Peer Review File

Manuscript Title: Asymmetric Activation of the Calcium Sensing Receptor Homodimer

Editorial Notes:

Redactions – Third Party Material

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Ref #1 Gao et al reported the determination of three cryo-electron microscopy structures of near full-length human CaSR. Several different conformational states, including inactive or active states bound to Ca2+ and various calcilytic or calcimimetic drug molecules. The resolutions for active-state-evocalcet + etelcalcetide, cinacalcet, and inactive-state with NPS2143 were at 2.5, 2.8 and 4.1 Å, respectively. The determined structures of CaSR homodimer complexed with 7TM-targeting calcimimetic drugs adopts an asymmetric 7TM configuration. Extensive mutations and assays were also performed. This work is significant in providing structural framework for understanding the activation, allosteric modulation mechanism and disease therapy for class C GPCR.

In the final summary of proposed working model of CaSR activation mechanism (Fig 5), "in the inactive state, CaSR is relatively flexible and the 7TMs are separated facing each other at the TM5-TM6 plane. The VFTs adopt inactive open-open or open-closed conformations. The open-closed conformation can be stabilized by amino acids such as L-Trp, which primes the receptor for activation. Upon activation by high Ca2+ concentration, the VFTs adopt an active closed-closed conformation, which can be stabilized by L-Trp bound at the cleft of each VFT and the ECD PAM etelcalcetide bound at the interface between LB2 of the VFTs. Closure of the VFTs leads to rearrangement of the CRDs, bringing the 7TMs together to form an asymmetric TM6-TM6 interface. The asymmetric configuration is stabilized by 7TM PAMs adopting distinct poses. The 7TM with a bent PAM is more tilted than the opposing 7TM with its C-terminus sequestered in the membrane, and likely unable to couple to G protein".

There are several major concerns about the proposed working model, definition of the status of the states and related conclusion.

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active condition. While it is interesting to observe the open-close VFT ECD in the presence of NAM complexed with 7TM, what is the physiological meaning of this determined structure? Please provide rationale and explanation.

3. The two "active-state" CaSR structures were determined with 10 mM CaCl2, 10 mM L-Trp and 20 μM of cinacalcet or a combination of 20 μM of evocalcet and 50 μM etelcalcetide. Both determined structures have positive modulators at the 7TM. As pointed by the authors (page 4 line 3 to line 7), in Extended Data Figure 4f, the complexed structure has much larger distance between the C-terminal of CRD (32 Å) vs that by Geng et al (23 Å). This difference is likely due to 1) binding of Etelcalcetide at the dimer interface or 2) binding of PAM at the 7TM. To justify their working model, it is important to determine the active state of the full length structures 1) without Etelcalcetide, and 2) without PAM at the 7TM.

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Ref #2

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The only thing that dampens my enthusiasm is that these are yet again membrane protein structures in detergent… Are these really representative for a lipid environment? Is the asymmetry observed upon activation impacted by the detergent environment? Maybe yes, maybe not… I know there is some functional backing, but such experiments can always be twisted and interpreted as one likes, there is nothing like a good quality structural evidence. My hope is that the GPCR field will eventually move away from detergents, including GDN. Nanodiscs are at least a sensible compromise. The authors have shown in the past (with another class C GPCR, mGluR5, PMID: 3067506) what is possible and I believe that has to be the way forward. That being said, I've been guilty of solving membrane protein structures in detergent in the past, so I should be the last one

to throw stones ☹ In the ion channels field we now have to solve again and again old detergent-based structures because they are inaccurate, and I am quite sure that the same will happen with many GPCR ones. At the same I fully appreciate that these structures didn't come cheaply, and as mentioned above the paper has a lot of merit. My point here is more of a wish for the field, especially from one of the leading labs, rather than a criticism.

Here are my specific suggestions for improving this manuscript:

1. I was (like the authors presumably) puzzled by the open/closed VFT conformations in the inactive state. Especially when reading the paragraph contrasting this to the open/open crystal structure(s) and looking at Fig 1. My suggestion is to mention from the start that there is a ligand in one of the VFTs, difficult to explain (and very unlikely to be some amino acid picked from the TC medium, considering the purification protocol…). This is probably some contaminant/breakdown product from the detergent solution. Building Trp in that density (and showing it as such in Fig 1a), is very confusing and not justified I think. Personally I would hesitate to model anything specific in that density. Some "ligand X" could be shown in the Fig5 cartoon.

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Author Rebuttals to Initial Comments:

We thank the reviewers for their valuable critiques and the overall favorable reviews of our work. Addressing their comments enabled us to produce an improved revised manuscript. Below is a point-by-point response to their comments:

Referee #1 (Remarks to the Author):

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Thank you for these positive comments on the execution of the study and the impact of the findings.

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There are several major concerns about the proposed working model, definition of the status of the states and related conclusion.

We have now determined, per reviewer's suggestion, a globally 3.2 Å structure of CaSR in complex with NAM under high Ca^{2+} and high Trp and incorporated the new findings into our revised manuscript. This structure lends further support to our proposed mechanism. Below we have elaborated on the details regarding the points raised by Reviewer #1:

1. The author did not determine any forms of two proposed inactive states with complexed with NAMs in the 7TM. Instead, their reported "inactive form" was determined in 20 mM HEPES 7.5, 150 mM NaCl, 0.5 mM CaCl2, 1 mM sodium phosphate (pH 7.5), 20 µM NPS-2143 and 0.005% GDN, 0.0005% CHS. This is a complex form with a negative modulator (NAM) NPS-2143 located at each of 7TM of CaSR. What is the evidence that this state is an inactive form? Additional evidence is needed to justify this claim. This determined "inactive form" with NAM has an asymmetric VFT (open-close) ECD that is largely different from the reported structure by Geng et al (open-open). It is also possible that the binding of NAM to 7TM reversely affect the conformation of the VFT ECD. It is important to determine a true inactive state with open-open form at VFT and without NAM at 7TM.

Based on the IP_1 accumulation assays titrating Ca^{2+} responses of CaSR in the presence of various NAM concentrations (Extended Data Fig. 1f), there is no detectable signaling of CaSR under the condition of 0.5 mM $CaCl_2$, 10 µM NAM (lower than the 20 µM used in structural determination), and thus we believe it is appropriate to refer to this state/structure as inactive in our manuscript.

The reason our inactive-state structure differs from the previous ECD crystal structure is two-fold: (1) our cryoEM structure was determined in solution where the conformation is not affected by crystal lattice packing and harsh crystallization conditions (in the case of inactive CaSR ECD crystal structure, the crystals were grown in 1.5 M Li_2SO_4, 2 mM $CaCl_2$, 100 mM Tris pH 8.5 and flash-frozen in 3.0 M Li_2SO_4, 2 mM $CaCl_2$, 100 mM Tris pH 8.5); (2) as importantly, the presence of 7TMs in our structure would restrict the conformation that can be adopted by the ECD. Furthermore, similar open-closed ECD conformations have been observed in the crystal structures of inactive mGlu1 ECD (Kunishima *et al. Nature* 407, 971–977 (2000)), another Family C GPCR. It is rarer to observe these conditions in crystal structures, which further underlines the strength of visualizing near full length constructs under more physiological conditions by cryoEM.

Although we cannot rule out the possibility that NAM-binding at the 7TM might affect the conformation of the ECD, the observation that the ECD in our CaSR-NAM-Ca^{2+}-Trp structure adopts identical conformation as in our active-state structures (RMSD = 0.3 Å) would suggest that this NAM

likely does not affect the ECD conformation. We also note that the 7TM in GPCRs is intrinsically flexible and dynamic, and the stabilization provided by a ligand is needed to achieve high resolution structures. This is also necessary to overcome challenges due to the pseudosymmetry of the homodimer and properly visualize differences in the relative conformation of the two protomers in the CaSR.

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We thank Reviewer #1 for pointing out the need for a structure of NAM-bound CaSR under a high calcium and high Trp condition. As note above, per reviewer's suggestion, we obtained a 3.2 Å cryoEM structure of NAM-bound CaSR in the presence of 10 mM $CaCl_2$ and 10 mM L-Trp and incorporated this new finding in our revised manuscript (Fig. 1c and Extended Data Fig. 4). The ECD in this structure adopts identical conformation as in the active-state structures (RMSD = 0.3 Å). Notably the 7TMs adopt a fully symmetric configuration with a NAM bound at each protomer and the 7TM interface is significantly different from the asymmetric interface observed in the active-state structures. Comparison between this new structure and our active-state structures sheds light on the mechanism of how NAM inhibits CaSR signaling. The CaSR-NAM-Ca^{2+}-Trp structure illustrates how a NAM would limit CaSR signaling in cases of abnormally high extracellular calcium levels or activating mutations in the ECD, while the inactive-state NAM-bound structure represents how NAM would help ameliorate the effects of activating mutations in the 7TMs.

3. The two "active-state" CaSR structures were determined with 10 mM $CaCl_2$, 10 mM L-Trp and 20 µM of cinacalcet or a combination of 20 µM of evocalcet and 50 µM etelcalcetide. Both determined structures have positive modulators at the 7TM. As pointed by the authors (page 4 line 3 to line 7), in Extended Data Figure 4f, the complexed structure has much larger distance between the C-terminal of CRD (32 Å) vs that by Geng et al (23 Å). This difference is likely due to 1) binding of Etelcalcetide at the dimer interface or 2) binding of PAM at the 7TM. To justify their working model, it is important to determine the active state of the full-length structures 1) without Etelcalcetide, and 2) without PAM at the 7TM.

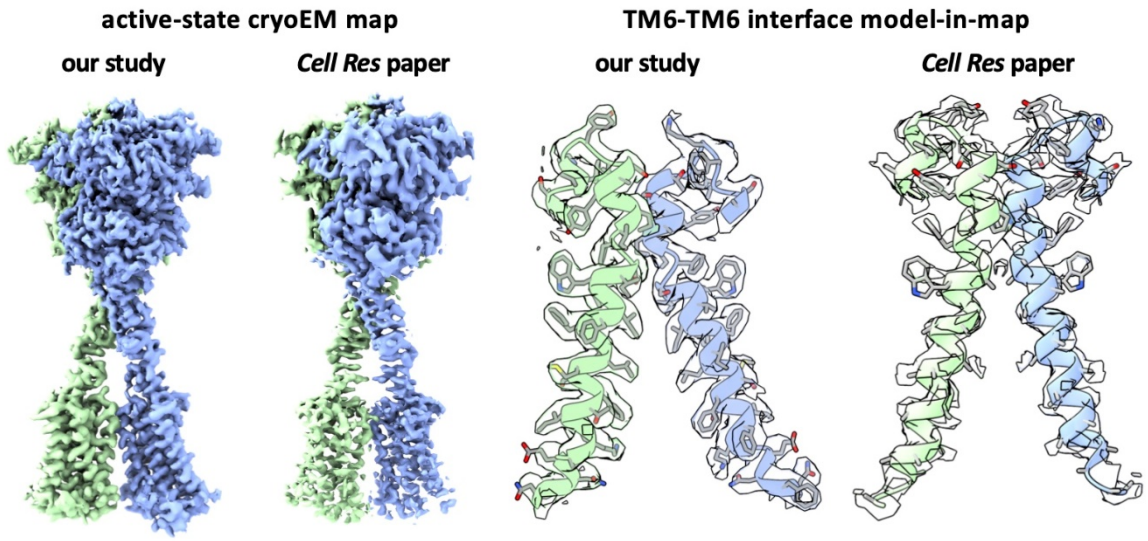
The 9 Å difference in CRD C-termini distance between our near full-length cryoEM structure and the previous ECD crystal structure is most likely due to the restraints posed by the presence of

the 7TMs. Analogous differences have been observed between crystal structures of ECD alone and cryoEM structures of near full length mGlu5 (Koehl *et al. Nature* 566, 79-84 (2019)). We have revised lines 9-12 on page 4 to clarify that. As discussed earlier, our cryoEM structures were determined in solution, and the difference may have arisen from the presence of crystal lattice packing and the use of crystallization conditions for the X-ray structure (the crystals were grown in 1.6 M NaH_2PO_4, 0.4 M K_2HPO_4, 100 mM Na_2HPO_4/citric acid pH 4.2, 10 mM $CaCl_2$, 10 mM L-Trp and flash-frozen in the presence of 20% glycerol). The ECD conformations in the CaSR-cinacalcet complex and CaSR-evocalcet-etelcalcetide complex structures agree very well with each other (RMSD = 0.2 Å). Therefore, the presence of etelcalcetide only facilitates the ECD to adopt the same active-state closed-closed conformation as in the CaSR-cinacalcet complex without etelcalcetide. The 7TMs of GPCRs are inherently flexible and ligands or other stabilizing agents are needed for high-resolution structural determination. Therefore, our active-state structures were determined with either a 7TM PAM or a combination of 7TM and ECD PAMs. Furthermore, the CaSR PAMs we used are in clinical use and of significant pharmacological interest. We believe that the additional layer of information on the mode of PAM (and NAM) binding is a significant strength of our study.

4. The observed asymmetry of 7TM in the presence of PAM is interesting and can be important. However, it is not clear that such 7TM asymmetry can be induced solely by the activation of the ECD by calcium and Trp without PAM binding at the 7TM? Additional information is needed to justify the importance of 7TM asymmetry in activation.

We thank Reviewer #1 for appreciating the importance of 7TMs asymmetry observed in our study. Previous biochemical studies have suggested that the dimeric Family C GPCRs couple to one G protein at a time, implying that there might be a coded asymmetry in a homodimer. Gratifyingly, in our accompanying manuscript of the mGlu2-Gi complex, we have also observed a similar asymmetric 7TM configuration in *both* the active-state mGlu2 alone and mGlu2-Gi complex structures (Extended Data Fig. 9b,c). We believe that collectively these findings provide further support for our results and analysis, and the validity of our interpretations. Our structural results of two different Family C GPCR members, CaSR and mGlu2, point to the importance of 7TM asymmetry in Family C GPCR activation. Given the intrinsically high flexibility of GPCR 7TMs, it would be very challenging to obtain *high resolution* structures of CaSR without the presence of ligands or other stabilizing agents. While our manuscript was under revision, another paper describing CaSR structures without drug molecules were published in *Cell Research* (http://doi.org/10.1038/s41422-021-00474-0). In that work, the use of enforced C2 symmetric averaging in cryoEM refinement together with the much lower resolution have prevented the observation of 7TMs asymmetry. Below is a figure comparing the 7TMs map and

model from our study and the ones from the *Cell Res.* paper, where due to the poor quality of the map most of the side chains in the deposited structure were not modelled.



5. It is not clear the local resolutions for the determined ligand binding sites and calcium binding sites at the ECD domain.

This is a good suggestion. We have now updated Extended Data Fig. 3 to show local resolutions and listed B factors for each ligand type in Extended Data Table 1.

Minor: 1. Line 15, active-state ECD adopts a symmetric closed-closed conformation (Fig.1d) should be Fig 1b, & e

Thank you for catching this. We have corrected this error.

Referee #2 (Remarks to the Author):

Gao et al report cryo-EM structures of CaSR, a homodimeric class C GPCR, in either inactive (VFTs open/closed, TMDs apart) or active (VFTs closed, TMDs in contact) states. The active states are of high resolution and small molecule PAMs (including a peptide) are present. These structures reveal an unexpected asymmetric arrangement of the TMDs, prompting the authors to speculate possible implications for downstream signaling (G protein coupling). The topic is clearly of utmost importance and structures very timely. I very much enjoyed reading this paper, my favorite experiment being the investigation of which subunit (within the asymmetric dimer) favours G protein activation. The use of GABAB coiled coil C-terminal helices to ensure cell surface trafficking of heteromers that harbour mutations meant to favour defined TMD conformations is super clever and the functional outcomes compelling. The fact that PAMs are present in EM maps of 2.5 and 2.8A resolution can also guide design of therapeutics, in particular the potential improvements suggested to the D-peptide

etelcalcetide are very sensible and should be straightforward to test (I hope the authors are doing this already). These structures could really make a positive impact. I have also enjoyed a lot the frequent references to human disease-linked mutations, peppered through the text and, in most cases, providing very interesting mechanistic insights.

We thank Reviewer #2 for the positive comments on the execution of our study and the impact of the findings. Indeed, we plan to test potential improvements on etelcalcetide guided by our structure.

The only thing that dampens my enthusiasm is that these are yet again membrane protein structures in detergent… Are these really representative for a lipid environment? Is the asymmetry observed upon activation impacted by the detergent environment? Maybe yes, maybe not… I know there is some functional backing, but such experiments can always be twisted and interpreted as one likes, there is nothing like a good quality structural evidence. My hope is that the GPCR field will eventually move away from detergents, including GDN. Nanodiscs are at least a sensible compromise. The authors have shown in the past (with another class C GPCR, mGluR5, PMID: 3067506) what is possible and I believe that has to be the way forward. That being said, I've been guilty of solving membrane protein structures in detergent in the past, so I should be the last one to throw stones J In the ion channels field we now have to solve again and again old detergent-based structures because they are inaccurate, and I am quite sure that the same will happen with many GPCR ones. At the same I fully appreciate that these structures didn't come cheaply, and as mentioned above the paper has a lot of merit. My point here is more of a wish for the field, especially from one of the leading labs, rather than a criticism.

We thank Reviewer #2 for his appreciation of the challenges in membrane protein structural determination and share his view of the need for more membrane protein structures determined in lipid environments. Although the few recent GPCR structures determined in nanodiscs are quite similar to the same structures solved in detergent (Yin *et al. Nature* 584, 125-129 (2020), Zhang *et al. Nat. Struct. Biol*. 28, 258-267 (2021)), we agree that the field requires a more systematic effort towards nanodisc reconstitutions. To this end we also plan on exploring the structure of CaSR and its complexes in nanodiscs.

Here are my specific suggestions for improving this manuscript:

We have elaborated on the details regarding the points raised by Reviewer #2 below:

1. I was (like the authors presumably) puzzled by the open/closed VFT conformations in the inactive state. Especially when reading the paragraph contrasting this to the open/open crystal structure(s)

and looking at Fig 1. My suggestion is to mention from the start that there is a ligand in one of the VFTs, difficult to explain (and very unlikely to be some amino acid picked from the TC medium, considering the purification protocol…). This is probably some contaminant/breakdown product from the detergent solution. Building Trp in that density (and showing it as such in Fig 1a), is very confusing and not justified I think. Personally I would hesitate to model anything specific in that density. Some "ligand X" could be shown in the Fig5 cartoon.

We agree with Reviewer #2 and have revised the manuscript accordingly on page 5, lines 7-14, and Figs. 1 and 5. We have refrained from modeling the density as Trp. A previous crystal structure of active CaSR ECD purified without the addition of Trp also revealed densities at the LB1-LB2 cleft and these densities were determined to be the Trp derivative, L-1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA), through mass spec (Zhang *et al. Sci Adv*. 2016; 2:e1600241). We postulate that the density observed in our open-closed ECD could be of a similar origin and are now referring to it as an aromatic amino acid or its derivative.

2. Another thing that I find surprising is to see so many ions in random places. Yes this is a calcium sensing receptor, but the maps (and models) provide absolutely no evidence for calcium presence at 6 out of 8 sites shown in a dimer. Sites 1 and 5 are OK. Sites 4 and 8 very questionable. Sites 2, 3, 6, 7 definitely not, it is simply impossible for calcium atoms to sit in that environment, the coordination sphere makes no sense. These structures, in my opinion, do not explain how CaSR senses calcium and this should be stated in the text. Neither do the crystal structures to be honest, which are of very poor quality, and have probably guided the models presented here. Therefore I would remove the calcium ions that are neither supported by the maps, nor by chemistry.

This is a good point. We agree with Reviewer #2 and have removed the calcium at sites 2, 3, 6, 7 from our models. Indeed, the initial placement of calcium atoms was based on the previous ECD crystal structure in which the calcium sites were identified with anomalous scattering and mutagenesis studies on residues at these sites suggested the importance of these regions. We can see small densities at positions identical to the calcium sites shown in the crystal structure, but we do agree that the coordination sphere does not support the presence of calcium at sites 2, 3, 6, 7 and we thus no longer include those calcium ions in the structure.

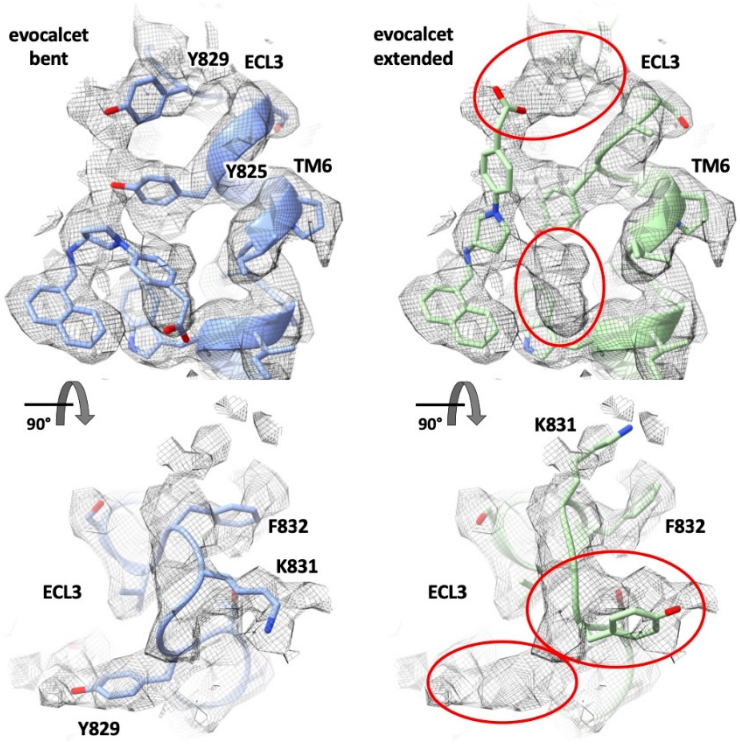
3. Prompted by the calcium/Trp situation, I started to look around the maps/models (and I very much appreciate the fact that authors have provided these!!). The problem is that models are poorly built, despite the impressive stats in EDT1. There are errors in the register (see for example region 552-559 in chain B or, more importantly, in the 825-832 region of chain A, which is key to coordinating the

PAM) in the 2.5A structure; some glycans are built in one chain but not in the other, despite clear density in the maps (see N261 and N541 in chain B of the 2.5A structure, or in chain A of the 2.8A structure); the C-terminus of chain A (which plays a role in explaining why one TMD but not the other couples with g proteins) has very poor density, even playing with different sharpening levels… an obvious mistake is the way Phe 881 and surrounding region was built in the 2.8A model, in the 2.5A the map and model are even worse… It seems to me that some errors are carried over from the X-ray models, or introduced by Swiss-model. It would be really important, I think, to have somebody experienced in building protein models go carefully through all three structures and fix as many problems as possible.

Thank for the careful examination of our maps and models. Indeed some errors were carried over from previous models, and we apologize for this oversight. We have very carefully looked at all regions, corrected the errors, further refined our models, and modelled all the glycans. We agree that the densities for the C-terminus of chain A is relatively weak, but we believe it is identifiable. We have refined the modelling in this region, stubbed the sidechains where the density doesn't allow for confident modelling of rotamers, and refrained from discussing specific interactions with Phe881 in the manuscript.

4. As alluded above, I am not convinced that the evocalcet PAM in chain A (2.5A structure) is built correctly as a "bent" conformation. At the very best, we see a mixture of the up and down conformations there. This is clearly reflected in the surrounding protein density. Tyr 825 should be down, Tyr829 up, Lys831 and Phe832 are not in the right density. To my eyes, this particular loop adopts a conformation consistent with an extended evocalcet conformation, much more so than a bent one. In contrast, the bent conformation of the cinacalcet PAM of the 2.8A structure is convincing.

We have tried Phenix density modification on the 7TMs local map of CaSR-etelcalcetide-evocalcet as suggested by Reviewer #2 in point 5 below, and tried modeling either a bent or extended evocalcet into the densities. As illustrated in the figure below, when this region is modelled with an extended evocalcet conformation, several strong densities (circled) cannot be accounted for while a bent conformation agrees better with the map. Although the ligands have differences, evocalcet and cinacalcet have appreciable similarities, and the bent evocalcet pose is consistent with the bent pose of cinacalcet. In addition, both poses are further supported by our ligand docking approach (GemSpot). We thus believe evocalcet adopts a bent conformation in this protomer, underlined by the ligand flexibility and the structure of the binding pocket.



5. I would suggest that many issues above would be helped by processing data in Relion rather than Cryosparc (especially an old version). Although CS reports more impressive nominal resolutions, and it's quick & easy to run, this comes at a cost: maps are rarely (OK, never…) as good as those output by Relion. I would also try density modification in Phenix (Resolve), they helped us clarify small molecule binding modes multiple times.

Thank you for these suggestions. Our data was processed with the latest version of cryosparc available at the time (v3.0 for the active- and inactive- state CaSR and v3.1 for the new CaSR-NAM-Ca^{2+}-Trp). In our case, the non-uniform refinement in cryosparc really helped resolve the 7TMs region. As suggested, we have tried Phenix density modification for the 7TMs local map of CaSR-etelcalcetide-evocalcet as illustrated above.

Smaller issues:

1. Page 3, rows 10/11: it is not clear which structure was solved to which resolution;

We have revised these sentences to make the resolution statement clearer.

2. Page 4, row 15: I believe Fig. 1e should be called (not 1d);

We have corrected this in the revised manuscript. Thank you.

3. Page 8, row 2: the asymmetry “suggests” (rather than “implies”), there is no clear evidence in this paper;

We have adjusted the wording here. The results of the heterodimer signaling studies following this sentence provide support that the protomer with an extended PAM is the G-protein-coupling one.

4. EDF 3 (or a separate figure?): there are no model vs map correlation curves… I think these are essential, and could have helped highlight problems with the models.

We have now added model-vs-map curves in the revised manuscript.

5. Methods, model building+refinement: refinement of the 4.1A model would benefit a lot from restraints provided by a high quality CaSR model (once built). The stats in EDT1 show this clearly, only 88% favoured Rama residues cannot be justified. The map at 4.1A is just bad, and it should not weigh too much in refinement.

We have improved the statistics of the inactive-state CaSR model with restraints from the 7TMs of our newly determined CaSR-NAM-Ca^{2+}-Trp structure and ECD of previous crystal structures.

6. EDT1: I suggest the model composition and B factor description should separate glycals from the other ligands, which must be listed one by one so readers could better judge their quality.

We have listed the B factors for each individual ligand and glycan.

7. EDT1: a B factor of 3.68 for the NAL (which barely has any density, to be honest…) makes no sense. This is either a typo or a refinement error.

This was indeed an error. We have now corrected it.

8. EDF5 seems to have no figure legend? Apologies if I missed it.

We apologize for this oversight and have added the figure legend.

I very much hope that the authors will find the comments above helpful. Irrespective of them this clearly is a most impressive piece of work!!

We very much appreciate Reviewer #2’s constructive comments! Addressing them has really helped produce a much better manuscript. Thank you!

Reviewer Reports on the First Revision:

Ref #1

This manuscript has been significantly improved with the additional requested work from the reviewers. Here are some addition questions:

1. It's interesting and promising to see the 7TM domain differences between inactive state and CaSR-NAM-Ca2+-Trp complex. The working model has been much improved. However, under low calcium concentration, how CaSR can be activated by cinacalcet is still needed to be fully understood for the activation mechanism of CaSR. Will the 7TM be asymmetric under the condition of low calcium with cinacalcet?

2. In Fig 3b, I agree that "The mutant favoring the bent conformer showed substantially blunted PAM potentiation of Ca2+ response, while the mutant favoring the extended PAM conformer showed enhanced PAM potentiation"
However, without cinacalcet, the EC50 of L773W V833W (bent) appears to be smaller than both WT and C781W I822W (extended)? What's the explanation of this?

Ref #2

I would like to thank the authors for addressing most of the points I have raised in my original review. There is no doubt that this work makes a very important contribution to the GPCR field and beyond.

I still disagree on some aspects of map interpretation. I understand the point that some elements of density are not explained by an "extended" evocalcet conformation. At the same time, the ECL3 in the "bent" evocalcet conformation doesn't match the density well either. To my eyes, this map illustrates a mixture of states and further classification would be beneficial. Careful reprocessing in a new Cryosparc version or indeed in Relion may lead to an improved map. I personally think one should always strive to use the best software available for the job. At the same time, this is a fairly minor issue and the authors have clearly spent much more time than me thinking about their data :-)) Map interpretation, especially at such resolution, is a subjective issue.

The aymmetric activation model proposed is much more interesting. I note that another group, working with a similar construct, seem to observe (as far as I can tell) a symmetric active state (https://www.biorxiv.org/content/10.1101/2021.03.30.437720v1). It is not clear whether such differences come from data processing (i.e. whether symmetry might have been imposed by Chen et al, which would be a mistake) or sample preparation differences. Both analyses reflect structures in detergent, which may or not be meaningful. On balance though, weighing the evidence currently available, I think the model presented by Gao et al is more likely to be correct. Especially because a similar asymmetric arrangement seems to be observed in the mGlu2 structures (complementary manuscript).

After all, the sooner these results become available for the benefit of (and scrutiny from) the wider community the better.

Author Rebuttals to First Revision:

Referee #1 (Remarks to the Author):

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Author Rebuttals to First Revision:

We thank the reviewers for their valuable critiques and the favorable reviews of our work. Below is a point-by-point response to their comments:

Referee #1 (Remarks to the Author):

This manuscript has been significantly improved with the additional requested work from the reviewers.

Thank you for the favorable review of our revision. Addressing the reviewers' comments has indeed helped improve the manuscript.

Here are some addition questions:

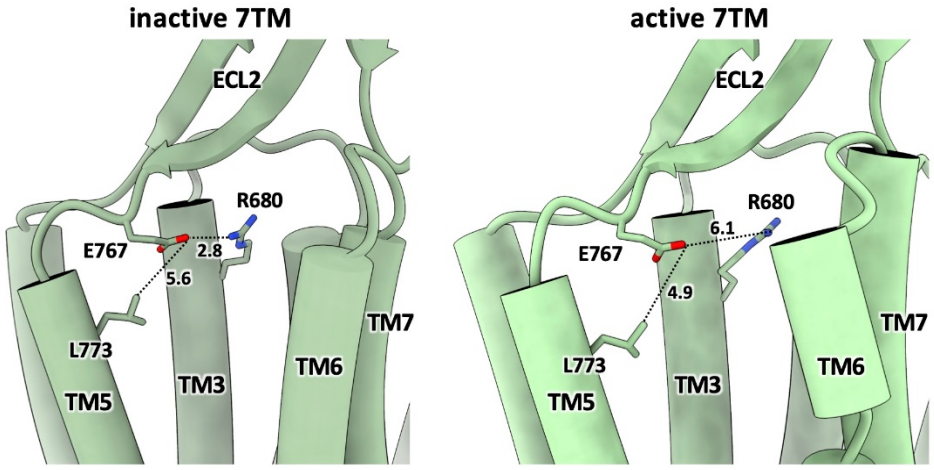
1. It's interesting and promising to see the 7TM domain differences between inactive state and CaSR-NAM-Ca^{2+}-Trp complex. The working model has been much improved. However, under low calcium concentration, how CaSR can be activated by cinacalcet is still needed to be fully understood for the activation mechanism of CaSR. Will the 7TM be asymmetric under the condition of low calcium with cinacalcet?

Cinacalcet has weak agonist activity toward CaSR under low calcium concentration (Extended Data Fig. 1c). It could arise from weak binding of the PAM in the 7TM with an extended conformation, transiently stabilizing an active conformation, but without further stabilization from an opposing 7TM (as observed in the active-state structures) this potentiation would be much weaker. In the low-calcium inactive state, the two 7TMs are separated and symmetric (Extended Data Figs. 1g,4a-b). We do not expect the 7TMs to come together and form a stable asymmetric 7TM configuration under low-calcium condition upon cinacalcet addition, as the CRDs would remain separated by the inactive ECD. However, it is possible that one cinacalcet-bound 7TM could transiently adopt an active conformation giving rise to weak agonist activity, as previous studies have shown that CaSR PAMs act as agonists for truncated 7TM-only CaSR (Leach *et al. Cell Res* 26, 574-592 (2016)).

2. In Fig 3b, I agree that "The mutant favoring the bent conformer showed substantially blunted PAM potentiation of Ca2+ response, while the mutant favoring the extended PAM conformer

showed enhanced PAM potentiation" However, without cinacalcet, the EC50 of L773W V833W (bent) appears to be smaller than both WT and C781W I822W (extended)? What's the explanation of this?

The lower EC50 of L773W V833W (bent) could potentially have arisen from L773W slightly perturbing the environment of the nearby E767 residue. E767 contributes to the stabilization of inactive conformation through interaction with R680 (figure below) and E767K is an activating ADH mutation (Uçkun-Kitapçi *et al*. *Am J Med Genet A* 132A, 125-129 (2005)).



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We appreciate (truly!) the reviewer's careful examination of the cryoEM maps and models. Indeed, the densities surrounding the bent evocalcet are not as clear as the bent cinacalcet despite our extensive efforts and various classification approaches, including also with Relion. This could be due to the significant flexibility of the particles (as illustrated in our 3D variability analysis, supplementary videos 4-6) and the inherent challenge in discerning small variations in a ligand pocket located within a large flexible homodimer. We essentially cannot rule out some residual heterogeneity in the classified particles that may be the reason behind this. Nevertheless, the comparison of the densities between the two related PAMs and the residues forming the allosteric binding site enhance our confidence in our model.

The aymmetric activation model proposed is much more interesting. I note that another group, working with a similar construct, seem to observe (as far as I can tell) a symmetric active state (https://www.biorxiv.org/content/10.1101/2021.03.30.437720v1). It is not clear whether such differences come from data processing (i.e. whether symmetry might have been imposed by Chen et al, which would be a mistake) or sample preparation differences. Both analyses reflect structures in detergent, which may or not be meaningful. On balance though, weighing the evidence currently available, I think the model presented by Gao et al is more likely to be correct. Especially because a similar asymmetric arrangement seems to be observed in the mGlu2 structures (complementary manuscript). After all, the sooner these results become available for the benefit of (and scrutiny from) the wider community the better.

This is a good point. In the recently published in *Cell Res* study (Ling *et al*. *Cell Res* 31, 383-394 (2021)), the use of enforced C2 symmetric averaging in cryoEM refinement together with the much lower resolution have prevented the observation of 7TMs asymmetry. In the newly deposited *biorxiv* preprint (doi.org/10.1101/ 2021.03.30.437720) C2 symmetry was not enforced according to their method section, but the resolution of the active-state was much lower (4.3Å at 7TMs) preventing the modeling of the PAM cinacalcet. Interestingly, in Figs. 1 and 6 of the preprint displaying the map and model of the active CaSR, the 7TMs do seem to be asymmetric (figure below), however this asymmetry has not been mentioned or discussed about by the authors. [REDACTED]