nature portfolio

Peer Review File

Energetic diversity in retinal ganglion cells is modulated by neuronal activity and correlates with resilience to degeneration

Corresponding Author: Dr Philip Williams

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)
This study by Wang, Williams, and colleagues investigates the metabolic diversity among retinal ganglion cells (RGCs) and how it correlates with their resilience to degeneration. RGCs are unique neuronal cell type in both their anatomy, signaling/firing properties, and their high metabolic demands. As such, metabolic diseases typically affect RGCs (in addition to other degenerative pathology, or as the sole affected cell type in diseases such as DOA or LHON). RGCs are also structurally, metabolically, and electrically diverse - and this diversity might contribute to their resilience to disease-related stressors (e.g. >30 subtypes identified in mice). The authors use in vivo 2-photon imaging to measure ATP levels, and identified differences in energy maintenance among various RGC types. An important finding is that alpha RGCs had lower ATP levels and were more susceptible to ATP decline when ATP synthesis was inhibited. The study further explored the role of oxidative phosphorylation in maintaining ATP levels and the correlation between low ATP levels and increased survival after axon injury.

The manuscript is well written, with a clear and logical narrative, and encompasses a large volume of work. I have the following comments designed to improve the readability and citeability of current manuscript.

Comment 1: powering, n numbers, and heterogeneity in the data
- Were mice injected and imaged mono- or bi- laterally? (Provide information in the methods and results.)
- What are the number of cells per retina analyzed? (Provide a supplementary table of the number of cells for EACH individual retina imaged.)
- P values are all very significant, most likely due to the large dataset sizes. Side-by-side boxplots should be shown using the averages of RGCs within a single retina imaged (i.e. for most of the data this will be n = 4-5). As the within mouse and across mice retina diversity is large, it is important to see that this data is not skewed by 1 or 2 outlier retinas.

Comment 2: RGC diversity
- Mice have a large RGC phenotypic diversity, which is not (well) matched in humans. Although the data presented is well designed and well analyzed, it doesn't escape the fact that mice are not human. Of course, it would be unreasonable to ask the authors to repeat these experiments in NHP or human ex vivo tissue. However, this should be a major topic of discussion.

Comment 3: CONC
- CONC is severe injury to the RGC, and is non-recoverable. It is unsurprising that there are changes to metabolism following such a severe injury. This isn't an issue in itself, but it would important to understand why the authors didn't pick a more moderate and clinically relevant injury such as ocular hypertension or even continuing rotenone injections to induce RGC death. This might be of relevance given the shuttling of molecules and metabolites in and out of the soma.
- The surviving vs. dying cells is a very nice piece of data, however it is poorly described how the authors identified a cell as dying (assuming not present in the next imaging session) and whether the data is time-point or time-point or simply alive on day 0 and dead on day 14. Summary statistics of the number of alive / dead cells at each time point would be beneficial to understand the time course of disease in these authors' hands.

Comment 4: metabolism, mitochondria, and metabolic treatments
- Sup Fig 1 is important to the story, and is a great figure. Consider 'promoting' to the main text.
- Line 155 the authors say "suggesting ... are enriched for mitochondria". I would argue that the data is pretty strong and that the authors have used the right markers. I think it is sufficient to say that in B6J mice alpha RGCs have a high total mitochondrial volume (may be best to state this way since the authors have not done individual mitochondria analyses).
- The authors discuss metabolic treatments for RGCs (citing nicotinamide, pyruvate as examples). Why was one of these treatments not chosen as a treatment arm? (Esp. relevant as both these treatments are progressing to Phase III clinical trials.)

Figure comments:
- Overlay text in figures is lost within the immunofluorescence, they should be placed outside.
- In most figures the ATP level / Intensity (purple green plots) are lost on the overlay (e.g. Fig 1 B, E) - is there a way to improve these or show insets?

Additional questions for discussion points (in addition to those mentioned above):
1. What are the specific mechanisms that cause the observed baseline ATP differences among RGC types? The study suggests that these differences are not solely due to neuronal activity, but the exact pathways remain unclear.
2. How does mitochondrial uncoupling contribute to the energetic diversity among RGCs? Are various RGC subtypes more or less susceptible to certain OXPHOS perturbations?
3. Beyond neuronal activity, what other cellular processes contribute significantly to ATP consumption in RGCs? The authors mention potential roles for cytoskeletal and membrane remodeling but is not fully explored or explained..
4. What are the precise mechanisms that enable RGCs to maintain ATP homeostasis in response to rapid changes in activity? The authors appear to observe this and then move on without further explanation. What other agents could drive this? Would intravitreal ATP or NAD drive these changes rapidly?
5. Why do RGCs with lower baseline ATP levels exhibit greater resilience to injury?
6. What are the predicted mechanisms or causes for the transient ATP elevation observed days after optic nerve crush?

Invited to review: 2025-03-04
Accepted review: 2025-03-04
Returned review: 2025-03-07

Congratulations on the excellent study.

Reviewer #2

(Remarks to the Author)
In this study by Wang et al., the authors investigated the diversity in ATP levels that distinguished different RGC types under homeostatic conditions and during circuit activity, in vivo. They found that mitochondrial oxidative phosphorylation fuels circuit activity. They also found that low baseline ATP levels correlated with resilience to mechanical injury and observed a transient ATP elevation immediately following injury. Although interesting, the results lack substantial evidence and mechanistic details to make this study comprehensive. Please address the following major and minor concerns:
Major concerns:
1. Fig. 2. Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types? Or, given that ATeam is a pH-sensitive sensor, are the low baseline ATP levels in αRGC due to lower pH in this RGC type? Please explain.
2. Fig. 3 and Fig. 4. What is the rationale for measuring ATP levels from all RGCs instead of αRGCs? Since αRGCs have low baseline ATP levels compared to all other RGCs, how do their ATP levels change upon str/bic or NBQX/AP5 injection, and light stimulation? Show ATP level changes in αRGCs pre- and post-injection and light stimulation after immunostaining with SPP1.
3. Fig. 5E. How does this result differ from the other RGC cell types with higher baseline ATP?
4. Fig. 5E. Explain how the experiments are linked to AMPK. Does blocking AMPK show a decline in ATP levels? How is this mechanism connected to resilience?

Minor concerns:
5. Fig.1B-D. For the heterogeneous distribution of ATP levels across RGC populations, mention the coefficient of variation.
6. Fig. 1B-D. What does the white color represent in the images? Most somas show white signal instead of either green or red. Describe the color gradient.
7. Fig. 1F. Use a color code for baseline and day 7 datapoints to distinguish the respective ATP levels reflecting the heterogeneity.
8. Fig. 1G- Mention what % of the RGCs were RGCs, ipRGCs, and ooDSGCs in the text. Explain why the population of SPP1-labelled RGCs is low - is it known to be low in the retina, or is it due to low SPP1 immunostaining efficacy?
9. Fig. 1G, H. Follow the same sequence as in the text, i.e., SPP1, TBR2, and CART instead of CART, TBR2, and SPP1, or change the sequence in the text.
10. Fig. 2F-U. Add a color legend in the figure to define each color.
11. Fig. 2J-M. Color code data points according to RGC type.
12. Fig. S2F. Why do the arrows point at the cells with high calcium levels for NBQX/AP5?
13. Fig. S2A, F, K. Explain why the images look out of focus.
14. Fig. 2. Mention the dose (in mg/kg body weight) injected for each inhibitor.
15. Fig. 2. Since there is a recovery in ATP levels post-injection 10-15 mins, what is the clearance rate of ROT, TTFA, AA,

and KCN after intraocular injection in mice? Discuss this in terms of the half-life of each of these inhibitors in mice with relevant references.
16. Fig. 2F-I, N-Q, R-U. Define the time points of the blue, gray, and purple shaded areas in the legend.
17. Fig. S2K. Add the time stamps to the image labels for scan onset and steady state.
18. Fig. 3H. Pre vs 140s ATP levels were not significantly different, but 15 m ATP levels were significantly higher than pre. Explain why the ATP levels remained unchanged at 140s upon NBQX/AP5 injection when the Ca2+ levels were significantly low (Fig. S2H).
19. Fig. 4A, C. Add time stamps in the image labels for ROT and ROT & NBQX/AP5.
20. Fig. 4I. How do the ATP levels respond in the presence of NBQX/AP5?
21. Fig. 5E. Summarize the data into box plots as in D to compare ATP levels for dying vs surviving at pre, 4th day, and 14th day.
22. Fig. 1D, F, H; 2J-M; 5C-E. Mention how many animals/biological replicates in the legend.
23. Fig. S2A, F, K; 3A, F, K; 4 A, C, F. Make image borders thicker, so the color scheme is more clearly visible.
24. Mention the p-values for all correlation coefficients throughout the manuscript.
25. Pg. 13, Line 272-273. Could this be measured by looking at baseline ATP in these different RGC types upon activity inhibition?
26. Does anesthesia change the homeostatic ATP levels compared to awake animals? Explain in the discussion section.

Reviewer #3

(Remarks to the Author)
This interesting study investigates metabolic heterogeneity in RGCs under physiological and pathological conditions that include light stimulation, pharmacologically induced energy depletion and optic nerve injury. The approach combines ATP detection using transgenic expression of a FRET sniffer with post hoc immunostaining for aRGCs, ipRGCs and ooDSGCs RGC subtypes. ATP measurements stand out for real time single cell resolution in the intact living retina, correlation between activity (calcium) and metabolism (ATP) and state-of-the-art analyses relying on high-throughput machine learning. The authors found that the 4 RGC subtypes differ vs. baseline [ATP], with aRGCs showing the lowest levels and greatest sensitivity to ETC inhibition. That somatic ATP levels are comparable across aRGC subtypes (that include M4 cells) but different from other classes of ipRGCs is an intriguing observation. As expected, ATP levels declined in response to excessive activity while the observation that 'physiological' activity does not impact ATP content suggests involvement of homeostatic compensation. Perhaps unexpectedly, RGCs that survived nerve crush had lower [ATP] than the less resilient cohort.

Potential weaknesses are methodological, mechanistic and conceptual. The study ignores the effect of pharmacological manipulations (ETC inhibitors) on the retina even though those effects must have been substantial as the inhibitors compromised presynaptic (excitatory and inhibitory) and inflammatory machinery and almost certainly disrupted phototransduction, neurotransmitter release, induced massive pan-retinal release of ROS together with cytokine/purine release from the glia. It is unclear if OXPHOSP inhibition differentially compromised viability or apoptosis in RGC subtypes. ATP changes in the cell body might be independent from dendritic or axonal responses and thus activity independence within the cell body could masks large activity-evoked changes in dendrites. Immunostaining for mitochondrial proteins would benefit from validation by an independent method and perhaps supplemented by direct measurements of the mitochondrial membrane potential. The study largely ignores glycolysis. Finally, impact would be strengthened if the authors tested NAD supplementation during ONC.

In summary: the study employs state-of-the-art high-throughput recordings and analyses to bring insight into RGC metabolism in the intact retina. The topic is important in part because of the extensive NAD supplementation literature which shows neuroprotection in mammalian glaucoma and ONC models. Mechanistic insight into the properties of metabolic regulation in RGCs and understanding why certain subtypes of RGC resist injury would be very useful from basic and translational perspectives. The concerns include descriptiveness, significant side effects of ETC manipulation and modest advance in mechanistic understanding.

Additional comments:

P3 line 51 Intro. The authors state "energetic diversity across neuronal populations is much less understood" without elaborating on this key claim. What is known about the energetic diversity in neurons? What do we know about the energetic diversity among neurons in the retina?

Ibid. Why should we study energetic diversity.

Fig 1H. The authors conclude that "natively resilient aRGCs had lower homeostatic ATP levels" than ipRGCs and ODSS cells yet ipRGCs are arguably more resilient than aRGCs.

P6. Intraocular injection of ROT, AA and other ETC drugs may have compromised the viability of photoreceptors and many RGCs, disrupted ON vs OFF signaling, induced massive oxidative stress, excitotoxicity from extracellular glutamate accumulation and inflammation. Can the authors provide evidence (TUNEL, GFAP etc) that this was/was not the case? The authors should make an explicit case for the cell-autonomy behind the stated 'diversity' of observed ATP effects.

The paradoxical observation of higher mitochondrial protein (NDUFB8, SDHA, UQCRC2, and ATP5A) expression in low-ATP aRGCs (Fig. S1) merits independent validation. Are the data congruent with subtype-specific transcriptomic evidence

available from databases (Tran et al., etc)? Would changes in mitochondrial protein expression predict subtype-specific changes in the mitochondrial membrane potential, calcium transport and dehydrogenase activity?

The assumption that glycolysis does not play a role in activity appears reasonable even though nerve crush is known to promote glycolytic activity. However, it would be interesting to confirm the assumption with 2-deoxyglucose or iodoacetate.

P11 line 233. “Since we observed that natively resilient aRGCs had lower homeostatic ATP levels”…. Please define resilience. Many studies (Della Santina et al.,; El Danaf et al.; Feng et al., Zapadka et al; Guttenplan et al) demonstrated that light responses, dendritic/synaptic architecture etc in aRGCs are impacted by short exposures to modest IOP elevations.

Might activity-independence of baseline [ATP] be compartment-specific? Light-evoked ATP changes in dendritic, prelaminar and (glial) laminar regions could be missed by somatic recordings, and compartmentalization might be more pronounced for cells with large dendritic trees (eg, aRGCs).

Discussion. The protective effect of NAD+ supplementation suggests that IOP- and ONC-stressed RGCs experience energy deficits that are compensated by energy supplementation. Hence, the authors observation that high-ATP cells seem to be preferentially killed by ONC that leads them to suggest that “low homeostatic ATP translates to increased resilience’ seems counterintuitive and could be tested with NAD supplementation.

Minor:
Recommend Figures be introduced by one-sentence summary.

Reviewer #4

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

Reviewer #5

(Remarks to the Author)
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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for clarifying the points made in the revision. One final point to make in the Discussion is regarding NAM and lack of neuroprotection - it needs to be pointed out clearly that these experiments were done subcutaneously (whereas oral has been shown as therapeutically viable for RGCs and oral is what has progressed into the Phase III clinical trials) and at a lower dose than has shown to be neuroprotective in glaucoma models (500-2000 mg/kg/d).

(Remarks on code availability)
I confirm that I can access the code, but I have not tried to run it.

Reviewer #2

(Remarks to the Author)
Thanks to the authors for addressing most of my comments. However, some of the major concerns and a few of minor concerns are still not addressed. Please see below.

Major concerns:

1. Fig. 2. Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types? Or, given that ATeam is a pH-sensitive sensor, are the low baseline ATP levels in αRGC due to lower pH in this RGC type? Please explain.

Authors’ response: Ateam1.03 is built on a mseCFP and mVenus FRET pair, which are relatively insensitive to physiological pH levels (3.5 and 5.3 pKa respectively). Estimates of intracellular pH in active neurons vary, but most observations do not drop below 6.5. Additionally, the ATeam1.03 biosensor has been applied to RGC degeneration in culture and in vivo and did not observe issues with pH sensitivity (Quintero et al., 2022). Lastly, we also used the FRET

biosensor Twitch2b, which is built with the more pH sensitive Citrine (pKa 5.7), and in this case YFP/CFP ratios increased with increasing activity (Previous Supplementary Figure 2). And our previous work showed that Twitch2b ratios are higher in αRGCs than other RGCs in contrast to ATeam ratios (McCracken et al., 2023).

Reviewer's response: If a pH-sensitive ATP reporter (Twitch2b) is showing a different relationship, i.e, αRGCs have a higher baseline than other RGCs, contradicting what they measured with ATeam, it does mean that the baseline pH is different between the RGC types. Please clarify. Add an explanation clarifying the pH sensitivity of Ateam1.03 to the results section, including references for the pH levels (greater than 6.5) in RGCs. The authors did not address the question, "Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types?" Please explain.

2. Fig. 3 and Fig. 4. What is the rationale for measuring ATP levels from all RGCs instead of αRGCs? Since αRGCs have low baseline ATP levels compared to all other RGCs, how do their ATP levels change upon str/bic or NBQX/AP5 injection, and light stimulation? Show ATP level changes in αRGCs pre- and post-injection and light stimulation after immunostaining with SPP1.

Authors' response: We did not perform type Immunostaining for these experiments because ATP changes resulting from activity manipulations were relatively small (or absent) and quite consistent across the RGC population (Fig 3D,E,I,J,N and O). This contrasts both with the Ca2+ response to activity manipulations, which were large inconsistent and in some case bidirectional across RGCs (Supp Fig 2D,E,I and J). And also contrast with pharmacological inhibition of electron transport chain function, where cause much stronger and varied changes in ATP level (Fig 2 J-M). For these reasons, we do not think it is necessary to perform RGC type analysis of circuit activity manipulations.

Reviewer's response: Explanation does not answer the question. Individual traces of single RGCs in an example retina in Fig. 3B (+Str/Bic), 4B (+ROT), and 4H (+ROT + dark adaptation) show a wide range of pre-ATP levels. It would be good to confirm whether the traces with lower pre-ATP levels correspond to αRGCs (due to their lower baseline ATP levels) and then measure how the ATP levels of these αRGCs are affected by Str/Bic, ROT, and ROT + dark adaptation. Since the paper characterizes the ATP heterogeneity in the RGC population, identifies type-specific ATP differences with αRGCs having low baseline ATP levels, and shows that αRGCs (Fig. 5D, Surviving +SPP1) are more resilient to injury, the authors are recommended to perform experiments measuring ATP levels specifically in αRGCs under the same conditions as in Fig. 3B, 4B, and 4H (currently not done in the paper) to show how αRGCs with low baseline ATP levels respond to the above conditions.

4. Fig. 5E. Explain how the experiments are linked to AMPK. Does blocking AMPK show a decline in ATP levels? How is this mechanism connected to resilience?

Authors' response: Since similar elevations in ATP levels were observed in both dying and surviving RGC cohorts, we do not believe that this is a feature that is related to resilience.

Reviewer's response: Line 275 – 276 mentions: "Energetic depletion and subsequent AMPK activation have been implicated in traumatic and glaucomatous degeneration of RGCs". Line 426 – 427 mentions: "RGC survival and morphology can be improved by inhibiting AMPK signaling". Additionally, the link between post-injury AMPK activation, as implicated in other studies, is mentioned in the results and discussion sections. Given these references and the observation in Fig. 5E that there is a transient increase in ATP levels post-injury, this paper does not show any experiments with AMPK manipulations, such as blocking AMPK followed by injury, to confirm that AMPK is responsible for this transient elevation in ATP levels post-injury or the resilience of surviving RGCs. How is the AMPK pathway linked to the results in Fig. 5E? Add the explanation to the results/discussion section or clarify that the observed elevation does not relate to AMPK signaling.

Minor concerns:

15. Fig. 2. Since there is a recovery in ATP levels post-injection 10-15 mins, what is the clearance rate of ROT, TTFA, AA, and KCN after intraocular injection in mice? Discuss this in terms of the half-life of each of these inhibitors in mice with relevant references.

Authors' response: From our previous experience with intraocular pharmacological injections (McCracken et al., 2023), and work in this paper, we feel that the rates of recovery are much more likely to be an issue of clearance than one of half-life because most modulators (whether related to circuit activity or metabolism) recover over the course of 10 to 30 minutes. This is much faster than the half-life of these agents at body temperature. Additionally, the affective dosage is also important as we have observed longer duration of inhibition from higher concentration injections (not shown). We feel like our data is rather difficult to relate to other publications of half-life in relation to systemic delivery and would likely be more confusing than helpful as a point of discussion.

Reviewer's response: Clarify if this recovery in ATP levels post-injection is due to the clearance of the inhibitors. Add references for the clearance times of each inhibitor. Add this explanation to the results section.

18. Fig. 3H. Pre vs 140s ATP levels were not significantly different, but 15 m ATP levels were significantly higher than pre. Explain why the ATP levels remained unchanged at 140s upon NBQX/AP5 injection when the Ca2+ levels were significantly low (Fig. S2H).

Authors' response: Ca2+ levels are further depressed at 15min (Fig. S2H). We believe this delayed ATP elevation is due to an accumulated effect of circuit inactivity. Thus, the RGCs are homeostatically producing a set amount of ATP to meet the expected demands that would exist even in a 'quiet' circuit. As these demands are lowered over a prolonged period, ATP then builds up.

Reviewer's response: Add this explanation to the results/discussion section.

22. Fig. 1D, F, H; 2J-M; 5C-E. Mention how many animals/biological replicates in the legend.

Authors' response: This information is already present in the Figure Legends.

Reviewer's response: n = __ RGCs from __ retinas mentioned, but the number of animals/biological replications is not mentioned in any of the legends. Add this information.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
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(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
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(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
All the comments are now addressed in this second revision. Please incorporate the revised text in the appropriate Results and Discussion sections.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
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(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
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(Remarks on code availability)

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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The manuscript is well written, with a clear and logical narrative, and encompasses a large volume of work. I have the following comments designed to improve the readability and citeability of current manuscript.

Comment 1: powering, n numbers, and heterogeneity in the data
- Were mice injected and imaged mono- or bi- laterally? (Provide information in the methods and results.)
We have clarified where experiments were performed mono- or bi- laterally in the Methods and Results.

- What are the number of cells per retina analyzed? (Provide a supplementary table of the number of cells for EACH individual retina imaged.)
We have added a supplementary table that includes all retinas used for in vivo imaging experiments and the number of cells derived from them. It should be noted that some experiments have more manipulations or repeated imaging time points, so sample quality is not the only variable affecting the number of viable cells.

- P values are all very significant, most likely due to the large dataset sizes. Side-by-side boxplots should be shown using the averages of RGCs within a single retina imaged (i.e. for most of the data this will be n = 4-5). As the within mouse and across mice retina diversity is large, it is important to see that this data is not skewed by 1 or 2 outlier retinas.
We have added in Sample mean graphs and statistics to all figures. In most cases, experiments remained statistically significant. In some instances, sample numbers were small which limited power. Also, variability of drug injections from sample to sample drove more of the differences than other measured variables like RGC type. This was shown in previous Figure 2 J-M, which has been moved to Supplementary Figure 2.

Comment 2: RGC diversity
- Mice have a large RGC phenotypic diversity, which is not (well) matched in humans. Although the data presented is well designed and well analyzed, it doesn't escape the fact that mice are not human. Of course, it would be unreasonable to ask the authors to repeat these experiments in NHP or human ex vivo tissue. However, this should be a major topic of discussion.
We have added a section to the Discussion elaborating on this topic.

Comment 3: CONC
- CONC is severe injury to the RGC, and is non-recoverable. It is unsurprising that there are changes to metabolism following such a severe injury. This isn't an issue in itself, but it would important to understand why the authors didn't pick a more moderate and clinically relevant injury such as ocular hypertension or even continuing rotenone injections to induce RGC death. This might be of relevance given the shuttling of molecules and metabolites in and out of the soma.
We agree with Reviewer 1 that CONC is a severe and perhaps less clinically relevant model of RGC degeneration. However, CONC has many advantages as an experimental model. Primarily, for time-lapse *in vivo* imaging experiments, CONC ensures that all RGCs are effected by the degenerative stimulus simultaneously. In contrast, intraocular pressure or genetic models of glaucoma are not time-locked and cellular responses would be quite difficult to quantify and interpret. For example, we identified a transient

elevation of ATP in the early time points after CONC. We believe that this would be difficult to observe in a slow and staggered degeneration context. Indeed, microbead models of glaucoma have identified reduced ATP levels in some RGCs at chronic degeneration time points (Quintero et al., 2022). Taken together, while we agree that it would be exciting to examine intrinsic metabolism in RGC during other models of degeneration, we feel it is beyond the scope of this individual study.

- The surviving vs. dying cells is a very nice piece of data, however it is poorly described how the authors identified a cell as dying (assuming not present in the next imaging session) and whether the data is time-point or time-point or simply alive on day 0 and dead on day 14. Summary statistics of the number of alive / dead cells at each time point would be beneficial to understand the time course of disease in these authors' hands.
Thanks for the suggestion. We have added the number of RGCs surviving at each time point to Figure 5 and clarified the labels of dying or surviving RGCs in the Figure caption.

Comment 4: metabolism, mitochondria, and metabolic treatments
- Sup Fig 1 is important to the story, and is a great figure. Consider 'promoting' to the main text.
We appreciate the suggestion, and are happy to promote this figure. However, given this was not suggested by other reviewers, we have left this as supplementary data.

- Line 155 the authors say "suggesting ... are enriched for mitochondria". I would argue that the data is pretty strong and that the authors have used the right markers. I think it is sufficient to say that in B6J mice alpha RGCs have a high total mitochondrial volume (may be best to state this way since the authors have not done individual mitochondria analyses).
We have rephrased this section to make the statement stronger, and to appropriately indicate higher mitochondrial volume in αRGCs and not mitochondrial number.

- The authors discuss metabolic treatments for RGCs (citing nicotinamide, pyruvate as examples). Why was one of these treatments not chosen as a treatment arm? (Esp. relevant as both these treatments are progressing to Phase III clinical trials.)
We agree with Reviewer 1 that it would be of interest to examine how our observations of homeostatic differences in surviving ATP might interact with treatments targeting metabolic deficiencies. To do this, we delivered 300 mg/kg nicotinamide mononucleotide (NAM) daily subcutaneously, which has previously been used to protect photoreceptors from animal models of retinitis pigmentosa, retinal detachment and ischemia reperfusion (Bai and Sheline, 2013; Chen et al., 2020). This dosage is also similar to experiments that protected RGCs from optic nerve crush using nicotinamide riboside, except we dosed daily instead of every three days (Zhang et al., 2021). In models of glaucoma, although here nicotinamide was delivered orally in drinking water (Williams et al., 2017). For three days prior to optic nerve crush, we pretreated mice with NAM for three days imaging ATeam prior to and after this pre-treatment. This pretreatment did not lead to measurable changes in ATP levels (Δ zYFP/CFP = 0.002 +/- 0.78 mean +/- s.d. n = 291 RGCs from 5 retinas). After optic nerve crush, we imaged samples every 2 days for the next 14 days, and collected retinas and stained for SPP1 to label αRGCs imaged *in vivo*, and performed Rbpms staining to quantify overall RGC survival independent of ATP measurements. Similar to our previous observations in Figure 5, we found that in both vehicle and NAM treated groups surviving RGCs had lower ATP levels than those that died even if αRGCs were removed from the analysis. Additionally, we observed increases in ATP levels after optic nerve crush beginning by day 2 after crush. These increases were higher in NAM treated samples. Surprisingly, although NAM treatment has been found to protect RGCs from optic nerve crush and glaucoma, we did not observe increased RGC survival following NAM treatment, as measured by Rbpms immunostaining. However, in previous experiments nicotinamide only protected RGCs from optic nerve crush at 3 days after injury, but was not protective from 7 days onwards (Zhang et al., 2021). We have added these experiments to the Results section and Figure 5.

Figure comments:
- Overlay text in figures is lost within the immunofluorescence, they should be placed outside.
We have placed black boxes behind the labels to improve clarity.

- In most figures the ATP level / Intensity (purple green plots) are lost on the overlay (e.g. Fig 1 B, E) - is there a way to improve these or show insets?

We have increased the weight of the yellow lines to improve their legibility.

Additional questions for discussion points (in addition to those mentioned above):
1. What are the specific mechanisms that cause the observed baseline ATP differences among RGC types? The study suggests that these differences are not solely due to neuronal activity, but the exact pathways remain unclear.
We discuss this topic in more detail below addressing questions 2 and 3. Essentially, we find some evidence for more UCP1&2 expression in alpha RGCs. Additionally, maintenance of the plasma membrane and cytoskeleton may represent other factors. We have added these points to the Discussion section of the manuscript and as Supplementary Figure 6.

2. How does mitochondrial uncoupling contribute to the energetic diversity among RGCs? Are various RGC subtypes more or less susceptible to certain OXPHOS perturbations?
Indeed, in Figure 2 we show that different RGC subtypes are more or less susceptible to certain OXPHOS perturbations. Generally, αRGCs were showed reduced ATP to more mild inhibitions of either Complex I or Complex II, whereas ooDSGCs showed lower ATP levels in response to more complete mitochondrial inhibition caused by potassium cyanide.
To examine the role of mitochondrial uncoupling in RGC ATP diversity, we immunostained retina sections for UCP1 or UCP2 along with the general RGC marker RBPMS, or the αRGC marker SPP1. We found that αRGCs had slightly but significantly higher expression of UCP1 (non-αRGC z-score -0.03 +/- 1.0 vs. αRGC z-score 0.29 +/- 0.91, $p = 0.02$, $n = 848$ RGCs from 3 retinas), but had higher expression of UCP2 that was visually obvious and significantly higher when quantified (non-αRGC z-score -0.09 +/- 0.94 vs. αRGC z-score 1.01 +/- 1.07, $p < 0.001$, $n = 966$ RGCs from 3 retinas). We have added this data to the manuscript as Supplementary Figure 6.

3. Beyond neuronal activity, what other cellular processes contribute significantly to ATP consumption in RGCs? The authors mention potential roles for cytoskeletal and membrane remodeling but is not fully explored or explained..
We have expanded this portion of the Discussion section to further elaborate on possible sources of homeostatic ATP consumption.

4. What are the precise mechanisms that enable RGCs to maintain ATP homeostasis in response to rapid changes in activity? The authors appear to observe this and then move on without further explanation. What other agents could drive this? Would intravitreal ATP or NAD drive these changes rapidly?
There are many adaptations that neurons likely use to maintain ATP homeostasis in response to changes in activity. Over rapid time scales, low ATP levels induce conversion of built-up ADP to AMP and ATP, mitochondrial Ca2+ uptake from elevated cytoplasmic Ca2+ levels stimulates the citric acid cycle and OXPHOS activity, glucose transporters in vesicles are delivered to the plasma membrane, and the malate-aspartate shuttle is activated (Ashrafi et al., 2017; Baeza-Lehnert et al., 2019; Connolly et al., 2014; Denton, 2009; Diaz-Garcia et al., 2021; Gellerich et al., 2013; Glancy and Balaban, 2012; Llorente-Folch et al., 2013; Perez-Liebana et al., 2022). Over somewhat longer timescales, AMPK signaling can compensate for reduced ATP levels, and transcription factors that include NRF-1, NRF-2, PGC-1A, SP1, and SP4 can help neurons to compensate for longer term deficiencies in ATP (Johar et al., 2014; Priya et al., 2013).

We believe that further experiments are beyond the scope of this study, but we have added these topics to the Discussion.

Regarding intravitreal delivery of ATP or NAD, intravitreal delivery of ATP would likely induce multiple off-target effects not related too boosting internal ATP levels since ATP is a potent signaling molecule that mediates RGC death in models of glaucoma (Reigada et al., 2008; Sakamoto et al., 2015). While we have not injected NAD into the eye directly, in our experiments added to Figure 5, we treated mice with subcutaneous NAM daily. This treatment did not chronically alter ATP levels in healthy retina, but did cause further elevations in ATP after optic nerve crush. While it is possible that higher and acute dosage with NAD into the vitreous may boost ATP levels, we feel it is beyond the scope of this study and will not add significantly to our overall findings.

5. Why do RGCs with lower baseline ATP levels exhibit greater resilience to injury?

We think that there are two most likely contributors to the lower baseline ATP level being linked to RGC survival. For one, OXPHOS generates a significant amount of toxic byproducts including ROS species. Mitochondria undergoing more ATP production may also be more stressed and have a higher turnover rate. Thus, maintaining lower homeostatic ATP levels could be protective by reducing cellular stress. Another possible factor is the idea of hermetic conditioning. Constant lower ATP levels could precondition neurons to metabolic stress. However, this seems somewhat less likely given that the lower ATP levels we observe are still within normal physiological ranges, and should not represent a stressful condition. Furthermore, we actually observed an overabundance of ATP after optic nerve crush suggesting that chronic lack of ATP is not present in RGCs after axon injury. We have expanded the Discussion section to expand on these points.

6. What are the predicted mechanisms or causes for the transient ATP elevation observed days after optic nerve crush?

We agree that this observation is curious, and we are confident in their accuracy given that our additional experiments with NAM treatments also noted ATP elevations after optic nerve crush, but our explanations for these ATP elevations are largely speculative. We hypothesize that two major signaling pathways likely contribute to the elevations of ATP seen in the days after optic nerve crush. Although, not reported after optic nerve crush, glaucoma models have established that AMPK activation precedes and drive RGC degeneration (Belforte et al., 2021). We expect that AMPK is similarly activated by optic nerve crush induced degeneration, although our attempts to immunostain for activated AMPK were unsuccessful (not shown). AMPK activation would lead to enzymatic activation of pathways to acutely produce more ATP *via* substrate import and enhanced glycolytic activity (Ashrafi et al., 2017; Kishton et al., 2016). Over longer time scales, AMPK signaling can boost mitochondrial biogenesis which would boost ATP production (Bergeron et al., 2001). mTOR signaling is decreased by optic nerve crush, which in turn reduces cellular biogenesis and could make ATP more abundant (Park et al., 2008). Taken together, we believe that AMPK activity and mTOR inhibition would be the most likely contributors to our observed ATP elevations after optic nerve crush. While we believe that further experiments would be beyond the scope of our study, we have added this to the Discussion section.

Invited to review: 2025-03-04
Accepted review: 2025-03-04
Returned review: 2025-03-07

Congratulations on the excellent study.

Reviewer #2 (Remarks to the Author):

In this study by Wang et al., the authors investigated the diversity in ATP levels that distinguished different RGC types under homeostatic conditions and during circuit activity, in vivo. They found that mitochondrial oxidative phosphorylation fuels circuit activity. They also found that low baseline ATP levels correlated with resilience to mechanical injury and observed a transient ATP elevation immediately following injury. Although interesting, the results lack substantial evidence and mechanistic details to make this study comprehensive. Please address the following major and minor concerns:

Major concerns:

1. Fig. 2. Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types? Or, given that ATeam is a pH-sensitive sensor, are the low baseline ATP levels in αRGC due to lower pH in this RGC type? Please explain.

Ateam1.03 is built on a mseCFP and mVenus FRET pair, which are relatively insensitive to physiological pH levels (3.5 and 5.3 pKa respectively). Estimates of intracellular pH in active neurons vary, but most observations do not drop below 6.5. Additionally, the ATeam1.03 biosensor has been applied to RGC degeneration in culture and *in vivo* and did not observe issues with pH sensitivity (Quintero et al., 2022). Lastly, we also used the FRET biosensor Twitch2b, which is built with the more pH sensitive Citrine (pKa 5.7), and in this case YFP/CFP ratios increased with increasing activity (Previous Supplementary Figure 2). And our previous work showed that Twitch2b ratios are higher in αRGCs than other RGCs in contrast to ATeam ratios (McCracken et al., 2023).

2. Fig. 3 and Fig. 4. What is the rationale for measuring ATP levels from all RGCs instead of αRGCs? Since αRGCs have low baseline ATP levels compared to all other RGCs, how do their ATP levels change upon str/bic or NBQX/AP5 injection, and light stimulation? Show ATP level changes in αRGCs pre- and post-injection and light stimulation after immunostaining with SPP1.

We did not perform type Immunostaining for these experiments because ATP changes resulting from activity manipulations were relatively small (or absent) and quite consistent across the RGC population (Fig 3D,E,I,J,N and O). This contrasts both with the Ca2+ response to activity manipulations, which were large inconsistent and in some case bidirectional across RGCs (Supp Fig 2D,E,I and J). And also contrast with pharmacological inhibition of electron transport chain function, where cause much stronger and varied changes in ATP level (Fig 2 J-M). For these reasons, we do not think it is necessary to perform RGC type analysis of circuit activity manipulations.

3. Fig. 5E. How does this result differ from the other RGC cell types with higher baseline ATP?

We are a bit unclear as to the question here. We have split the RGCs into those that were observed to die during the time lapse imaging experiment and those that survived the time lapse after ONC. This includes all RGC types since we can only include RGC typing after the time lapse has completed and cannot identify the types of dead RGCs.

4. Fig. 5E. Explain how the experiments are linked to AMPK. Does blocking AMPK show a decline in ATP levels? How is this mechanism connected to resilience?

Since similar elevations in ATP levels were observed in both dying and surviving RGC cohorts, we do not believe that this is a feature that is related to resilience.

Minor concerns:

5. Fig.1B-D. For the heterogeneous distribution of ATP levels across RGC populations, mention the coefficient of variation.

Across these samples it was 18.9 +/- 4.5% (mean +/- s.d.), which represents about 13% of the total dynamic range of ATeam1.03 and 0.5 mM ATP if assuming these differences operate over the linear range of the biosensor (Imamura et al., 2009). We have added the coefficient of variation to the Results section.

6. Fig. 1B-D. What does the white color represent in the images? Most somas show white signal instead of either green or red. Describe the color gradient.

As described in the Figure captions, we psuedocolored the CFP channel green and the YFP channel magenta. A white hue indicates a relatively equal balance between the two channels. However, if cells are saturated in an image they can also appear completely white. This is indicated in the look up table for each figure. We have done our best to represent the data while avoiding saturated RGCs wherever possible. However, since we are only using linear adjustments of minimum and maximum to alter the signal intensity, some saturated RGCs will be unavoidable. Generally, these saturated cells will have a white center with a hued halo. We can provide a more detailed description of this in the text.

To avoid confusion, we have added a brief description of the above in the Results and Methods sections.

7. Fig. 1F. Use a color code for baseline and day 7 datapoints to distinguish the respective ATP levels reflecting the heterogeneity.

This is a scatterplot of baseline on the y axis and 7 days on the x axis, so each point is graphing both values. If there is a relationship between repeat images, then the slope should equal about 1.

8. Fig. 1G- Mention what % of the RGCs were αRGCs, ipRGCs, and ooDSGCs in the text. Explain why the population of SPP1-labelled αRGCs is low - is it known to be low in the retina, or is it due to low SPP1 immunostaining efficacy?

We have added the proportions of each immunostained subtype to the Results section and compared them to numbers reported in the literature. Our analyzed RGCs were comprised of 29.2 +/- 4.8% $CART^+$ RGCs, 7.6 +/- 2.4% $TBR2^+$ RGCs, 9.3 +/- 5.7% $SPP1^+$ RGCs, consistent with previously reported native distributions by immunostaining (Chen et al., 2021; Duan et al., 2015; Kay et al., 2011; Mao et al., 2014; Ruff et al., 2021). These were all within the same range, with ipRGCs perhaps being a bit underrepresented. This could be due to a number of factors including AAV2 tropism, promoter efficiency, or cell soma size.

9. Fig. 1G, H. Follow the same sequence as in the text, i.e., SPP1, TBR2, and CART instead of CART, TBR2, and SPP1, or change the sequence in the text.
We have adjusted the text to match the sequencing of the figure.

10. Fig. 2F-U. Add a color legend in the figure to define each color.
We have added this legend to Figure 2.

11. Fig. 2J-M. Color code data points according to RGC type.
We have added pre vs. post scatterplots with points colored by RGC type to Figure 1. To make space for this addition, we have moved the sample coded plots to the Supplementary Figures. We feel this information is important as it shows that drug responses were variable in their intensity across different samples/injections.

12. Fig. S2F. Why do the arrows point at the cells with high calcium levels for NBQX/AP5?
We thought the OFF response was interesting, but agree this distracts from the purpose of the experiment. We have removed the arrows.

13. Fig. S2A, F, K. Explain why the images look out of focus.
Transpupillary imaging leads to rather strong aberrations, so most imaging with this approach is less crisp than histological imaging for example. We don't feel that these images are any less crisp than our previous images, but this might be in part due to the difference in look up tables. We think it is possible that the prevalent cyan in the Twitch2b images versus the prominent magenta in the ATeam images causes a different perception. Another possible difference is that, in our experience, Twitch2b is overall brighter than ATeam1.03. This would lead to higher density of labelled RGCs along with their dendrites and axons, which leads to more out of focus signal as compared to lower density labeling. We show in the Supplementary Table requested by Reviewer 1 that indeed more RGCs were observable in the Twitch2b samples than the ATeam samples.

14. Fig. 2. Mention the dose (in mg/kg body weight) injected for each inhibitor.
We have added the volume and dosage of each inhibitor to the Results and Methods section.

15. Fig. 2. Since there is a recovery in ATP levels post-injection 10-15 mins, what is the clearance rate of ROT, TTFA, AA, and KCN after intraocular injection in mice? Discuss this in terms of the half-life of each of these inhibitors in mice with relevant references.
From our previous experience with intraocular pharmacological injections (McCracken et al., 2023), and work in this paper, we feel that the rates of recovery are much more likely to be an issue of clearance than one of half-life because most modulators (whether related to circuit activity or metabolism) recover over the course of 10 to 30 minutes. This is much faster than the half-life of these agents at body temperature. Additionally, the affective dosage is also important as we have observed longer duration of inhibition from higher concentration injections (not shown). We feel like our data is rather difficult to relate to other publications of half-life in relation to systemic delivery and would likely be more confusing than helpful as a point of discussion.

16. Fig. 2F-I, N-Q, R-U. Define the time points of the blue, gray, and purple shaded areas in the legend.
Thanks for catching this. We have updated the figure legend accordingly.

17. Fig. S2K. Add the time stamps to the image labels for scan onset and steady state.
We have added time stamps to these image panels.

18. Fig. 3H. Pre vs 140s ATP levels were not significantly different, but 15 m ATP levels were significantly higher than pre. Explain why the ATP levels remained unchanged at 140s upon NBQX/AP5 injection when the Ca2+ levels were significantly low (Fig. S2H).
Ca2+ levels are further depressed at 15min (Fig. S2H). We believe this delayed ATP elevation is due to an accumulated effect of circuit inactivity. Thus, the RGCs are homeostatically producing a set amount of ATP to meet the expected demands that would exist even in a 'quiet' circuit. As these demands are lowered over a prolonged period, ATP then builds up.

19. Fig. 4A, C. Add time stamps in the image labels for ROT and ROT & NBQX/AP5.

These images are average intensity projections over variable amounts of time. The time frames line up with the shaded regions on the line trace graphs. We have made this more clear in in the Figure Captions.

20. Fig. 4I. How do the ATP levels respond in the presence of NBQX/AP5?
We are unclear what the question is here. If it is a suggestion to combine ROT with NBQX/AP5, we feel that this would be rather unnecessary. NBQX/AP5 blocked most circuit activity (Previous Supp Fig 2), so it is unlikely that we would expect much interaction between ROT and NBQX/AP5.

21. Fig. 5E. Summarize the data into box plots as in D to compare ATP levels for dying vs surviving at pre, 4th day, and 14th day.
We are unclear why specifically pre, 4^{th} and 14^{th} days are requested here. We have graphed pre values in Figure 5D, and by definition there will be no dying cells on the 14^{th} day. Thus, we feel that the sample mean and variance distribution plotted in Figure 5E is sufficient to represent this data. Our additional ATP measurements in vehicle and NAM treated samples further confirm and define the ATP dynamics after nerve crush.

22. Fig. 1D, F, H; 2J-M; 5C-E. Mention how many animals/biological replicates in the legend.
This information is already present in the Figure Legends.

23. Fig. S2A, F, K; 3A, F, K; 4 A, C, F. Make image borders thicker, so the color scheme is more clearly visible.
We have doubled the line thickness on image borders.

24. Mention the p-values for all correlation coefficients throughout the manuscript.
We have added p-values for correlation coefficients throughout the manuscript.

25. Pg. 13, Line 272-273. Could this be measured by looking at baseline ATP in these different RGC types upon activity inhibition?
After activity inhibition, ATP was subtly shifted by a relatively uniform degree across the RGC population after quite a long delay (Figure 3H-J). Thus, there is not evidence for differential alterations in ATP level as a result of activity inhibition, and we do not believe RGC types would be different here.

26. Does anesthesia change the homeostatic ATP levels compared to awake animals? Explain in the discussion section.
Anesthesia is well documented to suppress neuronal activity in response to stimulation. Thus it is possible that without anesthesia, circuit activity possibly being stronger could lead to observable differences in ATP in response to light. However, awake behaving fundus imaging of neuronal activity or energetic state is unprecedented. We are also not aware of any studies comparing homeostatic ATP levels in other neuronal populations comparing awake vs. anesthetized states. We have added the possible influence of anesthesia on our observations as a point of Discussion.

Reviewer #3 (Remarks to the Author):

This interesting study investigates metabolic heterogeneity in RGCs under physiological and pathological conditions that include light stimulation, pharmacologically induced energy depletion and optic nerve injury. The approach combines ATP detection using transgenic expression of a FRET sniffer with post hoc immunostaining for aRGCs, ipRGCs and ooDSGCs RGC subtypes. ATP measurements stand out for real time single cell resolution in the intact living retina, correlation between activity (calcium) and metabolism (ATP) and state-of-the-art analyses relying on high-throughput machine learning. The authors found that the 4 RGC subtypes differ vs. baseline [ATP], with aRGCs showing the lowest levels and greatest sensitivity to ETC inhibition. That somatic ATP levels are comparable across aRGC subtypes (that include M4 cells) but different from other classes of ipRGCs is an intriguing observation. As expected, ATP levels declined in response to excessive activity while the observation that ‘physiological’ activity does not impact ATP content suggests involvement of homeostatic compensation. Perhaps unexpectedly, RGCs that survived nerve crush had lower [ATP] than the less resilient cohort.

Potential weaknesses are methodological, mechanistic and conceptual. The study ignores the effect of

pharmacological manipulations (ETC inhibitors) on the retina even though those effects must have been substantial

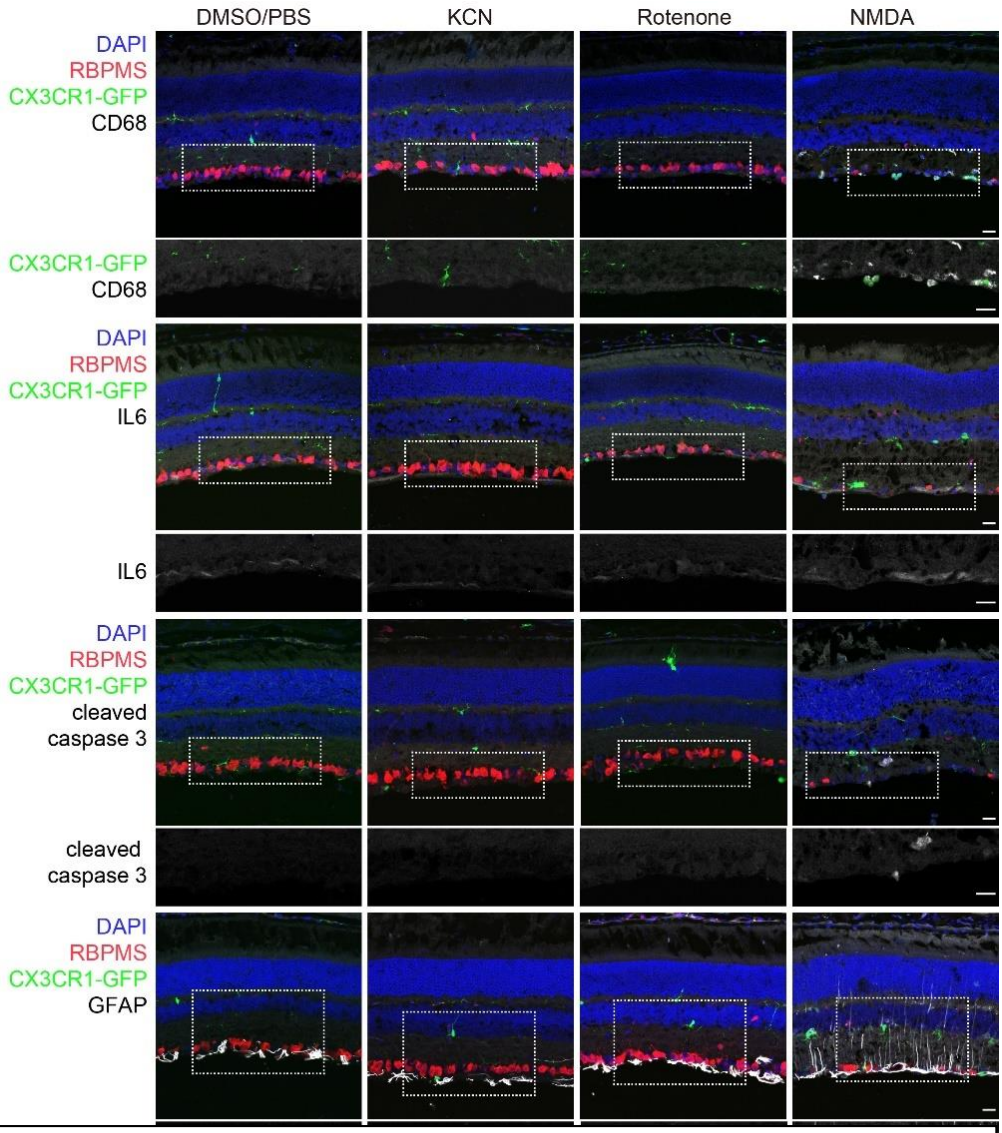
While we agree with Reviewer 3 that it is impossible to dissect out the complex interactions between the effects of ETC inhibition on the environment from direct actions on RGCs themselves, we strongly believe that *in vivo* observations of these pathways are critical to perform. More isolated systems where only RGCs are present (e.g. in primary culture) are highly artificial, represent cells already under extreme stress from dissection and isolation, and do not contain the full diversity of RGC types since many do not survive purification. The fact that we observe reproducible RGC type differences in response to ETC inhibition is most simply explained by cell intrinsic differences since there is little logic to the fact that the milieu differs in a type specific manner.

To further demonstrate that our ETC observations are reproducible, we chose a second Complex I inhibitor, etomoxir, and delivered it by intravitreal injection. We found that, similar to rotenone, etomoxir led to ATP reductions ATP that were more significant in αRGCs, and weak to non-existent in other RGC types. We believe this strongly supports our interpretation that the primary reason that RGC ATP levels are altered by ETC inhibition is a direct effect on these cells. This new data has been added to the Results as Supplementary Figure 1.

as the inhibitors compromised presynaptic (excitatory and inhibitory)

We performed ATP measurements in conditions of excitatory or inhibitory receptor blockade (Figure 3) and noticed mild effects as compared to mitochondrial inhibition (Figure 2). Additionally, combination of Complex I inhibition with excitatory receptor inhibition reduced the phenotype indicating that presynaptic excitatory drive is still active under rotenone delivery (Figure 4). These observations demonstrate that possible circuit alterations caused by ETC inhibitors are not likely to be a major contributing factor to downstream RGC ATP levels during inhibition.

and inflammatory machinery and almost certainly disrupted phototransduction, neurotransmitter release, induced massive pan-retinal release of ROS together with



Reviewer Figure 1. Acute ETC inhibitors do not lead to obvious defects in the retina. To examine the effects of ETC inhibitors on retinal inflammation, glial reactivity and possible induced cell death, we performed intravitreal injection KCN and rotenone as in Figures 2 and 4 of the manuscript. After waiting 1 hour (analogous to the timeline of ATP measurements) mice were perfused with 4% paraformaldehyde, globes were cryosectioned and samples were immunostained. DMSO in PBS was used as a negative control and 50 mM NMDA collected 2 days after injection was used as a positive control. We used CX3CR1-GFP transgenic mice to examine microglial location and morphology, CD68 to monitor phagocytosis, IL6 as a representative inflammatory cytokine, cleaved caspase 3 to monitor apoptosis and GFAP to examine Mueller glia reactivity. In all cases, retinas after acute ETC inhibition were relatively quiescent showing labeling similar to vehicle controls, whereas commonly accepted signs of damage and reactivity were present in NMDA positive controls. Scale bars = 20μm, dashed boxes are enlarged in monochrome panels below.

cytokine/purine release from the glia. It is unclear if OXPHOSP inhibition differentially compromised viability or apoptosis in RGC subtypes.

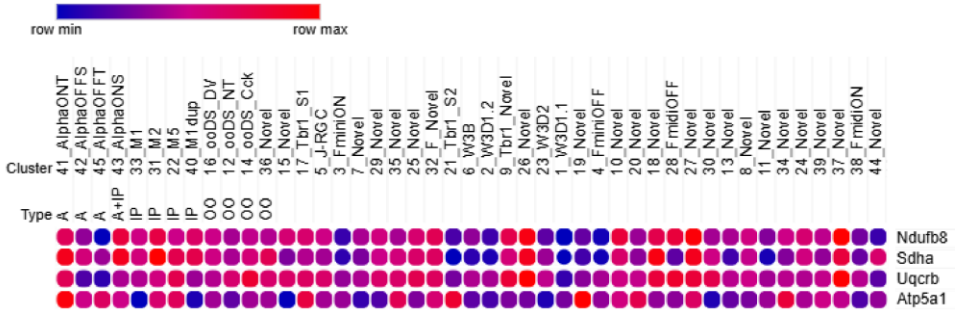
We have examined glial, inflammatory and degenerative responses to injection of our strongest ETC inhibitor, potassium cyanide (KCN), by performing histology of the microglial labeled transgenic line CX3CR1-GFP immunostained for GFAP, interleukin 6 (IL-6), CD68, and cleaved caspase-3. As a positive control, we included samples from excitotoxic lesions that we treated with intravitreal injection of 50mM NMDA and collected 2 days later. We show below in Reviewer Figure 1 that in all cases, retinas from KCN injected eyes were not noticeably different from vehicle injected negative controls. In contrast, NMDA treated positive controls showed microglial reactivity in that GFP+ microglia were swollen, ramified in the nuclear layers, and positive for CD68. KCN injected eyes also lacked Mueller Glia fibrosis as assayed by GFAP staining, lacked apoptotic RGCs as indicated by cleaved caspase-3, and did not show upregulation of the cytokine IL-6. Taken together, although degenerative cascades may eventually result from ETC inhibition, we did not notice any evidence that they are active during the time window acutely after injection during which we are performing measurements. Thus, they are unlikely to be a critical contributor to the measurements of RGC ATP levels.

ATP changes in the cell body might be independent from dendritic or axonal responses and thus activity independence within the cell body could masks large activity-evoked changes in dendrites.

We agree that cell compartments could definitely respond differently to activity or ETC inhibitors, and that this would be very interesting to examine. However, we would suggest that these proposed experiments are beyond the scope of this study.

Immunostaining for mitochondrial proteins would benefit from validation by an independent method and perhaps supplemented by direct measurements of the mitochondrial membrane potential.

As suggested we pulled out the transcriptomic data for the mitochondrial proteins immunostained for in our previous Supplemental Figure 1, shown below as Reviewer Figure 2.



Reviewer Figure 2. mRNA expression of electron transport chain components. Dot plots of genes immunostained for in Supplementary Figure 1. Data derived from Tran et al, 2019. RGCs are organized in clusters related to our study with aRGCs, ipRGCs, and ooDRGS clusters from left to right.

This analysis would suggest that the ON subset of αRGCs may be critical for driving the observed differences in mitochondrial protein expression by the αRGC cohort.

The study largely ignores glycolysis.

We agree with Reviewer 3 that we focused on ETC inhibitors, in part because with glycolysis feeding pyruvate into ETC it is possible that disrupting glycolysis would reduce ATP indirectly by disrupting mitochondrial pyruvate availability. However, we examined the importance of glycolysis in maintaining ATP levels by intravitreal injection of 2-deoxyglucose (2-DG). After injection of 2-DG, we did not observe any measurable reduction in RGC ATP levels (pre ATP -0.19 +/- 0.47, post 2-DG -0.39 +/- 0.18; mean +/- s.d.; p = 0.20). We have added these experiments to the Results as Supplementary Figure 3.

Finally, impact would be strengthened if the authors tested NAD supplementation during ONC.

We agree with Reviewer 3 that it would be of interest to examine how our observations of homeostatic differences in surviving ATP might interact with treatments targeting metabolic deficiencies. To do this, we delivered 300 mg/kg nicotinamide mononucleotide (NAM) daily subcutaneously, which has previously been used to protect photoreceptors from animal models of retinitis pigmentosa, retinal detachment and ischemia reperfusion (Bai and Sheline, 2013; Chen et al., 2020). This dosage is also similar to experiments that protected RGCs from optic nerve crush using nicotinamide riboside, except we dosed daily instead of every three days (Zhang et al., 2021). In models of glaucoma, although here nicotinamide was delivered orally in drinking water (Williams et al., 2017). For three days prior to optic nerve crush, we pretreated mice with NAM for three days imaging ATeam prior to and after this pre-treatment. This pretreatment did not lead to measurable changes in ATP levels (D zYFP/CFP = 0.002 +/- 0.78 mean +/- s.d. n = 291 RGCs from 5 retinas).

After optic nerve crush, we imaged samples every 2 days for the next 14 days, and collected retinas and stained for SPP1 to label aRGCs imaged *in vivo*, and performed Rbpms staining to quantify overall RGC survival independent of ATP measurements. Similar to our previous observations in Figure 5, we found that in both vehicle and NAM treated groups surviving RGCs had lower ATP levels than those that died even if aRGCs were removed from the analysis. Additionally, we observed increases in ATP levels after optic nerve crush beginning by day 2 after crush. These increases were higher in NAM treated samples. Surprisingly, although NAM treatment has been found to protect RGCs from optic nerve crush and glaucoma, we did not observe increased RGC survival following NAM treatment, as measured by Rbpms immunostaining. However, in previous experiments nicotinamide only protected RGCs from optic nerve crush at 3 days after injury, but was not protective from 7 days onwards (Zhang et al., 2021). We have added these experiments to the Results section and Figure 5.

In summary: the study employs state-of-the-art high-throughput recordings and analyses to bring insight into RGC metabolism in the intact retina. The topic is important in part because of the extensive NAD supplementation literature which shows neuroprotection in mammalian glaucoma and ONC models. Mechanistic insight into the properties of metabolic regulation in RGCs and understanding why certain subtypes of RGC resist injury would be very useful from basic and translational perspectives. The concerns include descriptiveness, significant side effects of ETC manipulation and modest advance in mechanistic understanding.

Additional comments:

P3 line 51 Intro. The authors state “energetic diversity across neuronal populations is much less understood” without elaborating on this key claim. What is known about the energetic diversity in neurons?
The vast majority of studies examining neuronal metabolism either use techniques with mesoscale non-cellular resolution, focus on the interactions between neurons and glia, and/or use a single neuronal type as a representative model system. For the most part, these foundational studies do not examine diversity across the neuronal population. Where diversity has been examined has generally been in the context of outlier populations. For example, photoreceptors in the retina where aerobic glycolysis supported by extremely dense vascular networks and two specialized support cells, the retina pigment epithelium and Mueller glia cells. Photoreceptor metabolism is very well characterized and is atypical compared to other neuronal types studied since photoreceptors primarily rely on aerobic glycolysis for their ATP production (Hurley, 2017; Hurley et al., 2015; Warburg, 1925). Other examples would be cells with dense mitochondrial contributions that can be identified by cytochrome oxidase labeling either following stimulation or at rest, like the whisker barrel cortex or αRGCs in cats that can be identified by cytochrome oxidase staining and are likely enriched for mitochondria and have high reliance on mitochondrial ATP generation (Kageyama and Wong-Riley, 1984; Wong-Riley and Welt, 1980).
Studies directly examining metabolic diversity across a broader neuronal population are less common. A large scale profile of mitochondrial DNA and respiratory chain activities found brain region specific signatures that were more correlated with their network partners than non-CNS tissues; however, analysis was not carried out at the level of individual cell or neuron types (Rosenberg et al., 2023). A recent preprint examined lactate dynamics in cultured *Drosophila* glutamatergic neurons using the laconic biosensor and demonstrated an anticorrelation between mitochondrial density and lactate consumption (Price et al., 2025).
We have added these points to the Introduction and Discussion sections.

What do we know about the energetic diversity among neurons in the retina?
As mentioned above, photoreceptors are by far the most studied retinal neurons in regards to their metabolic strategies. Photoreceptors largely rely on aerobic glycolysis to produce ATP, and were the first cell type identified to do so under normal conditions by Warburg (Warburg, 1925). Rod photoreceptors do this by highly expressing the enzyme isoforms PKM2 and LDHA, which favor the production of lactate during the final steps of glycolysis (Lindsay et al., 2014; Rueda et al., 2016). It is currently thought that photoreceptors maintain this reliance on aerobic glycolysis because of their massive biosynthetic requirements in the form of constantly shedding and reforming outer segments, placing photoreceptors in a state of constant cell growth (Viegas and Neuhauss, 2021). Broad histological screens in mice have been used to examine differences in metabolic function across major retinal cell types finding that beyond photoreceptor metabolism discussed above, inner retinal neurons have lower capacities for glycolysis; cone photoreceptors, horizontal cells and amacrine cells

possessed higher levels of adenylate kinase and creatine kinase to allow for ATP buffering; and that Mueller glia likely rely on anaplerosis and cataplerosis to generate GTP and maintain ATP levels (Rueda et al., 2016). Some diversity in RGCs has been noted mostly related to their abundance of mitochondrial capacity. In mice, COX histological activity is differential across the RGC population (Rueda et al., 2016). In monkeys, cats and rodents cytochrome C staining, a feature of highly active neurons classically described in the whisker barrel cortex, strongly labels αRGCs (Kageyama and Wong-Riley, 1984) suggesting a greater dependence on OXPHOS for their ATP demands. At the subcellular level, mitochondria cluster at RGC postsynaptic sites in their dendrites during development and likely support the energy demands of synaptic machinery (Faits et al., 2016). Interestingly, cone photoreceptor mitochondria may play a role beyond ATP generation as they form microlenses critical for image formation (Ball et al., 2022).
While we feel that some of these concepts are important, details of retina specific neuronal metabolism may be a bit niche for the broader readership of Nature Communications. Thus we have added a portion of this information to the Introduction and Discussion sections of the manuscript.

Ibid. Why should we study energetic diversity.
At the level of basic neuronal function, electrophysiological and modeling studies have demonstrated that the great diversity of neuronal electrophysiological phenotypes is heavily driven by efficiencies in metabolic consumption (Hasenstaub et al., 2010; Moujahid et al., 2014). Unique neuronal electrophysiological properties are the building blocks of circuit function diversity and are the result of the expression of specific subtypes of voltage- and ligand-gated ion channels. Focusing on Na+ channel types and density to model energy consumption during activity across different configurations and comparing this to the properties fast spiking interneurons found that neurons maximize their channel properties to carry out their signaling with the minimal energy expenditure (Hasenstaub et al., 2010). Similar concepts emerge for retinal ganglion cells, where OFF and ON pathway converge at a point of negative contrast, which ultimately leads to a maximized energetic efficiency of feature encoding (Soto et al., 2020). Given that the fundamental properties that govern signaling in neurons are established to maximize energetic efficiency, and that clear examples of energetic specializations are present in specific well-studied neuronal types, it stands to reason that more subtle optimizations of energy production and consumption are a critical feature of neuronal type diversity.
Additionally, metabolism is a key factor in neuronal degeneration. Diseases like amyotrophic lateral sclerosis and Parkinson's disease are directly driven by mitochondrial defects (Singh et al., 2021; Xiao et al., 2022). Altered metabolic states are present in most neurodegenerative conditions (Henn et al., 2022; Procaccini et al., 2016; Quintero et al., 2022). For example, in neurons derived from Alzheimer's Disease patient iPSCs, a switch from PKM1 to PKM2 expression pushed neurons into a Warburg-like metabolic phenotype, which drove neurodegeneration that could be prevented with PKM2 inhibition (Traxler et al., 2022). For retinal ganglion cell degeneration specifically, models of glaucoma have found reduced ATP levels and mitochondria transport rates (Quintero et al., 2022). RGC mitochondrial transport is also reduced after optic nerve crush (Cartoni et al., 2016). Treatments to boost mitochondrial transport protect RGCs from degeneration in both degeneration models, restore ATP levels in glaucoma, and promote axon regeneration after nerve crush (Cartoni et al., 2016; Quintero et al., 2022). Taken together, the significant connection between metabolism and neurodegeneration justifies the study of metabolic diversity across neuronal populations, especially given the clear difference in susceptibility to neurodegeneration.

Fig 1H. The authors conclude that "natively resilient aRGCs had lower homeostatic ATP levels" than ipRGCs and ODSS cells yet ipRGCs are arguably more resilient than aRGCs.
We agree this phrasing is misleading. We have removed "natively resilient" from this sentence.

P6. Intraocular injection of ROT, AA and other ETC drugs may have compromised the viability of photoreceptors and many RGCs, disrupted ON vs OFF signaling, induced massive oxidative stress, excitotoxicity from extracellular glutamate accumulation and inflammation. Can the authors provide evidence (TUNEL, GFAP etc) that this was/was not the case? The authors should make an explicit case for the cell-autonomy behind the stated 'diversity' of observed ATP effects.
As shown in Reviewer Figure 1 above, we did not notice cell death, glial reactivity via GFAP, or microglial reactivity by morphology or CD68 immunostaining due to ETC drug inhibition over the time course from which we recorded ATP responses.

The paradoxical observation of higher mitochondrial protein (NDUFB8, SDHA, UQCRC2, and ATP5A)

expression in low-ATP aRGCs (Fig. S1) merits independent validation. Are the data congruent with subtype-specific transcriptomic evidence available from databases (Tran et al., etc)? Would changes in mitochondrial protein expression predict subtype-specific changes in the mitochondrial membrane potential, calcium transport and dehydrogenase activity?
As discussed above, we have cross-referenced the transcriptomic measurements for the listed genes from the Tran 2019 dataset in Reviewer Figure 1.
Regarding how mitochondrial expression might predict type-specific diversity in mitochondrial traits like membrane potential, calcium transport and dehydrogenase activity, we do not think that our small immunostaining panel meant to examine representative ETC components is scale enough to infer this wide range of properties. We agree that it would be of interest to examine these traits across neuronal types, we believe that doing so would be beyond the scope of the current study.

The assumption that glycolysis does not play a role in activity appears reasonable even though nerve crush is known to promote glycolytic activity. However, it would be interesting to confirm the assumption with 2-deoxyglucose or iodoacetate.
We examined the importance of glycolysis in maintaining ATP levels by intravitreal injection of 2-deoxyglucose (2-DG). After injection of 2-DG, we did not observe any measurable reduction in RGC ATP levels (pre ATP -0.19 +/- 0.47, post 2-DG -0.39 +/- 0.18; mean +/- s.d.; p = 0.20). We have added these experiments to the Results as Supplementary Figure X.

P11 line 233. “Since we observed that natively resilient aRGCs had lower homeostatic ATP levels”…. Please define resilience. Many studies (Della Santina et al.,; El Danaf et al.; Feng et al., Zapadka et al; Guttenplan et al) demonstrated that light responses, dendritic/synaptic architecture etc in aRGCs are impacted by short exposures to modest IOP elevations.
We agree that resilient is a vague descriptor and have changed the phrasing to “well-surviving” throughout the manuscript.

Might activity-independence of baseline [ATP] be compartment-specific? Light-evoked ATP changes in dendritic, prelaminar and (glial) laminar regions could be missed by somatic recordings, and compartmentalization might be more pronounced for cells with large dendritic trees (eg, aRGCs).
We agree that cell compartments could definitely respond differently to alterations in circuit activity, and that this would be interesting to examine. However, our current methodology is not able to resolve dendritic or axonal compartments. We would also suggest that these proposed experiments are beyond the scope of this study.

Discussion. The protective effect of NAD+ supplementation suggests that IOP- and ONC-stressed RGCs experience energy deficits that are compensated by energy supplementation. Hence, the authors observation that high-ATP cells seem to be preferentially killed by ONC that leads them to suggest that “low homeostatic ATP translates to increased resilience’ seems counterintuitive and could be tested with NAD supplementation.
As discussed above, we have added NAM supplementation experiments to the manuscript. In accordance with previous publications (Zhang et al., 2021), we did not see protective effects of supplementation by 14 days after optic nerve crush. These data have been added to Figure 5. It thus seems that the metabolic contributions to RGC degeneration may be different between glaucoma and traumatic sources of RGC degeneration.

Minor:
Recommend Figures be introduced by one-sentence summary.
We have adjusted the Figure Titles to be more descriptive of the data contained within them.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for clarifying the points made in the revision. One final point to make in the Discussion is regarding NAM and lack of neuroprotection - it needs to be pointed out clearly that these experiments were done subcutaneously (whereas oral has been shown as therapeutically viable for RGCs and oral is what has progressed into the Phase III clinical trials) and at a lower dose than has shown to be neuroprotective in glaucoma models (500-2000 mg/kg/d).

While we pointed out this difference in NAM administration route in our Results, we agree with Reviewer #1 that this point should be emphasized when discussing the difference in outcome and have reinforced this difference in the Results section. Importantly, we do not believe that the route of administration should be the driver of this difference since subcutaneous NAM should be more bioavailable than oral (Yoshino et al., 2018). We instead believe that this is a difference in trauma induced neurodegeneration vs. glaucoma.

Reviewer #1 (Remarks on code availability):

I confirm that I can access the code, but I have not tried to run it.

Reviewer #2 (Remarks to the Author):

Thanks to the authors for addressing most of my comments. However, some of the major concerns and a few of minor concerns are still not addressed. Please see below.

Major concerns:

1. Fig. 2. Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types? Or, given that ATeam is a pH-sensitive sensor, are the low baseline ATP levels in αRGC due to lower pH in this RGC type? Please explain.

Authors' response: Ateam1.03 is built on a mseCFP and mVenus FRET pair, which are relatively insensitive to physiological pH levels (3.5 and 5.3 pKa respectively). Estimates of intracellular pH in active neurons vary, but most observations do not drop below 6.5. Additionally, the ATeam1.03 biosensor has been applied to RGC degeneration in culture and in vivo and did not observe issues with pH sensitivity (Quintero et al., 2022). Lastly, we also used the FRET biosensor Twitch2b, which is built with the more pH sensitive Citrine (pKa 5.7), and in this case YFP/CFP ratios increased with increasing activity (Previous Supplementary Figure 2). And our previous work showed that Twitch2b ratios are higher in αRGCs than other RGCs in contrast to ATeam ratios (McCracken et al., 2023).

Reviewer's response: If a pH-sensitive ATP reporter (Twitch2b) is showing a different relationship, i.e, αRGCs have a higher baseline than other RGCs, contradicting what they measured with ATeam, it does mean that the baseline pH is different between the RGC types. Please clarify. Add an explanation clarifying the pH sensitivity of Ateam1.03 to the results section, including references for the pH levels (greater than 6.5) in RGCs.

Twitch2b is not an ATP reporter, it is the Ca^{2+} reporter that we used in our supplementary experiments (Figure S5) to demonstrate our manipulations of circuit activity were functional, and that responses to scanning light and circuit activity changes were diverse across the RGC population.

The pH sensitivity of the ATeam1.03 reporter (mVenus pKa = 5.5 (Kremers et al., 2006)) is roughly a log10 less than the pH observed in active neurons (minimum pH 6.5). More specifically, intracellular pH has been studied extensively in different neuron types. Most report slightly basic pH levels (an incomplete summary: 7.29 +/- 0.01 rat hippocampus (Smith et al., 1998), 7.08 +- 0.37 rat hippocampus (Bevensee et al., 1996), 7.23 +/- 0.03 fetal rat hippocampus (Baxter and Church, 1996), 7.76 +/- 0.12 adult rat hippocampus (Raley-Susman et al., 1993), 7.17 +/- 0.03 fetal rat hippocampus (Raley-Susman et al., 1991), 6.81 +/- 0.02 rat hippocampus (Schwiening and Boron, 1994), 6.85 – 7.05 frog motoneuron (Endres et al., 1986), 7.2 – 7.3 turtle cerebellum (Chen and Chesler, 1992)). Tetanic or strong pharmacological glutamate receptor simulation induces slight acidification (ranging from 0.05 to 0.20 ΔpH; turtle cerebellum (Chen and Chesler, 1992), rat hippocampus (Kaila et al., 1992), frog motoneuron (Endres et al., 1986)). Thus, it is extremely unlikely that RGCs under mild stimulation from 2-photon scanning should have intracellular pH anywhere near the sensitivity range of ATeam1.03.

We have added a brief explanation of the ATeam1.03 pH sensitivities with references to observed pH levels in active neurons to the Results section.

The authors did not address the question, “Is it possible that the ATP reporter is highly sensitive in the low baseline ATP range in αRGC, resulting in a higher response to inhibitors than the other RGC types?” Please explain.

No, we do not believe this explains our observations that αRGCs are more susceptible to mitochondrial inhibition.

First, since we can observe increases in RGC population ATP levels minutes after silencing circuit activity with NBQX/AP5 injection, this indicates that we are likely recording ATP levels within the linear function of ATeam1.03 response. Additionally, our baseline ATP levels reported in Figure 1 are normally and symmetrically distributed. If we were recording from the exponential upper bound of ATeam FRET ratios, we would expect a long tail of lower ratios with a pile up of higher ratios at the upper bound. Thus, if we are within the linear range of the ATeam biosensor, there is no reason for the initial ratio to impact the change in magnitude of the ATP change observed in our population.

Additionally, αRGCs are not perfectly or exclusively low ATP RGCs. There are a number of non-αRGCs with lower baseline ATP observed in our experiments that do not respond to rotenone. If an artifact of baseline ATeam ratios is driving the magnitude of response to mitochondrial inhibition, we should see very strong correlations between baseline ATP and post inhibition ATP. We instead show a mild relationship (e.g. rotenone $R^2 = 0.24$).

We have added this point to the Results section.

2. Fig. 3 and Fig. 4. What is the rationale for measuring ATP levels from all RGCs instead of αRGCs? Since αRGCs have low baseline ATP levels compared to all other RGCs, how do their

ATP levels change upon str/bic or NBQX/AP5 injection, and light stimulation? Show ATP level changes in αRGCs pre- and post-injection and light stimulation after immunostaining with SPP1.

Authors' response: We did not perform type Immunostaining for these experiments because ATP changes resulting from activity manipulations were relatively small (or absent) and quite consistent across the RGC population (Fig 3D,E,I,J,N and O). This contrasts both with the Ca2+ response to activity manipulations, which were large inconsistent and in some case bidirectional across RGCs (Supp Fig 2D,E,I and J). And also contrast with pharmacological inhibition of electron transport chain function, where cause much stronger and varied changes in ATP level (Fig 2 J-M). For these reasons, we do not think it is necessary to perform RGC type analysis of circuit activity manipulations.

Reviewer's response: Explanation does not answer the question. Individual traces of single RGCs in an example retina in Fig. 3B (+Str/Bic), 4B (+ROT), and 4H (+ROT + dark adaptation) show a wide range of pre-ATP levels. It would be good to confirm whether the traces with lower pre-ATP levels correspond to αRGCs (due to their lower baseline ATP levels) and then measure how the ATP levels of these αRGCs are affected by Str/Bic, ROT, and ROT + dark adaptation. Since the paper characterizes the ATP heterogeneity in the RGC population, identifies type-specific ATP differences with αRGCs having low baseline ATP levels, and shows that αRGCs (Fig. 5D, Surviving +SPP1) are more resilient to injury, the authors are recommended to perform experiments measuring ATP levels specifically in αRGCs under the same conditions as in Fig. 3B, 4B, and 4H (currently not done in the paper) to show how αRGCs with low baseline ATP levels respond to the above conditions.

We are unclear what is meant by pointing out that the pre-ATP levels show a wide range. We previously address the pre-levels (baseline) in Figure 1 and show that the lower ratios are enriched for αRGCs, while other RGC types tend to be distributed more evenly across the pre-distribution. We have already demonstrated in both Figure 1, and Figure 2 repeatedly, that low pre-levels are enriched for αRGCs.

We demonstrate in Figure 3B and 3H that the ATP levels are shifting as a population, or in 4D not at all. This can be clearly observed both in our heatmap RGC traces that indicate little if any mixing of high and low pre-ATP level RGCs after injection, and in scatter plots in 3E,F and 3K,L that show a very strong relationship between pre and post injection ratios. For examples of what non-uniform traces look like, please refer to Figure 2, and Figure S5. This argues that RGCs are shifting their ATP levels uniformly in response to changes in circuit activation, αRGCs included.

Finally, the proposed experiments of imaging ATP responses to circuit activation in αRGCs specifically using KCNG4-Cre transgenic mice are extremely unlikely to demonstrate anything we do not already show in Figures 3B, 4B or 4H. In Figure 3B all RGCs shifted downward by a similar amount (see quantification in 4E), indicating αRGCs would shift downward by a similar amount. αRGC responses to rotenone shown in 4B were already presented in Figure 2B-V where we demonstrated that ATP decrease as a result of rotenone injection was largely a feature of αRGCs. Figure 4H is the same model as Figure 4B and Figure 2B-V, which would give the same results. If anything, experiments in KCNG4-Cre mice would be harder to interpret since comparison to other RGC types would not be possible.

4. Fig. 5E. Explain how the experiments are linked to AMPK. Does blocking AMPK show a decline in ATP levels? How is this mechanism connected to resilience?

Authors' response: Since similar elevations in ATP levels were observed in both dying and surviving RGC cohorts, we do not believe that this is a feature that is related to resilience.

Reviewer's response: Line 275 – 276 mentions: "Energetic depletion and subsequent AMPK activation have been implicated in traumatic and glaucomatous degeneration of RGCs". Line 426 – 427 mentions: "RGC survival and morphology can be improved by inhibiting AMPK signaling". Additionally, the link between post-injury AMPK activation, as implicated in other studies, is mentioned in the results and discussion sections. Given these references and the observation in Fig. 5E that there is a transient increase in ATP levels post-injury, this paper does not show any experiments with AMPK manipulations, such as blocking AMPK followed by injury, to confirm that AMPK is responsible for this transient elevation in ATP levels post-injury or the resilience of surviving RGCs. How is the AMPK pathway linked to the results in Fig. 5E? Add the explanation to the results/discussion section or clarify that the observed elevation does not relate to AMPK signaling.

AMPK is activated when cells build up AMP, specifically it reads out the AMP/ATP ratio. This can occur in conditions of cell stress and has been reported in models of RGC degeneration. The downstream effects of AMPK signaling are to correct the AMP/ATP ratio by both producing more ATP and consuming less ATP. We believe our results in 5E could represent an overcorrection of the metabolic state by AMPK signaling triggered by the stress of axon injury. We can include more detail to make our interpretation clearer in the Discussion.

Minor concerns:

15. Fig. 2. Since there is a recovery in ATP levels post-injection 10-15 mins, what is the clearance rate of ROT, TTFA, AA, and KCN after intraocular injection in mice? Discuss this in terms of the half-life of each of these inhibitors in mice with relevant references.

Authors' response: From our previous experience with intraocular pharmacological injections (McCracken et al., 2023), and work in this paper, we feel that the rates of recovery are much more likely to be an issue of clearance than one of half-life because most modulators (whether related to circuit activity or metabolism) recover over the course of 10 to 30 minutes. This is much faster than the half-life of these agents at body temperature. Additionally, the affective dosage is also important as we have observed longer duration of inhibition from higher concentration injections (not shown). We feel like our data is rather difficult to relate to other publications of half-life in relation to systemic delivery and would likely be more confusing than helpful as a point of discussion.

Reviewer's response: Clarify if this recovery in ATP levels post-injection is due to the clearance of the inhibitors. Add references for the clearance times of each inhibitor. Add this explanation to the results section.

We can add this description of drug clearance to our manuscript. There are no references for the clearance time of these inhibitors following intravitreal injection.

18. Fig. 3H. Pre vs 140s ATP levels were not significantly different, but 15 m ATP levels were significantly higher than pre. Explain why the ATP levels remained unchanged at 140s upon NBQX/AP5 injection when the Ca2+ levels were significantly low (Fig. S2H).

Authors' response: Ca2+ levels are further depressed at 15min (Fig. S2H). We believe this delayed ATP elevation is due to an accumulated effect of circuit inactivity. Thus, the RGCs are

homeostatically producing a set amount of ATP to meet the expected demands that would exist even in a 'quiet' circuit. As these demands are lowered over a prolonged period, ATP then builds up.

Reviewer's response: Add this explanation to the results/discussion section.

We have added this information to the manuscript.

22. Fig. 1D, F, H; 2J-M; 5C-E. Mention how many animals/biological replicates in the legend.

Authors' response: This information is already present in the Figure Legends.

Reviewer's response: n = __ RGCs from __ retinas mentioned, but the number of animals/biological replications is not mentioned in any of the legends. Add this information.

Our mistake, while most of our experiments were one retina from one mouse, there were some instances where both retinas from the same mouse were used. Effected experiments are:

Repeat imaging (Figure 1F): n = 7 retinas from 5 mice
TTFA (Figure 2KOS): n = 4 retinas from 3 mice
Antimycin A (Figure 2LPT): n = 4 retinas from 3 mice
NBQX/AP5 (Figure 3I-L): n = 4 retinas from 3 mice
Stimulation (Figure 3O): n = 4 retinas from 3 mice
Survival (Figure 5C-E): n = 5 retinas from 4 mice
Ca^{2+} Str/Bic (Figure S5C-F): n = 6 retinas from 5 mice
Ca^{2+} Stimulation (Figure S5NO): n = 13 retinas from 7 mice

We have corrected the n's throughout.

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