nature portfolio

# Peer Review File

# A membrane-targeted photoswitch restores physiological ON/OFF responses to light in the degenerate retina

Corresponding Author: Professor Fabio Benfenati

**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)
In this study, Ziraldo et al. investigated the potential of Ziapin2, a membrane-targeted photoswitch, in restoring physiological visual responses in degenerate retinas from models of retina degeneration. The main goal was to characterise the effect of Ziapin2 on the light-responses of retinal ganglion cells (RGC) in wild-type and two animal models of retina degeneration and to assess its ability to restore complex physiological light responses in retinal explants from blind animals as well as visually driven behaviour. The authors investigated the effects of Ziapin2 on light responses using retinal explants from rd10 mice and RCS rats, two photoreceptor degeneration models of different genetic origins, by recording light-evoked responses to different wavelengths using patch-clamp and high-density multielectrode array recordings from RGCs. In addition, the authors used pharmacological blockers of the ON and the OFF pathways to investigate the source of the light responses and elucidate the effects of Ziapin2 on the retinal network. Furthermore, they carried out in vivo experiments after intravitreal injection of Ziapin2 in blind mice to assess light-driven behaviour and optomotor reflexes. The authors convincingly demonstrate that Ziapin2 modulates neuronal excitability in response to light stimulation, restoring ON, OFF and ON-OFF responses in RGCs. Conductance measurements showed that Ziapin2 reactivates excitatory and inhibitory synaptic currents in RGCs, leading to the differential activation of ON and OFF bipolar cells and the emergence of responses in subpopulations of physiologically distinct RGC. Moreover, intravitreal injection of Ziapin2 restored light sensitivity, optomotor reflexes and RGC firing patterns in degenerate retinas, resembling responses in sighted animals. The methodologies, experimental design, and data analysis are robust and rigorous. The high-quality recordings clearly demonstrate a strong effect of Ziapin2 on the light responses of RGCs of retinal explants of blind animals. In summary, these groundbreaking findings demonstrate that Ziapin2 restores a greater diversity of retinal responses (ON, OFF and ON-OFF) compared to other approaches currently explored for vision restoration. The authors' conclusion that Ziapin2 shows promise in restoring physiological visual responses in degenerate retinas, regardless of the mutation causing blindness is well-supported by the findings.
Please find my specific comments below:
Major:
Line 131: The statement that Ziapin2 was tested for its ability to restore physiological phototransduction processes is misleading, given that Ziapin2 does not influence any steps of phototransduction and both rd and RCS retinas mostly lack photoreceptors.
Line 186: It is unclear how the statement about Ziapin2 in the RGC membrane; significantly synchronizes sub-threshold light-evoked membrane potential oscillations, reducing response variability; is supported. What evidence is there that Ziapin2 synchronises inputs from bipolar cells? How does it reduce response variability? Did the authors analyse the standard deviation or any other parameter?
Line 235 and some other sections: Although not critical, given the well-documented effects of Ziapin2 using other methods, the authors could expand their understanding of Ziapin2 effect by exploring how light activation of Ziapin2 affects the membrane time constant (tau) during voltage-clamp recordings with square pulses. Such analysis might help directly quantify Ziapin2s effect on membrane capacitance and provide deeper insights into the mechanisms.
Line 412: Could you clarify what is meant by bipolar cells.. are massively affected by light beams impinging on the retina?
Minor:
There are several expressions in the text that, although they do not detract from the quality of the study, seem awkward or unclear. Revising and correcting them could further improve the manuscript's readability.
Line 57: reactivation of light computation; seems a bit awkward
Line 59: with a concomitant activation of RGC; should read consistent with activation of RGC;

Line 72: more than 20 types; should read "more than 30 functional types;
Line 79: the use of the term antithetically in the sentence: Rod BCs of the peripheral retina antithetically modulate cone ON- and OFF-pathways, is a bit awkward. Please consider rephrasing
Line 89: The phrase affects ~8% of the world population that becomes ~25% above 70 years of age is a bit unclear and should be rephrased for better clarity
Line 131: the use of the term implemented in the sentence: complex responses to light stimuli implemented in healthy retinas is a bit awkward. Please consider rephrasing
Line 136: Please add between phagocytose and in the phrase to phagocytose shed outer rod and cone segments.
Line 233: concomitant to photoreceptor loss should read concomitant with photoreceptor loss
Line 299: was not virtually affected should read virtually unaffected;
Line 305: the use of "abrogated" in the sentence "CNQX notably abrogated the light-evoked synaptic inputs to RGC" appears somewhat awkward. Abolished may be a more suitable choice
Line 398: cyan light could activate a multiplicity of distinct RGC subpopulations should read cyan light is capable of activating multiple distinct RGC subpopulations
Line 427: more precocious should read faster;
Line 474: reactivates abundant excitatory and inhibitory synaptic currents should read reactivates excitatory and inhibitory synaptic currents
Please add units to Figures 1D, 3 C, D & E (change in peak firing rate?)

Reviewer #2

(Remarks to the Author)
This manuscript presents an original investigation on a novel amphiphilic azobenzene-based photoswitch Ziapin2 known to act at the membrane level modulating its capacitance and excitability bidirectionally. In the dark, Ziapin2 decreases membrane thickness leading to increased capacitance and making neurons less excitable while under cyan light illumination, it causes membrane and decrease in capacitance, increased intrinsic excitability and action potential (AP) firing. When applied directly onto retinal ganglion cells, it increased their light responses under blue illumination. When blind retinal explants from rd10 mice and RCS rat (two models of retinal dystrophy associated with mutations in the Pde6b and Mertk genes, respectively) were first bathed in a Zlapin2 solution, their recording showed many more light-evoked responses from retinal ganglion cells (RGCs) with patch-clamp and high density multielectrode arrays. The main results include recovery of the diverse ON/OFF responses in RGCs evoked by full-field or patterned stimuli with reactivation of both excitatory and inhibitory conductances. Finally, when Ziapin2 was injected in the vitreous of blind rd1 mice, they recovered a light/dark-behavior and optomotor test indicating recovered visual acuity. The results provide evidence of restored ON/OFF cell responses at the cellular level leading to in vivo recovery of visual function. The Authors conclude that Ziapin2 can be active on any retinal circuit that has lost photoreceptors and light-sensitivity.

Comments and suggestions:
1) In Introduction, readers would benefit of more information about the Ziapin2 mechanism of action investigated in other tissues. It will be very useful to explain that Ziapin2 acts on passive properties of the membrane, without interfering with ion channels or neurotransmitter receptors (compared to other photoswitches that trigger responses of voltage- or ligand-gated ion channels of RGCs by acting intracellularly at the channel site. For example, the intravitreal molecular photoswitch KIO-301 (Kiora Pharmaceuticals) that localizes within specific voltage-gated ion channels, is already in clinical trial in patients with retinitis pigmentosa.
2) The main conclusion of the paper, as indicated in the title, is a restoration of both ON and OFF responses. The authors suggest a potential effect on both the ON and the OFF bipolar cells unless the effect is selective to the ON bipolar cells and transmitted by ON rod bipolar cells to AII amacrine cells connected to OFF bipolar cells through a glycinergic synapse. This pathway was already used to restore the ON-OFF responses by optogenetic strategy in different studies (Mace et al. 2015, Khabou et al. 2023). The authors must test this hypothesis by applying strychnine, a glycinergic inhibitor, to define if it suppresses the OFF response. This question is very important because it defines the potential field of view for treated patients as rod bipolar cells and AII amacrine cells are not present in the human macula. The results will then have to be discussed with the above references.
3) On the WT retina, the application was only achieved locally at the retinal ganglion cells showing that it increases the light responses of retinal ganglion cells. However, it would be important to define if the observed Ziapin2 effect on the bathed blind retina can also be observed on the bathed WT retina. Using green light, retinal ganglion cells could be defined in great details such that then the Ziapin2 effect on top of the cyan light response could be compared to the natural responses. It would also allow to define it simply increase the light sensitivity of the retina.
Increasing the light sensitivity of the retina could allow light responses to become detectable in the pathological retina. In the rd1 retina, already 5% of the cells appear already to be naturally responsive to light. Therefore, the retina is not totally blind as indicated by the Authors and an increased sensitivity could explain the reported effect. In fact, the group of Kramer has shown an enhanced intrinsic excitability leading to accelerated spontaneous firing in the retinal ganglion cells of the degenerating retina preventing the detection of their light responses (Denlinger et al. 2020). Even, the Authors suggest that Ziapin2 could dampen this effect restoring the detection of light responses in retinal ganglion cells. It is therefore crucial to investigate this question because it would define patients to be included in a clinical trial.
The experiment would also solve the question whether the effect is specific to the degenerating retina. Furthermore, it is unclear if the effect on the blind retina is obtained from the vitreal side at the bipolar cell axons as proposed by the Authors or at another level. The experiment on the WT retina may answer this question because Ziapin2 diffusion from the outer retinal side would be hampered by the photoreceptors in the WT animal. These questions are crucial for the potential applications in patients because the effect of Ziapin2 may differ depending on the mode of application.

4) Question on animal age. RCS
5) In figure 3, it is unclear how the retinal layering was measured to assume the cells were ON or OFF cells. Please refer to Roska and Werblin 2001 for this retinal dissection of the IPL. In his case, he used antibody immunolabeling to dissect the different layers.
6) Using the MEA recording strategy, the 1% increase does not seem to be a highly validated measurement. One expects an increase in the spiking frequency above the at least two times the signal to noise ratio. In the presentation of the figure, it is important to magnify the cell recording and organize them by cell types. With the provided representation in Figure 4A, nothing can be seen at this scale. Furthermore, it is very difficult on the provided data to have information on the reproducibility of the experiments. Tables of individual MEA experiments have to be provided with the numbers of recorded cells, the responsive cells to green and blue light, their class, their time to first spike, the spiking frequency in the 500ms window in comparison to normal spiking frequencies in WT animals. In the present manuscript, it is unclear how many rd1 mouse retina were recorded with the DMSO vehicle and compared to how many rd1 mouse retina treated with Ziapin2. Such information is also needed for the in vivo experiments.
7) In the dystrophic RCS retina, the effect of synaptic blockers LAP4 and CNQX were investigated. However, no statistics for the MEA recordings are provided. Individual responses are not sufficient.
8) In the in vivo experiments, the behavior analysis was performed on the following day to the injection. First, there is no sham injected DMSO animals. Second, the effect duration was not analyzed by measuring the behavior at different time point post injection. If the visual restoration is only seen on a single day post-injection, how authors plan to apply this strategy in patients. Patients cannot be injected on a daily basis.
9) Regarding the cyan light (450-500 nm) stimulation, the Authors should underline that, as in many other restorative strategies, patients will have to wear goggles because their environment will have to be converted into a blue light intensities otherwise they would miss red objects!
10) Authors describe some limitations of Ziapin2 for therapeutic application, including a relatively narrow spectral sensitivity in the cyan region of the spectrum, partial solubility in physiological solutions, and short half-life on the plasma membrane due to membrane turnover. Do the Authors have ideas of how these problems could be solved? Please provide explanation and/or working hypothesis for a potential translational to clinic.
11) Figure S5, the OFF suppressed cell is not at all convincing.
12) Figure S7 on the RCS retina. It is unclear the age of the animals. Are they really blind and at least 11 months old? Furthermore, the recording are not convincing as the firing rate of the illustrated cells in Ziapin2 is very small (18 spikes) as compared to a normal RGC response (80 spikes or more).
13) The histological figure S10 clearly demonstrates the presence of many cone photoreceptors in the rd10 retina suggesting the animals were not totally blind. It is unclear how these surviving cones are distributed in the retina and if the retinal pieces for the recording were always coming from the same piece of the retina. Such a variability could explain that an enhanced sensitivity could restore some visual function.

Minor points
Lines 42-43 strategies to replace dead photoreceptors, including optogenetics and retinal prostheses: more correct would be strategies to substitute the function of dead photoreceptors.

Lines 73-74 in the rodent retina, more than 20 types of retinal ganglion cells (RGCs) characterized by distinct responses to light and dark can be identified. The Authors may consider also the paper by Goetz et al (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9364428/).

Line 181: it is unclear to what refer the comparison.

Reviewer #3

(Remarks to the Author)
The manuscript by Ziraldo et al. deals with an important and interesting topic that is vision restoration after photoreceptor degeneration. In this manuscript, the group of authors build upon their own (Bertarelli, Lanzani and Benfenati, PMID: 32015505) published paper demonstrating that Ziapin2 works as a membrane targeted photoswitch to modulate neuronal firing. In the present manuscript, the authors elegantly apply the Ziapin2 knowledge to restore/boost retina photosensitivity and perhaps useful vision after photoreceptor degeneration, by enhancing light evoked activity remaining phototransduction in retinas. The dark/light properties of Ziapin2 are recognizable. The controls done with green light indubitably attest the Ziappin2 effects other than just an optimized better excitation of all natural photochromes in the retina. Perhaps the authors should state that for the Ziapin2 to work, one needs neuronal activity to be initiated by retina inputs from natural phototransduction, since Ziapin2 slightly hyperpolarize membranes when isomerized to cis upon illumination, being the Ziapin2 mechanism based on a return of the membrane capacitance to the normal levels. Nevertheless, the manuscript is of high relevance, high impact, and high quality. It is well written, and therefore suitable for publication by Nature Communications after some revisions as shown below.
1) Ziapin2 significantly increases the membrane capacitance (Di Francesco et al., PMID: 32015505) which effectively hyperpolarizes the cell membrane. The rebound current when light stimulus is turned off is depolarizing and that gives a cell a depolarizing transient. That said, I would expect that, in the experiment with RGCs labelled with 200 uM Ziapin2 for 30 min (yielding deep retina layers unlabeled), and with no synaptic blockers used, the cyan light would not generate or improve RGC electric activity. Instead, Ziapin2 illumination would cause a hyperpolarization due to membrane thickening. The data is clear, but for this reviewer the mechanisms here is not clear.
2) The ON/OFF pathways in the retina were speculatively demonstrated, but since Ziapin2 indiscriminately labels cell membranes of all types of cells, how this molecule keeps that functionality is still a question. Some discussion about that

should be added to the manuscript for future readers.
3) Another way to slice this is the simple fact that Ziapin2, upon illumination, decreases the membrane capacitance, boosting the power of any ionic current to change the membrane potential. When capacitance suddenly decreases, there is an instantaneous hyperpolarization, but whatever synaptic input or membrane current will have better ability to change the membrane potential by the simple fact that the Q=CmVm is changed at the proportion of the capacitance. These effects are transient, and it appears again when another light stimulation comes after a new dark period.
4) In figure 2, the authors claim that Ziapin2 induces depolarization, contrary of what it is known as the mechanism of action for this photoswitch. If the matter is due to that rebound current showed for Ziapin2, long stimulation (very limited without blocking synaptic transmission).
5) Rd10 retinas are thought to be not completely degenerated. The recordings of ERGs/VEPs on all these mice and rat strains would greatly enrich the manuscript, although not be mandatory to keep its significance. These recordings would elucidate further the effects of dark/illuminated Ziapin2 on sighted and degenerated retinas. At least a comment on that regard would certainly enrich the study.
6) The phase plots clearly show the illuminated Ziapin2 restoring basal AP dynamic conditions (Fig 2A), which in one hand is good, but on the other hand, it may suggest illuminated Ziapin2 is not directly excitatory. In Figure 5C we have a demonstration that dark Ziapin2 suppress neuronal activity and the illumination with cyan actually remove that suppression back to basal levels.
7) It is tempting to state that On or OFF RGCs are at play when cyan excites and inhibits electric activity. Not the intention here to suggest the authors should research all types of RGCs (more than 16 described), but some rationale about this matter should be exercised in the discussion for readers to know the possibilities.
8) A test, or speculation, of how fast Ziapin2 can be used to boost cell excitability would boost the significance of the study. Many studies use 15Hz as the gold standard for ‘movement vision. Since Ziapin2 induces a capacitive, transient effect, this aspect is important for this methodology to be used as therapy.
9) To the point when the authors say that it is possible that rod/cone-ON BCs are more sensitive than cone-OFF BCs to the effects of Ziapin2 because of their higher overall cell capacitance and amount of Ziapin2 captured in the membrane. This explanation does not seem plausible in my opinion. Ziapin2 changes a % of capacitance whatever it is. Ziapin2 will change capacitance per labeled area, that ultimately is determined by Ziapin2 concentration and labeling time. The dose of light will induce capacitance change. Wherever Ziapin2 is, there will be a change in the local capacitance upon illumination, that changes the time constant of the membrane, effectively determining the speed of electrotonic decremental propagation in BC axons. This reviewer thinks it is worth checking if this mechanism would fit the data. In this model, the ON BCs would be more ‘quieted’ during the dark Ziapin2 because the electrotonic transmission would be too slow to reach the end of the axon in time. Upon illumination, speedy transmission would be restored. Therefore, for ON BCs, Ziapin2 would give more functional contrast dependent on light. With short axons OFF BCs, slow transmission would not effectively, maybe, affect transmission in a shorter axon distance.
10) A plethora of mechanosensitive channels has been reported to be expressed in retina neurons. Speculatively, it would be nice to add to the manuscript some words on the possibility that Ziapin2 may also actuate these channels by changing the geometry of the membrane, a possible effect that is irrespective to electric properties (or electric equivalent) of the membrane.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have reviewed the revised version of your manuscript, and I am pleased to see that the concerns raised in the initial review have been effectively addressed, with the changes introduced improving the clarity and overall quality of the manuscript. Thank you for your responsiveness to the comments.

Reviewer #2

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

Reviewer #3

(Remarks to the Author)
The authors succeeded in addressing my questions and by observing my suggestive comments they have made the manuscript even better than at the first submission. I have no further concerns about it and I am looking forward to seeing this paper out.

**POINT-BY-POINT RESPONSES TO REVIEWER COMMENTS**

**Reviewer #1**

In this study, Ziraldo et al. investigated the potential of Ziapin2, a membrane-targeted photoswitch, in restoring physiological visual responses in degenerate retinas from models of retina degeneration. The main goal was to characterise the effect of Ziapin2 on the light-responses of retinal ganglion cells (RGC) in wild-type and two animal models of retina degeneration and to assess its ability to restore complex physiological light responses in retinal explants from blind animals as well as visually driven behaviour. The authors investigated the effects of Ziapin2 on light responses using retinal explants from rd10 mice and RCS rats, two photoreceptor degeneration models of different genetic origins, by recording light-evoked responses to different wavelengths using patch-clamp and high-density multielectrode array recordings from RGCs. In addition, the authors used pharmacological blockers of the ON and the OFF pathways to investigate the source of the light responses and elucidate the effects of Ziapin2 on the retinal network. Furthermore, they carried out in vivo experiments after intravitreal injection of Ziapin2 in blind mice to assess light-driven behaviour and optomotor reflexes. The authors convincingly demonstrate that Ziapin2 modulates neuronal excitability in response to light stimulation, restoring ON, OFF and ON-OFF responses in RGCs. Conductance measurements showed that Ziapin2 reactivates excitatory and inhibitory synaptic currents in RGCs, leading to the differential activation of ON and OFF bipolar cells and the emergence of responses in subpopulations of physiologically distinct RGC. Moreover, intravitreal injection of Ziapin2 restored light sensitivity, optomotor reflexes and RGC firing patterns in degenerate retinas, resembling responses in sighted animals. The methodologies, experimental design, and data analysis are robust and rigorous. The high-quality recordings clearly demonstrate a strong effect of Ziapin2 on the light responses of RGCs of retinal explants of blind animals. In summary, these groundbreaking findings demonstrate that Ziapin2 restores a greater diversity of retinal responses (ON, OFF and ON-OFF) compared to other approaches currently explored for vision restoration. The authors' conclusion that Ziapin2 shows promise in restoring physiological visual responses in degenerate retinas, regardless of the mutation causing blindness is well-supported by the findings. Please find my specific comments below.

We thank the Reviewer for the very positive comments.

**Major:**

Line 131: The statement that Ziapin2 was tested for its ability to "restore physiological phototransduction processes" is misleading, given that Ziapin2 does not influence any steps of phototransduction and both rd and RCS retinas mostly lack photoreceptors.

The Reviewer is right, we replaced the statement with "*restore physiological light responses in degenerate retinas*".

Line 186: It is unclear how the statement about Ziapin2 in the RGC membrane "significantly synchronizes sub-threshold light-evoked membrane potential oscillations, reducing response variability" is supported. What evidence is there that Ziapin2 synchronises inputs from bipolar cells? How does it reduce response variability? Did the authors analyse the standard deviation or any other parameter?

When looking at Fig. 2C lower panels (representation of the $V_m$ changes in the 15 sweeps with 50-ms bins), it is immediately clear that Ziapin2 focuses the onset of depolarization and the return to basal $V_m$ levels exactly at the switching on/off of the light stimulus, while RGCs under basal conditions or treated with vehicle display a much wider temporal variability in $V_m$ changes. In this representative case, RGCs are directly puffed with Ziapin2, that rules out a confounding effect of Ziapin2 on bipolar cells. However, the Reviewer is correct in asking some more quantitative evaluation of this effect for all patched RGCs that have been recorded. We have now calculated, for each RGC, the standard deviation of the bins in which the maximum $V_m$ changes occurred over the 15 sweeps, as suggested. This analysis is now shown as Figure 2e and described in the text (page 5).

Line 235 and some other sections: Although not critical, given the well-documented effects of Ziapin2 using other methods, the authors could expand their understanding of Ziapin2 effect by exploring how light activation of Ziapin2 affects the membrane time constant (tau) during voltage-clamp recordings with square pulses. Such analysis might help directly quantify Ziapin2's effect on membrane capacitance and provide deeper insights into the mechanisms.
The biophysical effects of Ziapin2 were extensively characterized in Di Francesco et al., *Nat Nano* 2020 in primary hippocampal neurons and are comparable and fully consistent with the effects on the AP waveform. However, we measured the capacitance and membrane resistance in rd10 RGCs, as required. The results, fully confirming our earlier data, are now shown in the new Figure S7 and commented on page 7.

Line 412: Could you clarify what is meant by "bipolar cells…. are massively affected by light beams impinging on the retina"?
Bipolar cells are distributed radially along the incoming light axis, whereas the other retinal neuronal cells, excluding photoreceptors, are organized perpendicularly to the light axis. This arrangement contributes to the lateralization of the signals. As a result, bipolar cells absorb more light per unit area due to this structural difference. We tried to better explain this concept in the Discussion (page 11).

**Minor:**

There are several expressions in the text that, although they do not detract from the quality of the study, seem awkward or unclear. Revising and correcting them could further improve the manuscript's readability.

Line 57: "reactivation of light computation" seems a bit awkward
We rephrased in "*Pharmacological dissection of retinal processing revealed that the effects of Ziapin2 occurred at the bipolar cell level, followed by light-dependent reactivation of the entire retinal network*".

Line 59: "with a concomitant activation of RGC" should read "consistent with activation of RGC"
Done.

Line 72: "more than 20 types" should read "more than 30 functional types"
Done. We also add the Goetz *et al.*, 2022 reference.

Line 79: the use of the term antithetically in the sentence: "Rod BCs of the peripheral retina antithetically cone ON- and OFF-pathways", is a bit awkward. Please consider rephrasing
We rephrased in: "*Rod BCs of the peripheral retina modulate cone ON- and OFF-pathways in an opposite fashion*".

Line 89: The phrase "affects ~8% of the world population that becomes ~25% above 70 years of age" is a bit unclear and should be rephrased for better clarity
We rephrased in: "*affects ~8% of the general world population and ~25% of people above 70 years of age*".

Line 131: the use of the term implemented in the sentence: "complex responses to light stimuli implemented in healthy retinas" is a bit awkward. Please consider rephrasing
We rephrased in: "*Here, we tested the ex vivo and in vivo ability of Ziapin2 to restore physiological light responses in degenerate retinas*".

Line 136: Please add "the" between "phagocytose" and "shed" in the phrase "to phagocytose shed outer rod and cone segments."
Done.

Line 233: “concomitant to photoreceptor loss” should read “concomitant with photoreceptor loss”
Done.

Line 299: “was not virtually affected” should read “virtually unaffected”
Done.

Line 305: the use of "abrogated" in the sentence "CNQX notably abrogated the light-evoked synaptic inputs to RGC" appears somewhat awkward. “Abolished” may be a more suitable choice
Done.

Line 398: “cyan light could activate a multiplicity of distinct RGC subpopulations” should read “cyan light is capable of activating multiple distinct RGC subpopulations”
Done.

Line 427: “more precocious” should read “faster”
Done.

Line 474: “reactivates abundant excitatory and inhibitory synaptic currents” should read “reactivates excitatory and inhibitory synaptic currents”
Done.

Please add units to Figures 1D, 3 C, D & E (change in peak firing rate?)
Done.

**Reviewer #2**

This manuscript presents an original investigation on a novel amphiphilic azobenzene-based photoswitch Ziapin2 known to act at the membrane level modulating its capacitance and excitability bidirectionally. In the dark, Ziapin2 decreases membrane thickness leading to increased capacitance and making neurons less excitable while under cyan light illumination, it causes membrane and decrease in capacitance, increased intrinsic excitability and action potential (AP) firing. When applied directly onto retinal ganglion cells, it increased their light responses under blue illumination. When blind retinal explants from rd10 mice and RCS rat (two models of retinal dystrophy associated with mutations in the Pde6b and Mertk genes, respectively) were first bathed in a ZIapin2 solution, their recording showed many more light-evoked responses from retinal ganglion cells (RGCs) with patch-clamp and high density multielectrode arrays. The main results include recovery of the diverse ON/OFF responses in RGCs evoked by full-field or patterned stimuli with reactivation of both excitatory and inhibitory conductances. Finally, when Ziapin2 was injected in the vitreous of blind rd1 mice, they recovered a light/dark-behavior and optomotor test indicating recovered visual acuity. The results provide evidence of restored ON/OFF cell responses at the cellular level leading to in vivo recovery of visual function. The Authors conclude that Ziapin2 can be active on any retinal circuit that has lost photoreceptors and light-sensitivity.
We thank the Reviewer for the clear synthesis and appreciation of our results.

Comments and suggestions:
1) In “Introduction”, readers would benefit of more information about the Ziapin2 mechanism of action investigated in other tissues. It will be very useful to explain that Ziapin2 acts on passive properties of the membrane, without interfering with ion channels or neurotransmitter receptors (compared to other photoswitches that trigger responses of voltage- or ligand-gated ion channels of RGCs by acting intracellularly at the channel site. For example, the intravitreal molecular photoswitch KIO-301 (Kiora Pharmaceuticals) that localizes within specific voltage-gated ion channels, is already in clinical trial in patients with retinitis pigmentosa.
We thank the Reviewer for the useful suggestion. We already mentioned the properties of Ziapin2 in the Introduction, but it is certainly wise to better emphasize that Ziapin2 acts on passive properties of the membrane, without interfering with ion channels or neurotransmitter receptors. We already discussed the comparison of the effects of Ziapin2 with those of KIO-301 in the Discussion.

2) The main conclusion of the paper, as indicated in the title, is a restoration of both ON and OFF responses. The authors suggest a potential effect on both the ON and the OFF bipolar cells unless the effect is selective to the ON bipolar cells and transmitted by ON rod bipolar cells to AII amacrine cells connected to OFF bipolar cells through a glycinergic synapse. This pathway was already used to restore the ON-OFF responses by optogenetic strategy in different studies (Mace et al. 2015, Khabou et al. 2023). The authors must test this hypothesis by applying strychnine, a glycinergic inhibitor, to define if it suppresses the OFF response. This question is very important because it defines the potential field of view for treated patients as rod bipolar cells and AII amacrine cells are not present in the human macula. The results will then have to be discussed with the above references.
We now provide the required new data on the effects of strychnine on the ON/OFF RGC populations activated by illuminated Ziapin2. Strychnine indeed decreased the number of OFF and ON-OFF RGCs although only partially. The data are now reported in the new Figure S8, described in the Results (page 8) and commented for their significance in the Discussion section, also adding the suggested references on optogenetic targeting of bipolar cells.

3) On the WT retina, the application was only achieved locally at the retinal ganglion cells showing that it increases the light responses of retinal ganglion cells. However, it would be important to define if the observed Ziapin2 effect on the bathed blind retina can also be observed on the bathed WT retina. Using green light, retinal ganglion cells could be defined in great details such that then the Ziapin2 effect on top of the cyan light response could be compared to the natural responses. It would also allow to define it simply increase the light sensitivity of the retina.
Increasing the light sensitivity of the retina could allow light responses to become detectable in the

pathological retina. In the rd1 retina, already 5% of the cells appear already to be naturally responsive to light. Therefore, the retina is not totally blind as indicated by the Authors and an increased sensitivity could explain the reported effect. In fact, the group of Kramer has shown an "enhanced intrinsic excitability leading to accelerated spontaneous firing" in the retinal ganglion cells of the degenerating retina preventing the detection of their light responses (Denlinger et al. 2020). Even, the Authors suggest that Ziapin2 could dampen this effect restoring the detection of light responses in retinal ganglion cells. It is therefore crucial to investigate this question because it would define patients to be included in a clinical trial.
The experiment would also solve the question whether the effect is specific to the degenerating retina. Furthermore, it is unclear if the effect on the blind retina is obtained from the vitreal side at the bipolar cell axons as proposed by the Authors or at another level. The experiment on the WT retina may answer this question because Ziapin2 diffusion from the outer retinal side would be hampered by the photoreceptors in the WT animal. These questions are crucial for the potential applications in patients because the effect of Ziapin2 may differ depending on the mode of application.
This comment covers several aspects. Firstly, the mice used were rd10 and not rd1. They were in end-stage of photoreceptor degeneration, with no rods and no cones bearing external segments. We did not record any response to green light, even with intense stimuli, indicating that it is unlikely that light responses may become detectable in the pathological retina simply increasing the light sensitivity of the retina. We agree, by quoting the suggested paper (that however deals with acute photoreceptor degeneration), that receptor degeneration enhances intrinsic excitability leading to accelerated spontaneous firing, and that one of the effects of Ziapin2 in the dark could be that of decreasing it, making RGCs more prone to express time-locked light responses, as already mentioned in the previous version of the Discussion. Regarding the suggested experiments on WT retinas, we agree they can provide useful information. We have now bathed WT retinas in Ziapin2 solution, as done with blind rd10 retinas. Although the important point is the effect of Ziapin2 on denervated bipolar cells, the experiment in the WT retina was interesting and demonstrated that Ziapin2, without affecting the RGC functional subtypes, significantly potentiates the firing responses of both sustained ON and OFF RGCs and had a much smaller effect on the respective transient RGC responses. The data are now shown in the new Figure S6 and described in the Results section (page 7).

4) Question on animal age. RCS
The age of the RCS rats used in the experiments was correctly indicated (lines 279 and 529-531 of the previous version). As it has been overlooked, we now repeat it in the text and all figure captions.

5) In figure 3, it is unclear how the retinal layering was measured to assume the cells were ON or OFF cells. Please refer to Roska and Werblin 2001 for this retinal dissection of the IPL. In his case, he used antibody immunolabeling to dissect the different layers.
We now expressively refer to the method used by Roska and Werblin 2001, who however did not use immunolabeling, but simply nuclear staining as we did, for defining retinal layers.

6) Using the MEA recording strategy, the 1% increase does not seem to be a highly validated measurement. One expects an increase in the spiking frequency above the at least two times the signal to noise ratio. In the presentation of the figure, it is important to magnify the cell recording and organize them by cell types. With the provided representation in Figure 4A, nothing can be seen at this scale. Furthermore, it is very difficult on the provided data to have information on the reproducibility of the experiments. Tables of individual MEA experiments have to be provided with the numbers of recorded cells, the responsive cells to green and blue light, their class, their time to first spike, the spiking frequency in the 500ms window in comparison to normal spiking frequencies in WT animals. In the present manuscript, it is unclear how many rd1 mouse retina were recorded with the DMSO vehicle and compared to how many rd1 mouse retina treated with Ziapin2. Such information is also needed for the in vivo experiments.
The 1% increase referred to the light-evoked firing frequency versus basal spontaneous frequency (described by the Reviewer as "noise"). Although we think our thresholding was not incorrect, we now followed the Reviewer's suggestion by considering "responsive" RGCs only those whose light-

evoked firing was above a firing threshold defines as “mean spontaneous firing rate + 2 x SD” (pages 6 and 17). Being this method more restrictive, it indeed emphasized the effect of Ziapin2 and used it throughout in the analysis of HD-MEA firing data. We also thank the Reviewer for the suggestion to make the firing representations shown in Figure 4A more accessible: we slightly rescaled the panels and sorted the responses of ON, OFF and ON/OFF RGCs. Regarding the reproducibility of the experiments, we obviously run multiple HD-MEA experiments from retinas dissected from different animals and provide the required Source Data Table showing numbers of recorded cells, responsive cells to green and blue light, their class, their time to first spike, the spiking frequency in the 500 ms window in the Supplementary Materials. In all figures showing HD-MEA recordings (main Figures 4, 6 and 7; new supplementary Figures S5, S6, S8 and S12) we now provide the statistical evaluations and the number of independent experiments.

7) In the dystrophic RCS retina, the effect of synaptic blockers LAP4 and CNQX were investigated. However, no statistics for the MEA recordings are provided. Individual responses are not sufficient.
We now show the full statistics of the multiple *ex-vivo* experiments run on RCS retinas, as reported above (see the new Figure S12).

8) In the in vivo experiments, the behavior analysis was performed on the following day to the injection. First, there is no sham injected DMSO animals. Second, the effect duration was not analyzed by measuring the behavior at different time point post injection. If the visual restoration is only seen on a single day post-injection, how authors plan to apply this strategy in patients. Patients cannot be injected on a daily basis.
The Reviewer may have overlooked the experimental groups in the *in vivo* experiments. Indeed, we had sham-injected WT mice and sham-injected rd10 mice which received (as it was detailed in the Results, Figure 7, Figure S9, respective legends, and Materials and Methods of the previous version of the manuscript), intravitreal injection of vehicle. In the first version of the manuscript, the effects were investigated at both 1 and 2 days after the injection. We have now performed behavioral analysis of visual rescue up to two weeks after the intravitreal administration. The results demonstrate that the visual improvements are persistent, and we thank the Reviewer for the useful suggestion. She/he can find the results in the new versions of Figure 7 and Figure S13 and in the respective sections of Results and Discussion (pages 9-10 and 13).

9) Regarding the cyan light (450-500 nm) stimulation, the Authors should underline that, as in many other restorative strategies, patients will have to wear goggles because their environment will have to be converted into a blue-light intensities otherwise they would miss red objects!
This observation applies to all photochromic molecules and most retinal prostheses to date. In addition to the possibility of using goggles, there is the possibility to generate azobenzene derivatives with red shifted absorption spectrum. This topic was already discussed in the previous version of the manuscript (lines 476-480) that we now expanded also in the light of the prolonged behavioral effects shown in Figure 7 (page 13).

10) Authors describe some limitations of Ziapin2 for therapeutic application, including a relatively narrow spectral sensitivity in the cyan region of the spectrum, partial solubility in physiological solutions, and short half-life on the plasma membrane due to membrane turnover. Do the Authors have ideas of how these problems could be solved? Please provide explanation and/or working hypothesis for a potential translational to clinic.
Apart for the lack of sensitivity in the red region, the absorbance spectrum of Ziapin2 is not narrow (see Fig. 1A; see also the above answer). Also, the solubility in water can be easily addressed by modifying the polar heads. Regarding the short half-life on the plasma membrane due to membrane turnover we are currently working on the encapsulation of the molecule for sustained delivery, as mentioned in lines 479-480 of the previous version of the manuscript and on page 13 of the revised version. We are working on these matters, but it is not advisable to go into details not to screw up future intellectual property initiatives. The Reviewer would certainly be aware of these issues.

11) Figure S5, the OFF suppressed cell is not at all convincing.

We do not agree with the Reviewer's opinion on the former Figure S5 (now Figure S9). We would like to focus the Reviewer's attention not only to the trace, showing only a tiny portion of the baseline, but to the raster plots and the PSTHs. From these graphs, one can clearly appreciate that the spontaneous activity is suppressed by light without a clear response at the offset. Finally, the same cell is also suppressed by OFF stimuli, allowing us to classify it as "*suppressed by contrast*". The layout of the figure did not allow to show the responses to both polarities, and we chose to show only the responses to ON stimuli.

12) Figure S7 on the RCS retina. It is unclear the age of the animals. Are they really blind, and at least 11 months old? Furthermore, the recordings are not convincing as the firing rate of the illustrated cells in Ziapin2 is very small (18 spikes) as compared to a normal RGC response (80 spikes or more).
The former Figure S7 (now Figure S11) shows data from cells recorded from Ziapin2-incubated retinas of 10-month-old RCS rats, as correctly stated in the Materials and Methods (lines 529-531) and now reported in the legend. At this age, our pink-eyed RCS rats are at the end stage of RP and totally blind, with no surviving rods/cones and a significant inner retina rewiring. For better information, the Reviewer can read our 2022 *Nat Comm* paper (Francia et al., 2022) or the paper by the Palanker's group (Lorach et al., 2018) stating that RCS rats (in this case more slowly degenerating pigmented RCS rats) can be considered blind after 6 months of age. We added a sentence on this point to the Discussion (page 13). Figure S7 (now Figure S11) only compares RGCs of RCS rats with Ziapin2 to those with Ziapin2 and blockers and does not compare blind with sighted WT groups.

13) The histological figure S10 clearly demonstrates the presence of many cone photoreceptors in the rd10 retina suggesting the animals were not totally blind. It is unclear how these surviving cones are distributed in the retina and if the retinal pieces for the recording were always coming from the same piece of the retina. Such a variability could explain that an enhanced sensitivity could restore some visual function.
The histological figure S10 of the former version of the manuscript is now Figure S14. In the late stages of photoreceptor degeneration in the rd10 mouse, residual cone bodies can be identified. These photoreceptors are highly disorganized and unable to produce normal light responses, as documented by Ellis et al., *Current Biology*, 33, 1513-1522, 2023. Although the authors observed that abnormal cones still retain some ability to respond to light, they are at least two orders of magnitude less responsive than WT. This indicates that the limited number of abnormal cones and their quasi-absent functionality cannot account for the Ziapin2 results. The absence of any light response to green, also to high luminances, recorded in both patch-clamp and HD-MEA recordings, strongly supports this interpretation. We added these considerations, and the respective reference, while describing Figure S14 (pages 10 and 12).

Minor points
Lines 42-43 "…strategies to replace dead photoreceptors, including optogenetics and retinal prostheses": more correct would be "…strategies to substitute the function of dead photoreceptors".
Done.

Lines 73-74 "in the rodent retina, more than 20 types of retinal ganglion cells (RGCs) characterized by distinct responses to light and dark can be identified". The Authors may consider also the paper by Goetz et al (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9364428/).
"More than 20 types" is a formally correct statement. Following the suggestions of both Reviewers #1 and #2, we now state "*more than 30 functional types*" and quote the suggested paper by Goetz et al. (2022).

Line 181: it is unclear to what refer the comparison.
The comparison refers to the changes in AP dynamics induced by cyan light in RGCs puffed with Ziapin2 (shown in Figure 2a,b; lines 176-178 of the previous version). The response is virtually

absent both if RGCs are puffed with vehicle and stimulated by cyan light (Figure S2) and if they are puffed with Ziapin2 but stimulated with green light (Figure S3).

**Reviewer #3**

The manuscript by Ziraldo et al. deals with an important and interesting topic that is vision restoration after photoreceptor degeneration. In this manuscript, the group of authors build upon their own (Bertarelli, Lanzani and Benfenati, PMID: 32015505) published paper demonstrating that Ziapin2 works as a membrane targeted photoswitch to modulate neuronal firing. In the present manuscript, the authors elegantly apply the Ziapin2 knowledge to restore/boost retina photosensitivity and perhaps useful vision after photoreceptor degeneration, by enhancing light evoked activity remaining phototransduction in retinas. The dark/light properties of Ziapin2 are recognizable. The controls done with green light indubitably attest the Ziappin2 effects other than just an optimized 'better' excitation of all natural photochromes in the retina. Perhaps the authors should state that for the Ziapin2 to work, one needs neuronal activity to be initiated by retina inputs from natural phototransduction, since Ziapin2 slightly hyperpolarize membranes when isomerized to cis upon illumination, being the Ziapin2 mechanism based on a return of the membrane capacitance to the 'normal' levels. Nevertheless, the manuscript is of high relevance, high impact, and high quality. It is well written, and therefore suitable for publication by Nature Communications after some revisions as shown below.

We thank the Reviewer for the very positive comments.

1) Ziapin2 significantly increases the membrane capacitance (Di Francesco et al., PMID: 32015505) which effectively hyperpolarizes the cell membrane. The rebound current when light stimulus is turned off is depolarizing and that gives a cell a depolarizing transient. That said, I would expect that, in the experiment with RGCs labelled with 200 uM Ziapin2 for 30 min (yielding deep retina layers unlabeled), and with no synaptic blockers used, the cyan light would not generate or improve RGC electric activity. Instead, Ziapin2 illumination would cause a hyperpolarization due to membrane thickening. The data is clear, but for this reviewer the mechanisms here is not clear.

In the quoted paper, light stimulation induces firing in neuronal primary hippocampal neurons both in the absence and in the presence of synaptic blockers. Firing occurs before the end of the light stimulus and is believed to occur with an "anode break" type of mechanism due to the combination of hyperpolarization, that makes the entirety of $Na^+$ channels fully activatable, and the ensuing rebound depolarization. We added some words in the Introduction to make it clearer (page 4).

2) The ON/OFF pathways in the retina were speculatively demonstrated, but since Ziapin2 indiscriminately labels cell membranes of all types of cells, how this molecule keeps that functionality is still a question. Some discussion about that should be added to the manuscript for future readers.

We indeed found reactivation of both excitatory and inhibitory conductances in RGCs with a variety of resulting ON and OFF responses. In addition, the use of blockers localized the effect of Ziapin2 at the level of bipolar cells. The new experiments with strychnine (new Figure S8) and those on WT retinas (new Figure S6) may help understanding the basis for the Ziapin2 effect on the ON pathway. We expanded these points in the Discussion section (page 12).

3) Another way to slice this is the simple fact that Ziapin2, upon illumination, decreases the membrane capacitance, boosting the power of any ionic current to change the membrane potential. When capacitance suddenly decreases, there is an instantaneous hyperpolarization, but whatever synaptic input or membrane current will have better ability to change the membrane potential by the simple fact that the Q=CmVm is changed at the proportion of the capacitance. These effects are transient, and it appears again when another light stimulation comes after a new dark period.

This is the right explanation for the increased excitability induced by light stimulation of Ziapin2 and the changes in $V_m$ perfectly follow the RC model of the neuronal membrane (DiFrancesco et al., 2020). The increased effectiveness of any current to change $V_m$ and stimulate firing will be further potentiated by the anode break effect of the transient hyperpolarization. We spend few words in the Discussion on these points (page 11).

4) In figure 2, the authors claim that Ziapin2 induces depolarization, contrary of what it is known as the mechanism of action for this photoswitch. If the matter is due to that rebound current showed for Ziapin2, long stimulation (very limited without blocking synaptic transmission).
We agree that the depolarizing effect shown in Figure 2C refers to the rebound depolarization, given the relatively long stimulation time (250 ms).

5) Rd10 retinas are thought to be not completely degenerated. The recordings of ERGs/VEPs on all these mice and rat strains would greatly enrich the manuscript, although not be mandatory to keep its significance. These recordings would elucidate further the effects of dark/illuminated Ziapin2 on sighted and degenerated retinas. At least a comment on that regard would certainly enrich the study.
At the age of our rd10 mice, no ERG could be recorded. Although some residual cone photoreceptors could be surviving, they have lost the external segment and their contribution to the retina output was reported to be negligible. See, e.g., Ellis et al., *Current Biology*, 33, 1513-1522, 2023. We added a comment on this point in the Discussion (pages 10 and 12).

6) The phase plots clearly show the illuminated Ziapin2 restoring basal AP dynamic conditions (Fig 2A), which in one hand is good, but on the other hand, it may suggest illuminated Ziapin2 is not directly excitatory. In Figure 5C we have a demonstration that dark Ziapin2 suppress neuronal activity and the illumination with cyan actually removes that suppression back to basal levels.
The interpretation is correct, it is the interplay between the dark suppression of the activity and the sudden increase in intrinsic excitability during the light-induced return to basal levels that generate the excitatory effect of the *trans→cis* isomerization of Ziapin2.

7) It is tempting to state that ON or OFF RGCs are at play when cyan excites and inhibits electric activity. Not the intention here to suggest the authors should research all types of RGCs (more than 16 described), but some rationale about this matter should be exercised in the discussion for readers to know the possibilities.
Cyan light stimulation reactivates a few types of ON/OFF RGCs in the degenerate retinas that were studied, but not all the numerous subtypes that one can find in a healthy retina (see, e.g., Goetz et al., 2022). We now point out in the Discussion (page 11) that, due to the advanced stage of degeneration of the rd10 mice and RCS rats used in the study, the presence of a marked rewiring of the inner retina hinders the extensive variability of RGC features observed in healthy retinas.

8) A test, or speculation, of how fast Ziapin2 can be used to boost cell excitability would boost the significance of the study. Many studies use 15Hz as the gold standard for ‘movement’ vision. Since Ziapin2 induces a capacitive, transient effect, this aspect is important for this methodology to be used as therapy.
We thank the Reviewer for the interesting suggestion. Indeed, being the capacitance change transient, the Ziapin2 has the potential to respond to high frequency stimuli. By subjecting blind rd10 explants to progressively shorter cyan light flashes at increasing frequency, as compared to a single 250 ms light flash, we found that a significant increase in the RCG firing rate was still present up to 10 Hz light stimulation frequency. The data are shown in the new Figure S5 and mentioned in the Results and Discussion (pages 6 and 11, respectively).

9) To the point when the authors say that “it is possible that rod/cone-ON BCs are more sensitive than cone-OFF BCs to the effects of Ziapin2 because of their higher overall cell capacitance and amount of Ziapin2 captured in the membrane.”: This explanation does not seem plausible in my opinion. Ziapin2 changes a % of capacitance whatever it is. Ziapin2 will change capacitance per labeled area, that ultimately is determined by Ziapin2 concentration and labeling time. The dose of light will induce capacitance change. Wherever Ziapin2 is, there will be a change in the local capacitance upon illumination, that changes the time constant of the membrane, effectively determining the speed of electrotonic decremental propagation in BC axons. This reviewer thinks it is worth checking if this mechanism would fit the data. In this model, the ON BCs would be more ‘quieted’ during the dark Ziapin2 because the electrotonic transmission would be too slow to reach

the end of the axon in time. Upon illumination, speedy transmission would be restored. Therefore, for ON BCs, Ziapin2 would give more functional contrast dependent on light. With short axons OFF BCs, slow transmission would not effectively, maybe, affect transmission in a shorter axon distance.
The Reviewer is right in saying that the membrane surface of BCs should not matter for the specificity of the Ziapin2 effects on ON BCs, as it changes a % of capacitance. However, we have shown that the effect of Ziapin2 also depends on the presence and density of lipid rafts (DiFrancesco et al., 2020), which might be different between ON and OFF BCs. However, the explanation provided by the Reviewer is very convincing and we thank her/him for suggesting it. Indeed, a change in the time constant of the membrane will impact on the speed of electrotonic decremental propagation along the BC axon as a function of the axonal length, which can justify a detectable effect on ON-BCs while OFF-BCs by virtue of their short axons will not be significantly affected. We added this point to the Discussion (page 12).

10) A plethora of mechanosensitive channels has been reported to be expressed in retina neurons. Speculatively, it would be nice to add to the manuscript some words on the possibility that Ziapin2 may also actuate these channels by changing the geometry of the membrane, a possible effect that is irrespective to electric properties (or electric equivalent) of the membrane.
An excellent observation. Indeed, our collaborating group (Moschetta et al., 2023) has recently found that Ziapin2, by virtue of the induced membrane deformation modulates TRAAK mechanosensitive potassium channels overexpressed in HEK293 cells. The paper demonstrates that TRAAK channels are recruited in the dark, resulting in an outward hyperpolarizing current in the dark, and that membrane relaxation upon light stimulation closes the channels, generating a compensatory depolarization. Thus, retinal mechanosensitive channels can potentially participate in the light/dark Ziapin2 effects. We added this potential additional mechanism to the Discussion (page 12).

# Response to the Reviewers' comments

**Reviewer #1**
I have reviewed the revised version of your manuscript, and I am pleased to see that the concerns raised in the initial review have been effectively addressed, with the changes introduced improving the clarity and overall quality of the manuscript. Thank you for your responsiveness to the comments.

**Reviewer #2**
The authors have addressed my concerns.

**Reviewer #3**
The authors succeeded in addressing my questions and by observing my suggestive comments they have made the manuscript even better than at the first submission. I have no further concerns about it and I am looking forward to seeing this paper out.

**Answer**
We thank all three Reviewers for their constructive comments and suggestions that allowed us to improve the quality of the paper.