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Peer Review File

OPTN protects retinal ganglion cells and ameliorates neuroinflammation in optic neuropathies

Corresponding Author: Dr Feng Tian

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors have explored the effects of deletion of C-terminal domain of OPTN in the mouse retina. Their results show that OPTN knockout (C-terminal deletion) in the retina contributes to short-term astrogliosis, retinal ganglion cell (RGC) loss and long-term microglia activation, with the propagation of microglia activation spreading to the optic nerve. Loss of OPTN function does not further exacerbate RGC death induced by ocular hypertension, which is known to cause glaucoma. They have carried out gene expression analysis from the data from mouse and human patients with optic neuropathies, available from various databases; this analysis suggests that OPTN dysfunction drives NPY downregulation and induces the expression of CHOP. They conclude that their results reveal a neuroprotective role for the OPTN-NPY signalling pathway, and its dysfunction contributes to RGC loss in glaucoma pathology. They also suggest that OPTN-NPY-mediated neuroinflammatory pathway provides a potential therapy for IOP-resistant glaucoma and highlights a druggable target for CHOP-associated neurodegeneration. Overall, some of their findings are important and provide new insights about the cytoprotective role of OPTN in the retina, which have relevance to the pathogenesis of normal tension glaucoma (NTG). However, I would like the following points to be addressed.
Major points
1. A better description of their experimental model is required. From which amino acid of OPTN the deletion occurs in their mouse model? Is it OPTN470T? In which cells of the retina, OPTN is deleted? Supplementary fig. S1a shows only RGC layer image (RBPMS staining) but other layers of retina are not shown. Is OPTN staining seen in other layers of retina? OPTN staining for other layers should also be shown for control as well as mutant retina.
2. Lines 260-264, fig 7e, f. How the quantification of NPY expression was done in Fig. 7f? Is it done only in GCL or whole retina? They need to consider the possibility that reduced NPY level in OPTN-deficient retina may be due to reduced number of RGCs. Can they rule out this possibility?
3. They conclude that their results reveal a neuroprotective role for the OPTN-NPY signalling pathway. This is based on downregulation of NPY upon deletion of OPTN. This is over-interpretation of the data. More evidence is needed to show that in their model NPY is cytoprotecive; eg. by overexpression of NPY.
4. Fig. 7a. How do they define resilient and susceptible RGCs?
5. Lines 199-200. “Ocular hypertension resulted in a greater loss of RGCs compared to the loss observed 3-months after OPTN loss”. Data are not shown.
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1. Legend to Fig. 1b needs correction. This fig. shows images only for one month and not for 3 and 6 months. The images for 3 and 6 months are shown in a supplementary Fig.
2. English needs some improvement at a few places. Lines 298-300, “…the OPTN N-terminal coiled-coil domain (NTD), where gain-of-function mutations such as E50K and M98K have been identified, is responsible for the OPTN/TBK1”. Perhaps they are implying “…OPTN-TBK1 interaction”.

Reviewer #2

(Remarks to the Author)
The manuscript by Wang et al. describes the involvement of the multi-functional adaptor protein, optineurin (OPTN), in retinal ganglion cells loss and accompanying degenerative such as astrogliosis and microglial activation. The authors report the interesting finding that there is no exacerbation of IOP-mediated RGC loss in OPTN knockout out mice indicating that OPTN is necessary but probably not sufficient in promoting RGC survival particularly during IOP elevation. Including AAV-2-mediated expression of OPTN during IOP elevation would greatly enhance the clarity of the role of OPTN in promoting RGC neuroprotection. The findings have clinical relevance given the finding that 16% of normal tension glaucoma patients harbor mutations in the optineurin gene. The manuscript is well written, but the organization of the manuscript needs to be improved. Some of the key data has been moved to supplementary data and it would be better to combine them with the main data in Figures 1 to 7. Here are the key points that need clarification:
1. In the abstract, in line 36, the authors should state what the NPY stands for.
2. Reference 14 entitled, "Characterization of Normal Tension Glaucoma Mouse Over Expressing Mutant OPTN (E50K)" could not be found on PubMed. A similar issue was encountered for Reference 15. Please check the publication titles and journals for these two publications.
3. In line 106 of the introduction, the authors should delete the second part of the statement about "…whether OPTN dysfunction represents a shared pathological mechanisms between NTG and HTG" since this is not completely addressed in the current study.
4. In line 118, authors must explain the sequences contained in the control AAV2-PLAP virus.
5. In line 141 the authors must explain what they mean by enlargement of the cytoskeleton in glaucoma.
6. In the description of the data shown in Figure 3 about microglial activation in the optic nerve (line 178), the authors should mention the distance of the optic nerve region being examined from the optic nerve head.
7. In Figure 4, the authors must include a treatment group pf AAV-2-Cre + PBS and include the IOP plot and RGC counts to demonstrate it as a model of NTG.
8. In line 240, state that FAM19a4 is a chemokine.
9. In Supplemental Figure 5e the authors must label the panel as GO enrichment analysis Day 2 post-crush.
10. In line 700 the authors must indicate if all animal procedures were carried out in accordance with either the ARVO or SFN guidelines for the use of animals in research.
11. In line 278, the authors state that gain of function optineurin mutations such as E50K have been shown to induce RGC degeneration by enhancing the OPTN-TBK1 pathway. Is that the more plausible scenario in glaucoma pathogenesis?
12. In the discussion, the authors must include references that make a mechanistic connection between optineurin and NPY.
13. There is very little discussion of the GO enrichment analysis of the scRNA seq data. The authors must discuss the potential implications of the key pathways identified is supplemental Figure 5..

Reviewer #3

(Remarks to the Author)
In the manuscript entitled "OPTN protects retinal ganglion cells and ameliorates neuroinflammation in optic neuropathies" the authors studied the role of OPTN in glaucoma pathology. They specifically examined the effect of OPTN deficiency on promoting irreversible microglia activation in the retina, assuming a spread to the optic nerve. However, they excluded an exacerbating role for OPTN dysfunction in RGC death in the HTG model. Finally, they found that NPY, an important downstream inflammatory modulator of OPTN involved in RGC rescue from death, in addition to OPTN should be considered as a promising IOP-independent target to treat glaucoma. Although this is an interesting and important study, I have a few remarks/suggestions for the Authors:
- As you know, among the missense OPTN mutations involved in NTG (e.g., E50K, H26D, H486R, E322K), the E50K is the most common one. It seems that this mutation leads to RGC death via autophagy, trafficking dysfunction and an increase in oxidative stress. Do you have evidence of OPTN loss of function in glaucoma? Since OPTN loss of function is considered the principal disease mechanism for ALS phenotype, do you have found patients with both conditions (glaucoma and ALS)? An additional investigation about the effect of mutated OPTN and OPTN knockout in eliciting astrogliosis, microglia activation, and RGC death would make the work more complete.
- Why has NF-kB activity in OPTN ko mice not been analyzed? It has been reported that inappropriate NF-kB activation is the main pathogenic mechanism underlying OPTN loss-of-function mutation.
- This work shows well that OPTN plays a pivotal role in neuroimmune homeostasis and RGC vulnerability. Indeed, suppressing OPTN, a selective autophagic receptor and a signaling regulator, compromises removing a wide range of substrates and regulating innate immune response, respectively. However, in this way, it becomes difficult to study which functional interactions (e.g., TBK1, LC3, myosin VI, TAX1BP1, Rab8, Htt, transferrin receptor, RIP, SOD1, caspase 8, HACE1 metabotropic glutamate receptor 1 and 5, and so on) are compromised in mutated forms found in glaucoma. So, my question is: why did you focus on analyzing the lack of OPTN instead of its most common mutated form?
- In the supplementary figures where both astrogliosis and microglia activation are demonstrated at 3 and 6 months after OPTN ko, I would add IHC staining of OPTN at the same time.
- In my opinion, highlighting that the bioinformatic analysis you performed on previous results from Daniszewski et al. was from NTG patients rather than POAG has more impact
- Regarding the NPY, do you suggest this neuropeptide as a promising target for specifically treating glaucoma involving OPTN mutations or for sporadic glaucoma also?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my comments satisfactorily.

Reviewer #2

(Remarks to the Author)
The authors have addressed the reviewer's points of critique and generated a well written manuscript.

Reviewer #3

(Remarks to the Author)
I would like to thank all the authors for their responses and revisions to the main manuscript; I have no further comments.

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Reviewer #1 (Remarks to the Author):

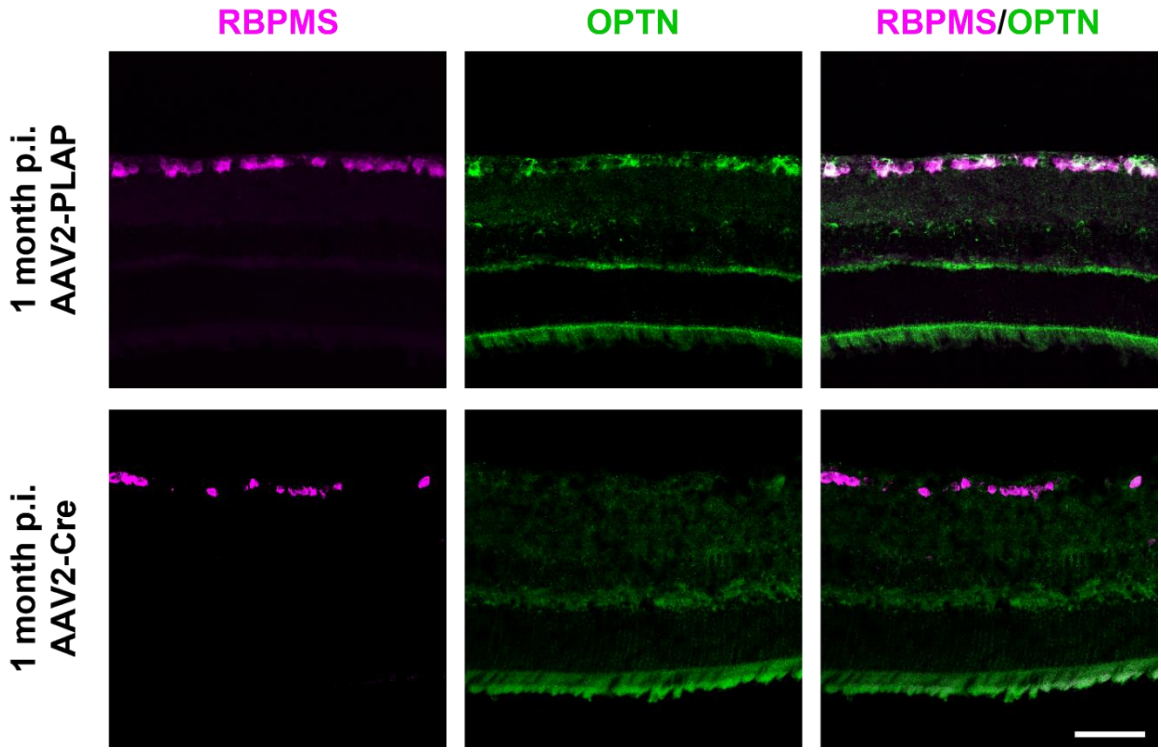
In this manuscript, the authors have explored the effects of deletion of C-terminal domain of OPTN in the mouse retina. Their results show that OPTN knockout (C-terminal deletion) in the retina contributes to short-term astrogliosis, retinal ganglion cell (RGC) loss and long-term microglia activation, with the propagation of microglia activation spreading to the optic nerve. Loss of OPTN function does not further exacerbate RGC death induced by ocular hypertension, which is known to cause glaucoma. They have carried out gene expression analysis from the data from mouse and human patients with optic neuropathies, available from various databases; this analysis suggests that OPTN dysfunction drives NPY downregulation and induces the expression of CHOP. They conclude that their results reveal a neuroprotective role for the OPTN-NPY signaling pathway, and its dysfunction contributes to RGC loss in glaucoma pathology. They also suggest that OPTN-NPY-mediated neuroinflammatory pathway provides a potential therapy for IOP-resistant glaucoma and highlights a druggable target for CHOP-associated neurodegeneration. Overall, some of their findings are important and provide new insights about the cytoprotective role of OPTN in the retina, which have relevance to the pathogenesis of normal tension glaucoma (NTG). However, I would like the following points to be addressed.

Major points

1. A better description of their experimental model is required. From which amino acid of OPTN the deletion occurs in their mouse model? Is it OPTN470T? In which cells of the retina, OPTN is deleted? Supplementary fig. S1a shows only RGC layer image (RBPMS staining) but other layers of retina are not shown. Is OPTN staining seen in other layers of retina? OPTN staining for other layers should also be shown for control as well as mutant retina.

We are grateful for this constructive suggestion. We confirm that this OPTN mutant is the truncated version from the 470th amino acid. In the floxed OPTN mouse model we have utilized in this paper, removal of the floxed OPTN exon 12 introduces a premature stop codon (TAG), resulting in a truncated 470 amino acid protein (OPTN^{470T}) that lacks the C-terminal 114 amino acids. This C-terminal region includes the entire ubiquitin-binding domain, which comprises the UBAN (ubiquitin binding region of ABIN proteins and NEMO) and a distal zinc finger (ZF) motif. Intravitreal injected AAV2 will transduce the majority of RGCs (~80-90%), while a few other cell types such as amacrine cells may be transduced too (Nawabi et al., Neuron, 2015). OPTN is also expressed in other retinal cells. This observation is confirmed by the immunofluorescence (IF) of OPTN across all layers of the retina (Reviewer Figure 1). We did not perform IF of other specific retinal cell markers to quantify the OPTN deletion efficiency in those cells, since the

cell-autonomous versus non-cell-autonomous effects are not the major focus of this paper and will not affect the rigor of our major conclusions.



Reviewer Figure 1 Representative images of OPTN staining across the full retinal layers at 1 month post OPTN knockout. Scale bar, 50 μm.

2. Lines 260-264, fig 7e, f. How the quantification of NPY expression was done in Fig. 7f? Is it done only in GCL or whole retina? They need to consider the possibility that reduced NPY level in OPTN-deficient retina may be due to reduced number of RGCs. Can they rule out this possibility?

We appreciate the reviewer's observation regarding this point. We assessed NPY expression levels by comparing the total fluorescence intensity of NPY in the retinal ganglion cell layer (GCL) and inner plexiform layer (IPL). Indeed, we acknowledge that we cannot fully rule out the possibility that reduced NPY level in OPTN-deficient retina may be partially due to the reduced number of RGCs. Future experiments such as single-cell RNA-seq on sorted RGCs from OPTN-deficient and control retinas may be performed to more accurately quantify the loss of RGCs and correlate with NPY expression in RGCs. Nevertheless, in the mouse optic nerve crush model, NPY expression in OPTN-negative RGCs was significantly lower than in OPTN-positive RGCs, supporting a regulatory role of OPTN in NPY expression (Supplementary figure 5b). We acknowledge this limitation in our current study and have included a discussion of this point in the revised manuscript.

3. They conclude that their results reveal a neuroprotective role for the OPTN-NPY signalling pathway. This is based on downregulation of NPY upon deletion of OPTN. This is over-

interpretation of the data. More evidence is needed to show that in their model NPY is cytoprotecive; eg. by overexpression of NPY.

We agree with the reviewer that this might be an overinterpretation and should be revised. In the previous version of our manuscript, we established the link between NPY and OPTN based on bioinformatics analysis and immunofluorescent staining, while citing previous studies (References 86 and 87) to support the neuroprotective role of NPY in retinal neuropathy. However, we agree that the current data does not directly demonstrate a cytoprotective role of NPY in our model. Accordingly, we have revised the over-interpreted statements to: “These findings highlight a neuroprotective role for OPTN as a key neuroimmune regulator. In addition, our study identifies promising downstream mediators, such as NPY and CHOP, which may serve as potential therapeutic targets for intraocular pressure (IOP)-resistant glaucoma.” Rather than overexpressing NPY, we focused on demonstrating the neuroprotective effect of wild-type OPTN. In light of the reviewer’s comment, we have modified the claim regarding the OPTN-NPY axis. We acknowledge that future studies are needed to elucidate the specific contribution of NPY to this pathway.

4. Fig. 7a. How do they define resilient and susceptible RGCs?

We appreciate the reviewer’s attention to this issue. Resilient versus susceptible RGCs have been defined based on a recently published single-cell RNA-seq dataset by Tran et al., published in Neuron in 2019 (Reference 61). In this study, the authors classified 46 subtypes of RGCs using defined resilient RGCs as those whose relative frequency increased by twofold 14 days after optic nerve crush, while susceptible RGCs were defined as those whose relative frequency decreased by twofold under the same conditions.

5. Lines 199-200. “Ocular hypertension resulted in a greater loss of RGCs compared to the loss observed 3-months after OPTN loss”. Data are not shown.

We are grateful for highlighting this issue. We apologize for this mistake of interpretating our data. We agree that our current evidence is insufficient to conclude that the high intraocular pressure model induces more severe RGC death compared to the OPTN knockout model. To address this, we have removed the corresponding statement from the manuscript.

Minor points
1. Legend to Fig. 1b needs correction. This fig. shows images only for one month and not for 3 and 6 months. The images for 3 and 6 months are shown in a supplementary Fig.

We have corrected it accordingly in the revised manuscript.

2. English needs some improvement at a few places. Lines 298-300, “…the OPTN N-terminal coiled-coil domain (NTD), where gain-of-function mutations such as E50K and M98K have been identified, is responsible for the OPTN/TBK1”. Perhaps they are implying “…OPTN-TBK1 interaction”.
In response to the reviewer’s correction, we have revised the corresponding section of the manuscript accordingly.

Reviewer #2 (Remarks to the Author):

The manuscript by Wang et al. describes the involvement of the multi-functional adaptor protein, optineurin (OPTN), in retinal ganglion cells loss and accompanying degenerative such as astrogliosis and microglial activation. The authors report the interesting finding that there is no exacerbation of IOP-mediated RGC loss in OPTN knockout out mice indicating that OPTN is necessary but probably not sufficient in promoting RGC survival particularly during IOP elevation. Including AAV-2-mediated expression of OPTN during IOP elevation would greatly enhance the clarity of the role of OPTN in promoting RGC neuroprotection. The findings have clinical relevance given the finding that 16% of normal tension glaucoma patients harbor mutations in the optineurin gene. The manuscript is well written, but the organization of the manuscript needs to be improved. Some of the key data has been moved to supplementary data and it would be better to combine them with the main data in Figures 1 to 7. Here are the key points that need clarification:

We appreciate the reviewer's thoughtful and detailed comments. We have included additional data on the in vivo overexpression of wild-type and mutant OPTN in this revised manuscript. Although certain key data currently in the supplementary information are important, we believe that including them as supplementary results would ensure clarity and keep our manuscript more focused. Nevertheless, we are grateful to this constructive suggestion.

1. In the abstract, in line 36, the authors should state what the NPY stands for.

We appreciate the reviewer for identifying this issue. The full name of NPY, neuropeptide Y, has been added to the abstract accordingly.

2. Reference 14 entitled, "Characterization of Normal Tension Glaucoma Mouse Over Expressing Mutant OPTN (E50K)" could not be found on PubMed. A similar issue was encountered for Reference 15. Please check the publication titles and journals for these two publications.

We acknowledge the reviewer's concern regarding the references cited, which were conference abstracts. Accordingly, we have replaced them with a peer-reviewed and published research article to enhance the rigor of this claim.

3. In line 106 of the introduction, the authors should delete the second part of the statement about "…whether OPTN dysfunction represents a shared pathological mechanisms between NTG and HTG" since this is not completely addressed in the current study.

We agree with the reviewer that the notion mentioned in this sentence cannot be fully addressed by this paper. Therefore, we have removed the corresponding content from the manuscript.

4. In line 118, authors must explain the sequences contained in the control AAV2-PLAP virus.

We appreciate the reviewer's valuable suggestion. The use of placenta alkaline phosphatase (PLAP)-expressing AAV as a control virus has been reported in multiple studies (Nawabi et al., 2015; Norsworthy et al., 2017). The full name of the AAV2-PLAP virus used in our study is AAV2-CAG-PLAP-WPRE.

5. In line 141 the authors must explain what they mean by enlargement of the cytoskeleton in glaucoma.

We appreciate the reviewer for carefully identifying this issue. The sentence is intended to describe reactive astrogliosis of astrocytes in glaucoma. Accordingly, we have revised the text as follows:

"In response to glaucoma, astrocytes upregulate the glial fibrillary acidic protein (GFAP), enlarge their cytoskeleton, and elongated their cellular processes, a process referred to reactive astrogliosis."

6. In the description of the data shown in Figure 3 about microglial activation in the optic nerve (line 178), the authors should mention the distance of the optic nerve region being examined from the optic nerve head.

We appreciate the reviewer's kind reminder. Astrogliosis and microglial activation were assessed at a location 1 to 2 mm from the optic nerve head. This detail has now been clarified in the corresponding sections of Methods in the manuscript. We are grateful for the reviewer's helpful feedback.

7. In Figure 4, the authors must include a treatment group pf AAV-2-Cre + PBS and include the IOP plot and RGC counts to demonstrate it as a model of NTG.

We appreciate the reviewer's valuable feedback. In Figure 4, the experimental and control groups were designed to evaluate whether OPTN knockout exacerbates RGC death induced by elevated intraocular pressure. Figure 1 has already demonstrated that OPTN knockout leads to RGC death independently of IOP changes. We hope that the data presented in Figure 1 adequately addresses the reviewer's concern.

8. In line 240, state that FAM19a4 is a chemokine.

We acknowledge the reviewer's comment. The sentence has been revised to "chemokine FAM19A4" in the corresponding section of the manuscript.

9. In Supplemental Figure 5e the authors must label the panel as GO enrichment analysis Day 2 post-crush.

We are grateful for the reviewer's suggestion. Supplemental Figure 5e has been updated and is now labeled as "GO enrichment analysis 2dpc RGCs OPTN+ vs OPTN-". 2dpc, 2 days post-crush.

10. In line 700 the authors must indicate if all animal procedures were carried out in accordance with either the ARVO or SFN guidelines for the use of animals in research.

We acknowledge the reviewer's reminder. We have now clarified in the manuscript that all animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with guidelines from the Office of Laboratory Animal Welfare (OLAW).

11. In line 278, the authors state that gain of function optineurin mutations such as E50K have been shown to induce RGC degeneration by enhancing the OPTN-TBK1 pathway. Is that the more plausible scenario in glaucoma pathogenesis?

We thank the reviewer for raising this important point. It is well recognized that the OPTN E50K mutation is a key pathogenic mutation in NTG, accounting for approximately 13.5% of NTG patients (Rezaie et al., Science, 2002). However, similar to our OPTN loss of function model, the premature stop mutation (c691_692insAG) has also been identified in 2.2% of NTG patients. Therefore, OPTN mutations in glaucoma may involve both gain-of-function and loss-of-function mechanisms. Our study focuses on the novel loss-of-function mechanisms of OPTN and proposes new therapeutic strategies for NTG, while our main conclusions do not rule out other gain-of-function mechanisms of OPTN in RGC degeneration

12. In the discussion, the authors must include references that make a mechanistic connection between optineurin and NPY.

We appreciate the reviewer's critical observation regarding this point. While previous studies have demonstrated the neuroprotective role of NPY, to our knowledge, no prior research has shown a direct mechanistic link between OPTN and NPY. In our current study, we have identified a potential association between OPTN and NPY for the first time through bioinformatic analysis (Supplementary Figure 5b) and immunofluorescence staining (Figure 7e, 7f). Nevertheless, a recent study has reported that NPY exerts significant neuroprotective effects in a glaucoma mouse model (Palanivel et al., PNAS Nexus, 2024).

13. There is very little discussion of the GO enrichment analysis of the scRNA seq data. The authors must discuss the potential implications of the key pathways identified is supplemental Figure 5.

In response to this constructive suggestion, we have included the interpretations of Supplementary Figure 5c-5f in both the Results and Discussion sections, as follows:

For result section: line 287-line 291: "Consistently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses between OPTN-positive and OPTN-negative RGCs indicate differential transcriptional and epigenetic programs associated with neurodegenerative disease-related pathways (Supplementary Fig. 5c-5f)."

For discussion section: line 351-line 357: "Our GO enrichment analysis revealed that the involvement of OPTN-positive RGCs in distinct signaling pathways in intact and injured conditions (Supplementary Figure 5c, 5e), suggesting that OPTN-related signaling may contribute to homeostatic regulation in response to injury. Consistent with this analysis, KEGG

analysis indicated an enrichment of neurodegeneration-associated pathways in OPTN-positive RGCs (Supplementary Fig. 5d, 5f), implicating a potential role of OPTN in modulating neuronal survival and regeneration."

We hope this revision responds to the reviewer's insightful comment.

Reviewer #3 (Remarks to the Author):

In the manuscript entitled "OPTN protects retinal ganglion cells and ameliorates neuroinflammation in optic neuropathies" the authors studied the role of OPTN in glaucoma pathology. They specifically examined the effect of OPTN deficiency on promoting irreversible microglia activation in the retina, assuming a spread to the optic nerve. However, they excluded an exacerbating role for OPTN dysfunction in RGC death in the HTG model. Finally, they found that NPY, an important downstream inflammatory modulator of OPTN involved in RGC rescue from death, in addition to OPTN should be considered as a promising IOP-independent target to treat glaucoma. Although this is an interesting and important study, I have a few remarks/suggestions for the Authors:

- As you know, among the missense OPTN mutations involved in NTG (e.g., E50K, H26D, H486R, E322K), the E50K is the most common one. It seems that this mutation leads to RGC death via autophagy, trafficking dysfunction and an increase in oxidative stress. Do you have evidence of OPTN loss of function in glaucoma? Since OPTN loss of function is considered the principal disease mechanism for ALS phenotype, do you have found patients with both conditions (glaucoma and ALS)? An additional investigation about the effect of mutated OPTN and OPTN knockout in eliciting astrogliosis, microglia activation, and RGC death would make the work more complete.

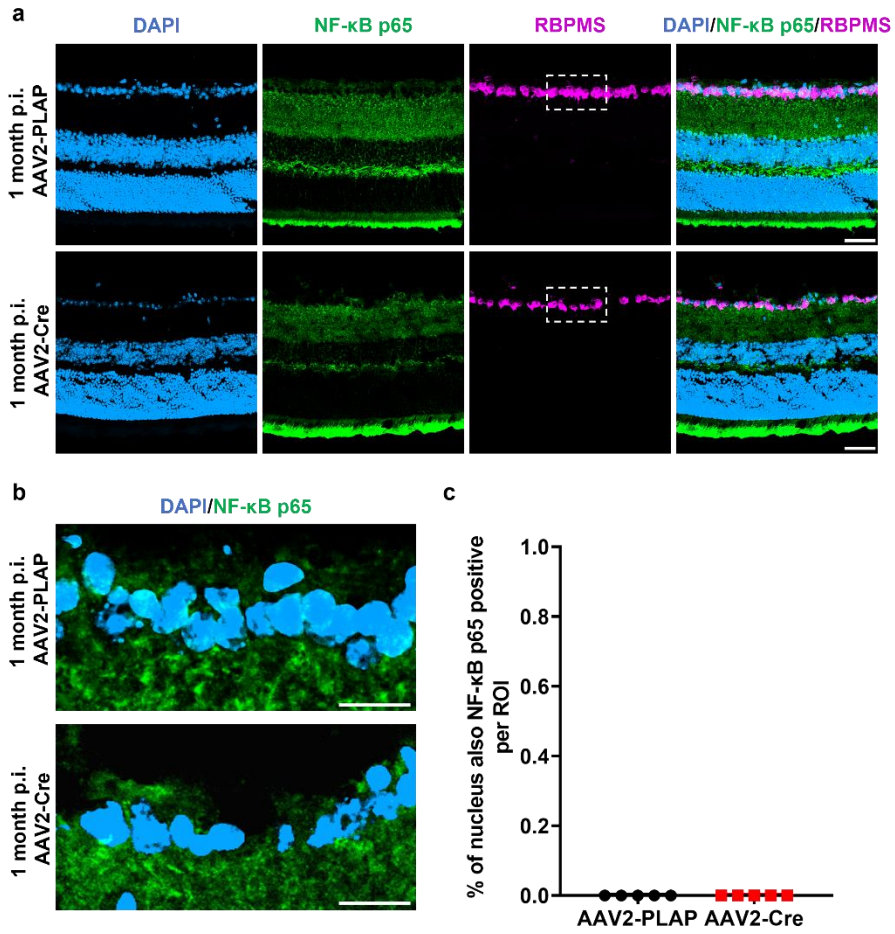
We appreciate the reviewer's insightful questions. The OPTN loss of function mutation (c691_692insAG) was identified in 2.2% of NTG patients (Rezaie et al., Science, 2002). Additionally, our bioinformatics analysis indicates a downregulation of OPTN expression in RGCs of NTG patients (Fig. 7d). Together, these findings support the involvement of OPTN loss of function in glaucoma pathology.

Regarding the second question, to our knowledge, there are no reported cases of patients diagnosed with both ALS and glaucoma. However, Retinal thinning without ophthalmic disease has been reported in ALS patients (Mukherjee et al., PLOS One, 2017). This may suggest that the rapid progression of ALS limits patients' lifespan, preventing the development of glaucoma pathology.

In response to the third constructive suggestion, we have overexpressed both wild-type OPTN and the OPTN E50K mutant in wild-type retinas and compared their effects on RGC death induced by elevated IOP. Furthermore, the role of the OPTN E50K mutation in neuroimmune regulation is indeed an important question, which we may explore in future studies.

- Why has NF-kB activity in OPTN ko mice not been analyzed? It has been reported that inappropriate NF-kB activation is the main pathogenic mechanism underlying OPTN loss-of-function mutation.

We have now examined NF-κB activity in the retina one month after OPTN knockout by assessing the nuclear localization of NF-κB p65. Our results revealed no significant changes in the nuclear translocation of p65, indicating that NF-κB signaling was not activated upon OPTN deletion (see Reviewer Figure 2 below). This finding is consistent with a previously published study showing that NF-κB activity did not significantly change in mice carrying a systemic C-terminal truncation of OPTN (OPTN^{470T}) (Munitic et al., J Immunol, 2013). Together, these results suggest that OPTN C-terminal truncation may promote neuroinflammation through pathways not necessarily dependent on NF-κB activity. Importantly, as mentioned in our manuscript, the wild-type OPTN protein also contributes to several other signaling pathways such as autophagy and membrane trafficking. This is an open question that merits further studies on the role of OPTN in neuroprotection and optic neuropathies.



Reviewer Figure 2 OPTN loss of function does not activate the NF-κB signaling pathway. **a** Representative IF images showing no nuclear colocalization of NF-κB p65 with DAPI-stained nuclei. Scale bar, 50 μm. **b** Enlarged views from **a** highlighting the DAPI-stained nuclei and NF-κB p65 localization. Scale bar, 20μm. **c** Quantification of NF-κB p65 positive nuclei.

- This work shows well that OPTN plays a pivotal role in neuroimmune homeostasis and RGC vulnerability. Indeed, suppressing OPTN, a selective autophagic receptor and a signaling regulator, compromises removing a wide range of substrates and regulating innate immune

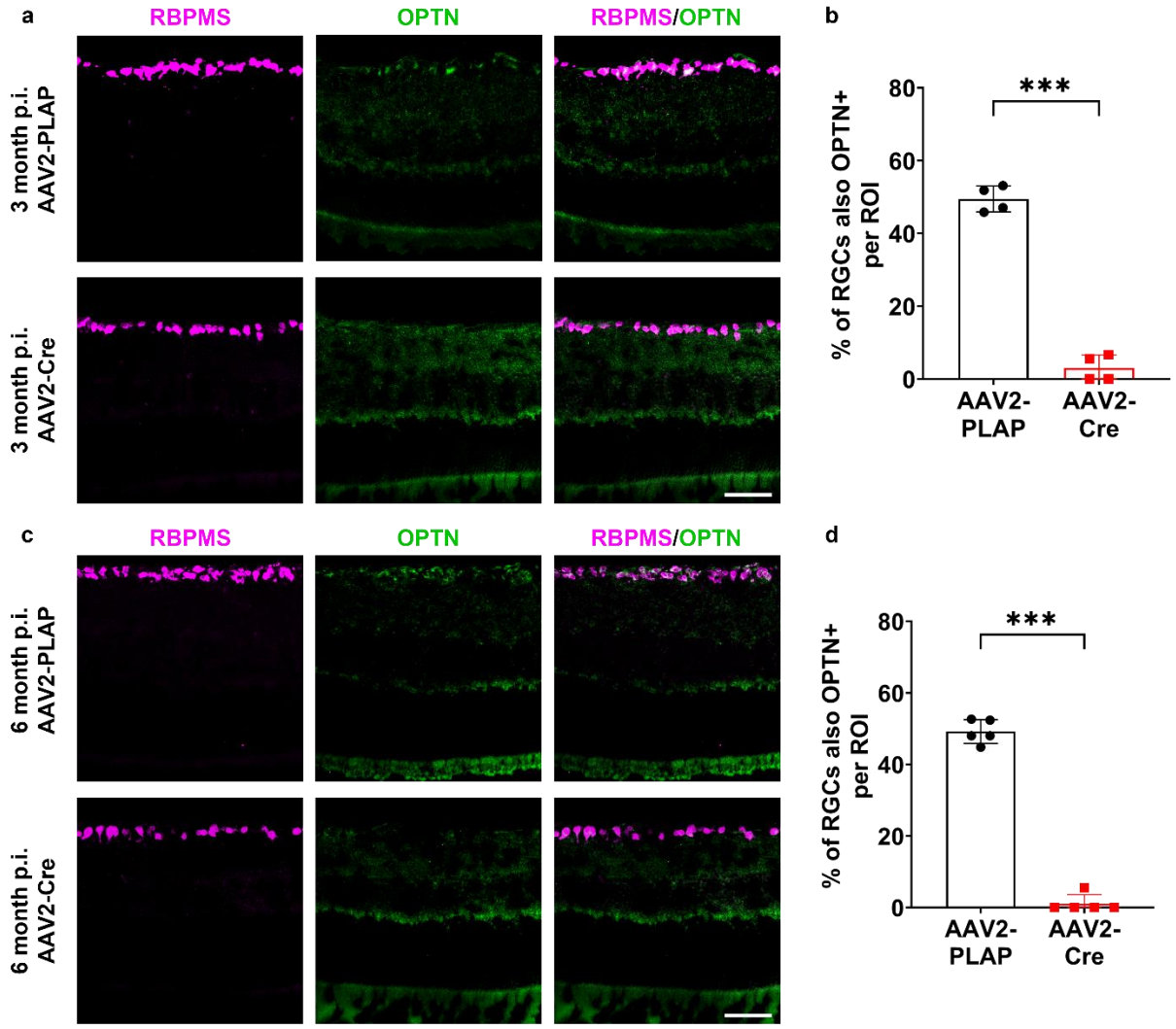
response, respectively. However, in this way, it becomes difficult to study which functional interactions (e.g., TBK1, LC3, myosin VI, TAX1BP1, Rab8, Htt, transferrin receptor, RIP, SOD1, caspase 8, HACE1 metabotropic glutamate receptor 1 and 5, and so on) are compromised in mutated forms found in glaucoma. So, my question is: why did you focus on analyzing the lack of OPTN instead of its most common mutated form?

We are grateful for this thoughtful question. OPTN gain of function mutations, particularly the E50K mutation, have indeed been widely studied and are well recognized for their significant contribution to the pathogenesis of glaucoma. However, loss of function mutations in OPTN have also been observed in glaucoma patients (Rezaie et al., Science, 2002), yet they remain underexplored. Our study aims to reveal the intrinsic OPTN-driven neuroprotective mechanism as the therapeutic target for a complementary strategy for glaucoma treatments.

- In the supplementary figures where both astrogliosis and microglia activation are demonstrated at 3 and 6 months after OPTN ko, I would add IHC staining of OPTN at the same time.

We acknowledge the reviewer's helpful suggestion. To verify that AAV-mediated OPTN knockout remains effective over the entire experimental period, we have now performed IF staining for OPTN in the same retinas examined for astrogliosis and microglia activation at both 3- and 6-months post-injection. The new images are presented below (Reviewer Figure 3). They demonstrate a sustained and nearly complete loss of OPTN signal in KO RGCs at each time point, while age-matched control retinas retain robust OPTN expression. These data confirm that the observed glial changes occur in the continued absence of OPTN.

To maintain figure clarity and avoid redundancy, we have not included these data in the main or supplementary figures; however, we will add them upon request if needed.



Reviewer Figure 3 Sustained OPTN knockout efficiency in RGCs at 3 and 6 months following AAV-mediated OPTN knockout. **a** Representative IF images showing reduced OPTN expression in the retina at 3 months post-knockout. Scale bar, 50 µm. **b** Quantification of OPTN expression in the retina at 3 months post-knockout. Data are presented as mean ± s.e.m., n = 4 biological replicates. **p < 0.001, unpaired t-test. **c** Representative IF images showing sustained OPTN knockout in the retina at 6 months post-knockout. Scale bar, 50 µm. **d** Quantification of OPTN expression in the retina at 6 months post-knockout. Data are presented as mean ± s.e.m., n = 5 biological replicates. **p < 0.001, unpaired t-test.

- In my opinion, highlighting that the bioinformatic analysis you performed on previous results from Daniszewski et al. was from NTG patients rather than POAG has more impact

We appreciate the reviewer's invaluable comment! We have emphasized in the corresponding section of the manuscript that our bioinformatics analysis results are derived from NTG patients.

- Regarding the NPY, do you suggest this neuropeptide as a promising target for specifically treating glaucoma involving OPTN mutations or for sporadic glaucoma also?

We acknowledge this insightful question. Based on our bioinformatics analysis and IF staining results, NPY may be involved in the OPTN-regulated neuroinflammatory pathway. However, we currently lack sufficient evidence to demonstrate that exogenous supplementation of NPY can significantly promote RGC survival under glaucomatous conditions. In this study, we primarily focus on the neuroprotective mechanism of OPTN through suppression of neuroinflammation. The therapeutic potential of NPY, including its effects in both NTG and HTG, will be explored in future studies.

Additionally, we have updated the statistical analysis to the most appropriate tests in some parts of the manuscript. We have also reorganized Supplementary Figure 6 and 7 to make the manuscript more coherent and easier to follow. Importantly, these modifications do not affect the conclusions corresponding to those analyses.