

Molecular basis of antagonism of the dimeric human arginine vasopressin receptor 1A

Corresponding Author: Dr Xudong Wu

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Zhong P et al. describes the cryo-electron microscopy (cryo-EM) three-dimensional (3D) structures of the human vasopressin V1a receptor (V1aR) in complex with three different types of antagonists, and in its apo state. These are the first high-resolution structures of V1aR ever described. Interestingly, all structures presented in this manuscript are those of homodimeric V1aR particles. In addition, the authors show that the apo state structure of V1aR displays a flat extracellular loop 2 (ECL2), a significant difference in conformation with that of the antagonist-bound receptor states (in which this extracellular loop exhibits a typical antiparallel beta-sheet).

This work is a nice combination of recombinant receptor expression and purification, nanobody selection and production, cryo-EM structure acquisition and analysis, site-directed mutagenesis and cell-based pharmacological assays. This study provides a comprehensive understanding of V1aR assembly and opens the way to structure-guided development of new antagonists for treating central nervous system disorders. It also gives information about antagonist receptor selectivity.

The methods developed are state-of-the-art and the results presented in the article are really interesting for G protein-coupled receptor (GPCR) research community. However, I have one major concern and also some other criticisms before the manuscript would be accepted for publication in Nature Communications.

1/ major comment:

My main criticism is focused on the ligand-free state of the V1aR. The author claim that the structures reveal a distinct conformation of ECL2 in the apo state compared to the antagonist-bound states. I agree with this nice idea but I think the present results do not support the conclusions.

The authors indicate that in the apo state, the ECL2 adopts a flat configuration whereas in the ligand-bound states this loop is in an open (up) conformation with a typical antiparallel beta-sheet conserved in most class A GPCRs. Moreover, the canonical disulfide bond between ECL2 and transmembrane domain 3 (TM3) is absent in the apo state while it is present in the antagonist-bound conformations. I think this disulfide bond is not seen because the ECL2 could not be constructed totally. Why the conserved disulfide bond would not be formed in the apo state? If I remember well, this post-translational modification should be done in the endoplasmic reticulum, before targeting the receptor to the plasma membrane and before interactions with ligands. Again, the antiparallel beta-sheet is not seen since part of the loop is missing in the density map, and amino-acid residues 191-201 are not present in the corresponding model. ECL2 is probably more flexible in the ligand-free state, thus I can understand it is more difficult to get a high-resolution density map in this region.

However, would it be possible to collect more movies (and substantially increase the number of particles) with this apo state sample in order to be able to solve completely the ECL2 and to conclude accurately? Indeed, authors collected only 4860 movies for the apo-state, and were able to collect 12117 or 6163 for the atosiban-bound state and the balovaptan-bound state, respectively.

If a new cryo-EM session is acquired and if the complete density of the ECL2 is seen, I would be happy with the authors conclusion. It would be quite interesting to discuss the role of the ECL2 in a "flat" conformation and its transfer into an "up" conformation in the presence of ligands. Could this loop close the solvent-exposed orthosteric binding pocket to help in maintaining a basal receptor state? Could this loop mimic a neutral ligand (no activation)? Since this apo state is the first one solved in the vasopressin/oxytocin receptor family as compared to active and inactive conformations of vasopressin V2 receptor (V2R) and oxytocin receptor (OTR) already available, the conclusions about the difference in ECL2 conformations

between ligand-free and antagonist-bound states are crucial.

2/ minor criticisms:

a) If we look at the density map of the apo state, there is a density on the intracellular side at the dimer interface between TM1 and TM7 which has not been assigned. This density is present in the two copies of the V1aR, symmetrically. Would this density correspond to a phospholipid? To a cholesterol molecule? Could this density correspond to part of helix 8 which is actually not seen?

b) Again, in the apo state structure, if we overlap the density and the corresponding three-dimensional model at the level of the extracellular loop 3 (ECL3), it is quite difficult to fit the residues in the density. I think some residues of the ECL3 should be removed.

c) All the structures solved in this study are those of dimeric V1aR. Indeed, size-exclusion chromatography (supplementary figure 1) and SDS-PAGE of the cryo-EM samples show purified dimeric species, in agreement with the homodimers seen in live cells. However, monomeric species are also seen in the samples that were pooled for subsequent cryo-EM grid preparations. I understand that image processing was started with an initial set of particles from which the software was trained to define those particles as templates containing only dimers, before picking the complete set of particles across all micrographs. Do the authors also see monomer particles in their cryo-EM preparations and micrographs? Would it be possible to process these particles like dimer particles, keeping in mind the size of these particles is perhaps too small for a single particle cryo-EM approach?

d) In absence of the nanobody 1 (Nb1), the resolution of the maps was very limited, whereas in the presence of Nb1, the overall resolution of the four maps is high (between 2.6 and 3 angstroms). Thus, the presence of Nb1 certainly stabilizes the complexes. Although Nb1 facilitated particle classification and alignment, its density was not resolved in the final maps. The authors suggest that Nb1 binds to a relatively flexible part of V1aR. Based on the stability of the complexes, is this conclusion not paradoxical?

e) To my opinion, the comparison of the different antagonist-bound V1aR structures is very interesting and is not sufficiently discussed. In supplementary figure 6, the structure of V1aR bound by atosiban, balovaptan and SRX246 were superimposed, but only the receptor is shown. Once the three structures are aligned based on the receptor structure, would it be possible to overlap the three ligands? I think it would be very useful to zoom on this ligand overlap and to present this illustration as a main figure. Comparing the docking poses of the three ligands in the orthosteric binding pocket in a single panel would be highly informative and would strengthen the discussion part on receptor antagonism and subtype selectivity. Since the orthosteric binding pocket is well conserved in the vasopressin/oxytocin receptor family, the binding sites of atosiban, balovaptan and SRX246 could be compared to that of retosiban in OTR, and those of tolavaptan and mambaquaretin in V2R.

Reviewer #2

(Remarks to the Author)

This paper presents the first high-resolution cryo-EM structures of the human V1aR in its apo state and in complex with three clinically relevant antagonists, atosiban, balovaptan, and SRX246. A major finding is the dimeric assembly of V1aR, which contrasts with previously resolved monomeric structures of related receptors and most of other class A GPCRs, providing crucial insights validated by cell-based assays. The study also elucidates significant conformational changes in ECL2, which transitions from a "flat" conformation in the apo state to an "up" conformation upon antagonist binding, creating a ligand-accommodating cavity. Furthermore, the structures and associated functional assays detail the distinct binding modes of the antagonists and identify key molecular determinants governing V1aR antagonism and subtype selectivity. This comprehensive mechanistic framework for V1aR regulation and assembly offers invaluable insights, paving the way for structure-guided design of novel antagonists.

However, while the structural data are of high quality and the reported V1aR dimer interface is intriguing, several major concerns regarding receptor function and pharmacology studies significantly limit the mechanistic impact and physiological relevance of the findings.

1. The only assay used to characterize the engineered V1aR as V1aR_{cryo} is the 3H-AVP radioligand binding assay. This does not assess whether the receptor is functionally competent. It is critical to perform signaling assays such as Ca²⁺ mobilization or G protein coupling to demonstrate that the engineered receptor retains signaling capacity. Without such data, it is unclear whether the receptor represents a physiologically relevant signaling-competent conformation.

2. For the radioligand binding assay, the saturation binding curve for 3H-AVP binding to V1aR_{cryo} (Supplementary Fig. 2a) does not reach a plateau and appears nearly linear across the concentration range tested. This calls into question the reliability of the reported K_d values and suggests that AVP binding is severely compromised (not slightly compromised). Accurate K_d determination under equilibrium conditions is essential for subsequent pharmacological analyses. It is commended to include more data points in the 3H-AVP saturation binding assay.

3. The competition binding assays for the antagonists are also highly problematic. The authors show that all three antagonists exhibit similar IC₅₀s (higher IC₅₀ for balovaptan) for V1aR_{cryo} compared to wtV1aR, and state that "Notably, the binding of antagonists atosiban, balovaptan, and SRX246 to V1aR_{cryo}, which are the focus of the current study, were almost unaltered" (line 109). However, according to basic pharmacological principles, if AVP binding is compromised (i.e., increased K_d), and the IC₅₀ values for antagonists are unchanged, it implies that the K_d values for the antagonists must also be compromised to the same extent. The authors should calculate and report the K_d values of all three antagonists using the

Cheng-Prusoff equation.

4. This issue also applies to the mutagenesis studies. In the mutagenesis studies, comparisons of IC50 values across V1aR mutants are presented without confirming whether AVP binding is preserved in each mutant. This is a critical oversight, as changes in AVP affinity would influence competition binding results. The proper approach is to first determine AVP Kd for each mutant and then calculate the corresponding antagonist Kd values to allow meaningful interpretation.

5. The observed dimer interface is structurally interesting, but its physiological significance remains uncertain. Class A GPCR dimerization is still a controversial topic. It has been well established that class A GPCRs do form clusters or loose oligomers under physiological conditions. However, whether they form stable dimers and how the oligomerization of class A GPCRs affect their signaling is still elusive. The authors should consider whether the observed dimerization may result from cryo-EM sample preparation artifacts such as the nanobody or the receptor modifications. In this regard, functional validation, such as ligand binding or signaling assays for dimer interface mutants like K52A and A59Y, is essential to demonstrate the relevance of dimerization to receptor function.

Moreover, it is important to report whether monomeric particles or highly oligomerized particles were also observed in the cryo-EM analysis. Please reference the recent study reporting higher-order oligomeric structures of a class A GPCR (doi.org/10.1038/s41594-024-01397-1). This would help clarify whether V1aR forms obligatory dimers, as seen in class C GPCRs, or if dimerization represents just one of several possible oligomeric states.

6. Previous studies have reported dimeric or oligomeric structures of other class A GPCRs, including the apelin receptor (Nat Struct Mol Biol 2022; Nat Commun 2024) and CXCR4 (Nat Commun 2023; Cell Rep 2025). Comparing the V1aR dimer interface with these receptors would help contextualize the findings and clarify whether this dimerization mode is unique or shared.

Other minor issues

7. It would be highly informative to discuss ligand selectivity in more details, as it is an important issue for developing antagonists for the AVP and OT receptor family. Atosiban is commonly used as a OXTR antagonist.

8. Since structures of V2R-AVP and OXTR-OT are available, it would be informative to model the binding of AVP and OT to V1aR and discuss its preference for the vasopressin and oxytocin peptides.

Reviewer #3

(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 #4

(Remarks to the Author)

In this manuscript, Zhong et al. present the structures of human V1aR in its apo state and in complexes with different antagonists. Overall, the manuscript is well-written. I am not a structural biologist and so am only commenting on the single molecule photobleaching experiment in Figure 2 b-f which I believe needs additional clarification and replicates to demonstrate statistical robustness.

Briefly - the authors perform single molecule photobleaching experiments in fixed cells that are transiently expressing GFP-tagged versions of the WT and K52A/A59Y versions of their protein. They conclude that the WT protein shows predominantly two-step photobleaching whereas the mutant protein shows single step photobleaching. Below are comments and suggestions for improvement:

Major Comments:

1) The authors describe a need to maintain “very-low” expression levels to minimize overexpression artifacts, yet it does not appear that they actually quantify the expression levels in cells. How do the fluorescently tagged protein levels compare to endogenous levels in WT cells?

2) It would be helpful if the authors could confirm that the GFP-tagged constructs are still functionally active. E.G. do cells expressing the GFP-tagged constructs respond similarly to agonism/antagonism compared to the non-fluorescently tagged proteins?

3) The authors show a representative image in Figure 2c, but do not state what the cell in the image is expressing. Furthermore, they indicate that only spots within the yellow circles were chosen for further analysis, but they do not state how these spots were selected or why they were chosen instead of the many other puncta-like objects in their image. I'm also concerned that the representative image shows many large aggregates of protein in addition to single diffraction-limited spots. What are these large aggregate structures? Are they an oligomerization artifact due to the tagged protein?

4) There are no error bars on Figure 2 e or f, nor do the authors provide information about the sample size (number of spots, number of cells, nor number biological replicates). Are these plots from just a single ROI in a single cell each?

5) At least in my experience, it is very unusual to have single molecule photobleaching defined by only monomers or dimers and not have any higher-order oligomerization states (even if only at a small percentage). Were these curves discarded from the analysis?

6) There is no information in the methods about how the number of bleaching steps for each trajectory was determined. Was this just done manually from the traces or did the authors utilize some objective criteria or algorithm?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised version of the manuscript by Zhong P et al. describes the cryo-electron microscopy (cryo-EM) three-dimensional (3D) structure of the apo state of the human vasopressin V1a receptor (V1aR) and those of the receptor in complex with three different types of antagonists. These are the first high-resolution structures of V1aR ever described. Interestingly, all structures presented in this manuscript are those of homodimeric V1aR particles. In addition, the authors show that the apo state structure of V1aR displays a flat extracellular loop 2 (ECL2) including an alpha helix, a significant difference in conformation with that of the antagonist-bound receptor states (in which this ECL2 exhibits a typical antiparallel beta-sheet). This study provides a comprehensive understanding of V1aR assembly and opens the way to structure-guided development of new antagonists for treating central nervous system disorders. It also gives information about antagonist receptor selectivity.

All the criticisms and comments I raised previously were adequately addressed in the revised version. Also, the remarks from the other reviewers were addressed (to my personal point of view).

My main criticism was focused on the ligand free state of the V1aR, for which the density map of the ECL2 was missing. Due to this absence, the previous results were not in agreement with the conclusions. Thanks to the authors who collected more cryo-EM images and describe now a conformation of the ECL2 which supports their conclusions. Indeed, the number of particles was increased, the overall resolution of the cryo-EM map was improved, and more importantly the majority of ECL2 was reliably modeled. This improvement allowed the authors to show that ECL2 adopts an alpha-helical conformation in the apo state and inserts into the ligand-binding pocket. These new data are now discussed, particularly about the transition from alpha-helical (apo state) to beta-sheet (ligand-bound) conformations upon ligand binding. New figures or new panels were also added to accommodate these changes.

Among the minor comments, since the orthosteric binding pocket is well conserved in the vasopressin/oxytocin receptor family, I also asked for a comparison between the binding sites of atosiban, balovaptan and SRX246 in the V1aR with those of retosiban in the OTR and those of tolvaptan and mambaquaretin in the V2R. The authors now introduced a new main figure and a new Supplementary figure, as well as text in the Result and Discussion sections.

All other minor criticisms were addressed adequately. And consequently, text and/or illustrations were added to the revised version.

I still have one remark about reference 39. Either it is incomplete, or it is wrong. Please complete or correct.

Providing this last modification (reference 39), I consider the manuscript is now acceptable for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)

The authors have addressed most of my concerns with additional pharmacological assays, and the new data look fine. I have only one remaining minor concern. The authors note that the two introduced mutations disrupt the signaling capacity of V1aR, although ligand binding is largely unaffected. In many GPCR structural studies that used engineered receptors with impaired signaling, the structural insights typically focused on ligand binding. In this case, the discussion of the potential dimer interface should be more cautious. It would be helpful to include a brief discussion of this limitation in the manuscript.

Reviewer #3

(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 #4

(Remarks to the Author)

The authors have generally addressed my comments by more clearly detailing the methods used for their selection of which cells and photobleaching spots to quantify, by more clearly describing their quantification procedure, and by verifying that the mStayGold-tagged proteins are functional. I do find their logic in describing the origin of and for excluding the

quantification of large aggregates to be a bit circular.

For example, the authors claim in the rebuttal that "stable expression systems or even CRISPR knock-in would lead to large aggregates, which would preclude reliable photobleaching analysis". Yet, they also state that "Overall, the puncta-like objects that were excluded from analysis most likely represent protein aggregates caused by transient transfection." So, if I understand the argument correctly, the authors anticipate that both endogenous expression (at native protein levels) and transient over expression lead to aggregates?

However, they exclude any large spots or traces with more than 2 photobleaching steps from further analysis, "Because there is currently no evidence that WT V1aR forms higher-order oligomers beyond dimers". I find this argument very confusing.

However, the authors do clearly state in the revised methods that they select only spots that show dimer or monomer photobleaching curves and discard any traces with more than 2 photobleaching steps. Given that this is a relatively minor part of the manuscript, I feel that this is probably sufficient.

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Point-by-point response to reviewers' comments

We sincerely appreciate the time and expertise dedicated by all the reviewers whose constructive comments have significantly strengthened our work. In response, we have carefully addressed all points raised using additional experiments and have revised the manuscript to incorporate the suggestions. All changes have been incorporated and are highlighted in the revised submission.

Below is a point-by-point response to the reviewers (our response is in blue):

Reviewer #1 (Remarks to the Author):

The manuscript by Zhong P et al. describes the cryo-electron microscopy (cryo-EM) three-dimensional (3D) structures of the human vasopressin V1a receptor (V1aR) in complex with three different types of antagonists, and in its apo state. These are the first high-resolution structures of V1aR ever described. Interestingly, all structures presented in this manuscript are those of homodimeric V1aR particles. In addition, the authors show that the apo state structure of V1aR displays a flat extracellular loop 2 (ECL2), a significant difference in conformation with that of the antagonist-bound receptor states (in which this extracellular loop exhibits a typical antiparallel beta-sheet).

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antagonist-bound conformations. I think this disulfide bond is not seen because the ECL2 could not be constructed totally. Why the conserved disulfide bond would not be formed in the apo state? If I remember well, this post-translational modification should be done in the endoplasmic reticulum, before targeting the receptor to the plasma membrane and before interactions with ligands. Again, the antiparallel beta-sheet is not seen since part of the loop is missing in the density map, and amino-acid residues 191-201 are not present in the corresponding model. ECL2 is probably more flexible in the ligand-free state, thus I can understand it is more difficult to get a high-resolution density map in this region.

However, would it be possible to collect more movies (and substantially increase the number of particles) with this apo state sample in order to be able to solve completely the ECL2 and to conclude accurately? Indeed, authors collected only 4860 movies for the apo-state, and were able to collect 12117 or 6163 for the atosiban-bound state and the balovaptan-bound state, respectively.

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Reply: Thank you for your suggestion. For the apo-state, we collected more images (from 4860 images to 8642 images now), which increases the final particles from 319,985 to 504,370. The overall resolution of the cryo-EM map has been improved from 2.9 Å to 2.8 Å. While the improvement in global resolution is modest, the local map quality has been significantly enhanced.

Using the new map of the apo state, the majority of ECL2 was reliably modeled, supported by well-defined local densities (please refer to **Fig. 3b** for local densities and **Supplementary Fig. 6c** for the exact protein sequence included in the final model). The model of the apo-state actually includes the protein segments of ECL2 that form the antiparallel beta-sheet observed in the antagonist-bound states (**Supplementary Fig. 6c**). Interestingly, in the apo structure, these regions adopt alpha-helical conformations: one becomes a continuation of TM4, while the other inserts into the ligand-binding pocket (**Fig. 3a, b, Supplementary Fig. 6c**). The two cysteines (one in TM3, and the other in ECL2) that form a disulfide bond in the antagonist bound states are both clearly resolved in the cryo-EM density in the apo form (**Fig. 3b**). They are spatially separated, with bulky residues such as F207 and W111 positioned between them (**Fig. 3b**). This arrangement provides strong structural evidence that the two cysteines remain unpaired in the apo state.

The region of ECL2 missing in the apo-state structure (residues 193-201; improved compared to the previous version) is not part of the antiparallel beta-sheet, but the flexible loop connecting the two beta-sheet segments. This region in the apo-state is visible as continuous and complete densities in the 6Å low-pass-filtered map (**Supplementary Fig. 5f**; the map has been uploaded for your evaluation). However, due to the limited resolution in this region, reliable atomic modeling is difficult, and thus, this segment was not included in the final model. Nevertheless, the absence of this region in the final model does not affect our conclusion that ECL2 in the apo-state adopts a planar orientation relative to the membrane in the apo structure, and thus is designated as the “flat” conformation.

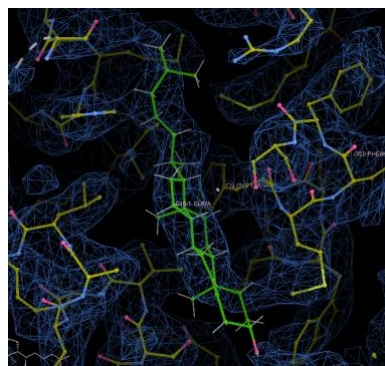
Indeed, the majority of disulfide bonds are formed in the ER. However, the oxidative extracellular environment, along with cell surface protein disulfide isomerase, can also facilitate disulfide bond formation or rearrangement, as demonstrated in studies (PMID: 22653978). In the case of GPCRs, a recent study on GPR4 (PMID: 39753131) demonstrated that ECL2 in the apo state has unpaired cysteines. We speculate that this apo state is a ground state. The transition from alpha-helical to beta-sheet could be very dynamic, which allows the selective recognition of the right ligand, while minimizing nonspecific interactions, particularly because the ligand-binding cavity in the “up” conformation is exceptionally large and exposed.

We modified **Fig. 3b, c**, made new **Supplementary Fig. 6c/Supplementary Fig. 5f**, and modified text (lines 265-276, lines 282-284) to accommodate the new changes.

2/ minor criticisms:

a) If we look at the density map of the apo state, there is a density on the intracellular side at the dimer interface between TM1 and TM7 which has not been assigned. This density is present in the two copies of the V1aR, symmetrically. Would this density correspond to a phospholipid? To a cholesterol molecule? Could this density correspond to part of helix 8 which is actually not seen?

Reply: Thank you for your question. We also noticed this density on the intracellular side. Based on the shape and size, we think it might be cholesterol (Please see the figure below for fitting). To be more conservative on the modeling building, we keep it unassigned.



b) Again, in the apo state structure, if we overlap the density and the corresponding three-dimensional model at the level of the extracellular loop 3 (ECL3), it is quite difficult to fit the residues in the density. I think some residues of the ECL3 should be removed.

Reply: Thank you for your suggestion. We reexamined the ECL3 density in the new map of the apo state and removed residue 325 from the final model.

c) All the structures solved in this study are those of dimeric V1aR. Indeed, size-exclusion chromatography (supplementary figure 1) and SDS-PAGE of the cryo-EM samples show purified dimeric species, in agreement with the homodimers seen in live cells. However, monomeric species are also seen in the samples that were pooled for subsequent cryo-EM grid preparations. I understand that image processing was started with an initial set of particles from which the software was trained to define those particles as templates containing only dimers, before picking the complete set of particles across all micrographs. Do the authors also see monomer particles in their cryo-EM preparations and micrographs? Would it be possible to process these particles like dimer particles, keeping in mind the size of these particles is perhaps too small for a single particle cryo-EM approach?

Reply: Thank you for your question. We also observed a small population of particles with a smaller diameter in raw images, likely corresponding to the monomeric species. We have tried to use different templates as well as particle diameter settings to select as many particles as possible during particle picking. However, due to their small size and low abundance, these particles were very likely lost during subsequent data processing and do not generate a reliable 3D reconstruction.

We modified the text (lines 192-195) to accommodate the new changes.

d) In absence of the nanobody 1 (Nb1), the resolution of the maps was very limited, whereas in the presence of Nb1, the overall resolution of the four maps is high (between 2.6 and 3 angstroms). Thus, the presence of Nb1 certainly stabilizes the complexes. Although Nb1 facilitated particle classification and alignment, its density was not resolved in the final maps. The authors suggest that Nb1 binds to a relatively flexible part of V1aR. Based on the stability of the complexes, is this conclusion not paradoxical?

Reply: Thank you for your question, and we apologize for any confusion. The limited resolution in the absence of Nb1 primarily arises from the lack of a sufficient fiducial mask outside the detergent micelle to guide particle alignment, which is a very common issue in cryo-EM studies of apo GPCRs. Although Nb1 is not well resolved in the final high-resolution reconstructions, the 6Å low-pass-filtered map reveals extra densities adjacent to the cytosolic face of V1aR that closely match the shape of Nb1 (**Supplementary Fig. 5e**). This indicates that Nb1 binds to a

conformationally heterogeneous epitope on the intracellular side of the receptor. This binding position is also far away from the dimer interface, making it unlikely that Nb1 stabilizes the complexes.

We therefore infer that Nb1 here primarily serves as a fiducial mask, facilitating **early** particle classifications and **global** low-resolution alignment. However, its flexible orientation relative to the receptor leads to partial signal averaging and eventual loss of nanobody density during high-resolution refinement.

We made a new **Supplementary Fig. 5e** and modified the text (lines 169-176) to explain this better.

e) To my opinion, the comparison of the different antagonist-bound V1aR structures is very interesting and is not sufficiently discussed. In supplementary figure 6, the structure of V1aR bound by atosiban, balovaptan and SRX246 were superimposed, but only the receptor is shown. Once the three structures are aligned based on the receptor structure, would it be possible to overlap the three ligands? I think it would be very useful to zoom on this ligand overlap and to present this illustration as a main figure. Comparing the docking poses of the three ligands in the orthosteric binding pocket in a single panel would be highly informative and would strengthen the discussion part on receptor antagonism and subtype selectivity. Since the orthosteric binding pocket is well conserved in the vasopressin/oxytocin receptor family, the binding sites of atosiban, balovaptan and SRX246 could be compared to that of retosiban in OTR, and those of tolvaptan and mambaquaretin in V2R.

Reply: Thank you very much for your suggestion. We have now introduced a new main figure, **Fig. 6**, to compare the binding poses of different antagonists, which include the three antagonists in the current study as well as retosiban (OTR), tolvaptan (V2R), and mambaquaretin (V2R). We also discussed in detail the specific interactions of these antagonists with the receptors, illustrated in the newly made **Supplementary Fig. 13**. The corresponding text has been added as a dedicated section (lines 466-488) in the Result section, as well as in the Discussion section (Lines 568-586).

Reviewer #2 (Remarks to the Author):

This paper presents the first high-resolution cryo-EM structures of the human V1aR in its apo state and in complex with three clinically relevant antagonists, atosiban, balovaptan, and SRX246. A major finding is the dimeric assembly of V1aR, which contrasts with previously resolved monomeric structures of related receptors and most of other class A GPCRs, providing crucial insights validated by cell-based assays. The study also elucidates significant conformational changes in ECL2, which transitions from a "flat" conformation in the apo state to an "up" conformation upon antagonist binding, creating a ligand-accommodating cavity.

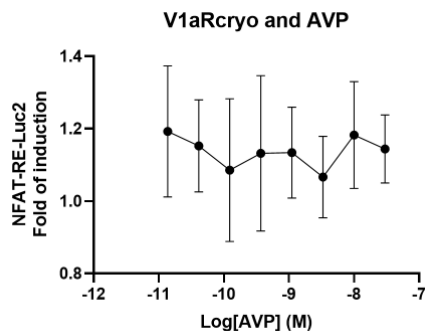
Furthermore, the structures and associated functional assays detail the distinct binding modes of the antagonists and identify key molecular determinants governing V1aR antagonism and subtype selectivity. This comprehensive mechanistic framework for V1aR regulation and assembly offers invaluable insights, paving the way for structure-guided design of novel antagonists.

However, while the structural data are of high quality and the reported V1aR dimer interface is intriguing, several major concerns regarding receptor function and pharmacology studies significantly limit the mechanistic impact and physiological relevance of the findings.

1. The only assay used to characterize the engineered V1aR as V1aRcryo is the 3H-AVP radioligand binding assay. This does not assess whether the receptor is functionally competent. It is critical to perform signaling assays such as Ca²⁺ mobilization or G protein coupling to demonstrate that the engineered receptor retains signaling capacity. Without such data, it is unclear whether the receptor represents a physiologically relevant signaling-competent conformation.

Reply: Thank you very much for your comment. The engineering of V1aR into V1aRcryo was adopted from a previous structural study of OTR (PMID: 32832646). In the previous study, eight mutations/truncations as well as fusion proteins were required to obtain the crystals, and the structure provides very valuable insights into the mechanism of OTR antagonism by retosiban. In our study, we aimed to preserve the wild-type sequence as closely as possible and significantly reduced the equivalent mutations from 8 to 2. The two adopted mutations used previously in OTR, S341C and N344K, were found to be essential for obtaining a stable sample suitable for structural studies; without them, V1aR remained refractory to high-resolution structure determination.

In the previous report (Logan, Richard Thomas (2013). The structure and function of the vasopressin V1a receptor. University of Birmingham. Ph.D. Table 4.3), the N344K mutation of V1aR was found not to be able to induce downstream signaling. We also used an NFAT-driven luciferase assay to assess the G-protein signaling of V1aRcryo and found it to be signaling-incompetent (Please see figure below).



However, V1aRcryo retains high sensitivity to antagonists atosiban, balovaptan, and SRX246 (Please see “reply to comment 3” below), validating its structural integrity. Since the current

study is focused on the molecular mechanism of antagonism, we deemed this engineering necessary and reasonable. To better inform the readers of the properties of V1aRcryo, we updated the text in (Lines 125-140).

2. For the radioligand binding assay, the saturation binding curve for 3H-AVP binding to V1aRcryo (Supplementary Fig. 2a) does not reach a plateau and appears nearly linear across the concentration range tested. This calls into question the reliability of the reported K_d values and suggests that AVP binding is severely compromised (not slightly compromised). Accurate K_d determination under equilibrium conditions is essential for subsequent pharmacological analyses. It is commended to include more data points in the 3H-AVP saturation binding assay.

Reply: Thank you very much for your suggestion. To improve the accuracy of the K_d measurement of AVP for V1aRcryo, we included more data points using higher concentrations of 3H-AVP (**Supplementary Fig. 2a**). The updated binding curve now reaches a plateau, and the new K_d value is ~19nM, compared with ~13nM in the previous version of the manuscript. The K_d value for V1aRcryo is updated in **Table 3**. In light of this change, we updated the description of AVP binding from “slightly compromised” to “modestly compromised” to better reflect the magnitude of the change and provide readers with a better understanding of the property of V1aRcryo.

We updated **Supplementary Fig. 2a**, **Table 3**, and modified the text (line 136) to accommodate the new changes.

3. The competition binding assays for the antagonists are also highly problematic. The authors show that all three antagonists exhibit similar IC₅₀s (higher IC₅₀ for balovaptan) for V1aRcryo compared to wtV1aR, and state that “Notably, the binding of antagonists atosiban, balovaptan, and SRX246 to V1aRcryo, which are the focus of the current study, were almost unaltered” (line 109). However, according to basic pharmacological principles, if AVP binding is compromised (i.e., increased K_d), and the IC₅₀ values for antagonists are unchanged, it implies that the K_d values for the antagonists must also be compromised to the same extent. The authors should calculate and report the K_d values of all three antagonists using the Cheng-Prusoff equation.

Reply: Thank you for your comments, and we apologize for this issue. Based on the updated K_d value for AVP, we converted the IC₅₀ values for antagonists into K_i using the Cheng-Prusoff equation. These values are now reported in the revised **Supplementary Table 3**. In light of this change, we updated the statement of antagonists binding from “almost unaltered” to “V1aRcryo remains highly sensitive to antagonists atosiban, balovaptan, and SRX246, enabling investigation of their molecular mechanisms, which are the focus of the current study.”.

We updated **Supplementary Table 3**, and modified the text (lines 138-140) to accommodate the new changes.

4. This issue also applies to the mutagenesis studies. In the mutagenesis studies, comparisons of IC₅₀ values across V1aR mutants are presented without confirming whether AVP binding is preserved in each mutant. This is a critical oversight, as changes in AVP affinity would influence competition binding results. The proper approach is to first determine AVP K_d for each mutant and then calculate the corresponding antagonist K_d values to allow meaningful interpretation.

Reply: Thank you for your comments, and we apologize for this oversight. To address this concern, we determined the K_d value of AVP for each mutant using the ligand-binding assays (**Supplementary Fig. 9, Table 3**) and confirmed that the AVP binding for each mutant is preserved, with no substantial changes compared to wild-type V1aR. Then, we converted the previous IC₅₀ values into K_i using the Cheng-Prusoff equation (**Supplementary Table 3**). All previous interpretations of the data remain the same.

We made a new **Supplementary Fig. 9** and modified the **Supplementary Table 3** to accommodate the changes.

5. The observed dimer interface is structurally interesting, but its physiological significance remains uncertain. Class A GPCR dimerization is still a controversial topic. It has been well established that class A GPCRs do form clusters or loose oligomers under physiological conditions. However, whether they form stable dimers and how the oligomerization of class A GPCRs affect their signaling is still elusive. The authors should consider whether the observed dimerization may result from cryo-EM sample preparation artifacts such as the nanobody or the receptor modifications. In this regard, functional validation, such as ligand binding or signaling assays for dimer interface mutants like K52A and A59Y, is essential to demonstrate the relevance of dimerization to receptor function.

Moreover, it is important to report whether monomeric particles or highly oligomerized particles were also observed in the cryo-EM analysis. Please reference the recent study reporting higher-order oligomeric structures of a class A GPCR (doi.org/10.1038/s41594-024-01397-1). This would help clarify whether V1aR forms obligatory dimers, as seen in class C GPCRs, or if dimerization represents just one of several possible oligomeric states.

Reply: Thank you very much for the suggestion. We are not the first to report the existence of V1aR dimers in the AVP/OT family. There has been a substantial amount of evidence showing the dimer formation among the AVP/OT family (PMID: 12554793) and their roles in beta-arrestin association and trafficking (PMID: 14757828), and this has guided the design of symmetric antagonists (PMID: 27420737). All of these underscore the physiological significance of the dimer and its importance for pharmacology. Our current study provides the structural insights into the dimer formation.

Following the reviewer's suggestion, we used the NFAT-driven luciferase assay to assess the G-protein signaling of wild-type V1aR and the K52A/A59Y mutant. The EC₅₀ for AVP is 5-fold

higher for the K52A/A59Y mutant than for wild-type, and the IC₅₀ values for all tested antagonists are higher for the mutant as well (**Supplementary Figure 7, Table 4**), which suggests that the dimer is physiologically relevant.

We did observe a small population of particles with a smaller diameter in raw images, likely corresponding to the monomeric species. However, due to their small size and low abundance, these particles were very likely lost during subsequent data processing and do not generate a reliable 3D reconstruction. In contrast, no clear trimeric or tetrameric particles were detected in our cryo-EM datasets, unlike CXCR4, for which recent cryo-EM studies have visualized trimeric and tetrameric assemblies. Now, we reported those findings in the text and referenced the paper of CXCR4 as well.

We made a new **Supplementary Fig. 7, Table 4**, and modified the text (lines 192-197, lines 247-249) to accommodate the new changes.

6. Previous studies have reported dimeric or oligomeric structures of other class A GPCRs, including the apelin receptor (Nat Struct Mol Biol 2022; Nat Commun 2024) and CXCR4 (Nat Commun 2023; Cell Rep 2025). Comparing the V1aR dimer interface with these receptors would help contextualize the findings and clarify whether this dimerization mode is unique or shared.

Reply: Thank you very much for your suggestion. We now compared the dimer interface of V1aR with that of β 1AR, rhodopsin, CXCR4, GPR3, and APJR and illustrated the comparison in the newly made **Supplementary Fig. 8** and contextualized our findings. The corresponding text has been added in the Result section (lines 250-256).

Other minor issues

7. It would be highly informative to discuss ligand selectivity in more details, as it is an important issue for developing antagonists for the AVP and OT receptor family. Atosiban is commonly used as a OXTR antagonist.

Reply: Thank you very much for your suggestion. We have now discussed the selectivity in more detail in the Discussion section (lines 568-586) and made a new **Supplementary Fig. 13** to make it more informative. Atosiban's dual antagonism is noted and discussed.

8. Since structures of V2R-AVP and OXTR-OT are available, it would be informative to model the binding of AVP and OT to V1aR and discuss its preference for the vasopressin and oxytocin peptides.

Reply: Thank you for your suggestion. We generated OT-bound V1aR and AVP-bound V1aR using Modeller based on V2R-AVP and OTR-OT structures (**Supplementary Fig. 11**). Using the computationally generated models, we discussed the residues that might contribute to the preference of AVP over OT. Phe³ of AVP inserts more deeply into the TM5–TM6 hydrophobic

cleft than Ile³ of oxytocin, enhancing hydrophobic interactions with V1aR. Moreover, Met200^{5,43} of V1aR will clash with Ile³(oxytocin), whereas in OTR and V2R, isoleucine at this position can accommodate Ile³(oxytocin).

We made a new **Supplementary Fig. 11** and modified the text (lines 385-396) to accommodate the new changes.

Reviewer #3 (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.

Reply: We sincerely thank Reviewer #3 for the thoughtful evaluation of our manuscript. As an Early Career Researcher participating in Nature Communications' peer review training initiative, your perspective is invaluable to the scientific community. We appreciate the rigor of your methodological insights. Your contributions exemplify how emerging scholars enhance academic discourse, and we commend the journal's commitment to fostering new voices in peer review.

Reviewer #4 (Remarks to the Author):

In this manuscript, Zhong et al. present the structures of human V1aR in its apo state and in complexes with different antagonists. Overall, the manuscript is well-written. I am not a structural biologist and so am only commenting on the single molecule photobleaching experiment in Figure 2 b-f which I believe needs additional clarification and replicates to demonstrate statistical robustness.

Briefly - the authors perform single molecule photobleaching experiments in fixed cells that are transiently expressing GFP-tagged versions of the WT and K52A/A59Y versions of their protein. They conclude that the WT protein shows predominantly two-step photobleaching whereas the mutant protein shows single step photobleaching. Below are comments and suggestions for improvement:

Major Comments:

1) The authors describe a need to maintain “very-low” expression levels to minimize overexpression artifacts, yet it does not appear that they actually quantify the expression levels in cells. How do the fluorescently tagged protein levels compare to endogenous levels in WT cells?

Reply: Thank you for your comment. V1aR exhibits very low, or even negligible, expression in the common cell lines available to us. We acknowledge that we did not perform absolute

quantification to directly compare protein levels in our experimental system with those in a subset of cells from normal tissues, as this is technically very challenging. Conventionally, GPCR dimerization is assessed using BRET assays following 24-48 hours of transfection, an approach that has also been applied to confirm the dimer formation of AVP/OT family members (PMID: 12554793). However, higher expression levels increase the likelihood of dimer or even higher-order oligomer formation. Therefore, we attempted to probe the dimerization behavior of V1aR under a much lower expression level compared with the traditional method. In our experimental setup of the single-molecule photobleaching approach, the transfection period was restricted to 3 hours, a substantially shorter duration, under which V1aR remained predominantly in the dimeric state.

2) It would be helpful if the authors could confirm that the GFP-tagged constructs are still functionally active. E.G. do cells expressing the GFP-tagged constructs respond similarly to agonism/antagonism compared to the non-fluorescently tagged proteins?

Reply: Thank you for your suggestion. GFP-tag was changed into mStayGold-tag for better photostability behaviors. We used the NFAT-driven luciferase assay to assess the G-protein signaling of wild-type V1aR and the mStayGold-tagged version. In all cases, the mStayGold-tagged construct responds similarly to agonism by AVP and antagonism by atosiban/balovaptan/SRX246, compared to the wild-type proteins (**Supplementary Fig. 7**).

We made a new **Supplementary Fig. 7, Table 4**, and modified the text (lines 207-209) to accommodate the new changes.

3) The authors show a representative image in Figure 2c, but do not state what the cell in the image is expressing. Furthermore, they indicate that only spots within the yellow circles were chosen for further analysis, but they do not state how these spots were selected or why they were chosen instead of the many other puncta-like objects in their image. I'm also concerned that the representative image shows many large aggregates of protein in addition to single diffraction-limited spots. What are these large aggregate structures? Are they an oligomerization artifact due to the tagged protein?

Reply: Thank you for these insightful questions, and we apologize for the missing information. In the updated **Fig. 2c**, the cell is expressing mStayGold-tagged V1aR K52A/A59Y (previously shown as mEGFP-tagged WT V1aR in the original figure). This information is now provided in the legend of the updated **Fig. 2c**.

Using a single-molecule photobleaching assay to distinguish monomers from dimers proved to be extremely challenging for two main reasons:

- (1) **Stringent requirement for low expression levels.** The expression level needs to be sufficiently low to minimize both oligomerization and overlap of individual molecules. We anticipated that stable expression systems or even CRISPR knock-in would lead to

large aggregates, which would preclude reliable photobleaching analysis. Therefore, as mentioned above, we restricted the transient transfection period to less than 3 hours. During imaging, we manually inspected the fluorescence intensity of individual cells and selected those with relatively low fluorescence signals to reduce the probability of overlapping molecules. However, even under these conditions, expression levels still varied between cells within the same sample. Moreover, within a single cell, protein density was not uniform across the entire plasma membrane. Consequently, even in low-expression cells, many fluorescent spots likely correspond to large protein aggregates, which is a common caveat of transient transfection; these spots were excluded from further analysis.

- (2) **Photobleaching during cell screening reduces the apparent dimer fraction.** As described above, we needed to manually inspect cells to identify those with suitably low expression levels, which were relatively rare. During this inspection, photobleaching inevitably occurred. This process preferentially reduces the number of detectable dimeric events: once one fluorophore in a dimer bleaches, the remaining signal becomes indistinguishable from that of a monomer. In contrast, the number of monomeric events is largely unaffected, because spots that are fully photobleached are simply not selected for analysis. This concern motivated our switch from mEGFP to mStayGold, the most photostable fluorescent protein available to date. Although the photostability of mStayGold is significantly compromised after fixation, this change allowed us to obtain more data of sufficient quality for statistical analysis. Nevertheless, the fraction of dimeric events observed in our study is still likely underestimated.

Overall, the puncta-like objects that were excluded from analysis most likely represent protein aggregates caused by transient transfection. However, they are not artifacts of the tagging using mStayGold, as we have confirmed that the mStayGold-tagged constructs retain normal function. Furthermore, we have additional evidence supporting that WT V1aR predominantly exists in a dimeric state as observed in the cryo-EM structures. The dimeric assembly was functionally validated using an NFAT-driven luciferase assay to assess the G-protein signaling of wild-type V1aR and the K52A/A59Y mutant. The EC₅₀ for AVP is 5-fold higher for the K52A/A59Y mutant than for wild-type, and the IC₅₀ values for all tested antagonists are higher for the mutant as well (**Supplementary Fig. 7, Table 4**), which suggests that the observed dimer is physiologically relevant.

4) There are no error bars on Figure 2 e or f, nor do the authors provide information about the sample size (number of spots, number of cells, nor number biological replicates). Are these plots from just a single ROI in a single cell each?

Reply: Thank you for the great question. After switching from mEGFP to mStayGold, we were able to obtain more data of sufficient quality for statistical analysis (300-400 events in total). In the updated **Fig. 2e and 2f**, the data are presented as mean $\pm$ SEM. For each group, the data were

obtained from three cells across two independent experiments. This information has been included in the legend of **Fig. 2** and in the **Supplementary Information**.

5) At least in my experience, it is very unusual to have single molecule photobleaching defined by only monomers or dimers and not have any higher-order oligomerization states (even if only at a small percentage). Were these curves discarded from the analysis?

Reply: Thank you for the great question. We did observe a small fraction of spots that exhibited more than two photobleaching steps in their fluorescence intensity profiles (less than 10% of all inspected spots). However, as mentioned above, transient transfection inevitably led to non-uniform protein expression and overlapping molecules. Consequently, we could not reliably determine whether spots with more than two steps represented genuine higher-order oligomeric events, overlapping molecules, or simply protein aggregates. Because there is currently no evidence that WT V1aR forms higher-order oligomers beyond dimers, we chose to exclude all spots that exhibited more than two photobleaching steps from our analysis.

We have now explicitly mentioned this point in the **Supplementary Information**.

“Disputed spots, including those showing more than two photobleaching steps or lacking clear, well-defined transitions in their intensity profiles, were excluded from further analysis.”

6) There is no information in the methods about how the number of bleaching steps for each trajectory was determined. Was this just done manually from the traces or did the authors utilize some objective criteria or algorithm?

Reply: Thank you for your comment. Because we were unable to identify a reliable algorithm for automatic analysis, we manually inspected the fluorescence intensity profiles to determine the number of photobleaching steps. We have now added a detailed description of how the number of bleaching steps was determined in the **Supplementary Information** under “*Single-molecule photobleaching assay*”.

“Raw image stacks comprising 3,000 frames were acquired at a frame rate of 5 Hz. Two researchers independently selected approximately 100 non-overlapping, single-spot regions from each stack using ImageJ software. The summed fluorescence intensity within a 3×3-pixel region of interest (ROI), centered on each spot, was computed. Fluorescence intensity profiles across the entire stack were then plotted to identify photobleaching steps for individual spots. One researcher manually classified the bleaching curve types, and a second researcher performed an independent review. Disputed spots, including those showing more than two photobleaching steps or lacking clear, well-defined transitions in their intensity profiles, were excluded from further analysis. The number of spots exhibiting distinct bleaching steps was quantified for each experimental group and subjected to statistical analysis. Each group included three replicates derived from two independent experiments.”

Point-by-point response to reviewers' comments

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript by Zhong P et al. describes the cryo-electron microscopy (cryo-EM) three-dimensional (3D) structure of the apo state of the human vasopressin V1a receptor (V1aR) and those of the receptor in complex with three different types of antagonists. These are the first high-resolution structures of V1aR ever described. Interestingly, all structures presented in this manuscript are those of homodimeric V1aR particles. In addition, the authors show that the apo state structure of V1aR displays a flat extracellular loop 2 (ECL2) including an alpha helix, a significant difference in conformation with that of the antagonist-bound receptor states (in which this ECL2 exhibits a typical antiparallel beta-sheet). This study provides a comprehensive understanding of V1aR assembly and opens the way to structure-guided development of new antagonists for treating central nervous system disorders. It also gives information about antagonist receptor selectivity.

All the criticisms and comments I raised previously were adequately addressed in the revised version. Also, the remarks from the other reviewers were addressed (to my personal point of view).

My main criticism was focused on the ligand free state of the V1aR, for which the density map of the ECL2 was missing. Due to this absence, the previous results were not in agreement with the conclusions. Thanks to the authors who collected more cryo-EM images and describe now a conformation of the ECL2 which supports their conclusions. Indeed, the number of particles was increased, the overall resolution of the cryo-EM map was improved, and more importantly the majority of ECL2 was reliably modeled. This improvement allowed the authors to show that ECL2 adopts an alpha-helical conformation in the apo state and inserts into the ligand-binding pocket. These new data are now discussed, particularly about the transition from alpha-helical (apo state) to beta-sheet (ligand-bound) conformations upon ligand binding. New figures or new panels were also added to accommodate these changes.

Among the minor comments, since the orthosteric binding pocket is well conserved in the vasopressin/oxytocin receptor family, I also asked for a comparison between the binding sites of atosiban, balovaptan and SRX246 in the V1aR with those of retosiban in the OTR and those of tolavaptan and mambaquaretin in the V2R. The authors now introduced a new main figure and a new Supplementary figure, as well as text in the Result and Discussion sections.

All other minor criticisms were addressed adequately. And consequently, text and/or illustrations were added to the revised version.

I still have one remark about reference 39. Either it is incomplete, or it is wrong. Please complete or correct.

Providing this last modification (reference 39), I consider the manuscript is now acceptable for publication in Nature Communications.

Reply: Thank you very much for you time and expertise in improving this manuscript. Reference 39 is a dissertation. We have manually made it more complete.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns with additional pharmacological assays, and the new data look fine. I have only one remaining minor concern. The authors note that the two introduced mutations disrupt the signaling capacity of V1aR, although ligand binding is largely unaffected. In many GPCR structural studies that used engineered receptors with impaired signaling, the structural insights typically focused on ligand binding. In this case, the discussion of the potential dimer interface should be more cautious. It would be helpful to include a brief discussion of this limitation in the manuscript.

Reply: Thank you very much for you time and expertise in improving this manuscript. We now added a sentence in the disscussion section to discuss the potential limitation. (whether the dimeric assembly persists within the signaling complex remains to be seen.)

Reviewer #3 (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.

Reply: Thank you very much for you time and expertise in improving this manuscript.

Reviewer #4 (Remarks to the Author):

The authors have generally addressed my comments by more clearly detailing the methods used for their selection of which cells and photobleaching spots to quantify, by more clearly describing their quantification procedure, and by verifying that the mStayGold-tagged proteins are functional. I do find their logic in describing the origin of and for excluding the quantification of large aggregates to be a bit circular.

For example, the authors claim in the rebuttal that "stable expression systems or even CRISPR knock-in would lead to large aggregates, which would preclude reliable photobleaching analysis". Yet, they also state that "Overall, the puncta-like objects that were excluded from analysis most likely represent protein aggregates caused by transient transfection." So, if I understand the argument correctly, the authors anticipate that both endogenous expression (at native protein levels) and transient over expression lead to aggregates?

However, they exclude any large spots or traces with more than 2 photobleaching steps from further analysis, "Because there is currently no evidence that WT V1aR forms higher-order oligomers beyond dimers". I find this argument very confusing.

However, the authors do clearly state in the revised methods that they select only spots that show dimer or monomer photobleaching curves and discard any traces with more than 2

photobleaching steps. Given that this is a relatively minor part of the manuscript, I feel that this is probably sufficient.

Reply: We sincerely thank you for your time and expertise in helping to improve this manuscript, and we apologize for any confusion caused.

As mentioned, there is no available cell line that expresses V1aR at endogenous level, we had to rely on recombinant expression systems, including transient transfection, stable expression or CRISPR knock-in of an expression cassette. However, these methods typically lead to expression levels much higher than endogenous, often resulting in aggregation of the expressed membrane proteins. Notably, there is currently no evidence that wild-type V1aR forms higher-order oligomers beyond dimers, consistent with our structural data and previous studies. Therefore, a fraction of large fluorescent spots is likely attributable to protein aggregates in our over expression system.

Moreover, the intensity pattern of a single molecule spans an area of approximately 5×5 pixels, corresponding to $\sim 600 \times 600$ nm in this study. Therefore, under our transient overexpression conditions, there is still a probability that two to three dimers could fall within the same diffraction-limited region. This explains why a fraction of spots exhibit more than two photobleaching steps.

We therefore conclude that regions showing more than two steps are more likely due to higher local protein density/expression as well as protein aggregates. Accordingly, we excluded large spots and any traces displaying more than two photobleaching steps from further analysis.