nature portfolio

Peer Review File

Channelrhodopsins with distinct chromophores and binding patterns

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Structure-function of light sensitive channelrhodopsins have been extensively investigated. All-trans retinals (ATRs) bind to the channelrhodopsins and light-induced isomerization of ATRs triggers conformational changes and open the ion conductive pore of the channelrhodopsins. In this study, Shan et al used Cryo-EM and electrophysiology to characterize how endogenous and exogenous retinals operate/gate the more sodium permeable ChR2 and the more potassium permeable kalium channelrhodopsin (KCR). The authors showed that ChR2 and KCR differentially respond to endogenous and exogenous retinals and proposed two provocative models to explain the differences. Although the observations are intriguing, current set of structural and functional evidence does not seem to be sufficient to support the models and the conclusions.
Major issues:
1. In the abstract, the authors wrote "ChR2 requires exogenous all-trans retinal to maintain its photosensitive channel activity". This is a strong claim, which is not supported by the authors' own result in Fig. 1a where EndoChR2 can be light activated, albeit with smaller current.
2. Fig. 1 & 5c-d electrophysiology has serious flaws.
a. The current was recorded from HEK293 cells overexpressing the channelrhodopsins. How do we know the current differences seen in a-b and e-f are not due to heterogeneity of CHR2 or KCR1 expression levels? N=5 is apparently not sufficient unless a-b and e-f were recorded from the exactly the same cells. Otherwise, the expression levels may differ; or incubation of exogenous ATRs could alter channelrhodopsin expression (see example doi: 10.1038/srep16542). Alternative approaches therefore are required to show that the recorded cells express the same/similar levels of the channelrhodopsins.
b. Why ChR2 amplitude is about 10 fold smaller than KCR1? Due to expression differences? Is this well documented?
c. Details in electrophysiology are lacking. Please disclose detailed voltage protocols including holding voltage. Use a dotted line to label your zero current level.
d. Panel 1c and 1g, it's unclear the IV was derived from peak current or steady state current. Please clarify.
e. The raw current traced from EndoChR2 in a and b do not match the IV in panel 1c. The current in a has a strong outward current, but in c there was almost no outward current. The same issue applies to ExChR2 in b and c. The ExChR2 current was very leaky. There was huge current before and after light stimulation. Did you record a very leaky cell? If yes, how reliable this recording was? What were the reversal potentials for ChR2? In your Na-out/K-in condition, why the current reversed at around 0 mV?
f. Fig. 5. C110T is constitutively open even without light. This means at resting membrane voltage, K+ will constantly leak out. How can the cells survive after expression the GOF mutant?
3. The rigor of prior research was not sufficiently introduced and discussed. For instance, this study is mainly about ATR binding and control to the channelrhodopsins. However, there was almost not description on the previous findings and comparison of the current finding with previous reports.
4. Based on the authors' model, both endogenous and exogenous ATRs will compete for the Schiff base linkage to the lysine residue. Before the exogenous ATRs were added, the channelrhodopsins (ChR2 and KCR1) should, in theory, be covalently linked to the endogenous ATRs. The authors mentioned the exogenous ATRs were added 2-6 hours before patch clamp recording, while for Cryo-EM protein purification, the incubation time was 2 days. Will 2-6 hours be enough for the exogenous ATRs to exchange with the covalent linked endogenous ATRs? Any evidence other than electrophysiology (see my comment 2a) to show this is the case?
5. It is interesting to solve an N-retinylidene PE-like density in EndoChR2 structures. Do the authors have evidence that this endogenous retinal is indeed expressed in HEK293 cells? Why it is not observed in the EndoKCR1 structures despite the fact that endogenous retinals have a much bigger effect on KCR1? Please also discuss how N-retinylidene is cleaved from the molecule and moves from one binding pocket to covalently link to the lysine residues. More importantly, please design mutagenesis experiments in the peripheral binding pocket to show the mutations could disrupt

endogenous retinal effects on ChR2 and potentially KCR1, as well.
Minor issues:
1. Define KCR1 in the abstract
2. Line 65-66: Na bath is very vague. It almost means nothing to a reader if it is not defined.
3. Fig. 5b. The fonts for the scales were too small to see.
4. Please tune down the claims about utilizing the channelrhodopsins for treating human diseases. To the best of my knowledge, this has not been successfully done yet.
5. Check grammar issues, eg. Line 48. Line 63-68, a super long sentence.
6. Line 114-116: This is too speculative. It's known ligands can allosterically regulate channel gating. Not necessary to be in the pore to prevent ion flux.
7. Line 141: add reference

Reviewer #2 (Remarks to the Author):

This manuscript by Shan et al. described the role of endogenous and exogenous retinal in the channelrhodopsin. A main conclusion of the manuscript is that "in the most common human kidneyHE293T cells, the endogenous retinal chromophore is more likely to be the retinal precursor or analogues, located in the other retinal binding pocket or forming the unconventional opsin-retinal interaction, which functions differently from all-trans retinal." is supported by the data presented. As I am not an expert in channelrhodopsin, I do not judge the significance of this conclusion.

Overall, this is not a very well written manuscript, with mistakes in writing that make the sentences rather confusing. Nonetheless, these could potentially be improved by revision.

Here are some specific comments:

Line 64 – 68: The writing is confusing or even wrong. "with and without" I think here author meant only "without", and the following sentence refer to "with". Otherwise, these two sentences contradict with each other, and reads very confusing. Similarly, it seems that panel Figure 1f is mis-labeled. In the figure panel, it is labeled as ExoChR2, same as panel b, but the legend says that it is expoKCR1.

Line 97-99: concerning the additional retinal binding pocket: does the endoChR2 contains two retinal? From the Figure, it seems that there is only one retinal (9-cis N-retinylidene-PE) bound to the protein. Is the other retinal pocket empty, without any structural changes? It is hard to think an empty binding pocket but without any changes. Or do authors mean that 9-cis N-retinylidene-PE binds to the same pocket but in a different form?

The title of this section: "The classic retinal binding pocket prevents the endogenous N-retinylidene-PE from hydrolysis and loading into the canonical retinal binding pocketin endoChR2." Is disconnected from the content of the section. The section does not conclude the "prevents" from "hydrolysis" and "loading into". If this title is the conclusion of this section, it is entirely unsupported by the data presented.

A related question: authors made a conclusion or speculation in the related manuscript (ref 21, also being reviewed by me) that retinal blocks the ion permeation pathway thus close the channel, and hydrolysis of the retinal opens the channel. Now that without the retinal in the binding pocket, the ion permeation pathway should be clear without blocking. But the channel is not open, as shown in patch clamp experiment. How does the ion permeation pathway look like, could the pore profile tell if the channel is still closed, which presumably should be but by what?

Line 109 – 110: As shown in ED Fig 7a-c, this density is a mixture of different binding form of the same retinal. The statement "likely presenting the process of N-retinylidene PE from hydrolysis to

loading into the canonical retinal binding pocket" is very speculative. If it is indeed intermediate states of loading the retinal into its canonical binding pocket, some density (even weak) should be seen within the canonical pocket. It is possible to better resolve this density by symmetry expansion followed by focused classification, which may provide better clue. And I would suggest authors to do so.

Same goes to endoKCR1, as shown in Figure 3b, c, d, where different forms of retinal can be modeled into the density. It is possible that the density reflects mixture of different retinal form. Again, symmetry expansion followed by focused classification could potentially sort this out. The statement "This suggests that the endo-retinal is different from the all-trans retinal" is a reasonable conclusion supported by the data. However, the statement "These models are referred to as practices transitioning from pre-hydrolyzed to hydrolyzed." is pure speculation.

Line 163-166: "Like before, we determined the cryo-EM structure of the endoC110T mutant in a potassium environment without exogenous all-trans retinal. Moreover, we acquired two classes of cryo-EM structures of exoC110T in a potassium environment, with overall resolutions of 2.57 Å and 2.56 Å, respectively". This description is confusing. Is this endoC110T or exoC110T, or you determined structures from both?

Minor technique points:

There is no need to state the resolution in the text to 2 digits after decimal point. It is fine to state this way in the method part.

Yifan Cheng

Reviewer #3 (Remarks to the Author):

The manuscript "Distinctive gating mechanisms of channelrhodopsin-2 and kalium channelrhodopsin-1" by Shang et al. describes the results of electrophysiology and structural studies of channelrhodopsins: nonselective ChR2 from Chlamydomonas reinhardtii and kalium selective KCR1 from H. catenoides. The authors heterologously expressed the proteins in human kidney cells HE293T with and without exogeneous all-trans-retinal. They showed that under the conditions of their experiment ChR2 requires exogenous all-trans-retinal to maintain its photosensitive channel activity. The absence of exogeneous retinal prohibited the ChR2 current. On the contrary, the supplementation of HEK293T cells with exogenous all-trans-retinal inhibits the KCR1 channel currents. Without exogenous all-trans-retinal KCR1 displayed photocurrents.
My first concern is that the corresponding 'inhibitions' of the currents are not absolute. In EndoChR2 the light activated current is smaller, nevertheless it is not equal to zero (it is about a factor of 7 less than the current of ExoChR2). A similar remark relates to the case of the KCR1 channel. The ratio is small (about 0.25), however, it is not equal to zero. It means that all variants of the proteins (Endo and ExoChR2 and also Endo and ExoKCR1) are displaying properties of light-gated channels.
To rationalize their results the authors performed cryo-EM structural studies of both proteins expressed in HEK293T cells in the presence and absence of exogenous all-trans-retinal. They interpreted electron densities in the retinal pocket of ChR2 expressed with exogenous retinal as those corresponding to all-trans-retinal in the case of the protein expressed in the presence of all-trans-retinal. When ChR2 was expressed without exogeneous retinal the authors did not observe the retinal in the conventional retinal pocket, but they claim its (more precisely, that of 9-cis N-retinylidene-PE) presence on the surface of the protein (Figure 2a and b). My second concern is that at the present resolution (around 2.5 Å) it is very problematic to ab initio identify the conformation retinal. It is supported by numerous crystallographic studies of microbial rhodopsins such as rhodopsin BR and also by the fact that even in the ground state of ChR2 the retinal is in two conformations (Kuhne et al.

Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin. Proc Natl Acad Sci U S A 116 (19) 9380-9389 (2019)). The authors cannot see this and even do not discuss this fact. The authors should present the proofs of their assignment using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal (Zhang et al. Photic generation of 11-cis-retinal in bovine retinal pigment epithelium. J. Biol. Chem. 294(50) 19137–19154 (2019)). The same concern is applicable to very smeared densities on the surface of EndoChR2 which were assigned to 9-cis N-retinylidene-PE. One cannot exclude that 1/7 of the value of the current of ExoChR2 observed in "EndoChR2" can be attributed to ChR2 with all-trans-retinal with the occupancy 1/7. The electron densities in the region of a classical retinal pocket of ChR2 in the case of EndoChR2 are not shown by the authors. In this case they can also be weak to reliably detect/interpret in endoChR2 at the present resolution. Moreover, there is a lot of evidence that endoChR2 (and many other similar channelrhodopsins) expressed in mammalians for optogenetic studies behaves as a classical exoChR2. This means that in such cases that EndoChRs comprise the all-trans-retinal in the ground state and the retinal is attached to Lys in a classical way along the photocycle (as it was (Nagel et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channe. Proc Natl Acad Sci U S A 100 (24), 13940-5 (2003)).

For basically the same reasons my concerns are applicable to the KCR1 data. The assignment of retinal conformations to densities as shown in Figure 3 is not convincing. Once again, accurate assignment is especially problematic when the chromophore processing, insertion and covalent binding to the lysine is not homogeneous (is not simultaneously completed in the proteins). This can be the case of endogenous stepwise retinal biochemical processing and insertion. I would like to mention once again that the authors should also present the proofs of their assignments using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal and it must be used in both cases.

Last but not least, the differences in the behavior observed by the authors of Endo and Exo ChR2 on the one hand and Endo and ExoKCR1 on the other may have an alternative interpretation. HEK293T is not a host system for both proteins. The host systems are very different (algae and fungi) and may have different from each other and from HEK293T retinoid processing machinery which is usually complex (Brueggemann and Sullivan. HEK293S Cells Have Functional Retinoid Processing Machinery. J. Gen. Physiol. 119, 593–612 (2002)). In addition, both proteins have different structural features and all this together may result in the differences of kinetics of the processing of the proteins and chromophore insertion in HEK293T cells. However, time dependence of the observed 'effects' has not been discussed in the manuscript

To summarize, I respect very much the basic idea of the authors, however, the experimental data should be sufficient to reliably make such a conclusion. Ehe work in its present form will not be convincing for the readers and requires additional experiments.

Point-by-Point Response to Reviewer's Comments

Journal: Nature Communications

Manuscript ID: NCOMMS-22-35109-T

Dear Reviewers:

We would like to express our great gratitude to the reviewers for their valuable comments and suggestions. Our co-authors have conducted a series of experiments to address the raised concerns in the past several weeks. Our response to review comments together with new experimental results were given in this letter and the revised manuscript. In the following, we present our response (marked in blue) to each review comment in detail. We hope the revised manuscript and figures can satisfy your and the reviewers' concerns.

We request your kind consideration of this work for publication in *Nature Communications*.

Reviewer #1 (Remarks to the Author):

Structure-function of light sensitive channelrhodopsins have been extensively investigated. All-trans retinals (ATRs) bind to the channelrhodopsins and light-induced isomerization of ATRs triggers conformational changes and open the ion conductive pore of the channelrhodopsins. In this study, Shan et al used Cryo-EM and electrophysiology to characterize how endogenous and exogenous retinals operate/gate the more sodium permeable ChR2 and the more potassium permeable kalium channelrhodopsin (KCR). The authors showed that ChR2 and KCR differentially respond to endogenous and exogenous retinals and proposed two provocative models to explain the differences. Although the observations are intriguing, current set of structural and functional evidence does not seem to be sufficient to support the models and the conclusions.

We thank reviewer #1's comments. We have tried to tune down the claim of the "provocative photo-hydrolysis" gating model and rewrite the paper.

Major issues:

1. In the abstract, the authors wrote "ChR2 requires exogenous all-trans retinal to maintain its photosensitive channel activity". This is a strong claim, which is not supported by the authors' own result in Fig. 1a where EndoChR2 can be light activated, albeit with smaller current.

We agree with the reviewer's comment. We have revised the relevant sentences in the electrophysiological results.

2. Fig. 1 & 5c-d electrophysiology has serious flaws.

a. The current was recorded from HEK293 cells overexpressing the channelrhodopsins. How do we know the current differences seen in a-b and e-f are not due to heterogeneity of CHR2 or KCR1 expression levels? N=5 is apparently not sufficient unless a-b and e-f were recorded from the exactly the same cells. Otherwise, the expression levels may differ; or incubation of exogenous ATRs could alter channelrhodopsin expression (see example doi: 10.1038/srep16542). Alternative approaches therefore are required to show that the recorded cells express the same/similar levels of the channelrhodopsins.

We thank this constructive comment. The results of fluorescence-detection size-exclusion chromatography (FSEC) show that the yields of EGFP-tagged CHR2 or KCR1 before and after incubation with ATR (Supplementary Fig. 1). As we only incubated with ATR for less than 6 hours, the expression levers do not significantly change.

b. Why ChR2 amplitude is about 10 fold smaller than KCR1? Due to expression differences? Is this well documented?

The expressed ChR2 in HEK293T cell showed around 400-500 pA photocurrent activated with a cw HeCd laser of 442 nm wavelength (up to 100 mW/mm^2) at the voltage of -100 mV (PMID

14615590). HcKCR1 in HEK293T exhibits more photocurrent magnitude (around 15 nA) than ChR2 in response to 540 nm and 6.3 mW mm^{-2} light pulses at the voltage of 100 mV (PMID 35726059). Our results are relatively consistent with previously reported results, and we added the reference to the main text.

c. Details in electrophysiology are lacking. Please disclose detailed voltage protocols including holding voltage. Use a dotted line to label your zero current level.

We thank you for reminding us to provide detailed electrophysiological experimental procedures. We have modified the legend of Figure 1. And the zero current level in Panels 1a-1b and 1e-1f is labeled as dotted lines.

d. Panel 1c and 1g, it's unclear the IV was derived from peak current or steady state current. Please clarify.

In panels 1c and 1g, the IV curves are derived from peak current. We have modified the legend of Figure 1.

e. The raw current traced from EndoChR2 in a and b do not match the IV in panel 1c. The current in a has a strong outward current, but in c there was almost no outward current. The same issue applies to ExChR2 in b and c. The ExChR2 current was very leaky. There was huge current before and after light stimulation. Did you record a very leaky cell? If yes, how reliable this recording was? What were the reversal potentials for ChR2? In your Na-out/K-in condition, why the current reversed at around 0 mV?

The currents without photo pulse from -100 mV to +100 mV upon 20 mV voltage increments are produced by native ion channels in HEK293. The photocurrents used for Panel c and g are subtracted mean of current derived from native ion channels. Therefore, both endoChR2 and exoChR2 exhibit inward currents. The membranes were held at -60 mV with a current around 250-500 pA which reached 1 GΩ resistance. Thus, our currents from the whole-cell patch clamp are reliable without leaky. The reversal potential for ChR2 is around +20 mV and has been labeled in panel 1c.

f. Fig. 5. C110T is constitutively open even without light. This means at resting membrane voltage, K+ will constantly leak out. How can the cells survive after expression the GOF mutant?

The environment of cell culture and electrophysiological experiment is dark to avoid the K^+ constantly leaky. The image of HEK293T with transient expression of C110T mutant below shows that the cell with GFP signal is healthy sticky in the glass slide. Another work also identified this mutant by electrophysiological experiment (PMID 37652010).

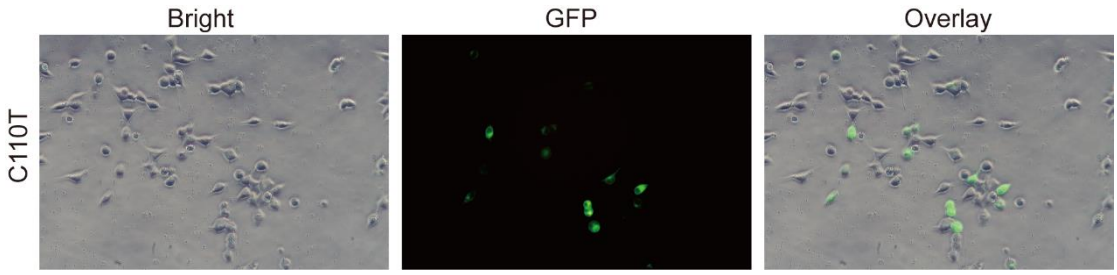
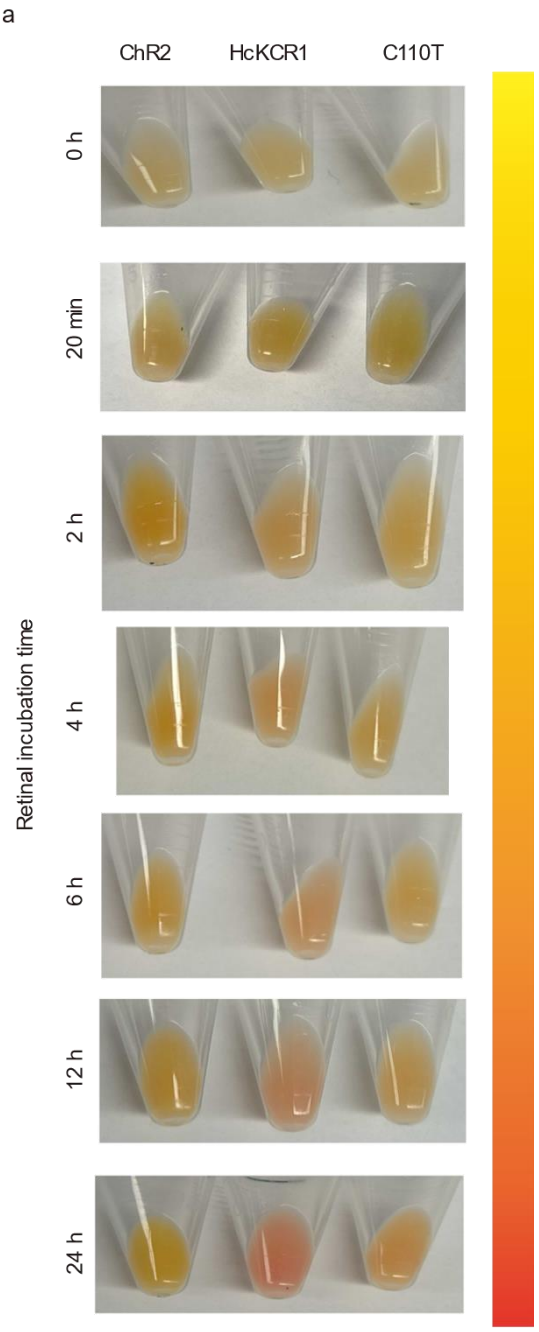


Image of HEK293T with transient expression of C110T mutant.

3. The rigor of prior research was not sufficiently introduced and discussed. For instance, this study is mainly about ATR binding and control to the channelrhodopsins. However, there was almost not description on the previous findings and comparison of the current finding with previous reports.

Thanks for this constructive suggestion. We have added the prior research in the introduction and discussion.

4. Based on the authors' model, both endogenous and exogenous ATRs will compete for the Schiff base linkage to the lysine residue. Before the exogenous ATRs were added, the channelrhodopsins (ChR2 and KCR1) should, in theory, be covalently linked to the endogenous ATRs. The authors mentioned the exogenous ATRs were added 2-6 hours before patch clamp recording, while for Cryo-EM protein purification, the incubation time was 2 days. Will 2-6 hours be enough for the exogenous ATRs to exchange with the covalent linked endogenous ATRs? Any evidence other than electrophysiology (see my comment 2a) to show this is the case?



We have added another piece of evidence for exogenous and endogenous retinal competition by cell color change assay. As you can see, the colour of the cell expressing ChR2 or KCR1 changes with the incubation of the exogenous ATR. This phenomenon is clear and dominant.

5. It is interesting to solve an N-retinylidene PE-like density in EndoChR2 structures. Do the authors have evidence that this endogenous retinal is indeed expressed in HEK293 cells? Why it is not observed in the EndoKCR1 structures despite the fact that endogenous retinals have a much bigger effect on KCR1? Please also discuss how N-retinylidene is cleaved from the molecule and moves from one binding pocket to covalently link to the lysine residues. More importantly, please design mutagenesis experiments in the peripheral binding pocket to show the mutations could disrupt endogenous retinal effects on ChR2 and potentially KCR1, as well.

It is reported that N-retinylidene-phosphatidylethanolamine need to be synthesis by the reaction of ATR generated by phototransduction and excess 11-cis retinal not required for the regeneration of the visual pigments reversibly with phosphatidylethanolamine (PE) in disc membranes. And N-Ret-PE can flip from the lumen to the cytoplasmic leaflet of photoreceptor membranes by A BCA4, an ATP-binding cassette (ABC) transporter (PMID 34625547) and ABCA4 is highly expressed in kidney from the data of HPA RNA-seq normal tissues.
Actually, in HEK293 cells or other mammalian systems, the retinoid metabolic pathway may be more complicated. We show that the lateral retinal binding pocket mutant can largely decrease the light response of the ChR2 without exogenous ATR (Fig 2g). However, in this study, we still cannot rule out what the real chemical formula of endo-retinal in KCR1 is. One hypothesis or most reasonable hypothesis is that N-retinylidene PE undergoes hydrolysis by ChR2 itself or a chaperone protein and releases the N-retinylidene. Further chemical study of the endo-retinal modulating channelrhodopsins remains an interesting study.

Minor issues:
1. Define KCR1 in the abstract
We have defined HcKCR1 as *H. catenoides* kalium channelrhodopsins in the abstract.

2. Line 65-66: Na bath is very vague. It almost means nothing to a reader if it is not defined.
We have revised the statement in the electrophysiological parts.

3. Fig. 5b. The fonts for the scales were too small to see.
The scales of the fonts have been bigger in Fig 5b.

4. Please tune down the claims about utilizing the channelrhodopsins for treating human diseases. To the best of my knowledge, this has not been successfully done yet.
We have tuned down the claims about the channelrhodopsins based therapy in the introductions.

5. Check grammar issues, eg. Line 48. Line 63-68, a super long sentence.
The sentences have been revised.

6. Line 114-116: This is too speculative. It's known ligands can allosterically regulate channel gating. Not necessary to be in the pore to prevent ion flux.
We have changed the statement.

7. Line 141: add reference
We have added the references in about the sentence in line 141.

Reviewer #2 (Remarks to the Author):

This manuscript by Shan et al. described the role of endogenous and exogenous retinal in the channelrhodopsin. A main conclusion of the manuscript is that "in the most common human kidneyHE293T cells, the endogenous retinal chromophore is more likely to be the retinal

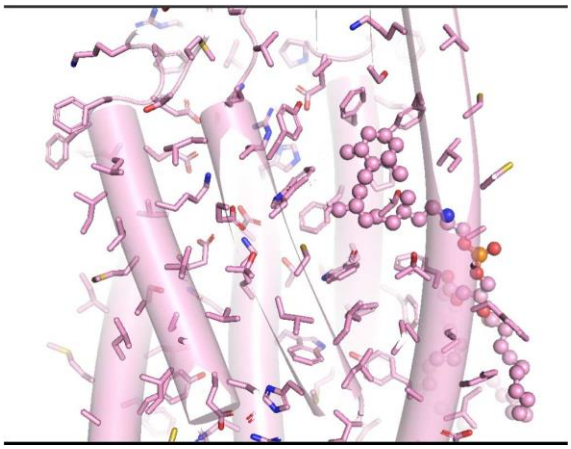
precursor or analogues, located in the other retinal binding pocket or forming the unconventional opsin-retinal interaction, which functions differently from all-trans retinal." is supported by the data presented. As I am not an expert in channelrhodopsin, I do not judge the significance of this conclusion.
Overall, this is not a very well written manuscript, with mistakes in writing that make the sentences rather confusing. Nonetheless, these could potentially be improved by revision.
Here are some specific comments:

Line 64 – 68: The writing is confusing or even wrong. "with and without" I think here author meant only "without", and the following sentence refer to "with". Otherwise, these two sentences contradict with each other, and reads very confusing. Similarly, it seems that panel Figure 1f is mis-labeled. In the figure panel, it is labeled as ExoChR2, same as panel b, but the legend says that it is expoKCR1.

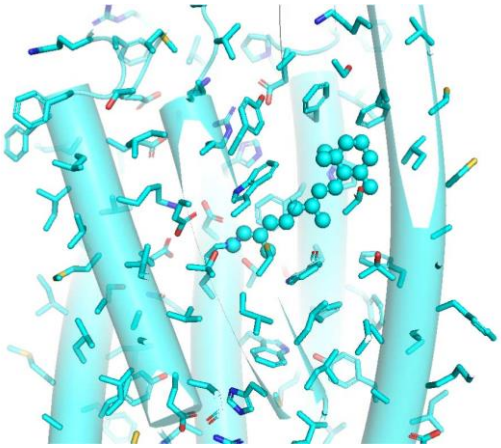
We apologize for the misleading statements. The sentences in line 64-68 have been revised. Also, in Figure 1f, the mis-labeled ExoChR2 have been displaced with ExoKCR1.

Line 97-99: concerning the additional retinal binding pocket: does the endoChR2 contains two retinal? From the Figure, it seems that there is only one retinal (9-cis N-retinylidene-PE) bound to the protein. Is the other retinal pocket empty, without any structural changes? It is hard to think an empty binding pocket but without any changes. Or do authors mean that 9-cis N-retinylidene-PE binds to the same pocket but in a different form?

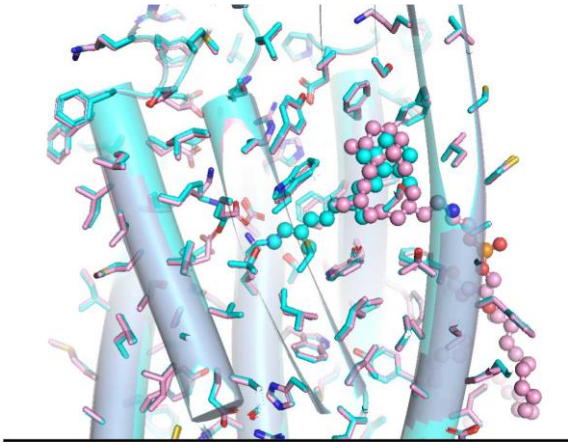
endoChR2



exoChR2



Overlay



Comparing the structure of endoChR2 with that of exoChR2, the main chain and even side chain of endoChR2 with that of exoChR2 are almost the same (RSMD=0.2), except the difference between the ATR and 9-cis N-retinylidene-PE. The lateral retinal binding pocket and the canonical retinal binding pocket share only the same location of the β-ionone ring, they are the two relatively independent but overlapping pockets.

The title of this section: “The classic retinal binding pocket prevents the endogenous N-retinylidene-PE from hydrolysis and loading into the canonical retinal binding pocket in endoChR2.” Is disconnected from the content of the section. The section does not conclude

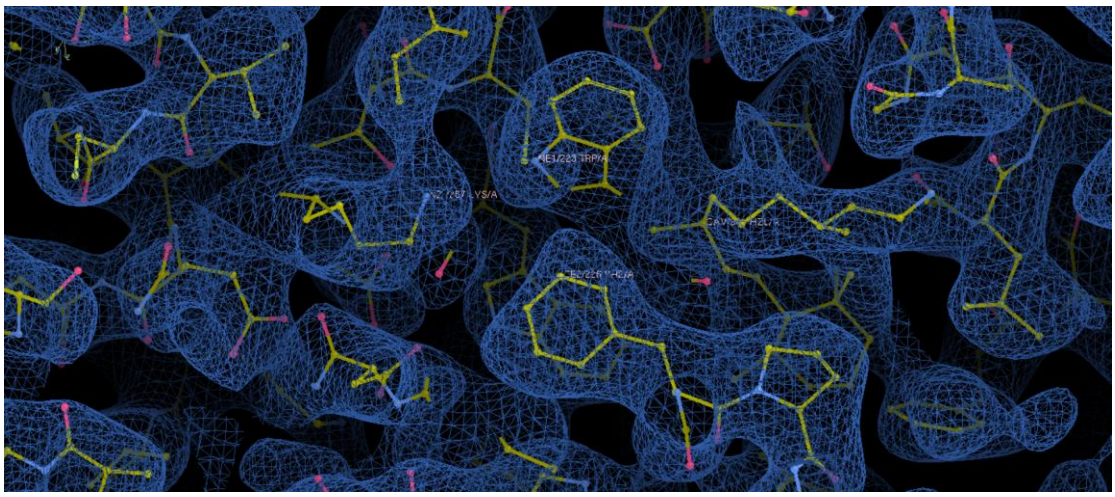
the “prevents” from “hydrolysis” and “loading into”. If this title is the conclusion of this section, it is entirely unsupported by the data presented.

We have changed the title to “EndoChR2 recruits an endogenous N-retinylidene-PE-like molecule to a lateral retinal binding pocket in HEK293 cells” to fit the content of the section.

A related question: authors made a conclusion or speculation in the related manuscript (ref 21, also being reviewed by me) that retinal blocks the ion permeation pathway thus close the channel, and hydrolysis of the retinal opens the channel. Now that without the retinal in the binding pocket, the ion permeation pathway should be clear without blocking. But the channel is not open, as shown in patch clamp experiment. How does the ion permeation pathway look like, could the pore profile tell if the channel is still closed, which presumably should be but by what?

The channelrhodopsins are not the conventional channels but more likely the transporter-like channels such as the CLC-1 channel. In the CLC-1 channel, only a side chain of glutamate (E-gate) rotation can drive the ion flow (PMID: 31022181). In channelrhodopsins, despite solving several structures, including the endo-KCR1 (In fact, the side chain of the ion permeation pathway of endoKCR1 already shows significant differences from exoKCR1, as shown by two other groups (PMID: 37652010, PMID: 37474513) who only solved exoKCR1.) and leaky conducting mutant C110T, it still cannot accept our gating hypothesis. We realized that this gating hypothesis was preliminary and removed that part.

Line 109 – 110: As shown in ED Fig 7a-c, this density is a mixture of different binding form of the same retinal. The statement “likely presenting the process of N-retinylidene PE from hydrolysis to loading into the canonical retinal binding pocket” is very speculative. If it is indeed intermediate states of loading the retinal into its canonical binding pocket, some density (even weak) should be seen within the canonical pocket. It is possible to better resolve this density by symmetry expansion followed by focused classification, which may provide better clue. And I would suggest authors to do so.



For ChR2, we indeed cannot see any density in the canonical pocket (shown above). We thank the reviewer’s constructive suggestion. As ChR2 or KCR1 is a small, non-soluble transmembrane

protein, and the tiny conformational changes only happen in the retina, it is really a challenge to isolate them. In fact, we've been trying to distinguish between these conformations, but we still haven't been able to get these interesting results.

Same goes to endoKCR1, as shown in Figure 3b, c, d, where different forms of retinal can be modeled into the density. It is possible that the density reflects mixture of different retinal form. Again, symmetry expansion followed by focused classification could potentially sort this out. The statement "This suggests that the endo-retinal is different from the all-trans retinal" is a reasonable conclusion supported by the data. However, the statement "These models are referred to as practices transitioning from pre-hydrolyzed to hydrolyzed." Is pure speculation.

We removed the statement "These models are referred to as practices transitioning from pre-hydrolyzed to hydrolyzed." As discussed above, despite the fact that we cannot isolate the mixture states of the endoKCR1, the results of exoKCR1 presenting the canonical ATR at least suggest that the endo-retinal is different from the canonical ATR. The deciphering of the heterogeneities of the endo-retinal may require the help of other methods.

Line 163-166: "Like before, we determined the cryo-EM structure of the endoC110T mutant in a potassium environment without exogenous all-trans retinal. Moreover, we acquired two classes of cryo-EM structures of exoC110T in a potassium environment, with overall resolutions of 2.57 Å and 2.56 Å, respectively". This description is confusing. Is this endoC110T or exoC110T, or you determined structures from both?

We mean we can obtain one high-resolution map from the cryo-EM sample of endoC110T mutant in a potassium environment without exogenous all-trans retinal supplement. Meanwhile, we can acquire two high-resolution maps (2.57 Å and 2.56 Å, respectively) from the cryo-EM sample of exoC110T in a potassium environment. One structure determined by 2.57-Å map shows a "hydrolyzed retinal", while another structure determined by 2.56-Å map displays the conventional Schiff-base linked all-trans retinal containing state. To avoid the confusion, we revised the sentences in the main text.

Minor technique points:

There is no need to state the resolution in the text to 2 digits after decimal point. It is fine to state this way in the method part.

The resolution in the text has been change to 1 digit after decimal point.

Reviewer #3 (Remarks to the Author):

The manuscript "Distinctive gating mechanisms of channelrhodopsin-2 and kalium channelrhodopsin-1" by Shang et al. describes the results of electrophysiology and structural studies of channelrhodopsins: nonselective ChR2 from Chlamydomonas reinhardtii and kalium selective KCR1 from H. catenoides. The authors heterologously expressed the proteins

in human kidney cells HE293T with and without exogeneous all-trans-retinal. They showed that under the conditions of their experiment ChR2 requires exogenous all-trans-retinal to maintain its photosensitive channel activity. The absence of exogeneous retinal prohibited the ChR2 current. On the contrary, the supplementation of HEK293T cells with exogenous all-trans-retinal inhibits the KCR1 channel currents. Without exogenous all-trans-retinal KCR1 displayed photocurrents.

We thank reviewer #3's comments.

My first concern is that the corresponding 'inhibitions' of the currents are not absolute. In EndoChR2 the light activated current is smaller, nevertheless it is not equal to zero (it is about a factor of 7 less than the current of ExoChR2). A similar remark relates to the case of the KCR1 channel. The ratio is small (about 0.25), however, it is not equal to zero. It means that all variants of the proteins (Endo and ExoChR2 and also Endo and ExoKCR1) are displaying properties of light-gated channels.

We have revised the sentence.

To rationalize their results the authors performed cryo-EM structural studies of both proteins expressed in HEK293T cells in the presence and absence of exogenous all-trans-retinal. They interpreted electron densities in the retinal pocket of ChR2 expressed with exogenous retinal as those corresponding to all-trans-retinal in the case of the protein expressed in the presence of all-trans-retinal. When ChR2 was expressed without exogeneous retinal the authors did not observe the retinal in the conventional retinal pocket, but they claim its (more precisely, that of 9-cis N-retinylidene-PE) presence on the surface of the protein (Figure 2a and b). My second concern is that at the present resolution (around 2.5 Å) it is very problematic to ab initio identify the conformation retinal. It is supported by numerous crystallographic studies of microbial rhodopsins such as rhodopsin BR and also by the fact that even in the ground state of ChR2 the retinal is in two conformations (Kuhne et al. Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin. Proc Natl Acad Sci U S A 116 (19) 9380-9389 (2019)). The authors cannot see this and even do not discuss this fact. The authors should present the proofs of their assignment using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal (Zhang et al. Photic generation of 11-cis-retinal in bovine retinal pigment epithelium. J. Biol. Chem. 294(50) 19137–19154 (2019)). The same concern is applicable to very smeared densities on the surface of EndoChR2 which were assigned to 9-cis N-retinylidene-PE. One cannot exclude that 1/7 of the value of the current of ExoChR2 observed in "EndoChR2" can be attributed to ChR2 with all-trans-retinal with the occupancy 1/7. The electron densities in the region of a classical retinal pocket of ChR2 in the case of EndoChR2 are not shown by the authors. In this case they can also be weak to reliably detect/interpret in endoChR2 at the present resolution. Moreover, there is a lot of evidence that endoChR2 (and many other similar channelrhodopsins) expressed in mammalians for optogenetic studies behaves as a classical exoChR2. This means that in such cases that

EndoChRs comprise the all-trans-retinal in the ground state and the retinal is attached to Lys in a classical way along the photocycle (as it was (Nagel et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channe. Proc Natl Acad Sci U S A 100 (24), 13940-5 (2003)).

We agree with the comments of reviewer #3 that a small fraction of endoChR2 is light sensitive. However, in our cryo-EM analysis we only obtained the N-retinylidene PE containing ChR2. Here we want to say that some of the small less soluble transmembrane protein is a little difficult to resolve small heterogeneity. We add the discussion of the reference paper provided by the reviewer.

For basically the same reasons my concerns are applicable to the KCR1 data. The assignment of retinal conformations to densities as shown in Figure 3 is not convincing. Once again, accurate assignment is especially problematic when the chromophore processing, insertion and covalent binding to the lysine is not homogeneous (is not simultaneously completed in the proteins). This can be the case of endogenous stepwise retinal biochemical processing and insertion. I would like to mention once again that the authors should also present the proofs of their assignments using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal and it must be used in both cases.

We thank reviewer #3 for his suggestion. We are also curious and have tried to isolate the different cryo-EM maps of endoKCR1 as it may show large retinal heterogeneities. However, as discussed before, KCR1 is also a small non-soluble transmembrane protein, and we are still unable to separate the tiny differences of retinal heterogeneities in endoKCR1 by cryo-EM technology. For highly sensitive HPLC approach, our lab is not expert in this field, we have toned down the retinal description by "not well fitted ATR".

Last but not least, the differences in the behavior observed by the authors of Endo and Exo ChR2 on the one hand and Endo and ExoKCR1 on the other may have an alternative interpretation. HEK293T is not a host system for both proteins. The host systems are very different (algae and fungi) and may have different from each other and from HEK293T retinoid processing machinery which is usually complex (Brueggemann and Sullivan. HEK293S Cells Have Functional Retinoid Processing Machinery. J. Gen. Physiol. 119, 593–612 (2002)). In addition, both proteins have different structural features and all this together may result in the differences of kinetics of the processing of the proteins and chromophore insertion in HEK293T cells. However, time dependence of the observed ‘effects’ has not been discussed in the manuscript.

We agree with reviewer #3 that the HEK293T cell may have a different retinoid metabolism and discuss this hypothesis in the Discussion section. In Supplementary Figure 1, we add the time-dependent color changes of the ATR binding assay, which support the constitutive binding model.

To summarize, I respect very much the basic idea of the authors, however, the experimental data should be sufficient to reliably make such a conclusion. The work in its present form will

not be convincing for the readers and requires additional experiments.

We are grateful for the comments of the reviewer and we have toned down our claim about the light sensing mechanism of channelrhodopsins.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate the authors' effort to improve the manuscript. I have no further questions.

Reviewer #3 (Remarks to the Author):

I appreciate very much the efforts of the authors, however, the experimental data are still not sufficient to reliably make major conclusion. First of all, two of my previous comments have not been clearly addressed:

(1)"To rationalize their results the authors performed cryo-EM structural studies of both proteins expressed in HEK293T cells in the presence and absence of exogenous all-trans-retinal. They interpreted electron densities in the retinal pocket of ChR2 expressed with exogenous retinal as those corresponding to all-trans-retinal in the case of the protein expressed in the presence of all-trans-retinal. When ChR2 was expressed without exogeneous retinal the authors did not observe the retinal in the conventional retinal pocket, but they claim its (more precisely, that of 9-cis N-retinylidene-PE) presence on the surface of the protein (Figure 2a and b). My second concern is that at the present resolution (around 2.5 Å) it is very problematic to ab initio identify the conformation retinal. It is supported by numerous crystallographic studies of microbial rhodopsins such as rhodopsin BR and also by the fact that even in the ground state of ChR2 the retinal is in two conformations (Kuhne et al. Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin. Proc Natl Acad Sci U S A 116 (19) 9380-9389 (2019)). The authors cannot see this and even do not discuss this fact. The authors should present the proofs of their assignment using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal (Zhang et al. Photic generation of 11-cis-retinal in bovine retinal pigment epithelium. J. Biol. Chem. 294(50) 19137–19154 (2019)). The same concern is applicable to very smeared densities on the surface of EndoChR2 which were assigned to 9-cis N-retinylidene-PE. One cannot exclude that 1/7 of the value of the current of ExoChR2 observed in "EndoChR2" can be attributed to ChR2 with all-trans-retinal with the occupancy 1/7. The electron densities in the region of a classical retinal pocket of ChR2 in the case of EndoChR2 are not shown by the authors. In this case they can also be weak to reliably detect/interpret in endoChR2 at the present resolution. Moreover, there is a lot of evidence that endoChR2 (and many other similar channelrhodopsins) expressed in mammalians for optogenetic studies behaves as a classical exoChR2. This means that in such cases that EndoChRs comprise the all-trans-retinal in the ground state and the retinal is attached to Lys in a classical way along the photocycle (as it was (Nagel et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channe. Proc Natl Acad Sci U S A 100 (24), 13940-5 (2003))."

(2) "My first concern is that the corresponding 'inhibitions' of the currents are not absolute. In EndoChR2 the light activated current is smaller, nevertheless it is not equal to zero (it is about a factor of 7 less than the current of ExoChR2). A similar remark relates to the case of the KCR1 channel. The ratio is small (about 0.25), however, it is not equal to zero. It means that all variants of the proteins (Endo and ExoChR2 and also Endo and ExoKCR1) are displaying properties of light-gated channels".

Some additional efforts are still required otherwise the work in its present form will not be convincing for the readers.

Point-by-Point Response to Reviewer's Comments

Reviewer #1 (Remarks to the Author):

I appreciate the authors' effort to improve the manuscript. I have no further questions.

We thank reviewer #1 and #2 for kindly reviewing our paper.

Reviewer #3 (Remarks to the Author):

I appreciate very much the efforts of the authors, however, the experimental data are still not sufficient to reliably make major conclusion. First of all, two of my previous comments have not been clearly addressed:

(1)"To rationalize their results the authors performed cryo-EM structural studies of both proteins expressed in HEK293T cells in the presence and absence of exogenous all-trans-retinal. They interpreted electron densities in the retinal pocket of ChR2 expressed with exogenous retinal as those corresponding to all-trans-retinal in the case of the protein expressed in the presence of all-trans-retinal. When ChR2 was expressed without exogeneous retinal the authors did not observe the retinal in the conventional retinal pocket, but they claim its (more precisely, that of 9-cis N-retinylidene-PE) presence on the surface of the protein (Figure 2a and b). My second concern is that at the present resolution (around 2.5 Å) it is very problematic to ab initio identify the conformation retinal. It is supported by numerous crystallographic studies of microbial rhodopsins such as rhodopsin BR and also by the fact that even in the ground state of ChR2 the retinal is in two conformations (Kuhne et al. Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin. Proc Natl Acad Sci U S A 116 (19) 9380-9389 (2019)). The authors cannot see this and even do not discuss this fact. The authors should present the proofs of their assignment using standard crystallographic approaches such as omit maps and difference electron density maps. Moreover, in such cases a highly sensitive HPLC approach is used to determine real conformation(s) of the retinal (Zhang et al. Photic generation of 11-cis-retinal in bovine retinal pigment epithelium. J. Biol. Chem. 294(50) 19137–19154 (2019)). The same concern is applicable to very smeared densities on the surface of EndoChR2 which were assigned to 9-cis N-retinylidene-PE. One cannot exclude that 1/7 of the value of the current of ExoChR2 observed in "EndoChR2" can be attributed to ChR2 with all-trans-retinal with the occupancy 1/7. The electron densities in the region of a classical retinal pocket of ChR2 in the case of EndoChR2 are not shown by the authors. In this case they can also be weak to reliably detect/interpret in endoChR2 at the present resolution. Moreover, there is a lot of evidence that endoChR2 (and many other similar channelrhodopsins) expressed in mammalians for optogenetic studies behaves as a classical exoChR2. This means that in such cases that EndoChRs comprise the all-trans-retinal in the ground state and the retinal is attached to Lys in a classical way along the photocycle (as it was (Nagel et al. Channelrhodopsin-2, a directly

light-gated cation-selective membrane channe. Proc Natl Acad Sci U S A 100 (24), 13940-5 (2003))."

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Some additional efforts are still required otherwise the work in its present form will not be convincing for the readers.

We thank reviewer #3's constructive comments again. We further modified the 9-cis N-retinylidene-PE as 9-cis N-retinylidene-PE-like molecule in the revised manuscript and further tune down statement of the precise chemical formula. And we applied LC-MS to detect ATR in samples of endoChR2, exoChR2, endoKCR1 and exoKCR1 with a standard sample of commercial ATR. The results show that purified endoKCR1 and endoChR2 is lack of ATR while exoKCR1 and exoChR2 present high peaks with same retention time as standard sample of ATR (Supplementary Fig. 11), which confirms that the ligand of endoKCR1 and endoChR2 is distinct from ATR in exoKCR1 and exoChR2. We have also added discussion of the potential hypothesis to explain the unconventional results of endoChR2 and endoKCR1 in the Discussion.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors performed suggested additional experiments, addressed my comments and properly revised the text of the manuscript.

I would suggest to publish this work.