nature portfolio

Peer Review File

Structural basis of orientated asymmetry in a mGlu heterodimer

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.
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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have used single-molecule Forster resonance energy transfer (smFRET) to probe the conformational states of the mGlu2-mGlu4 receptor. Sophisticated methods are used to label the mGlu2 and mGlu4 subunits with donor and acceptor fluorophores. The experimental smFRET data and analysis are of high quality and generally support the inferences drawn. However, I have a few comments that I would like the authors to address.

1. Were the smFRET experiments performed using purified receptors that were reconstituted in detergent micelles or membrane mimetics ? This should be stated at the beginning of the smFRET data presentation, for example on line 234.
2. While the E=0.56 and E=0.3 appear to be the major FRET states present, the histograms also reveal minor populations at very low or very high FRET efficiency. There is no explanation offered for these species. The authors should offer suggestions as to the origin of these minor FRET states.
3. It would be helpful to include a table in the SI summarizing the peak positions and peak areas obtained from the four-state fits to the FRET histograms, for each sample condition.
4. The abstract states “Single-molecule FRET data illustrate the constant oscillation of monoliganded mGlu2-4 between the intermediate states and an active one”. In fact, there are no results presented that reveal dynamic transitions between different FRET states. This statement should be removed.

Reviewer #2

(Remarks to the Author)
The study sought to elucidate the structural basis for the preferential activation of G proteins by mGlu4 within the mGlu2/mGlu4 heterodimers, despite the intrinsic capability of both mGlu2 and mGlu4 to form homodimers that can activate G proteins independently. The authors presented cryo-EM structures of the mGlu2/mGlu4 heterodimers bound with two specific antagonists (targeting mGlu2 and mGlu4 respectively), an agonist for mGlu2, an agonist for mGlu4, and the endogenous agonist Glu combined with an mGlu4-specific positive allosteric modulator (PAM) and Gi protein. These structures unveiled the PAM VU0155041 binding site on mGlu4 and the functional significance of the interacting residues was assessed. Notably, the activation process involved a transition in the dimer interface from a TM5-TM5 interaction to a TM6/7-TM6 interaction. The team further explored the partially activated states of the mGlu2/mGlu4 dimer induced by agonists for either mGlu2 or mGlu4, reinforcing the importance of the CRD-ELC2 interaction in dimer activation. A comparison with previously reported structures revealed consistent intra-subunit conformational changes within both mGlu2 and mGlu4. Through functional analysis, they suggested that G protein activation within the heterodimer was mediated by mGlu4. The manuscript culminates with an examination of Gi protein's interaction with mGlu4.

Overall, the manuscript provides useful structural information on the mGlu2/mGlu4 heterodimers in different states. However, it does not fully elucidate the initial question it poses regarding why mGlu4 preferentially interacts with and activates G proteins within the heterodimer context.

Main Comments:

1. There appears to be a discrepancy between the data presented in Figures 3 (d-g) and 4 (f-g), where Figure 3 suggests

that an mGlu2-specific agonist promotes a more efficient transition to an active conformation compared to an mGlu4-specific agonist, conflicting with the conclusion that mGlu4 is the primary mediator of G protein signaling within the heterodimer. Clarification and further analysis of these observations would be beneficial.

2. Are there differences in the binding affinity and stability between mGlu2 and mGlu4 for Gi in the presence of glutamate? Such data could provide valuable insights into the preferential interaction of mGlu4 with Gi.

3. It remains unclear whether complexes involving mGlu2/mGlu4 heterodimers and Gi can be effectively formed in the presence of either an mGlu2-specific or an mGlu4-specific agonist. Additional experiments to investigate these potential complexes would enhance the understanding of the activation mechanism.

Minor Comments:

1. The observation that point mutations entirely negate the effect of VU0155041 in Figure 1h is intriguing and warrants further discussion regarding the critical interactions of this PAM with mGlu4.

2. Given the reported poor density for VU0155041 in the complex, the reliability of its orientation should be evaluated by MD simulations.

3. The high constitutive activity observed with T793C + P803C mutants in Figure 2e is surprising and merits additional context, particularly in relation to the structural basis of this phenomenon.

4. While the smFRET data provide valuable insights into the equilibrium among various conformations, the claim of revealing the structural basis for partial activation seems overstated.

5. The similar effects of the mGlu2 agonist (LY379268) and the endogenous agonist (Glu) on activation, contrasted with the near-control level effect of the mGlu4 agonist (L-AP4), in Figure 3 (d-g) requires explanation. Elaboration on the differential efficacy of these agonists could clarify the activation mechanism of the heterodimer.

Reviewer #3

(Remarks to the Author)
Huang et al., 2024 report on structural analyses of a class C GPCR heterodimer comprising mGlu2 and mGlu4 in four different states: inactive (antagonist bound to each protomer), 2x intermediate inactive states (with subtype selective agonists bound), and an active complex (mGlu2/4+glutamate+PAM+Gi protein). The cryo-EM analyses report on heterogenous sample populations, with each state ultimately being solved with map resolutions ranging from 3.7 to 5.95Angstroms. Local refinement has allowed discrete domains of the active complex to achieve slightly higher resolution. Domain architecture in these new structures is compared within the new structures presented as well as with prior work including mGlu2 and 4 homodimers as well as mGlu2/4 heterodimer with G protein complexed with mGlu2 (as opposed to mGlu4 herein). Biochemical (cys cross-linking) and biophysical (smFRET) studies are included to cross-validate and enrich structural observations. Mutagenesis studies complement the structural information and largely support the observations. Collectively, the study provides insights into how agonist binding in the VFT domains of these dimeric proteins is transmitted to yield asymmetric G protein coupling.

Regarding specific claims made:
It is a stretch to refer to the PAM binding mode as entirely ‘new’. mGlu4 PAM (VU0155041) binding pocket in the 7TM domain overlaps in part with the common allosteric binding site observed across multiple class C GPCR family members. With VU0155041 bound to mGlu4 is the lower part of the pocket empty? Or is the resolution too low to ascertain? For completeness this comparison to allosteric modulator binding poses across class C GPCRs (Extended Fig 5a) should also include the mGlu2/4 heterodimer: mGlu2 PAM: JNJ-40411813/ADX-71149 & mGlu4 PAM: ADX-88178 [PDB: 8JD5].
The VU0155041 binding mode is supported by mutagenesis testing the effect on a single concentration of PAM. Noting quantification of PAM data is limited.

smFRET data is used in an attempt to explain different agonist occupied states and partial agonism from single occupancy of VFT dimer. These experiments do how transitions between states and different proportions in an agonist dependent manner. However, without adding G proteins into the system these are incomplete and cannot truly explain partial agonism as claimed (lines 241, 244, 362).

line 244-245: “Of note, the mGlu2 specific agonist is more likely to induce a reorientation than a mGlu4 specific agonist.” Is this observation linked to relative intrinsic efficacy of the agonists tested? Do mGlu2 and mGlu4 orthosteric agonists achieve similar or different maximal responses relative to glutamate?

Extending findings to general conclusions about mGlu2 not being able to optimally couple G proteins (line 392). There is a need to acknowledge caveats regarding the artificial systems being used here, and the limited use of Gi3 only in BRET G protein recruitment assays.

Regarding data presentation, analysis and visualisation:
Applicable to all statistical representations, generally a single threshold level of significance is predefined. Therefore,

reporting on different levels of significance is not recommended. In addition, there a number of issues/queries regarding the presentation of the pharmacological datasets.
Fig 2, how was one-way ANOVA performed on these data sets? Do these data report on multiple ANOVAs comparing the three groups for each mutation? Or some other approach? What post-test was used to determine differences in dimers between conditions and mutations?

For data reported in extended table 2, expression and functional Emax data are expressed as % of control. How were one-way ANOVA statistical tests performed given the control used for comparison have been normalised and do not have associated error? The non-normalised data should be used to determine if Emax differences are significant. If this was the case, the methods should be updated accordingly. This impacts data presented in Figures 3c, 4b-g, 5e, Extended Figures 7e-f

Fig 4, net BRET data have been normalised to the control data for each agonist. However, this normalisation obscures any potential differences in the maximal effect of the different agonists used. If the raw data are compared, do the maximal responses of glutamate, L-AP4 or LY379268 differ at the mGlu2/4 heterodimer or homodimers? This is relevant to interpretation on Emax increases for glutamate at mGlu2/2 mutations, if glutamate is a partial agonist at mGlu2/2 WT.

IP1 assay data are normalised to 100% of control, but not clear how 0% was defined from the description. Typically, these types of data are presented as actual IP1 concentrations or as fold change from basal. Since the IP1 HTRF assay relies on competition which has a non-linear relationship. Therefore, how 0% is defined can have considerable effects on how the data are presented and any apparent effects on baseline levels. Somewhat related, in Extended Fig 8g, why does the baseline of Ca2+ release assay begin above zero? Typically, constitutive activity is not evidence in transient Ca2+ release assays.

Minor points:
- Control data in Fig 1h appears to be missing data sets for effect of VU0155041 on glutamate potency, as there is a different number of data points compared to the reference data set.
- Cys cross-linking used to evaluate changes in dimer interface between 7TM domains. To improve clarity in the description of the data presented, lines 163-166: specify these are 7TM dimer contacts under non-stimulated conditions.
- Line 254-255: “Functional data show that substitutions of these residues in either mGlu4 or mGlu2 to alanine substantially reduced 28%-40% of the maximal Gi activation (Emax) induced by glutamate in the heterodimer” This analysis is incorrectly presented and the effect of these mutations is moderate, with the Emax of these mutations achieving 60-72% relative to WT. May be missing “by” when describing the reduced Emax.
- Entry for L353A EC50 ratio is incorrectly calculated in extended table 2. EC50 should be 21uM, and a ratio of 4.7.
- Both mGlu2 and mGlu4 are canonically coupled to Gi/o proteins, such that the chimera Gqi9 was transfected in for calcium assays. Was this chimeric G protein also transfected into cells when measuring IP1? Or is the IP1 measured arising from coupling to endogenous G proteins in the recombinant cells? Please clarify in methods as appropriate to enable reproducibility.
- Fig 5g: if G667W is significantly different to the control, then would expect A668W and G667W+A668W to also be significantly different given the responses are larger.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided satisfactory responses to all of my comments in the initial review. As a result, the description of the smFRET methods and results have been greatly clarified.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my previous concerns.

Reviewer #3

(Remarks to the Author)
My previous critiques have largely been satisfactorily addressed by the revisions/responses, with the following exceptions:
1) Claim the binding pocket for VU0155041 is “novel” still seems like an overstatement given evidence supplied in Supp Fig 6 of overlapping binding site with FITM in mGlu1 and CaSR PAMs (cinacalcet, R-568).
2) Regarding Supp Fig 8d – the statistical description here does not make sense. If each group of three experiments is compared to the no CuP treatment using Dunnett’ post-test, then there should not be differences indicated between +CuP/no Glu and +CuP&Glu. As the Dunnett’s test compares to a single control and not between other groups. The revised figure legend here seems to indicate multiple unpaired student’s t tests have also been performed, which is inappropriate since the

authors are comparing three groups.
3) As previously mentioned, to my understanding it is not appropriate to report multiple levels of significance once a threshold has been set. For e.g. if the authors have set the significance threshold at $P<0.05$ when performing statistical tests, then reporting $P<0.001$ etc is redundant and gives the impression of different degrees of significance. Unless, the authors have a scientific justification for why different levels of significance are chosen for different analyses/types of experimental data. Reporting exact P values is unusual, and not the standard approach in the field for these types of experimental data. If this is required to meet with editorial policy for this journal, please disregard.
4) Please check calculation of EC50's, EC50 ratios relative to pEC50's derived from all curve fits. In Supplementary table 4, many of these do not match, i.e. converting pEC50 to EC50 does not give the reported value. This applies to all of the values under "G protein dissociation induced by Glu on Galphai3 WT and mutants" as well as mGlu2C1-FS + mGlu4C2 in Ca2+ release when co-transfected with Gqi9 and Gqo; as well as many other values in this table. Notable because the same pEC50 value is linked to different EC50 values in a way that is not attributable to rounding. Since EC50 values are logarithmically distributed, the ratio should be derived based on the mean pEC50 values. As pEC50 values were used for statistical testing, conclusions are unlikely to be affected.

Reviewer #4

(Remarks to the Author)
Huang et al. report new structure of the mGlu2/mGlu4 dimer in different conformational states, shedding light on the molecular mechanism of activation. The topic is of extreme relevance for the GPCR community and we enjoyed very much the reading, which results clear and precise.

The structure in complex with the VU0155041 underwent MD simulations with the aim to clarify the orientation of the ligand.

- The MD protocol should be reported in the Methodology. And we would suggest to add standard MD analyses in the supplementary, to show the overall stability of the simulated systems.

- It would be good to make the MD trajectories available via open-source repositories (i.e. Zendo).

- We agree with the authors that pose 1 seems more stable that pose 2. However, the ligand is small and in replicas 1 and 3, the average RMSD is above 3.5 Angstrom (Figure S5), which could also be due to a rearrangement of the pose. As we see same tendency in the last 50 ns of replica 2, we would suggest to prove that the pose is maintained with additional analyses. The author could monitor the interactions and show that they are maintained during the simulation (contact analysis), and/or cluster the ligand conformations along the trajectories. In case the ligand pose rearranges, the new coordinates from MD could be compared to the density maps.

- Typos in Figure 4e.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
We appreciate the great work of the authors in addressing all our comments.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #1 (Remarks to the Author):

The authors have used single-molecule Forster resonance energy transfer (smFRET) to probe the conformational states of the mGlu2-mGlu4 receptor. Sophisticated methods are used to label the mGlu2 and mGlu4 subunits with donor and acceptor fluorophores. The experimental smFRET data and analysis are of high quality and generally support the inferences drawn. However, I have a few comments that I would like the authors to address.

1. Were the smFRET experiments performed using purified receptors that were reconstituted in detergent micelles or membrane mimetics ? This should be stated at the beginning of the smFRET data presentation, for example on line 234.

As requested, we have added the following sentence at the beginning of the smFRET data presentation, line 213-221: " Receptors were produced and solubilized as previously described[31, 32], and smFRET acquisitions were performed in detergent micelles with 0.0025% LMNG (w/v), 0.00025% CHS tris (w/v), and 0.0025% GDN (w/v). Under these conditions, the receptor is fully functional and sensitive to allosteric modulators[31, 32]. Receptors were labeled with d2 on a SNAP-Tag[29] on the N-terminus of the mGlu4 subunit, and with Cy3B-tetrazine on a trans-Cyclooct-2-en-L-Lysine (TCO*A) incorporated at position 358 of mGlu2[33]. This FRET sensors reports on the reorientation of the VFT dimer from the resting to the active state as a decrease in FRET, similar to our previously used SNAP/SNAP and ncAA/ncAA sensors for mGlu2 homodimers[29, 31, 32, 34].".

2. While the E=0.56 and E=0.3 appear to be the major FRET states present, the histograms also reveal minor populations at very low or very high FRET efficiency. There is no explanation offered for these species. The authors should offer suggestions as to the origin of these minor FRET states.

These species have been observed previously when using SNAP tags in our published experiments on mGlu2 dimers (Cao A.M. et al., Nat Commun., 2021). Of note, when using two ncAA as targets for fluorophore attachment, these populations are almost totally absent (Lecat-Guillet N et al., Sci Adv., 2023). In the present paper, we kept one subunit with a SNAP tag for the smFRET analysis to remain as close as possible to the ensemble experiments. Importantly the conclusions on the states explored by the receptors remain unchanged if we take them into account. As these very low and very high FRET are very minor, and are not influenced by the presence of the ligands, they are included in the fitting to properly represent the data, but can be neglected in the quantitative analysis of the populations.

To clarify this point, we have now added the following sentence in line 223-234:

"We fitted all distributions with four Gaussians (corresponding to the very low FRET (VLF), the low FRET (LF), the high FRET (HF) and the very high FRET (VHF) populations). The major changes in response to ligands were found to result from the depopulation of the HF state accompanied by an increase of the LF state, reporting on the reorientation of the VFT dimer toward the active state. To gain a quantitative view of this reorientation, we calculated the fraction of active molecules, defined as the fractional amplitude of the molecules found in the LF state relative to the HF + LF states. As no notable changes were observed in the two minor populations at VLF, E ~ 0.02 and VHF, E ~ 0.88, **(Supplementary Table 2, Supplementary Fig. 11a, b)** that are only observed with SNAP tagged subunits[32], these were not included in the analysis. We nevertheless verified that

calculating the fraction of the active molecules as VLF + LF relative to all molecules led to similar results."

3. It would be helpful to include a table in the SI summarizing the peak positions and peak areas obtained from the four-state fits to the FRET histograms, for each sample condition.

We thank the reviewer for this suggestion. We have now added the requested tables as Supplementary Tables 2 and 3. To quantify the relative population of resting and active states, the mean FRET efficiency E of LF and HF populations was determined by fitting histograms obtained in the presence of L-AP4 and LY37. For fitting of the apo condition, the mean E of the LF population, determined in the presence of L-AP4 and LY37, was fixed and the HF population was fitted to account for a small but noticeable difference in mean FRET efficiency. VLF and VHF FRET values were determined from all conditions and their mean was used in the final fitting.

4. The abstract states “Single-molecule FRET data illustrate the constant oscillation of monoliganded mGlu2-4 between the intermediate states and an active one”. In fact, there are no results presented that reveal dynamic transitions between different FRET states. This statement should be removed.

We have now rewritten the sentence in the abstract as : line 40-41: "Single-molecule FRET data show that the monoliganded mGlu2-4 can be found in both intermediate states and an active one."

Reviewer #2 (Remarks to the Author):

The study sought to elucidate the structural basis for the preferential activation of G proteins by mGlu4 within the mGlu2/mGlu4 heterodimers, despite the intrinsic capability of both mGlu2 and mGlu4 to form homodimers that can activate G proteins independently. The authors presented cryo-EM structures of the mGlu2/mGlu4 heterodimers bound with two specific antagonists (targeting mGlu2 and mGlu4 respectively), an agonist for mGlu2, an agonist for mGlu4, and the endogenous agonist Glu combined with an mGlu4-specific positive allosteric modulator (PAM) and Gi protein. These structures unveiled the PAM VU0155041 binding site on mGlu4 and the functional significance of the interacting residues was assessed. Notably, the activation process involved a transition in the dimer interface from a TM5-TM5 interaction to a TM6/7-TM6 interaction. The team further explored the partially activated states of the mGlu2/mGlu4 dimer induced by agonists for either mGlu2 or mGlu4, reinforcing the importance of the CRD-ELC2 interaction in dimer activation. A comparison with previously reported structures revealed consistent intra-subunit conformational changes within both mGlu2 and mGlu4. Through functional analysis, they suggested that G protein activation within the heterodimer was mediated by mGlu4. The manuscript culminates with an examination of Gi protein's interaction with mGlu4.

Overall, the manuscript provides useful structural information on the mGlu2/mGlu4 heterodimers in different states. However, it does not fully elucidate the initial question it poses regarding why mGlu4 preferentially interacts with and activates G proteins within the heterodimer context.

Main Comments:

1. There appears to be a discrepancy between the data presented in Figures 3 (d-g) and 4 (f-g), where Figure 3 suggests that an mGlu2-specific agonist promotes a more efficient transition to an active conformation compared to an mGlu4-specific agonist, conflicting with the conclusion that mGlu4 is the primary mediator of G protein signaling within the heterodimer. Clarification and further analysis of these observations would be beneficial.

The referee is correct, and this point led us to further examine the effect of LY379268. We found that this reported group-II mGluR agonist can activate mGlu4 at high concentrations (see new Supplementary Fig. 11). The same has been reported with another group-II mGluR agonist LY354740 (Moreno-Delgado D et al., eLife, 2017). Accordingly, at the LY379268 concentration used in our first sets of smFRET experiments (100 µM), activation of both mGlu2 and mGlu4 VFTs is expected, as the effect at mGlu4 is likely potentiated by the closed mGlu2 VFT (Kniazeff J et al., Nat Struct Mol Biol., 2004). We then repeated all our smFRET experiments using a concentration of LY379268 of 1 µM, a concentration sufficient to almost saturate the mGlu2 VFTs, but not sufficient for activating mGlu4 (Supplementary Fig. 11).

Under such condition, we observed that LY379268 increases the proportion of mGlu2-4 in the LF state to a similar extend as L-AP4, in agreement with the partial activation of the mGlu2-4 heterodimer by mGlu2 agonists (Moreno-Delgado D et al., eLife, 2017).

2. Are there differences in the binding affinity and stability between mGlu2 and mGlu4 for Gi in the presence of glutamate? Such data could provide valuable insights into the preferential interaction of mGlu4 with Gi.

Understanding why mGlu2-4 mainly activates G proteins through the mGlu4 subunit is important. It can come either from an oriented asymmetry of the dimer, or to a preferential association (faster ON rate, and/or better affinity) of the G protein for mGlu4 compared to mGlu2. Determination of the glutamate bound state of the mGlu2-4 heterodimer in the absence of the G protein would help clarify this issue, but we have not been able to solve it. However, our data illustrating an asymmetric intermediate state dimer in the absence of the G protein, rather support an oriented asymmetry. Moreover, the observation that the coupling efficacy of mGlu4 homodimers is lower than that of mGlu2 (Liu J et al., eLife, 2017), and that of mGlu2-4 is equivalent to that of mGlu2 further supports an oriented asymmetry, the mGlu2 subunit enhancing the coupling efficacy of mGlu4, rather than a preferential coupling of mGlu4 to the G protein relative to mGlu2.

This point is now mentioned in the revised discussion: line 384-389 “These data strengthen our view that the oriented asymmetry of the active form of mGlu2-4 is likely responsible for the G protein activation by mGlu4, rather than a better G protein recruitment of the G protein by mGlu4 leading to the observed asymmetry. Indeed, mGlu4 homodimer is less efficient in activating G proteins than mGlu2 homodimers, but the mGlu4 subunit becomes as efficient to activate G protein in the heterodimer, than mGlu2 in the homodimer[8, 29].”

We agree with the reviewer that these proposed data would further strengthen our proposal. For us to perform it, it would require the large-scale purification of mGlu2 and mGlu4 homodimers, as well as the heterotrimeric Gi protein, and setup of a binding assay to determine the G protein affinity on each receptor. Such a task will require several months of intense work without a

guaranteed success, and we think it goes beyond the scope of the present study.

3. It remains unclear whether complexes involving mGlu2/mGlu4 heterodimers and Gi can be effectively formed in the presence of either an mGlu2-specific or an mGlu4-specific agonist. Additional experiments to investigate these potential complexes would enhance the understanding of the activation mechanism.

Unfortunately, we have not been able to obtain a good resolution of an mGlu2-4-Gi complex in the presence of either an mGlu2 or an mGlu4 selective agonist. However, in our previous study (Liu J et al., eLife, 2017) examining the coupling of each subunit of the mGlu2-4 heterodimer to G proteins we reported that: i) stabilizing the mGlu2-4 VFT dimer in its active orientation through an inter-subunit disulfide bridge leads to G protein activation by the mGlu4 7TM, and ii) both selective mGlu2 and mGlu4 agonists applied separately lead to G protein activation through the mGlu4 7TM, and not the mGlu2 one. These data nicely illustrate that G protein activation by mGlu2-4 heterodimer originates from the mGlu4 7TM, whether either one, or the other of both VFTs are agonist occupied.

Although we could not provide a structure of the receptor activated by a single agonist in complex with the G protein, we provided more information on the effect of the G protein on the stabilization of the active form of the receptor occupied by a single agonist.

Minor Comments:

1. The observation that point mutations entirely negate the effect of VU0155041 in Figure 1h is intriguing and warrants further discussion regarding the critical interactions of this PAM with mGlu4.

We tested the response of different concentrations of VU0155041 with EC_{20} of glutamate in the revised version. As shown in Supplementary Fig. 7k, the mutations all clearly decrease the VU0155041 potency, which is consistent with our previous results.

2. Given the reported poor density for VU0155041 in the complex, the reliability of its orientation should be evaluated by MD simulations.

We thank the reviewer for this suggestion. To investigate the debate surrounding the two possible conformations (Pose-1 and Pose-2) of VU0155041, we conducted 500 ns Molecular Dynamics (MD) simulations with three replicates for each conformation to determine which exhibits higher binding stability. Analysis indicated that PAM in Pose-1 shows superior stability, as evidenced by a relatively conserved RMSD distribution. Conversely, PAM in Pose-2 displays greater flexibility, reflected in a wider RMSD distribution. This comparison underscores the enhanced stability of PAM in Pose-1, which supports the orientation of VU0155041 shown in our structure. This information has been added in Supplementary Fig. 5 in the revised version, and are presented in the paragraph "**A novel PAM binding site in the mGlu2-4 heterodimer"** in the results section, line 115-117 “The conformation of VU0155041 was also confirmed by MD simulations **(Supplementary Fig. 5)**, which supports the orientation of VU0155041 shown in our structure.”.

3. The high constitutive activity observed with T793C + P803C mutants in Figure 2e is surprising and merits additional context, particularly in relation to the structural basis of this phenomenon.

The referee is correct. The T793C + P803C mutant displays a higher constitutive activity than the control. This is consistent with the two introduced Cys residues facing each other in the active state of the mGlu2-4 heterodimer. Indeed, our cryo-EM data show that mGlu2 TM7 come in close proximity to the mGlu4 TM6, making a disulfide bridge between the two introduced Cys residues possible, then stabilizing the dimer in an active state. As not every receptor is cross-linked, this effect is partial. To clarify this point, we added a new panel in Fig 3d displaying the basal IP_1 concentrations measured in mock cells, cells expressing the mutant in which both natural Cys involved in the covalent disulfide bridge are missing (CA-CA), and cells expressing the mGlu2-4 mutant carrying a Cys in mGlu2 TM7 $T793^{7.31}C$, and another one in mGlu4 TM6 $P803^{6.55}C$. These data illustrate the high basal production of IP_1 by the Ctr-CA/Ctr-CA receptor, and an even higher production with the $T793^{7.31}C$ + $P803^{6.55}C$ mutant.

4. While the smFRET data provide valuable insights into the equilibrium among various conformations, the claim of revealing the structural basis for partial activation seems overstated.

Our cryo-EM data with a single bound ligand per dimer reveal mGlu2-4 VFTs in a resting orientation with one VFT closed, and the other open. Such structural data were not in agreement with our previous and present observation that either mGlu2 selective, or mGlu4 selective agonist generate a partial response (Liu J et al., eLife, 2017, Scholler P et al., Nat Chem Biol., 2017). However, our structural data are consistent with our observation that the partial activity measured with a single agonist, is due to an open-closed conformation of the VFT dimer, as the antagonist targeting the unoccupied subunit, known to stabilize the open state of the VTF have no effect (Supplementary Fig. 10 c,d). To clarify this issue, we examined the conformation of the VFT dimer using smFRET, and observed that a significant fraction of the dimers are in an active orientation, consistent with the partial activity. However, we have not observed such conformation in our cryo-EM images, possibly due to specific constraints that further limit the active form population under our cryo-EM experimental conditions. We now clarify this point, as we agree that the smFRET data being those providing the explanation for the partial activity of the mono-liganded dimers.

5. The similar effects of the mGlu2 agonist (LY379268) and the endogenous agonist (Glu) on activation, contrasted with the near-control level effect of the mGlu4 agonist (L-AP4), in Figure 3 (d-g) requires explanation. Elaboration on the differential efficacy of these agonists could clarify the activation mechanism of the heterodimer.

This point is related to the first point raised by this referee. See our answer above. Note that when using a concentration of LY379268 that almost saturate mGlu2 VFT only, the response is similar to that obtained with the mGlu4 agonist L-AP4.

Reviewer #3 (Remarks to the Author):

Huang et al., 2024 report on structural analyses of a class C GPCR heterodimer comprising mGlu2 and mGlu4 in four different states: inactive (antagonist bound to each protomer), 2x intermediate

inactive states (with subtype selective agonists bound), and an active complex (mGlu2/4+glutamate+PAM+Gi protein). The cryo-EM analyses report on heterogenous sample populations, with each state ultimately being solved with map resolutions ranging from 3.7 to 5.95Angstroms. Local refinement has allowed discrete domains of the active complex to achieve slightly higher resolution.
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Regarding specific claims made:
It is a stretch to refer to the PAM binding mode as entirely 'new'. mGlu4 PAM (VU0155041) binding pocket in the 7TM domain overlaps in part with the common allosteric binding site observed across multiple class C GPCR family members. With VU0155041 bound to mGlu4 is the lower part of the pocket empty? Or is the resolution too low to ascertain? For completeness this comparison to allosteric modulator binding poses across class C GPCRs (Extended Fig 5a) should also include the mGlu2/4 heterodimer: mGlu2 PAM: JNJ-40411813/ADX-71149 & mGlu4 PAM: ADX-88178 [PDB: 8JD5].

We thank the reviewer for this comment. The position of VU0155041 binding in mGlu4 has been validated through MD simulation as shown in Supplementary Fig. 5 (See above our answer to the referee #2's 2nd point). The mGlu2 PAM: JNJ-40411813/ADX-71149 and mGlu4 PAM: ADX-88178 [PDB: 8JD5] have been added in the comparison as suggested. When comparing the mGlu4 PAM VU01550411 with these reported class C GPCR family members, VU0155041 is located in the upper site in mGlu4, as shown in the Supplementary Fig. 6 of the revised manuscript.

The VU0155041 binding mode is supported by mutagenesis testing the effect on a single concentration of PAM. Noting quantification of PAM data is limited.

We agree with the reviewer that a single dose does not provide enough information on the effect of the point mutations. This point was also raised by the second referee (minor point #1). As indicated in our answer to the second referee, we did perform full dose-response curves of VU0155041 on an EC_{20} concentration of glutamate for the WT receptor and all mutants. These data are included in the new Supplementary Fig. 7. The new data show that most mutants do decrease the PAM potency, as expected for mutations of single residues involved in the PAM binding, and consistent with our cryo-EM observation.

smFRET data is used in an attempt to explain different agonist occupied states and partial agonism from single occupancy of VFT dimer. These experiments do how transitions between states and different proportions in an agonist dependent manner. However, without adding G proteins into the system these are incomplete and cannot truly explain partial agonism as claimed (lines 241, 244, 362).

As requested by the referee, we repeated the smFRET analysis in the presence of the Gi heterotrimer ($G\alpha_{i1}\beta_1\gamma_2$). The data are now presented in the new revised Fig. 4. In the presence of the Gi protein, there is no increase in the active fraction (low FRET) of the mGlu2-4 receptor in the absence of ligand (Apo), but a significant increase in the fraction of active receptor bound to either the mGlu2 selective LY379268, or the mGlu4 selective agonist L-AP4. Under these conditions, about 50% of the receptors are in the active state, while 50% remains in the resting state, consistent with the partial agonist action of these two agonists.

These data are presented in the revised results section (new Figure 4c-e, and new Supplementary Fig. 11, and Supplementary Table 2 and 3), line 241-244: " In the presence of the Gi protein heterotrimer, the fraction of receptors in the active state (LF state) in the presence of either LY379268 or L-AP4 largely increases **(Fig. 4d, e, Supplementary Table 3)**, consistent with the G protein stabilizing the active conformation of the monoliganded mGlu2-4 heterodimer."

line 244-245: "Of note, the mGlu2 specific agonist is more likely to induce a reorientation than a mGlu4 specific agonist." Is this observation linked to relative intrinsic efficacy of the agonists tested? Do mGlu2 and mGlu4 orthosteric agonists achieve similar or different maximal responses relative to glutamate?

This question was also raised by the second referee (main point #1 and minor point #5). As explained in our answer to the second referee, the larger efficacy of LY379268 was due to the too high concentration of this ligand, allowing it to not only activate mGlu2, but also the mGlu4 subunit. When used at a sufficient concentration to saturate mGlu2 VFT only. Under such conditions, this ligand has an effect similar to that of the mGlu4 selective agonist L-AP4, both agonists being partial relative to the maximal effect obtained with saturating concentrations of glutamate.

This sentence has been modified accordingly: line 235-239: "LY379268 or L-AP4 used at a concentration that selectively activate the mGlu2 or mGlu4 subunit, respectively (1 µM and 10 µM, respectively) (**Supplementary Fig. 11c-e**) applied alone are also able to increase the population of this low FRET active state, but not as efficiently as glutamate **(Fig. 4c)**, consistent with the partial activation of the mGlu2-4 heterodimer by these selective agonists (**Supplementary Fig. 12)**."

Extending findings to general conclusions about mGlu2 not being able to optimally couple G proteins (line 392). There is a need to acknowledge caveats regarding the artificial systems being used here, and the limited use of Gi3 only in BRET G protein recruitment assays.

We thank the reviewer for this comment. We performed Ca^{2+} assay to confirm that the mGlu2 subunit does not couple to Gi and Go proteins in the mGlu2-4 heterodimer in the revised version. The chimeric Gqi9 and Gqo proteins were used to enable mGlu2-4 to couple to the PLC pathway and induce Ca^{2+} release. Mutation of the conserved Phe residue into Ser in the third intracellular loop was introduced in mGlu4 (F781S) or mGlu2 (F756S) to abolish the G protein coupling as reported (Kniazeff J et al., Nat Struct Mol Biol. 2004; Liu J et al., eLife, 2017; Lin S et al., Nature, 2021). As shown in new Supplementary Fig. 15e, f, the FS mutation in mGlu4 abolished the agonist-induced mGlu2-4 heterodimer activity, while the mGlu2-4 heterodimer with mGlu2-FS is still functional. These results suggest that both Gi and Go couple to mGlu4 and not mGlu2 in the mGlu2-4 heterodimer in HEK293 cells. These results are consistent with our previous work (Liu J et al.,

eLife, 2017).

Our manuscript has been modified. Line 297-299: " In agreement with mGlu4 being responsible for Gi and Go activation in the heterodimer, the mGlu2-4 heterodimer carrying the F781S mutation in mGlu4 only is not functional **(Supplementary Fig. 15e-f)**. "

Regarding data presentation, analysis and visualisation:
Applicable to all statistical representations, generally a single threshold level of significance is predefined. Therefore, reporting on different levels of significance is not recommended. In addition, there a number of issues/queries regarding the presentation of the pharmacological datasets.
Fig 2, how was one-way ANOVA performed on these data sets? Do these data report on multiple ANOVAs comparing the three groups for each mutation? Or some other approach? What post-test was used to determine differences in dimers between conditions and mutations?

We thank the reviewer to point out this issue. For Fig. 2c, data of the percentage of dimer in the three treatments (without CuP and Glu, with CuP or with CuP and Glu) of each mutation are analyzed using one-way ANOVA with a Dunnett's post-hoc multiple comparison test to determine significance. This description has been added in the legend of the indicated figure (now present as Supplementary Fig. 8d) in the revised version, line 107-119: "Cross-linking of cell surface mGlu2 and mGlu4 subunits between the indicated residues mutated into cysteine (Cys), in the indicated TM helices, without or with CuP (blue) and with Glu (yellow). Ctr, $mGlu2_{C1}$-$mGlu4_{C2}$ heterodimer. Ctr-CA, Ctr with C121A mutation in mGlu2 and C136A-C715A mutation in mGlu4 to avoid disulfide bonds between mGlu4 subunits. Ctr-CA + Cys mutation, Ctr-CA with an additional single Cys mutation to alanine in the indicated TMs of mGlu2 or mGlu4. Data of the percentage of dimer in the three treatments (without CuP and Glu, with CuP or with CuP and Glu) of each mutation are mean ± s.e.m. from at least three independent biological replicates. The number of independent experiments (n) for Ctr and indicated receptors are n = 3, 16, 4, 6, 5, 6, 6, 7, 7, 7, 7, 5, 5. Data are analyzed using one-way ANOVA with a Dunnett's post-hoc multiple comparison test compared with the treatment without Cup in each group to determine significance. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, not significant (ns) > 0.05."

For data reported in extended table 2, expression and functional E_{max} data are expressed as % of control. How were one-way ANOVA statistical tests performed given the control used for comparison have been normalised and do not have associated error? The non-normalised data should be used to determine if E_{max} differences are significant. If this was the case, the methods should be updated accordingly. This impacts data presented in Figures 3c, 4b-g, 5e, Extended Figures 7e-f.
We agree with the reviewer that it is better to use non-normalized data. We now determined the statistical significance of the E_{max} in each independent experiment using the non-normalized data for previous Figures 3c, 4b-g, 5e and Supplementary Figures 7e-f. All these data have been added in the revised version of figures present as Figures 3d, 5b-c, 6c-d, 7e, Supplementary Fig. 11c-e. The statistical significance did not change.

Fig 4, net BRET data have been normalised to the control data for each agonist. However, this normalisation obscures any potential differences in the maximal effect of the different agonists used. If the raw data are compared, do the maximal responses of glutamate, L-AP4 or LY379268 differ at the mGlu2/4 heterodimer or homodimers? This is relevant to interpretation on Emax increases for glutamate at mGlu2/2 mutations, if glutamate is a partial agonist at mGlu2/2 WT.

The differences in Emax of different agonists at mGlu2-2, mGlu4-4 and mGlu2-4 were already reported in several papers (Moreno Delgado D et al., eLife, 2017; Yin S et al., J Neurosci. 2014; Liu J et al., eLife, 2017). These studies show that glutamate and LY379628 are full agonist of mGlu2-2 homodimer and full at mGlu2-4 heterodimer but the related compound LY354740 is partial. Note that the full agonist effect of LY379628 very likely results from an agonist action at the mGlu4 subunit as discussed above. On mGlu4-4 homodimer, L-AP4 and glutamate are full agonists but L-AP4 is partial at mGlu2-4 heterodimer.

Table from published paper (Yin S et al., J Neurosci. 2014) [REDACTED]

In our revised manuscript, we now include a comparison of the Emax values from our experiments and confirmed that L-AP4 is partial at the mGlu2-4 heterodimer (Supplementary Fig. 12 in the revised version), while LY379628 can fully activate the heterodimer due to its capability to activate the mGlu4 subunit at high concentrations (also see our responses above, when analyzing the smFRET data).

IP1 assay data are normalised to 100% of control, but not clear how 0% was defined from the description. Typically, these types of data are presented as actual IP1 concentrations or as fold change from basal. Since the IP1 HTRF assay relies on competition which has a non-linear relationship. Therefore, how 0% is defined can have considerable effects on how the data are presented and any apparent effects on baseline levels.

We agree with the reviewer's comment. In the related figures, all values of IP_1 assays were calculated as IP_1 concentration (nM) deduced from a standard curve before normalization. We also make sure the IP_1 concentrations were in the correct range for their accurate determinations. To clarify the basal IP_1 concentration in our system (HEK293 cells), we detected the IP_1 accumulation in mock cells, cells transfected with mGlu2-4 or mGlu2-4 mutants (See above our answer to the referee #2's minor, 3rd point). The values of IP_1 accumulation from three independent experiments were presented in the graph. An increase of IP_1 accumulation in mGlu2-4 mutant (mGlu2-T793$^{7.31}$C + mGlu4-P803$^{6.55}$C) was shown when compared with mGlu2-4 control and analyzed using one-way ANOVA with a Dunnett's post-hoc multiple comparison test. It indicates an increase of the basal activity in this mutant, which is consistent with our previous description. These data are presented in the new Figure 2d of the revised manuscript. We also agree the definition of the 0 % and 100 % is not clear in the previous Fig. 2e, but we did not test IP_1 production in mock cells in parallel. As the the new Figure 2d shows an obvious increased of basal activity already, we removed this curve.

Somewhat related, in Extended Fig 8g, why does the baseline of Ca2+ release assay begin above zero? Typically, constitutive activity is not evidence in transient Ca2+ release assays.

Here the baseline of Ca^{2+} is due to the basal signal measured, when the buffer was injected into the cells. It is a variation of the assay and not related to constitutive activity. The avoid this confusion, we re-calculated the values and used the net Ca^{2+} release in the revised version (new Figure 8b, e). First, the values of Ca^{2+} release were calculated by the maximum value measured after drug or buffer injection subtracted of the minimal value measured before drug or buffer addition (define as Ca^{2+}_{drug} and Ca^{2+}_{buffer}). The average was calculated from triplicates in each individual experiment. This description has been added in the Methods in the revised version, line 557-558 "All the values of drug treatment were subtract the value in buffer treatment as the net Ca^{2+} release. "

Minor points:

- Control data in Fig 1h appears to be missing data sets for effect of VU0155041 on glutamate potency, as there is a different number of data points compared to the reference data set.

We thank the reviewer to point out this issue. This mistake has been corrected in the revised version, new Fig. 2c.

- Cys cross-linking used to evaluate changes in dimer interface between 7TM domains. To improve clarity in the description of the data presented, lines 163-166: specify these are 7TM dimer contacts under non-stimulated conditions.

We thank the reviewer's comment. We modified this sentence as line 149-150 "be symmetrically cross-linked in reducing conditions with CuP treatment under non-stimulated conditions" in the revised version.

- Line 254-255: "Functional data show that substitutions of these residues in either mGlu4 or mGlu2 to alanine substantially reduced 28%-40% of the maximal Gi activation (Emax) induced by glutamate in the heterodimer" This analysis is incorrectly presented and the effect of these mutations is moderate, with the Emax of these mutations achieving 60-72% relative to WT. May be missing "by" when describing the reduced Emax.

We thank the reviewer for this comment. We added "by" in this sentence in the revised version, line 255.

- Entry for L353A EC50 ratio is incorrectly calculated in extended table 2. EC50 should be 21uM, and a ratio of 4.7.

We thank the reviewer for this comment. It has been corrected in the revised version, now present as Supplementary Table 4.

- Both mGlu2 and mGlu4 are canonically coupled to Gi/o proteins, such that the chimera Gqi9 was transfected in for calcium assays. Was this chimeric G protein also transfected into cells when measuring IP1? Or is the IP1 measured arising from coupling to endogenous G proteins in the recombinant cells? Please clarify in methods as appropriate to enable reproducibility.

We agree with the reviewer's comment. The chimera Gqi9 was also transfected in the IP

measurement and the IP_1 measured arising is not from coupling to endogenous G proteins in the recombinant cells.

We clarified it in Methods in the revised version: line 540-543, “HEK293 cells were co-transfected with the plasmids of receptor, glutamate transporter EAAC1 and a chimeric G protein $G\alpha_{qi9}$, which allows efficient signalling through the phospholipase C pathway, at a ratio of 2:1:1. ”

- Fig 5g: if G667W is significantly different to the control, then would expect A668W and G667W+A668W to also be significantly different given the responses are larger.

We thank the reviewer for this comment. The reviewer is right. We have analyzed the statistic and shown the significance now in Fig. 8f indicating in the revised version.

Reviewer #1 (Remarks to the Author):

The authors have provided satisfactory responses to all of my comments in the initial review. As a result, the description of the smFRET methods and results have been greatly clarified.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my previous concerns.

Reviewer #3 (Remarks to the Author):

My previous critiques have largely been satisfactorily addressed by the revisions/responses, with the following exceptions:
1) Claim the binding pocket for VU0155041 is “novel” still seems like an overstatement given evidence supplied in Supp Fig 6 of overlapping binding site with FITM in mGlu1 and CaSR PAMs (cinacalcet, R-568).

The referee is right when he says there is an overlap between the lower part of the VU0155041 site with those of FITM in mGlu1 and Cinacalcet, R-568 in CaSR. However, VU0155041 is located close to ECL2, a situation not observed with any other class C PAMs. We then modified the paragraph title, replacing "novel" by "unique", and introduced the following within the paragraph: "only the lower part of this site overlapping those of FITM and cinacalcet in mGlu1 and CaSR, respectively,…".
.

2) Regarding Supp Fig 8d – the statistical description here does not make sense. If each group of three experiments is compared to the no CuP treatment using Dunnett’ post-test, then there should not be differences indicated between +CuP/no Glu and +CuP&Glu. As the Dunnett’s test compares to a single control and not between other groups. The revised figure legend here seems to indicate multiple unpaired student’s t tests have also been performed, which is inappropriate since the authors are comparing three groups.

We thank the reviewer for pointing this mistake. We now corrected the figure legend of Supp Fig 8d in the revised version as “Data are analyzed using one-way ANOVA with a Dunnett’s post-hoc multiple comparison test for each group of three treatments (-CuP/no Glu, +CuP/no Glu, +CuP&Glu), through comparing the mean of each treatment with the mean of every other treatment.”

3) As previously mentioned, to my understanding it is not appropriate to report multiple levels of significance once a threshold has been set. For e.g. if the authors have set the significance threshold at $P<0.05$ when performing statistical tests, then reporting $P<0.001$ etc is redundant and gives the impression of different degrees of significance. Unless, the authors have a scientific

justification for why different levels of significance are chosen for different analyses/types of experimental data. Reporting exact P values is unusual, and not the standard approach in the field for these types of experimental data. If this is required to meet with editorial policy for this journal, please disregard.

We thank the reviewer for this comment. Reporting the exact P values is required by the journal.

4) Please check calculation of EC50's, EC50 ratios relative to pEC50's derived from all curve fits. In Supplementary table 4, many of these do not match, i.e. converting pEC50 to EC50 does not give the reported value. This applies to all of the values under "G protein dissociation induced by Glu on Galphai3 WT and mutants" as well as mGlu2C1-FS + mGlu4C2 in Ca2+ release when co-transfected with Gqi9 and Gqo; as well as many other values in this table. Notable because the same pEC50 value is linked to different EC50 values in a way that is not attributable to rounding. Since EC50 values are logarithmically distributed, the ratio should be derived based on the mean pEC50 values. As pEC50 values were used for statistical testing, conclusions are unlikely to be affected.

As pointed by the referee, the EC50 values are logarithmically distributed, such that their means not necessarily correspond to those of the pEC50 values. To avoid any confusion, we have corrected the value of EC50s, by calculating them using the mean pEC50 values of independent experiments. Then, we used these deduced EC50 values to calculate the ratios. This has been modified in the entire Supp Table 4.

Reviewer #4 (Remarks to the Author):

Huang et al. report new structure of the mGlu2/mGlu4 dimer in different conformational states, shedding light on the molecular mechanism of activation. The topic is of extreme relevance for the GPCR community and we enjoyed very much the reading, which results clear and precise.

We wish to thank the reviewer for his/her kind and encouraging comments.

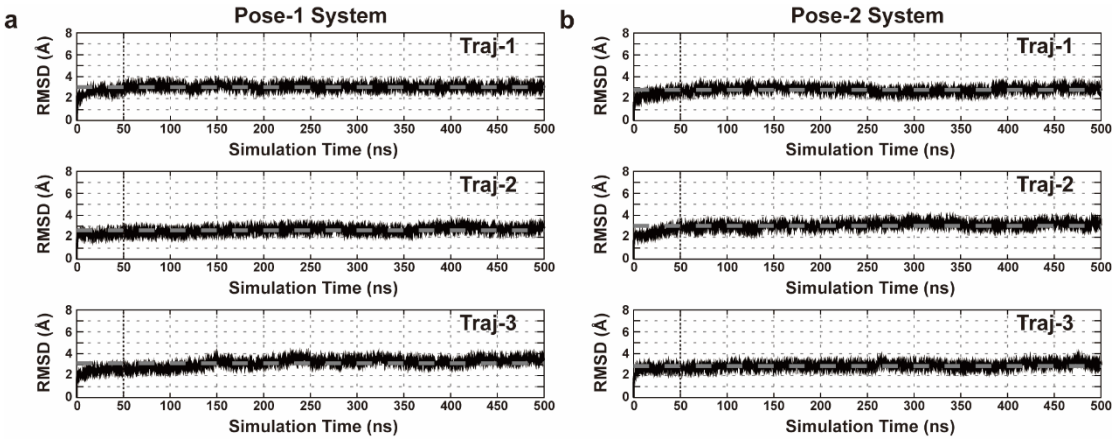
The structure in complex with the VU0155041 underwent MD simulations with the aim to clarify the orientation of the ligand.

- The MD protocol should be reported in the Methodology. And we would suggest to add standard MD analyses in the supplementary, to show the overall stability of the simulated systems.

We thank the reviewer for this important suggestion. To address these issues, we have added the detailed description of the MD protocol in the Methodology and the standard MD analyses to show the overall stability of the simulated systems, as listed below.

"**Molecular dynamics simulations**
From the structure of the mGlu2-4 heterodimer complex (PDB ID: 8WGB), the ECD regions were

directly removed, and the retained mGlu2-4 heterodimer complex with two possible poses of VU0155041 (recognized as PAM), pose-1 with inward-aromatic ring and pose-2 with outward-aromatic ring, was used as the initial configurations for all-atom molecular dynamics (MD) simulations, respectively **(Supplementary Fig. 5a-c)**. The mGlu2-4 heterodimer complex was embedded into a lipid bilayer (232 POPC molecules), which was built by the Membrane Builder module in the CHARMM-GUI server[51]. The protonation state of the complex was determined by H++[52]. Subsequently, the system was solvated in a box ($10.0\times10.0\times15.6$ nm^3) with TIP3P waters and 0.15 M Na^+/Cl^- ions **(Supplementary Table 5)**. The CHARMM36m forcefield was used to describe the system[53], and all MD simulations were performed using GROMACS-2019.4[54]. After a 5000 steps of energy minimization performed by the steepest descent algorithm, then a 250 ps NVT equilibration simulation was performed at 310 K. Subsequently, a cumulatively 1.65 ns NPT equilibration to 1 atm was performed by using the Berendsen barostat. During the pre-equilibrium MD simulation, a harmonic restraint potential of 10 kCal/mol·Å^{-2} was adopted and gradually reduced to zero. Long-range electrostatic interactions were treated by the particle-mesh Ewald method. The short-range electrostatic and van der Waals interactions both used a cutoff of 10 Å. All bonds were constrained by the LINCS algorithm. Finally, to mitigate the uncertainties associated with sampling results, three replicates of the production run with different initial velocities were performed. The overall stability of the system was evaluated by calculating the RMSD of the heavy atoms of the mGlu2-4 heterodimer complex (except the loops in the extracellular and intracellular regions), and the Cα atoms of the transmembrane helices were used for structural alignment **(Supplementary Fig. 6)**. GROMACS's rms function was used to calculate RMSD. For the stability analysis of ligand pose of pose-1 system, the first 50 ns was ignored and only the last 450 ns MD simulation data was used **(Supplementary Fig. 6)**. The stability was evaluated by overall structural alignment of the representative conformations (saved every 100 ns for each trajectory) for the three replicas and the distance distribution of important contacts **(Supplementary Fig. 7)**. The distances between the chloride atoms (Cl_A and Cl_B) of ligand and the Cα atoms of R655/R656 were applied to monitor part-1 stability of ligand, and the distance between the C14 atom of ligand and the Cα atom of T819 was applied to monitor part-2 stability of ligand. The analyses results confirmed the stability of ligand pose in pose-1 system. All the analysis of the structures from the calculation results were displayed by using ChimeraX[46] and PyMol." (Lines 526-558, page 15).



Supplementary Fig. 6. Stability evaluation of MD simulations. a, b, The RMSD of all heavy

atoms of mGlu2-4 heterodimer complex (except the loops in the extracellular and intracellular regions) for the three replicas of Pose-1 system (**a**) and Pose-2 system (**b**).

Supplementary Table 5. Details of the all-atomistic molecular dynamic simulations

System Name		mGlu2-4 complex (Pose-1)	mGlu2-4 complex (Pose-2)
System size		10.0×10.0×15.6 nm^3	10.0×10.0×15.6 nm^3
Number of lipids	POPC	232	232
Number of Waters		34,914	34,827
Ions	Na^+	94	94
	Cl^-	115	115

- It would be good to make the MD trajectories available via open-source repositories (i.e. Zendo).

We thank the reviewer for this important suggestion. We have released all the detailed information and data of MD simulation (cleaned trajectories, start structure, MD simulation parameters) and added the following sentence in the "Data availability".

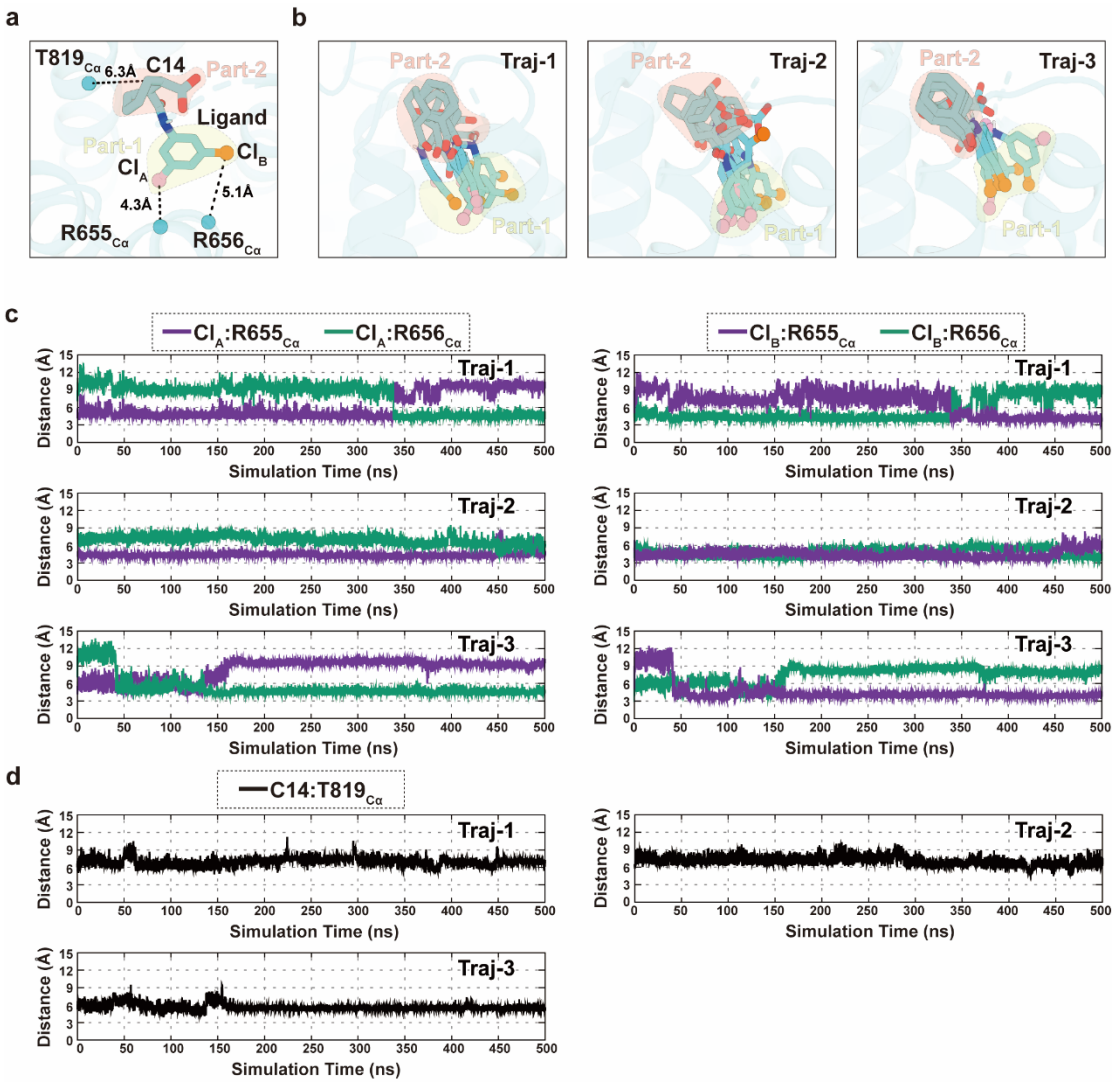
"The MD simulation data (cleaned trajectories, start structure, simulation parameters) generated in this study have been deposited in the github (https://github.com/Yanzhang-ZJU/mGluDimer.git) and Zenodo (https://doi.org/10.5281/zenodo.13293875)." (Lines 716-719, page 19).

- We agree with the authors that pose 1 seems more stable that pose 2. However, the ligand is small and in replicas 1 and 3, the average RMSD is above 3.5 Angstrom (Figure S5), which could also be due to a rearrangement of the pose. As we see same tendency in the last 50 ns of replica 2, we would suggest to prove that the pose is maintained with additional analyses. The author could monitor the interactions and show that they are maintained during the simulation (contact analysis), and/or cluster the ligand conformations along the trajectories. In case the ligand pose rearranges, the new coordinates from MD could be compared to the density maps.

We thank the reviewer for this comment. We have carefully analyzed the MD simulation data for the three replicas of pose-1 MD simulation, and added the following analyses to demonstrate that the ligand pose is maintained during the simulation. We have calculated the important contacts between the two parts of the ligand (part-1 and part-2, **Supplementary Fig. 7a)** and their neighbor contact residues to monitor the stability of the ligand pose, as well as the structural alignment of the representative conformations (saved every 100 ns for each trajectory) of the three replicas of pose-1 MD simulation **(Supplementary Fig. 7b-d)**.

"For the stability analysis of ligand pose of pose-1 system, the first 50 ns was ignored and only the last 450 ns MD simulation data was used **(Supplementary Fig. 6)**. The stability was evaluated by overall structural alignment of the representative conformations (saved every 100 ns for each trajectory) for the three replicas and the distance distribution of important contacts

(Supplementary Fig. 7). The distances between the chloride atoms (Cl_A and Cl_B) of ligand and the Cα atoms of R655/R656 were applied to monitor part-1 stability of ligand, and the distance between the C14 atom of ligand and the Cα atom of T819 was applied to monitor part-2 stability of ligand. The analyses results confirmed the stability of ligand pose in pose-1 system." (Lines 549-557, page 15).



Supplementary Fig. 7. Ligand pose stability evaluated for Pose-1 systems. a, The important contacts used to evaluate the stability of the ligand pose: the distances between the chloride atoms (Cl_A and Cl_B) and the Cα atoms of R655/R656 (Part-1 of ligand), the distance between the C14 of ligand and the Cα atom of T819 (Part-2 of ligand). **b,** The snapshots of representative conformations (saved every 100 ns for each trajectory) of the three replicas of pose-1 MD simulations. **c,d,** The distance distribution of the important contacts to monitor the pose stability of ligand for the three replicas of pose-1 MD simulation.

- Typos in Figure 4e.

Thank you for this comment. We have corrected the typing error in Figure 4e.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.