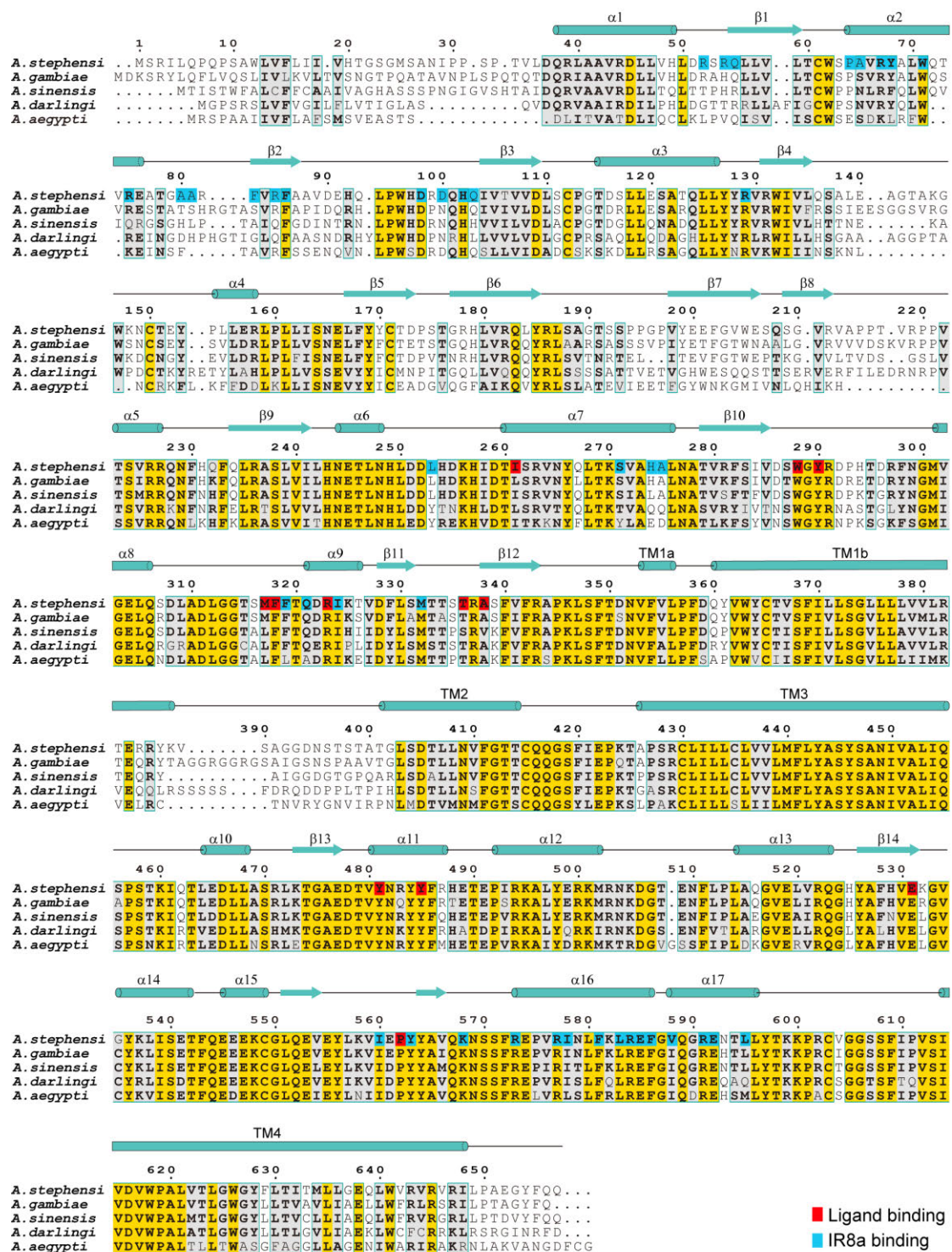
enabling the mechanical linker to transmit this motion to the TMD, ultimately tugging the channel opening. **b**, Model of ligand induced channel activation of ORs. The OR complex is composed of three co-receptor subunits and one tuning receptor. Upon ligand binding, the tuning receptor undergoes a conformational shift that is transmitted to the S7b helix, triggering its rearrangement and the opening of the ion channel pore.

6. In the results section the authors present highly interesting evolutionary data when comparing different anthropophilic species. It would have been very interesting to see the same comparison with some closely related non-anthropophilic species. Is the difference in host preference mirrored in more drastic changes in the critical, ligand-binding locations of the receptor? Such a comparison might even provide further insights regarding target sites for manipulation of the system.

We thank the reviewer for this keen observation. We agree that comparing receptor sequences across species with different host preferences is important for understanding the evolution of ligand specificity.

To this end, our sequence alignment (Extended Data Fig. 9) already includes mosquito species spanning a range of host preferences, including anthropophilic species (e.g., *Anopheles gambiae*, *Aedes aegypti*), opportunistic feeders (e.g., *Aedes albopictus*, *Culex quinquefasciatus*), and more zoophilic species (e.g., *Anopheles sinensis*). Across this diverse set of species, we observe strong conservation of the key residues forming the ligand-binding pocket. This conservation suggests that IR75k's fundamental capacity to recognize medium-chain carboxylic acids is an ancestral trait, and that host preference is more likely shaped by higher-order mechanisms, such as combinatorial receptor expression and neural circuit integration, rather than by sequence variation at the ligand-binding site itself.

To avoid any ambiguity regarding the species coverage, we have revised the results section to explicitly list the species included in our analysis: “To assess whether this ligand recognition mechanism is evolutionarily conserved, we examined the conservation of key binding residues across mosquito species with diverse host preferences, including anthropophilic species (e.g., *Anopheles gambiae*, *Aedes aegypti*), opportunistic feeders (e.g., *Aedes albopictus*, *Culex quinquefasciatus*), and more zoophilic species (e.g., *Anopheles sinensis*) (Extended Data Fig. 9). The key residues (W288, Y290, F318, R323, Y481, Y485 and E531) are strongly conserved across all examined mosquito species, suggesting that the core ligand recognition mechanism is evolutionarily maintained.” We have also clarified this point in the discussion section to emphasize that the conserved ligand-binding pocket across both anthropophilic and non-anthropophilic species points to an ancestral function, with host preference likely emerging at higher levels of olfactory processing. We hope this addresses the reviewer's concern.



Extended Data Fig. 9 | Sequence alignment of IR75k among mosquitoes.

The sequences were aligned using Clustal Omega. Secondary structural elements of *Anopheles stephensi* IR75k are indicated above the sequences. Invariant residues are shaded yellow, and the conserved ones are shaded gray.

The residues involved in ligands binding are shaded red, while the residues participated in IR8a extracellular domains' binding are shaded light blue. The sequence IDs or Uniprot IDs for the aligned sequences are: *Anopheles stephensi*: XP_035916906.1; *Anopheles culicifacies*: ACUA022596; *Anopheles arabiensis*: AARA21_012763; *Anopheles gambiae*: Q7PT48; *Anopheles sinensis*: A0A084WNB9; *Anopheles darlingi*: W5JST1; *Aedes aegypti*: A0A6I8TFY9; *Aedes albopictus*: AALFPA_056866; *Culex quinquefasciatus*: CQUJHB006394. Among all the species, *Anopheles culicifacies*, *Anopheles arabiensis* and *Anopheles sinensis* are non- anthropophilic, others are anthropophilic.

7. In general, I find the discussion lacking. It is totally concentrated on the “druggable sites.” There is so much more to discuss in this highly interesting study. Especially, I lack a deeper discussion regarding the evolution of receptors, and, in connection to point 6 above, I would like to see a specific discussion regarding the evolution of receptor specificity in connection to host-shifts. The data is at the fingertips of the authors.

We thank the reviewer for this insightful comment. We fully agree that the Discussion in our original submission was overly focused on druggable sites and did not adequately explore the broader evolutionary implications of our structural findings.

In the revised manuscript, we have expanded the Discussion to include a dedicated section on receptor evolution and its relationship to host preference. Specifically, we now discuss the conservation of key ligand-binding residues across mosquito species with divergent host preferences and interpret this as evidence for evolutionary constraint on the core ligand recognition and gating functions of IR75k. This conservation suggests that the capacity to recognize medium-chain carboxylic acids is an ancestral trait, and that human host preference is unlikely to be determined by sequence variation in IR75k alone.

We further propose that behavioral diversification, such as shifts in host preference, is more likely achieved through higher-order mechanisms, including combinatorial receptor co-expression, variation in receptor repertoires across species, and neural circuit integration. Our single-nucleus RNA-seq data, which show that IR8a is expressed in a broader population of neurons than IR75k, are consistent with a model in which a conserved core receptor function is combined with flexible tuning and wiring at the systems level, enabling evolutionary adaptation without compromising receptor functionality.

We believe this expanded Discussion enhances the significance of our work and directly addresses the reviewer's concern. The revised text has been incorporated into the manuscript.

Minor comments:

1. I recommend the authors provide some more background on prior research regarding the function of the IR8a-IR75k complex. Specifically, what ligands are known to interact with IR8a-IR75k, and what are the associated behavioral responses? This additional context would offer compelling evidence for why the authors chose the IR8a-IR75k complex to study the structural mechanisms of the IR complex via cryo-EM.

We thank the reviewer for this suggestion. We fully agree that providing sufficient biological context, particularly regarding the known ligands and associated behaviors, is essential for understanding why the IR8a-IR75k complex represents an ideal model for structural investigation.

IR75k is known to directly bind medium-chain carboxylic acids (e.g., nonanoic acid, octanoic acid), which are human skin odor components, while IR8a acts as an obligatory co-receptor required for its *in vivo* function. Behaviorally, this complex is linked to host-seeking in mosquitoes.

Importantly, despite the functional significance of the IR family, no high-resolution structure of any IR complex has been reported. This structural void, combined with the well-characterized ligand and behavioral readouts of IR8a-IR75k, made it an ideal model for our cryo-EM study. We have expanded the Introduction to include this rationale. We hope this addresses the reviewer's concern.

2. Line 58, The term "olfactory receptors" should be clarified, as it does not include gustatory receptors. It may be helpful to explicitly differentiate these receptor types to avoid any confusion.

We thank the reviewer for this careful observation. We agree that the term "olfactory receptors" is imprecise in this context, as it excludes gustatory receptors. To avoid any confusion, we have replaced "olfactory receptors" with "chemosensory receptors" in the revised manuscript

3. Line 61, The statement that mosquitoes detect key stimuli such as CO₂ and body heat should specify that this detection occurs at close distances. This is important for understanding the context of mosquito sensory behavior.

We thank the reviewer for this important clarification. We agree that the spatial context of sensory detection should be specified, as CO₂ is detected at longer ranges, whereas skin odors are relevant at close proximity. Accordingly, we have revised the sentence to read:

" Key stimuli such as CO₂, enables mosquito GRs to detect hosts from more than ten meters, while skin odors, detected by ORs and IRs, allow them to discriminate humans from non-human hosts at close range."

We believe this revision more accurately reflects the spatial context of mosquito host-seeking behavior.

4. Line 64, It is important to note that gustatory receptors (GRs) generally detect non-volatile compounds or contact stimuli. This distinction will help clarify their role compared to olfactory receptors.

We thank the reviewer for this important distinction. We agree that clarifying the difference between olfactory and gustatory receptor families is essential for reader comprehension. In the revised manuscript, we have rephrased the sentence to read:

"Structural studies have shown that insect OR/OR co-receptor (ORco) complexes form heterotetramers for the detection of volatile odorants, whereas GRs have been reported to form homotetramers in recombinant expression systems for non-volatile sugar and fructose recognition. However, in vivo evidence suggests that CO₂-sensitive GRs may also assemble as heteromultimers, although the precise stoichiometry and functional relevance of these assemblies remain to be determined "

We believe this revision more clearly distinguishes the functional roles of ORs and GRs.

5. Line 68-69, There is a logical gap in the transition from discussing IRs to introducing ionotropic glutamate receptors (iGluRs) from Drosophila. The authors should explain the similarity between these gene families to make the transition clearer and more relevant to the current study.

We agree that the reference to the *Drosophila* iGluR ligand-binding domain structure was not directly relevant to our study. To maintain focus and logical flow, we have removed this reference from the revised manuscript.

6. Line 150, There is some confusion in this section. In the abstract, the authors mention reporting the cryo-EM structures of the Anopheles stephensi IR8a-IR75k complex in ligand-free (apo), nonanoic acid (NA)-bound, and octanoic

acid (OA)-bound states. However, in this section, the authors state that they identified only two primary classes of particles: one representing the apo form of the complex and the other representing the OA-bound form. Could the authors clarify this discrepancy?

We apologize for the confusion caused by our previous description. To clarify: in the original submission, we had successfully resolved the ligand-free full length, NA-bound, and OA-bound structures of the extracellular domain (ECD) only. However, the full-length NA-bound structure had remained elusive at that time. After nearly one year of intensive efforts, we are now pleased to report that we have successfully determined the full-length NA-bound open-state structure. In the revised manuscript, we therefore present a complete set of structures, including: (1) the ligand-free full-length closed state (apo state), (2) the NA-bound full-length open state, (3) the NA-bound ECD, and (4) the OA-bound ECD. We believe this expanded structural repertoire significantly strengthens our analysis and clarifies the conformational transitions underlying ligand-induced gating (Extended Data Figs. 2 and 6, Table 1).

Summary of Cryo-EM data

		Apo	NA bound	NA bound (ECD)	OA bound (ECD)
		IR8a-IR75k	IR8a-IR75k	IR8a-IR75k	IR8a-IR75k
EMDB	Accession	81812	81813	81814	81815
ID					
PDB	Accession ID	43EF	43EG	43EH	43EI

7. Line 190, The authors mention that "evolutionary analysis revealed strong conservation of key residues (W288, Y290, F318, R323, Y481, Y485, and E531) among anthropophilic Anopheles species (Extended Data Fig. 7), indicating the conservation of the ligand recognition mechanism." If the conservation of the ligand recognition mechanism is being emphasized, it would be beneficial to validate this by testing it in these Anopheles species. If such testing is not

conducted, I suggest rephrasing this statement to avoid implying direct functional conservation without supporting evidence.

We thank the reviewer for this important suggestion. We fully agree that without experimental validation across species, a claim of functional conservation should be stated with appropriate caution. In response, we have rephrased the original statement in the revised manuscript to avoid over-interpretation.

In addition, we have expanded our sequence alignment analysis to include IR75k orthologs from multiple mosquito genera with different host preferences, including *Anopheles*, *Culex*, and *Aedes* (see Extended Data Fig. 9). This extended alignment reveals that the key residues forming the aromatic cage (W288, Y481, Y485) and those coordinating the carboxylate group (R323, Y290) are largely invariant across these species. Based on this observation, we now interpret our findings as suggesting that the capacity to recognize medium-chain carboxylic acids is likely an ancestral trait, and that human host preference is unlikely to be determined solely by IR75k.

We have revised the text accordingly to ensure our conclusions are appropriately supported by the available evidence.

We are grateful to the reviewers for carefully reading our manuscript and for all the constructive comments.

Referee #3:

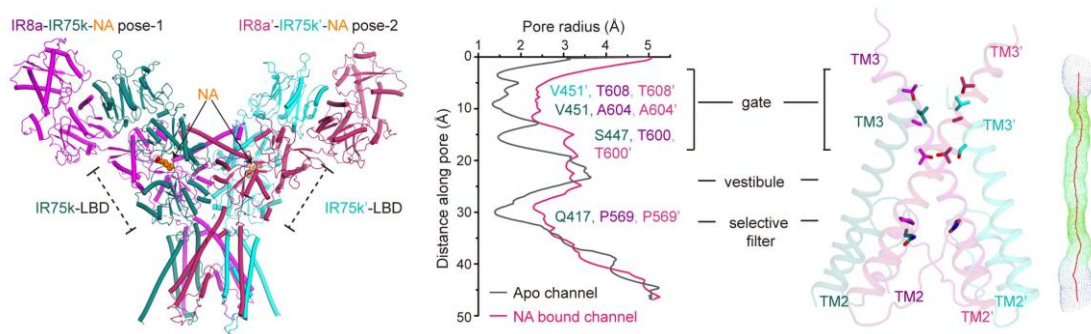
We sincerely thank the reviewer for their critical assessment of our work. We appreciate their acknowledgment of the importance of obtaining high-resolution structural data on insect IR complexes, a goal that has remained elusive since the discovery of these receptors in 2009. We also take seriously the reviewer's concerns regarding the evidentiary support for our conclusions. In response, we have addressed each gap by adding new experimental evidence, re-

analyzing our data, and revising the manuscript. Specifically, we have determined the full-length NA-bound open state structure, expanded our structural comparisons, and modified our interpretations to be more circumspect. We believe these revisions substantially strengthen our conclusions and broaden the appeal of our findings beyond the specialist audience. Below, we provide detailed point-by-point responses to each specific comment.

Major concerns:

1. The structures for the ligand bound forms do not include the transmembrane domains. This means that we are left with structures of only the extracellular ligand-binding domains bound to NA or OA. While it is interesting to see how these carboxylic acids interact with Ir75k, the structures do not and cannot provide any insights into how ligand binding impacts gating of these ion channels if the structure does not include any data from the transmembrane domains and the ion-conducting pore. Everything in Figure 4 is therefore purely speculative and needs to be removed or significantly toned down.

We sincerely thank the reviewer for this critical and important suggestion. Following their recommendation, we devoted substantial effort to determining the full-length NA-bound structure, which proved to be one of the most technically challenging aspects of this revision. We are pleased to report that we have now successfully obtained the open-state structure (see revised Fig. 2c-f). Consequently, all structural analyses presented in Fig. 4 are now based on experimentally determined structures rather than models. We believe this major advance not only addresses the reviewer's concern but also substantially strengthens the overall reliability and impact of our conclusions. The following figure shows the overall open-state structure (*left*), the pore radius calculated using HOLE (*middle*), and the key residues that form the gate and selectivity filter (*right*).



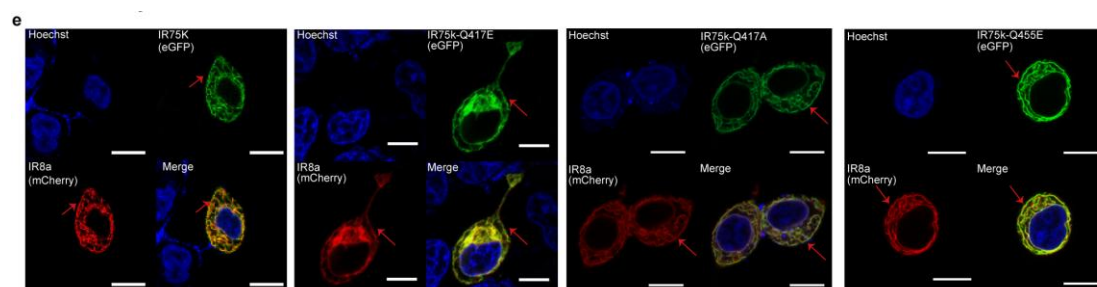
Adapted from Fig. 2 | Overall structure of IR8a-IR75k in complex with nonanoic acid (NA) presents an open state ion channel. *Left:* Structural of IR8a-IR75k heterodimer with NA-bound conformations. *Middle:* HOLE analysis of TMD pore architecture. *Right:* Side views of the channel pore arrangements, formed by the TM3 helices of the four subunits, with corresponding HOLE-generated pore profiles. Residues constituting the gate and selective filter are shown as sticks, as indicated in the middle panel.

2. The mutagenesis of the binding site produces severe impacts on ion conductance, but the authors do not adequately consider the alternative hypothesis that the mutations impact trafficking or protein expression. The photomicrographs in Supplementary Figures 4 and 6 show bright staining in likely intracellular intramembrane compartments but this technique would not resolve the amount of surface expression of the IRs. One would need an extracellular epitope and antibody. Furthermore single-channel recordings would provide much stronger resolution than the whole cell patch recordings provided here. So the interpretations about ligand binding being mediated by these residues is based on incomplete data.

We thank the reviewer for raising this important alternative hypothesis. We fully agree that mutations could potentially affect receptor trafficking or surface expression rather than ligand binding itself, and we have carefully considered this possibility.

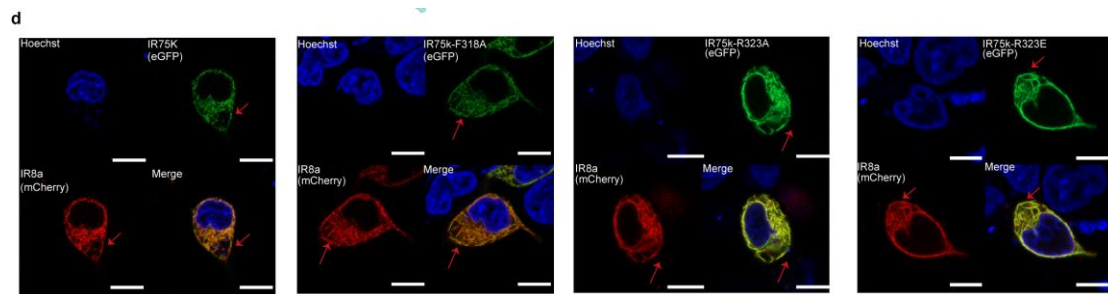
It is worth noting that for membrane proteins overexpressed in mammalian cells, it is entirely expected to observe strong intracellular fluorescence signals associated with the ER and Golgi membranes, as these are the normal biosynthetic compartments along the secretory pathway. Therefore, the presence of intracellular staining alone does not indicate defective trafficking.

To directly address whether the mutants reach the plasma membrane at levels comparable to WT, we performed confocal imaging with the imaging plane precisely focused on the cell surface. Under these conditions, all mutants exhibited surface fluorescence intensities comparable to those of WT (see revised Extended Data Fig. 4e and 8d), indicating that the mutations do not substantially impair membrane delivery.



Extended Data Fig. 4e | Confocal images of IR8a-IR75k variants constituting the ion channel.

Representative confocal microscope images of HEK293T cells transfected with IR8a and IR75k variants: wildtype IR8a-IR75k, IR8a-IR75k-Q417E, IR8a-IR75k-Q417A, and IR8a-IR75k-Q455E. The bar represents 10 μ m in all the confocal microscopy images.



Extended Data Fig. 8d | Confocal characterization of the IR75k variants on its ligand binding.

Representative confocal microscope images of HEK293T cells transfected with IR8a and IR75k variants: wildtype IR8a-IR75k, IR8a-IR75k-F318A, R8a-IR75k-R323A and IR8a-IR75k-R323E.

Following the reviewer's suggestion, we additionally attempted single-channel recordings to further characterize the electrophysiological properties of the receptor. However, we found that single-channel recordings of the IR8a-IR75k complex were technically challenging due to the relatively low channel activity of this receptor. For WT IR8a-IR75k, single-channel activity was detected in only 11 out of 71 independent recordings (~15.5%), with representative traces shown in the following figure. Given this low detection frequency for the WT receptor, successful recording of single-channel activity from non-functional mutants would be expected to be even more challenging. Indeed, for the R323A mutant, we performed 12 independent recordings and did not detect any channel-opening events.

WT: 11/71 positive patches (~15.5%)



R323A: 0/12 positive patches



Single-channel recording of IR8a and IR75k (top) and IR8a and IR75k variant (bottom)

These results are consistent with our whole-cell electrophysiological data showing a complete loss of ligand-induced currents for the R323A mutant. At the same time, we acknowledge that single-channel recording alone cannot distinguish whether the loss of activity results from impaired trafficking, altered gating, or defects in ligand activation. Therefore, surface expression analysis remains essential for addressing the trafficking possibility.

Taken together, our confocal imaging data demonstrates that the mutant receptors are delivered to the plasma membrane, while the absence of both whole-cell currents and detectable single-channel activity in mutants support a functional defect in ligand recognition or gating rather than a trafficking deficiency.

We have also revised representative confocal images with red arrows indicating surface expression in Extended Data Figs. 4e and 8d to better illustrate the comparable membrane localization of WT and mutant receptors.

3. Figure 5 and associated text is entirely speculative and adds little to the paper, especially not having the full structure of the dimer of dimers, it feels very premature to speculate about the 'drugability' of these proteins. I would recommend deleting Figure 5 and associated text.

We thank the reviewer for this constructive suggestion. In response, we have removed Figure 5 and its associated text from the revised manuscript.

In addition, we have also expanded the Discussion to include a mechanistic comparison of the gating mechanisms between IR and OR receptors in insects, which we believe adds valuable context to our structural findings. We are grateful to the reviewer for helping us focus on the most solid and rigorous aspects of our work.

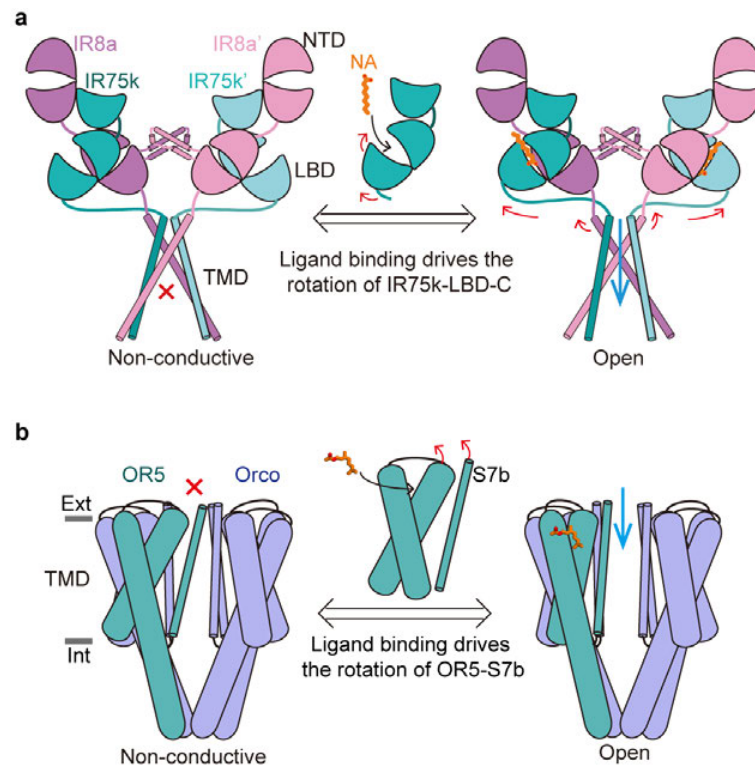


Fig. 5 | Different activation mechanisms of IRs and ORs.

a, Model of ligand induced channel activation of IRs. The subunits are represented with the corresponding colors as in previous figures. Extensive inter-subunit contacts between the extracellular domains of IR8a and IR75k form a largely rigid scaffold, except for the C-lobe of the IR75k-LBD. Upon ligand binding, the IR75k LBD undergoes closure with C-lobe's rotation, enabling the mechanical linker to transmit this motion to the TMD, ultimately tugging the channel opening. **b**, Model of ligand induced channel activation of ORs. The OR complex is composed of three co-receptor subunits and one tuning receptor. Upon ligand binding, the tuning receptor undergoes a conformational shift that is transmitted to the S7b helix, triggering its rearrangement and the opening of the ion channel pore.

4. The paper lacks key experimental details and it is therefore difficult for others to reproduce these experiments (see below) and the flow of the paper could be improved as it jumps around between different topics

We appreciate the reviewer's careful reading and constructive critique. To address the experimental details, we have substantially expanded the Materials and Methods section to include: Detailed purification protocols, including detergent concentrations at each step and the final protein and detergent concentrations in the sample applied to cryo-EM grids; Complete cryo-EM data processing parameters and refinement procedures; Detailed electrophysiology recording and MD simulation protocols. To improve the logical flow, we have restructured the Results section to follow a more coherent sequence: from structural determination to ligand recognition analysis, to activation mechanism, and finally to evolutionary implications and mechanism comparisons, minimizing unnecessary topic jumps. We trust these revisions will facilitate reproducibility and enhance the overall clarity of the work.

Minor points in order of occurrence:

1. Abstract – the authors should frame the problem in context of what is known about vertebrate iGluRs and why this family is different and interesting and worthy of study.

We thank the reviewer for this suggestion. In the revised manuscript, we have incorporated the following text into the Abstract to better frame the problem:

"Insect IRs are evolutionarily related to the ionotropic glutamate receptor (iGluR) superfamily, whose members adopt a tetrameric channel architecture. However, unlike iGluRs (NMDA, AMPA, or Kainate receptors), which are gated by glutamate in vertebrate neuronal systems, insect IRs have evolved to detect a diverse array of small molecules, including acids, amines, and temperature changes."

2. Line 25: most readers will not know that "Ionotropic Receptor" is a specific term for a class of iGluR-like proteins – this should be stated clearly

We agree with the reviewer. To ensure clarity for a broad readership, we have added a clear definition of IRs early in the Introduction: "Insect IRs are evolutionarily related to the ionotropic glutamate receptor (iGluR) superfamily "

We thank the reviewer for this suggestion.

3. Line 33: the C-lobe is never defined here or later in the paper.

We thank the reviewer for pointing this out. The definition of "C-lobe" has now been clearly stated in the revised manuscript.

4. Line 39: given the incomplete evidence of mechanism, it is premature to claim that these structures will be useful to prevent malaria.

We agree with the reviewer and have rephrased the sentence to avoid overstatement. The revised text now reads: "These insights provide an atomic-resolution view of IR-mediated host detection in mosquitoes and establish a foundation for the rational design of next-generation mosquito control strategies targeting host-seeking behavior."

5. Line 46: delete "hematophagous" – technical jargon and not needed here

We agree with the reviewer and we have removed it from the sentence to improve readability for a broader audience.

6. Line 49: delete the medical terms – the symptoms (itching, redness, swelling) are sufficient to convey the point

We thank the reviewer for this suggestion. The sentence has been revised to focus on the symptoms only.

7. Line 55: suggest to use "chemosensory receptor" as an overall term for insect ORs, IRs, and GRs because "olfactory receptor" is not precise given the broad ligand tuning of these three multi-gene families

We agree with the reviewer. Since GRs are not olfactory receptors, ORs and IRs represent a distinct family with broader chemosensory functions, we have replaced "olfactory receptor" with "chemosensory receptor" as suggested and revised other instances accordingly.

8. Line 56: Anopheles mosquitoes have olfactory neurons in the maxillary palp, proboscis in addition to the antenna

We agree with the reviewer. We have revised the sentence to explicitly include the maxillary palps and proboscis alongside the antenna: "Their chemosensory receptors, located on the sensory neurons of the antenna, maxillary palps and proboscis of *Anopheles* mosquitoes, detect a variety of human-associated chemicals."

9. Line 57-58: the authors introduce only the concept of the antenna but the maxillary palp expresses the GRs that respond to CO₂

We thank the reviewer for this important clarification. We have revised the text to specify that CO₂ is detected by GRs expressed in the maxillary palp "Key stimuli such as CO₂, enables GRs on the maxillary palps to detect hosts from more than ten meters."

10. Line 65-66: the structural biology of Ors and GRs is still at an early stage and neither the stoichiometry or mechanisms are at a point of consensus. Particularly GRs are likely to function as heteromultimers, this just has not yet been explored. So this part of the introduction should be more general.

We thank the reviewer for this important correction. We have revised the introduction to be more general, acknowledging that GR stoichiometry, including the possibility of heteromultimers, remains to be fully explored.

"Structural studies have shown that insect OR/OR co-receptor (ORco) complexes form heterotetramers for the detection of volatile odorants, whereas GRs have been reported to form homotetramers in recombinant expression systems for non-volatile sugar and fructose recognition. However, observations that multiple sugar- or bitter-sensitive GRs and CO₂-sensitive GRs are co-expressed in the same gustatory neurons suggest that these receptors may form heteromultimeric complexes, although the precise stoichiometry and functional relevance of these assemblies remain to be determined."

11. Line 68-69: this does not seem relevant - the insect iGluRs are highly related to the vertebrate iGluRs for which there are structures. The authors should emphasize here that the insect IRs are highly divergent from iGluRs and that there has been no structural insight (until this paper). I would delete references 35-36 and focus on the unknowns of the IRs rather than the known of the iGluRs

We thank the reviewer for this excellent suggestion. We have deleted references 35-36 and revised the text to emphasize that insect IRs are highly divergent from iGluRs, and that no structural insight has been available until the present study.

12. Line 75-76: this is not true - no one knows the mechanism of ligand recognition of IRs - all that is known is rather indirect information that they are activated by these ligands, but most of these data are indirect from in vivo measurements in mutants compared to wild type.

We thank the reviewer for this correction. We have revised the text to clarify that existing evidence for IR ligand recognition is depending on *in vivo* assays, and that the structural mechanisms have remained unknown.

13. Line 78-79: reference 37 does not use any mosquito receptors and is a pure characterization of IRs in Drosophila. The reference should be changed or the text aligned to the data in the reference

We thank the reviewer for catching this. We agree that reference 37 (*Drosophila* IR characterization) was not appropriate for a statement about mosquito receptors. We have replaced it with relevant mosquito IR references and rephrased the text.

14. Line 86: Please state that this is the major malaria vector in India, not in Africa, where Anopheles gambiae is the main vector

We thank the reviewer for this clarification. The text has been revised accordingly.

15. Line 90-92: this overstates what applications emerge from this paper – recommend to remove or significantly soften the claims

We thank the reviewer for this important suggestion. We agree that the original wording overstated the direct applications emerging from this study. To address this, we have significantly softened the claim in the revised manuscript. The sentence now reads: "These findings provide atomic-level insights into mosquito host-seeking and may inform the future design of strategies aimed at disrupting olfactory-driven behavior." We believe this revision more accurately reflects the scope of our study.

16. Line 97: mosquito genomes are heterogenous within a species. Was there any effort to determine if this is the major haplotype in this strain? What tissue was used to make the cDNA library – what method was used to make it? There are no experimental details anywhere that I could find.

We thank the reviewer for raising these important technical points. We have now added detailed experimental information to the Materials and Methods section, including: (1) RNA was extracted from adult female antennal tissue from the laboratory Hor strain of *Anopheles stephensi* (a gift from Jingwen Wang Lab at Fudan University, Shanghai, China; *Feng et al., Nature Microbiology, 2022*); (2) The CDS of IR8a and IR75k were initially amplified from antennal cDNA (synthesized using Superscript III Reverse Transcriptase System (Invitrogen)) but showed poor HEK293 expression, so we codon-optimized and commercially synthesized the genes; and (3) We have not systematically assessed haplotype variation. However, based on the six clones we sequenced, all of them showed identical CDS with no variations in the nucleotide sequences. We believe these additions address the reviewer's concerns.

17. Line 102-103: overstates the results – there is no TMD structure

For the apo structure, we did have the TMD resolved.

We thank the reviewer for this comment. In the revised manuscript, we have made this distinction clearer by specifying which structures include the TMD and which are limited to the ECD (Extended Data Figs. 2 and 6, Table 1).

Summary of Cryo-EM data

		Apo	NA bound	NA bound (ECD)	OA bound (ECD)
		IR8a-IR75k	IR8a-IR75k	IR8a-IR75k	IR8a-IR75k
EMDB	Accession	81812	81813	81814	81815
ID					
PDB	Accession ID	43EF	43EG	43EH	43EI

18. Line 108-109: this statement does not follow directly from the data. Ir8a is thought to be a lactic acid receptor – have the authors considered the possibility

that the IRs are flexible in contributing as structural elements in one complex and ligand-binding elements in another complex?

We thank the reviewer for this insightful comment. We agree that the role of a given IR subunit is likely context-dependent and may differ depending on its partner. In the IR8a-IR75k complex, our structural and functional data clearly demonstrate that IR75k is the ligand-binding tuning subunit, while IR8a serves as the co-receptor. We also note that, to our knowledge, IR8a has only been suggested to be involved in lactic acid detection in *Aedes aegypti*, but no direct evidence has demonstrated that it functions as a binding receptor for lactic acid (Raji et al., *Current Biology*, 2019). In *Drosophila*, IR8a has been suggested to function as potential co-receptor to form ion channel with IR64a in detecting acetic acid (Ai et al., *Journal of Neuroscience*, 2013). In this work, to avoid over-interpretation, we have revised the text to carefully state that our conclusions regarding IR8a as a co-receptor in the IR8a-IR75k complex. We have also added a sentence acknowledging the potential context-dependent functional flexibility of IR subunits. We thank the reviewer for helping us clarify this important point.

19. Line 111: typo – appears to refer to a single transmembrane domain but as the authors know this is a multi-TMD protein family

We thank the reviewer for this comment. We would like to clarify that "a transmembrane domain" is used to describe the entire membrane-spanning region of the protein, which comprises multiple transmembrane helices.

20. Line 127-128: there is a problem with the flow of the logic – data from the functional assay are mentioned here, but the assay is not introduced until Line 141

We thank the reviewer for pointing this out. We have reorganized the text to introduce the functional assay before presenting the corresponding data. We have also carefully reviewed the manuscript to ensure logical flow throughout.

21. Line 130-131: overinterpreted – no evidence that steric hindrance is responsible

We thank the reviewer for this important correction. We agree that attributing the enhanced ion influx to reduced steric hindrance was speculative without direct evidence. We have revised the text to simply state the observation without speculating on the underlying mechanism.

22. Line 133: these experiments do not provide evidence of correct trafficking – they only confirm that the proteins are expressed – most staining is intracellular.

As addressed in our response to Major Concern #2, confocal imaging confirms that all mutants reach the plasma membrane at WT levels, ruling out trafficking defects as the primary cause of the loss of function. The text has been revised accordingly.

23. Line 146: important caveat to mention is that these earlier studies used expression in Xenopus oocytes not the HEK293 cells used here. There should be some consideration of confounds of the two different heterologous expression systems.

We thank the reviewer for this important technical point. We fully agree that the different heterologous expression systems warrant consideration. We have now addressed this in the revised manuscript with two lines of evidence:

(1) Published precedent: HEK293 cells have been widely and successfully used for functional characterization of insect ORs and GRs (*Wang et al., 2026*;

Ma et al., 2024; Rinker et al., 2012; Liu et al., 2016), establishing this system as a well-validated platform for insect chemosensory receptor studies.

(2) Direct cross-system comparison: We compared the functional properties of our *Anopheles* IR8a/IR75k complex expressed in HEK293 cells with those of a functionally homologous *Aedes* IR8a/IR75k complex previously characterized in *Xenopus* oocytes (*Ray et al., PLoS Negl Trop Dis, 2023*). The EC₅₀ values are comparable between the two systems: for NA, ~16 μ M (our HEK293 system) vs. 8–18 μ M (*Xenopus*); for OA, ~46 μ M (our HEK293 system) vs. ~25 μ M (*Xenopus*). This cross-system consistency validates our HEK293-based approach.

REDACTED, this was a reproduction of Fig. 4 from Ray et al.,
<https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0011402>

We have incorporated this discussion into the revised manuscript to address the reviewer's concern.

24. Line 155-157: this is the key gap in the paper. Many of the strong conclusions that the authors reach are not evaluable without this information

25. Line 164-165: conclusions overstated given weak data

We thank the reviewer for highlighting these concerns. In the original submission, the TMD was indeed unresolved in the ligand-bound states. In the revised manuscript, however, we have now determined the full-length NA-

bound open-state structure, which includes the TMD and directly visualizes the pore in its dilated conformation. This new structure provides the critical information needed to support our conclusions. We have updated the relevant figures and text accordingly.

26. Line 188: the photomicrographs do not show “proper membrane localization” – they show mostly intracellular staining. Nothing can be interpreted from these images to speak to cell surface expression.

As addressed in Major Concern #2, confocal imaging confirms that all mutants reach the plasma membrane at WT levels, ruling out trafficking defects. The text has been revised accordingly.

27. Line 197-198: greatly overstates the conclusions relative to the available data

We thank the reviewer for this important correction. We agree that the original wording overstated the conclusions. We have revised the sentence to more accurately reflect the scope of our findings:

" Our structural insights provide a molecular framework for understanding how mosquitoes detect and differentiate host-derived volatile compounds."

28. Line 209: please define C-lobe

We thank the reviewer for pointing this out. "C-lobe" has now been defined at its first appearance in the revised manuscript.

29. Line 213-223: without a complete structure of the ligand-bound form of this channel, it is not possible to draw these strong conclusions about gating. This whole passage needs to be deleted or substantially toned down.

As detailed in our response to Major Concern #1, we have now determined the full-length NA-bound open-state structure. All gating-related analyses are now based on experimental data, and the text has been revised accordingly.

30. Line 256-302: I would delete all of this and the associated figure. It is pure speculation and detracts from the paper. There is also no consideration of how these theoretical disruptors of IR function will be deployed in the real world to actual mosquitoes.

We thank the reviewer for this suggestion. As discussed in our response to Major Concern #3, we have removed Fig. 5 and the entire associated discussion from the revised manuscript to eliminate speculation.

31. Line 313: what was the source tissue of the RNA? How was the library made?

We thank the reviewer for pointing out these omissions. We have now clarified two separate points in the Methods section:

(1) For recombinant expression in HEK293 cells, the coding sequences (CDS) of IR8a and IR75k were codon-optimized for mammalian expression and commercially synthesized.

(2) For single-nucleus RNA-seq analysis, RNA was extracted from dissected antennae of adult female mosquitoes.

We have updated the Methods section accordingly with these details.

32. Line 323: Please define "P/S"

We thank the reviewer for pointing this out. "P/S" has now been defined as "penicillin/streptomycin" in the revised manuscript.

33. Line 348: please provide the missing technical details of how these mutant plasmids were generated. Was the entire ORF sequenced to confirm that only the desired mutations were present?

We thank the reviewer for pointing this out. We have now added the missing details to the Methods section. Specifically, all mutants were generated using the site-directed two-step PCR mutagenesis method, and the entire ORF of each construct was sequenced to confirm that only the desired mutations were introduced.

34. Line 358: "identical conditions" relative to what?

We thank the reviewer for pointing out this ambiguity. We have revised the text to clarify that "identical conditions" refers to the same cell culture conditions (e.g., temperature, CO₂, medium) used for all transfected cells during protein expression.

35. Line 378: what was the supplier for NA and OA, the catalogue number, the purity etc?

We thank the reviewer for pointing this out. The catalog numbers and purity information for NA (HEOWNS, Cat# N-00494, 98% purity) and OA (HEOWNS, Cat# O-38930, 98% purity) have now been added to the Materials and Methods section.

36. Line 381-383: what was the supplier for the antibody, the catalogue number, the dilution, etc?

We thank the reviewer for pointing out this omission. We have now added the following antibody details to the Materials and Methods section: anti-Strep Tag II mouse monoclonal antibody (for IR8a detection, Sangon Biotech, Cat# D191106) and anti-Flag-Tag rabbit monoclonal antibody (for IR75k detection,

Abcam, Cat# ab205606), followed by HRP-conjugated goat anti-mouse IgG (Cwbio, Cat# D110087-0025) and HRP-conjugated goat anti-rabbit IgG (Proteintech, Cat# SA0001-2). Primary and secondary antibodies were both diluted 1:5,000.

37. Line 384: what was the protocol for ECL – no information provided

We thank the reviewer for pointing this out. We have now added the catalogue number for the kit of ECL experiment in the revised manuscript.

38. Line 600: 72 references is a lot of references for a brief paper, consider curating them to the most essential

We thank the reviewer for this suggestion. We note that the 72 references include 20 references that are cited exclusively in the Methods section. After separating the Methods references from the main text reference list, the revised manuscript now contains 55 references in the main text (including references for software). We believe this appropriately addresses the reviewer's concern.

39. Line 680-681: this is the source of the concern that these mutants are just poor expressers or folders, rather than it being LBD mutations.

We thank the reviewer for pointing this out. As detailed in our response to Major Concern #2, we have now directly addressed the trafficking question through confocal imaging focused on the plasma membrane, which confirms that all mutants reach the cell surface at levels comparable to WT. We have revised the text accordingly to clarify that the loss of function is due to defective ligand recognition/gating rather than impaired expression or trafficking.

40. Figure 4: without any data of the TMD in the ligand bound state, everything here is highly speculative and not backed up by experimental data.

We thank the reviewer for this concern. As detailed in our response to Major Concern #1, we have now determined the full-length NA-bound open-state structure, which resolves the TMD and directly visualizes the pore in its dilated conformation. Consequently, all structural analyses presented in Fig. 4 are now based on experimentally determined structures rather than inferences. We have revised the figure and the associated text accordingly.

41. Figure 5: suggest to delete – entirely speculative

We thank the reviewer for this suggestion. As noted in our response to Major Concern #3, we have removed Fig. 5 and its associated text from the revised manuscript.

We are grateful to the reviewer for carefully reading our manuscript and for all the constructive comments.

Referee #4:

We are deeply grateful to the reviewer for their generous and thoughtful assessment of our study. We greatly appreciate their acknowledgment of the importance of our work—providing the first atomic-resolution structures of a mosquito IR complex across multiple functional states—and its relevance to vector biology, insect neurobiology, and structural biology. We are also encouraged by their positive reception of our proposed "molecular lever" model for IR channel activation. The constructive criticisms and suggestions offered by the reviewer have been extremely helpful in refining our manuscript. We have responded to each point in detail below.

Major comments:

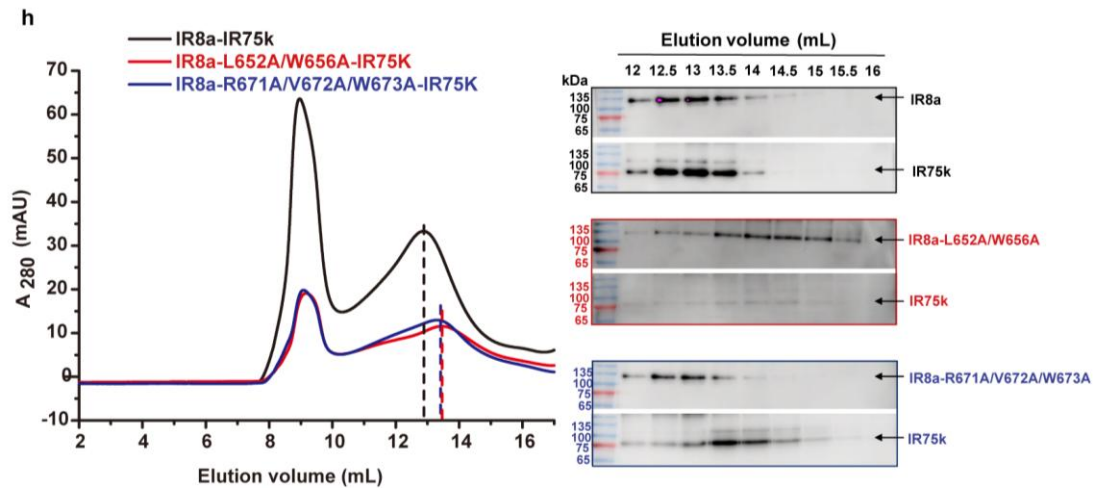
1. A major weakness of this study is the lack of direct biochemical validation for the reported 3D architecture and its proposed subunit interfaces. The model is

currently presented without the support of orthogonal experimental evidence. To validate the structural model, the authors must perform additional assays. For instance, cysteine cross-linking experiments to confirm the proximity of specific residues at the proposed interfaces in the functional receptor complex. Such validation is considered a minimal requirement in the field. Authors may see the seminal structural papers on the related iGluR receptors.

We appreciate the reviewer's emphasis on orthogonal biochemical validation of our structural model. We agree that such evidence strengthens confidence in the proposed architecture, and we have indeed performed two complementary experiments.

Extended Data Fig. 11h presents co-migration and western blot analyses demonstrating the formation of the IR8a-IR75k heterocomplex, which provides direct biochemical support for the complex formation.

We also pursued cysteine cross-linking as the reviewer recommended. However, we encountered a technical obstacle: in the absence of DTT, all subunits co-migrated irrespective of cysteine mutation, rendering the results inconclusive. Despite extensive optimization, we were unable to overcome this limitation. We trust that the combination of high-resolution cryo-EM and biochemical co-migration evidence provides a solid foundation for the proposed architecture.



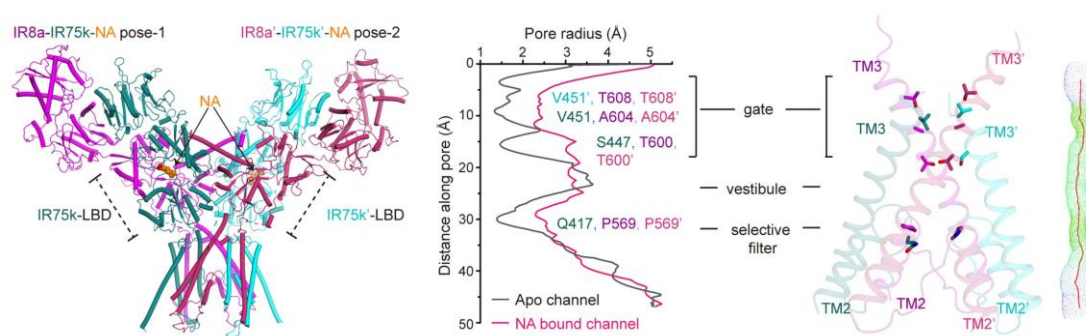
Extended Data Fig. 11 | IR8a and IR75k were stuck together except for the C-lobe of IR75k-LBD which can move freely.

h, Purification and analysis of IR8a-IR75k variants. *Left*: Final purification of the WT IR8a-IR75k (black curve), IR8a-L652A/W656A-IR75k (red curve) and IR8a-R671A/V672A/W673A-IR75k (blue curve) by SEC. Dotted line indicates the elution peak position. *Right*: Western blot of corresponding SEC fractions confirms complex integrity. L652A/W656A on IR8a disrupts its dimerization, impairing IR75k binding. R671A/V672A/W673A of IR8a directly abolishes the IR75k binding.

2. Another weakness of this manuscript is that the TMD structure could not be resolved in either the NA- or OA-bound states. Thus, the entire model of pore dilation is an inference based on superimposing the observed LBD movements onto the static, closed-pore TMD structure from the apo state. The causal link between LBD closure and TMD opening is hypothesized, not structurally demonstrated. This limitation must be more prominently acknowledged in the text, and the model should be presented as a well-supported hypothesis rather than a definitive conclusion. Authors may add some additional experiments to strengthen these claims, e.g., by MD simulations.

We thank the reviewer for this critical and constructive comment. We fully agree that a conclusive pore-opening model requires direct structural evidence of the open-pore TMD, rather than inference from LBD movements alone.

In response, we have now experimentally determined the full-length NA-bound open state structure, which resolves the TMD region and directly visualizes the pore in its dilated conformation (see new Figs. 2c-f, 4 and the flowing figure).



Adapted from Fig. 2 | Overall structure of IR8a-IR75k in complex with nonanoic acid (NA) presents an open state ion channel. *Left:* Structural of IR8a-IR75k heterodimer with NA-bound conformations. *Middle:* HOLE analysis of TMD pore architecture. *Right:* Side views of the channel pore arrangements, formed by the TM3 helices of the four subunits, with corresponding HOLE-generated pore profiles. Residues constituting the gate and selective filter are shown as sticks, as indicated in the middle panel.

In addition, following the reviewer's excellent suggestion, we have performed molecular dynamics (MD) simulations to further elucidate the conformational dynamics (comparison between close-state and open state) and ion permeation. These simulations corroborate our structural observations and provide independent support for the proposed gating mechanism.

We are particularly grateful to the reviewer for recommending the MD simulations, which have proven invaluable in strengthening our mechanistic model. We have revised the manuscript accordingly to present these new data

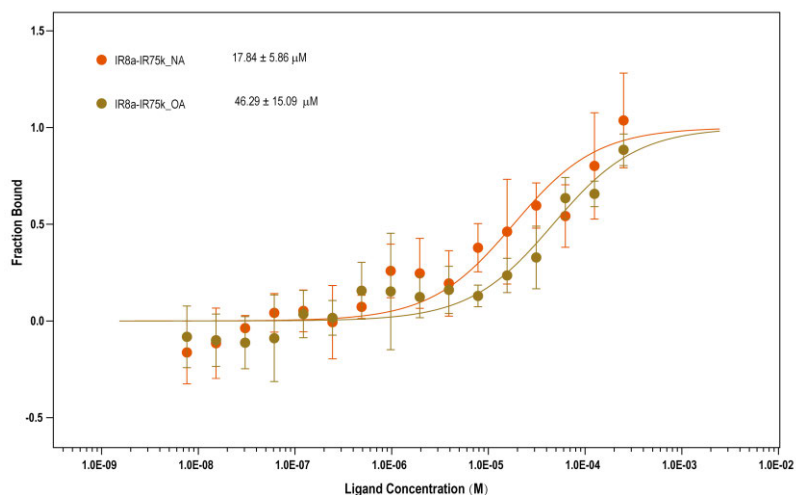
and to frame our model as a structurally validated mechanism. We hope this addresses the reviewer's concern.

3. The authors report the half-maximal effective concentration (EC₅₀) for nonanoic acid (NA) as ~17μM and for octanoic acid (OA) as ~46μM. The manuscript would be significantly strengthened by providing a direct measure of binding affinity (K_d), which is distinct from functional potency (EC₅₀). This would allow to deconvolve the energetic contributions of ligand binding from the mechanics of channel gating.

We appreciate the reviewer's insightful suggestion to distinguish binding affinity from functional potency. To directly measure binding affinity, we carried out microscale thermophoresis (MST) binding assays for NA and OA with the IR8a-IR75k complex. The estimated K_d values, approximately 17.8 μM for NA and 46.3 μM for OA, are closely aligned with the corresponding EC₅₀ values from our electrophysiological recordings.

However, the MST fitting curves show relatively large error bars. This is largely attributable to the technical challenges inherent to membrane protein binding assays—the detergent-solubilized IR complex is not in its native lipid environment, and the hydrophobic nature of the carboxylic acid ligands can lead to non-specific interactions. In addition, our IR samples contain multiple conformational states. These factors collectively limit the precision of the measurements.

We have therefore presented this MST data here for the reviewer's reference, but given the technical limitations, we have chosen not to include them in manuscript to avoid over-interpretation. Instead, we rely on the structural definition of the binding pocket and the rigorously determined EC₅₀ values as the primary evidence for ligand recognition. We hope this addresses the reviewer's concern. The following figure shows the MST results.



Microscale thermophoresis (MST) measurement about the binding affinities of IR8a-IR75k with NA and OA. n represents the replicates number of the experiment, and n=5. Data represent mean $\pm$ SEM.

Minor Comments:

1. The authors mention that NA-bound and OA-bound structures are at resolutions of 2.36 Å and 2.55 Å. It gives the impression that this is the resolution for the entire structure. However, in reality, it's just the ECD domains. This must be explicitly made clear.

We thank the reviewer for this important clarification. We agree that the original wording may have been misleading. In the revised manuscript, we have now clearly specified that the reported resolutions of 2.36 Å (NA-bound) and 2.55 Å (OA-bound) correspond to the extracellular domain (ECD) of the complex, rather than the entire structure. We have also explicitly stated the resolution of the full-length open structure where applicable. We appreciate the reviewer's careful attention to this important technical detail.

Summary of Cryo-EM data

Apo	NA bound	NA bound (ECD)	OA bound (ECD)
IR8a-IR75k	IR8a-IR75k	IR8a-IR75k	IR8a-IR75k

EMDB	Accession	81812	81813	81814	81815
ID					
PDB	Accession ID	43EF	43EG	43EH	43EI

2. A particularly important observation that deserves greater emphasis in the discussion is the unique, non-swapped architecture of the IR8a-IR75k complex. In vertebrate iGluRs, the subunit partners swap between the ATD and LBD layers, creating a complex "dimer of dimers" arrangement that is crucial for receptor assembly and allosteric modulation. The structures presented here reveal a simpler, non-swapped arrangement. This likely represents a significant evolutionary divergence. The authors should comment more explicitly on the functional implications of this architectural difference. Does it allow for greater promiscuity in co-receptor partnering? Does it streamline the receptor for a more direct ligand-gating response, bypassing the allosteric control layer provided by the NTD interactions in iGluRs? Highlighting these key differences would further underscore the novelty of the IR structure and likely its adaptation from a synaptic to a chemosensory role.

We appreciate the reviewer's thoughtful and constructive observation regarding the evolutionary significance of the non-swapped IR architecture. We agree that this aspect merits more explicit discussion.

In the revised manuscript, we have expanded the Discussion to address three key implications of this architectural divergence:

First, the non-swapped arrangement of IRs contrasts sharply with the domain-swapped "dimer of dimers" architecture of vertebrate iGluRs, suggesting a progressive structural simplification that may accompany the functional transition from synaptic neurotransmission to olfactory chemosensation.

Second, the modular organization, with a central IR8a scaffold and peripheral IR75k ligand-binding subunits, may enable flexible partnering between co-receptors and multiple tuning receptors, providing a structural basis for the combinatorial diversity observed in the IR olfactory system.

Third, the absence of an allosteric ATD layer suggests a more direct coupling between ligand binding and pore opening in IRs, bypassing the complex allosteric regulation characteristic of iGluRs, which may lead to much simpler and more efficient signal transduction.

We have incorporated these points into the Discussion and are grateful to the reviewer for highlighting this important direction.

3. The manuscript includes a discussion on several potential "druggable sites" that could be targeted to disrupt mosquito olfaction. While identifying such sites is a valuable outcome of structural work, the claims made in this section are currently without any experimental support and must be considered highly speculative. The authors have not presented any data such as compound screening, binding assays, or functional inhibition assays to validate any of these proposed targets. Therefore, this section should be significantly toned down to more accurately reflect the speculative nature of these ideas.

We thank the reviewer for this important suggestion. We agree that the druggable sites discussion was speculative without experimental validation. Accordingly, we have deleted this section entirely from the revised manuscript, including the associated figure.

In place of this material, we have expanded the Discussion to include a mechanistic comparison of insect IR and OR gating mechanisms, which we believe provides a more substantive and evidence-based contribution to the

field. We thank the reviewer for guiding us to present our findings with appropriate restraint and scientific rigor.

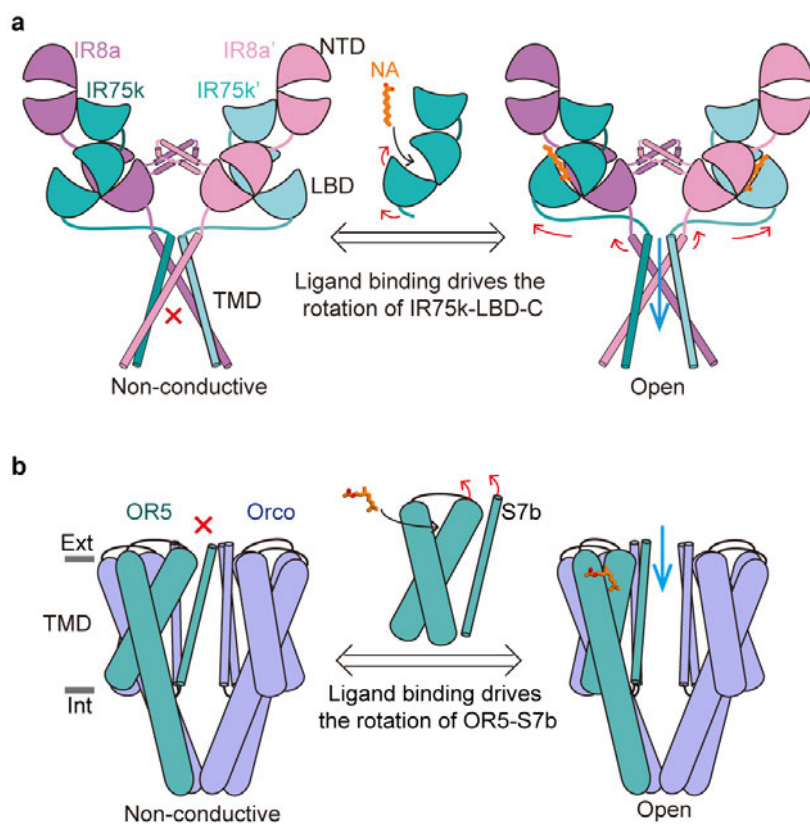


Fig. 5 | Different activation mechanisms of IRs and ORs.

a, Model of ligand induced channel activation of IRs. The subunits are represented with the corresponding colors as in previous figures. Extensive inter-subunit contacts between the extracellular domains of IR8a and IR75k form a largely rigid scaffold, except for the C-lobe of the IR75k-LBD. Upon ligand binding, the IR75k LBD undergoes closure with C-lobe's rotation, enabling the mechanical linker to transmit this motion to the TMD, ultimately tugging the channel opening. **b**, Model of ligand induced channel activation of ORs. The OR complex is composed of three co-receptor subunits and one tuning receptor. Upon ligand binding, the tuning receptor undergoes a conformational shift that is transmitted to the S7b helix, triggering its rearrangement and the opening of the ion channel pore.

4. For the benefit of readers working in the membrane protein biology field, a detailed purification protocol highlighting the approximate detergent concentration at the various steps of purification, and protein concentration would be useful. For example, what was the approximate protein and detergent concentration in the sample applied to the EM grids?

We appreciate the reviewer's suggestion. In the revised Materials and Methods section, we have now included a comprehensive description of buffer conditions throughout the purification workflow—from solubilization through to the final SEC step. We have also explicitly stated the catalog numbers for key reagents, as well as the approximate protein concentration of the sample applied to EM grids. We trust these additions will facilitate replication of our protocol by other laboratories.

5. The paragraph on page 11 (lines 247-254) is a summary of the activation mechanism that has already been detailed. It disrupts the narrative flow and may be removed.

We thank the reviewer for this suggestion. We have removed the redundant paragraph from the revised manuscript to improve the narrative flow.

6. On page 13, line 274, the text refers to "Fig. 7," but the manuscript only contains five figures. This must be corrected; the reference is likely to a panel within Figure 5.

7. The term "Ca atoms" is used throughout the manuscript (e.g., lines 117, 168, 171). The correct notation for alpha-carbons is "C α atoms" and should be corrected.

8. The manuscript contains several minor typographical and grammatical errors. These must be checked very carefully and corrected.

We thank the reviewer for carefully reading our manuscript and for pointing out these errors. We have now thoroughly proofread the entire manuscript and corrected all typographical and grammatical errors we could identify.

We are grateful to the reviewer for carefully reading our manuscript and for all the constructive comments.

Point-to-point responses

Referee #1 and Referee #2:

The authors have made substantial efforts to address the comments raised by all reviewers, and I am now happy to recommend the manuscript for publication. I have only one minor suggestion for the authors to consider. The findings presented in Extended Data Figs. 1d–e, concerning the functional validation and gene expression analysis of IR8a–IR75k in *Anopheles* mosquitoes, are currently described in the Introduction. As these data represent experimental results from the present study, I suggest that they be moved to and briefly described in the Results section rather than in the Introduction.

Response:

We thank the referees for this insightful suggestion and for the positive assessment of our revised manuscript. We agree that the data in Extended Data Figs. 1d–e are experimental findings from this study and belong in the Results section. We have therefore moved the corresponding description from the Introduction to the Results and adjusted the surrounding text accordingly.

Referee #3:

The authors have produced a revision that addresses all of the concerns I raised in the initial review. Most importantly they have addressed the gap that the original submission did not have the full structure of the ligand-bound IR, only the extracellular soluble domain. After what sounds like absolutely heroic work, the authors now come back with the full structure, which makes the paper complete and compelling. I also appreciate that the authors conducted a number of other important experiments requested by the reviewers, specifically exploring the ligand selectivity in more depth, and firming up the heterologous expression of the mutants. The second most important thing the authors have

done is to write a proper methods section that now explains all necessary experimental details. The third most important thing the authors have done is a substantial rewrite to improve the flow of the manuscript and to remove the highly speculative aspects that several reviewers noted. Overall this paper is an order of magnitude or more improved and I am pleased to offer my strong and unqualified support for publication.

Response:

We sincerely thank the referee for the very positive assessment of our revised manuscript and for recognizing the substantial experimental and textual revisions undertaken in response to the previous round of review. We are particularly grateful for the referee's constructive suggestions in the previous round, which proved instrumental in motivating us to determine the full-length ligand-bound structure, carry out additional functional experiments, expand the Methods section, and restructure the manuscript. These suggestions have significantly strengthened the study.

Referee #4:

The authors have successfully addressed the key concerns raised in the revised manuscript. I congratulate the authors for delivering a high-quality and impactful study that not only provides a structural blueprint for how mosquitoes detect human host odors but also insights into divergence from vertebrate iGluRs.

The determination of the full-length open-state structure, supported by molecular dynamics simulations and rigorous surface-localization controls, provides a solid foundation for their conclusions.

Response:

We thank the referee for the careful evaluation of our revised manuscript and for the positive assessment of the full-length open-state structure, molecular dynamics simulations, and functional validation. We have carefully considered

the remaining comments and have revised the manuscript accordingly.

[Comment:](#)

In my opinion, the authors should include the MST results in the supplementary information; omitting binding affinity metrics leaves a minor experimental gap, as they rely entirely on functional EC₅₀ values from heterologous cells to infer ligand sensitivity.

[Response:](#)

We thank the referee for this thoughtful suggestion. We agree that a direct measurement of ligand-binding affinity could, in principle, provide complementary information to the functional EC₅₀ measurements. We therefore carefully re-evaluated the MST experiments and the resulting datasets.

However, the MST measurements showed substantial experimental variability and did not yield sufficiently robust and reproducible quantitative affinity estimates. We are therefore concerned that including these measurements, even in the Supplementary Information, could lead to overinterpretation of binding-affinity values that we do not consider quantitatively reliable.

Importantly, throughout this study, ligand sensitivity is evaluated using electrophysiological measurements, which directly report receptor activation. The EC₅₀ values for NA and OA, as well as the functional characterization of the ligand-binding-pocket variants, were all obtained within the same electrophysiological framework. We believe that maintaining this internally consistent functional assay provides the most rigorous basis for comparing ligand responses in the present study. For these reasons, we have chosen not to include the MST data in the revised manuscript.

We nevertheless appreciate the reviewer's point and have carefully ensured that the manuscript does not interpret the electrophysiologically determined EC₅₀ values as direct equilibrium binding affinities.

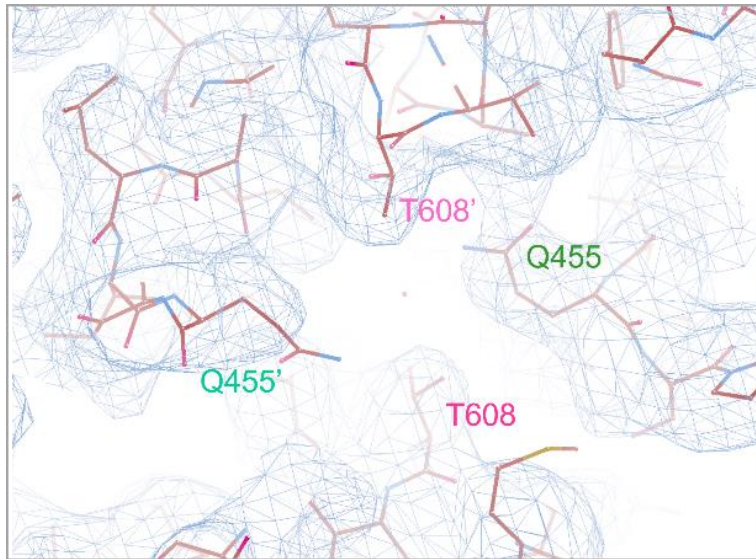
[Minor comments:](#)

Figure 1d: Please clarify whether rotameric flexibility was accounted for during minimum pore radius calculations?

Response:

We thank the referee for raising this important point. In our HOLE analysis, we used the single side-chain conformation observed in the cryo-EM map rather than performing explicit rotameric sampling. We consider this appropriate for the following reason: the side chains of Q455 in IR75k and T608 in IR8a, which define the narrowest constriction of the pore, are well resolved in the cryo-EM density, and the structure model shows close agreement with the corresponding local map densities. Thus, the conformations used for pore-radius calculation are directly supported by experimental density rather than by unconstrained rotamer selection.

To make this point more transparent, here, we have added local iso-mesh representations of the cryo-EM density surrounding Q455 and T608, showing the close agreement between the modeled side-chain conformations and the experimental density. We have also clarified the corresponding description in the revised manuscript. The reported minimum pore radius therefore reflects the experimentally supported side-chain conformations. We note that alternative rotamers could in principle affect the exact value of the minimum radius; we have therefore described the calculated radius as corresponding to the modeled conformation rather than as an absolute geometric property of the channel.



Local cryo-EM density (blue mesh, contoured at 6σ) for the pore-constricting residues Q455 of IR75k and T608 of IR8a in the apo structure, showing well-resolved side-chain conformations in the same viewing orientation as in Fig. 1d.

Figure 2b: provide specific Hill slope values, which are omitted from the figure legend.

Response:

We thank the reviewer for pointing out this omission. We have now added the Hill slope values to the Fig. 2b legend. The Hill Slopes are 0.84 ± 0.14 and 1.06 ± 0.21 for nonanoic acid (NA) and octanoic acid (OA), respectively.

The manuscript still needs a thorough proofreading to weed out some remaining typos and grammatical errors.

Response:

We thank the reviewer for carefully identifying these issues. We have thoroughly proofread the revised manuscript and corrected the indicated typographical, formatting, and grammatical errors, as well as additional issues identified during our proofreading.