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Transcriptomic profiling of retinal cells reveals a subpopulation of microglia/macrophages expressing Rbpms marker of retinal ganglion cells (RGCs) that confound identification of RGCs

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Abstract

Analysis of retinal ganglion cells (RGCs) by scRNA-seq is emerging as a state-of-the-art method for studying RGC biology and subtypes, as well as for studying the mechanisms of neuroprotection and axon regeneration in the central nervous system (CNS). Rbpms has been established as a pan-RGC marker, and Spp1 has been established as an α RGC type and macrophage marker. Here, we analyzed by scRNA-seq retinal microglia and macrophages, and found Rbpms⁺ subpopulations of retinal microglia/macrophages, which pose a potential pitfall in scRNA-seq studies involving RGCs. We performed comparative analysis of cellular identity of the presumed RGC cells isolated in recent scRNA-seq studies, and found that Rbpms⁺ microglia/macrophages confounded identification of RGCs. We also showed using immunohistological analysis that, Rbpms protein localizes to stress granules in a subpopulation of retinal microglia after optic nerve injury, which was further supported by bioinformatics analysis identifying stress granule-associated genes enriched in the Rbpms⁺ microglia/macrophages. Our findings suggest that the identification of Rbpms⁺ RGCs by immunostaining after optic nerve injury should exclude cells in which Rbpms signal is restricted to a subcellular granule, and include only those cells in which the Rbpms signal is labeling cell soma diffusely. Finally, we provide solutions for circumventing this potential pitfall of Rbpms-expressing microglia/macrophages in scRNA-seq studies, by including in RGC and α RGC selection criteria other pan-RGC and α RGC markers.

Graphical Abstract

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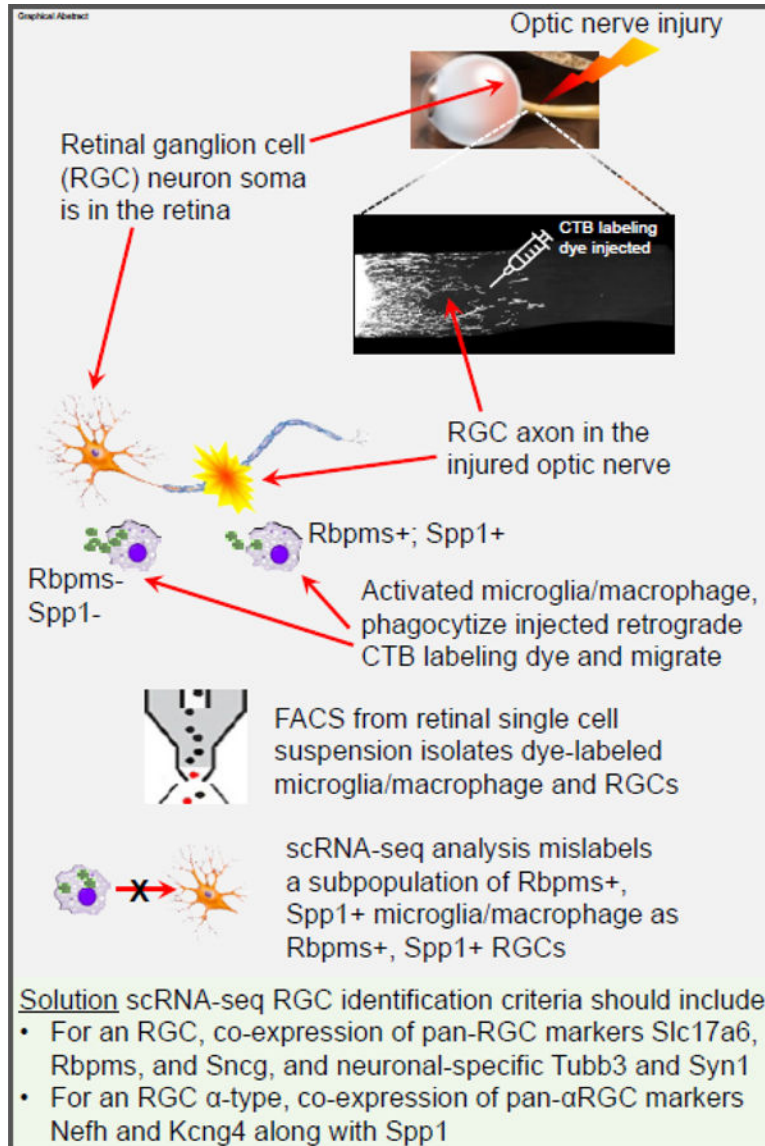
AUTHOR CONTRIBUTIONS

W.C.T and M.P.F. performed the experiments and analysis. E.F.T. wrote the manuscript.

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DECLARATION OF INTERESTS

The authors declare no competing interests.



INTRODUCTION

The *Pten* tumor suppressor gene is one of the most potent gene-regulators of CNS axon regeneration. *Pten* suppresses regeneration through inhibition of the mTOR pathway, and *Pten* knockout (KO) and knockdown (KD) have been shown to promote various extents of axon regeneration, after optic nerve crush (ONC) injury in mice, in the α and intrinsically photosensitive (ip) subsets of retinal ganglion cells (RGCs)¹⁻⁴. In order to understand the effect of *Pten* inhibition on the transcriptomes of RGCs that respond by regenerating axons of any length, recent studies by Li et al.⁵ and by Jacobi et al.⁶ isolated the RGCs with *Pten* KO that regenerated axons at least short-distance (1.5 mm) and profiled them by scRNA-seq. In contrast, we developed a new method for isolating specifically the rare subset of RGCs which respond to *Pten* inhibition by regenerating axons long-distance (~3 mm past the injury site or beyond) and also profiled them by scRNA-seq⁷. Here, we performed

comparative analysis of cellular identity of the presumed RGC cells isolated in these studies, along with scRNA-seq profiled retinal and optic nerve microglia/macrophages, and reveal the existence of an Rbpms+ subpopulation of microglia/macrophages, which could confound identification of RGCs in scRNA-seq studies.

RESULTS

We report that, the pan-RGC marker Rbpms^{8,9} is also expressed in a subset of microglia and macrophages (which infiltrate the CNS after injury). Therefore, Rbpms alone is insufficient for identifying RGCs in scRNA-seq analysis. For example, in the study by Li et al.⁵, Pten KO-treated RGCs were selected from within (fluorescently pre-labeled and FACS-sorted) scRNA-seq-profiled retinal cells based on the expression of at least one RGC marker (either Rbpms, Thy1, Slc17a6, or Pou4f1–3), rather than co-expression of multiple RGC markers. As injury-activated resident microglia and peripheral macrophages phagocytize fluorescent dye and migrate (including into the retina), the reliance on single RGC marker expression rather than co-expression of multiple markers resulted in ~94% of the cells selected as presumed RGCs to be identified as microglia/macrophages by retrospect analyses (see below), which is consistent with top enriched genes reported in that study⁵ being microglia/macrophage markers and the top enriched gene-ontology terms being microglia/macrophage-related (e.g., leukocyte migration, lymphocyte mediated immunity). Thus, in order to determine RGC cell type identity by scRNA-seq, several of the well-established pan-RGC markers (e.g., Slc17a6 and Sncg) and neuronal markers (e.g., Tubb3 and Syn1) need to be co-expressed in a cell.

To demonstrate this potential pitfall of relying only on expression of a single RGC marker rather than co-expression of several markers in scRNA-seq studies, we performed comparative analysis of the cells with Pten KO/KD labeled as RGCs in the studies by Li et al.⁵, Jacobi et al.⁶, and ourselves⁷. We also included in the analysis scRNA-seq-profiled microglia/macrophages, which we isolated after ONC from retinas and optic nerves, and separated based on the expression of Rbpms (Fig. 1A). In total, 6.6% of microglia/macrophage cells were Rbpms+. Rbpms detection was not a consequence of a piece of broken RGC sticking to microglia/macrophage as we applied doublet filters and did not find other RGC/neuronal markers expressed in either subpopulation of microglia/macrophages (Fig. 1A-H). The range of Rbpms expression in the Rbpms+ microglia/macrophages was equivalent to levels observed in uninjured and injured RGCs (Fig. 1A). Rbpms was expressed in the RGC-labeled cells from studies by Li et al.⁵, Jacobi et al.⁶, and ourselves⁷ (Fig. 1A). However, the other RGC-specific markers, Slc17a6, Sncg, and Brn3a, and neuronal-specific marker Syn1, were not enriched in the RGC-labeled cells in Li et al.⁵, but were enriched in cells from studies by Jacobi et al.⁶ and ourselves⁷ (Fig. 1A-H). We also examined expression of established ipRGC type markers, melanopsin (Opn4) and Tbr2 (Eomes)^{9–13}. Both Li et al.⁵ and Jacobi et al.⁶ cells were negative for these markers, whereas Opn4 and to a lesser extent Eomes were enriched in non- α RGC cells we isolated ourselves (which regenerated axons long-distance)⁷ (Fig. 1E-F; see in more detail below). Neuronal-specific marker Tubb3 (which is also RGC-specific within the retina⁹) was expressed in cells from our study⁷ and in uninjured and injured (untreated) RGCs, but not enriched in cells from the other studies^{5,6} (Fig. 1G). Only 17 cells, which represent 5.7% of all RGC-labeled

cells that regenerated axons in the study by Li et al.⁵, expressed the appropriate RGC markers.

We then analyzed the expression of established microglia/macrophage markers, Iba1, Cd45, Cd14, Cd16, and Cx3cr1. We found that these markers were enriched in microglia/macrophages (regardless of whether they expressed Rbpms) and in the presumed RGC cells from the study by Li et al.⁵, but not in any of the RGC conditions, even after Pten KO/KD in studies by Jacobi et al.⁶ and ourselves⁷ (Fig. 1I-M). For reference, UMAPs show that between them, these microglia/macrophage markers were expressed in all (and co-expressed in most of) the microglia/macrophages we isolated (Fig. 1N-R), along with Spp1 marker of macrophages¹⁴⁻¹⁶ (Fig. 1S). After ONC, injury-activated microglia and infiltrating peripheral macrophages migrate within the optic nerve and into the retina. They can phagocytize the fluorescent tracer dye injected into the injured optic nerve, and some of them migrate into the retina within a day (thus, they are FACS-sorted as positive for the labeling dye). We also show that, all Rbpms+ microglia/macrophages we identified are positive for established microglia/macrophages markers Iba1 and/or Cd14 (Fig. 1T-U) and nearly half of them do not express Pten (Fig. 1V). Taken together, these data demonstrate that, it is insufficient to rely on expression of only one pan-RGC marker to identify RGCs within a mixed population of scRNA-seq-profiled retinal cell types, as this can lead to inclusion of microglia/macrophages. Co-expression of pan-RGC markers (including Slc17a6 and Snca) is necessary, and the inclusion of neuronal-specific markers (e.g., Tubb3 and Syn1) is preferred, in order to identify RGCs by scRNA-seq.

Because Li et al.⁵ found expression of certain α RGC markers in their cells, we analyzed whether they are expressed in microglia/macrophages as well. The α RGC marker genes¹⁷, Nefh (which encodes Neurofilament-H containing a non-phosphorylated epitope recognized by an SMI-32 antibody)^{2,10}, Kcng4^{2,18}, and Etv1^{10,19}, were not enriched in cells from the study by Li et al.⁵, with only a handful of cells expressing it within the 5.7% of cells that were indeed RGCs (Fig. 2A-C). However, Nefh and Kcng4 were enriched in the Jacobi et al.⁶ RGCs. Furthermore, although Kcng4 and Etv1 were downregulated in α RGCs after ONC, Kcng4 downregulation was prevented by Pten KO pre-treatment in Jacobi et al.⁶ cells (Fig. 2B-C). Other α RGC markers, Spp1^{2,10,11} and subtype C42-specific Fes¹¹, however, were enriched in the Li et al.⁵ cells, but they were also enriched in the macrophages/microglia (Fig. 2D-E). Spp1 was also enriched in Jacobi et al.⁶ RGCs. As cells we isolated ourselves⁷ were non- α RGC subtypes (which regenerated axons long-distance in response to Pten KD), they did not express these α RGC markers except for low levels of Spp1, consistent with prior reports that Pten inhibition leads to Spp1 upregulation in some cell types²⁰⁻²². Spp1 expression was also substantially higher in the Li et al.⁵ cells compared to Jacobi et al. and our cells (Fig. 2D), consistent with that Spp1 is an established marker of macrophages¹⁴⁻¹⁶ (Fig. 1S). Another α RGC subtype-specific marker, Kit (for C41)¹¹, was not expressed except for in a few cells isolated by Li et al.⁵, and the remaining α RGC subtype-specific markers, Il1rapl2 (for C43) and Tpbp (for C45)¹¹, were not enriched in the Li et al.⁵ cells. Thus, when using these markers for α RGCs in a mixed population of scRNA-seq-profiled cells, it is important to first evaluate for co-expression of pan-RGC markers.

We also found that the neuroprotective genes, *Anxa2* and *Mpp1*, found by Li et al.⁵, are robustly expressed in the *Rbpms*+ microglia/macrophages (Fig. 2F-G), and along with *Rbpms* are also present in macroglia previously analyzed by bulkRNA-seq (see mouse microglia dataset at <https://www.brainrnaseq.org>)²³. We further confirmed that the presumed RGC cells from the study by Li et al.⁵ are microglia/macrophages, by analyzing global transcriptomes (and not only the canonical cell type markers) and finding that they correlated and clustered more strongly with our microglia/macrophage dataset than with any RGC dataset (Fig. 2H). Analysis of global transcriptomes also confirmed that *Rbpms*+ microglia/macrophages are near identical (by correlation and associated clustering) to the *Rbpms*- microglia/macrophages (Fig. 2H). Thus, inadvertently, Li et al.⁵ screened microglia/macrophage genes (instead of the genes enriched in the RGCs that regenerated axons after *Pten* KO), and found several neuroprotective hits, such as *Anxa2* and *Mpp1*, which are expressed in both *Rbpms*+ microglia/macrophage and RGCs (Fig. 2F-G).

To test whether *Rbpms* is translated in microglia/macrophages which express *Rbpms* mRNA, we co-immunostained the retinas after ONC for *Rbpms*, *Iba1*, and *Tubb3* (anti-Tuj1 antibody). All *Iba1*+ cells were Tuj1-, whereas *Rbpms*+/*Tuj1*+ RGCs were present in the ganglion cell layer (GCL), as expected. Amongst the *Iba1*+ cells, we found a subpopulation of cells containing subcellular *Rbpms*+ structures which morphologically resembled stress granules, in contrast to the diffusely distributed *Rbpms* signal in *Tuj1*+ RGCs in the same retinas (Fig. 3A-I). Thus, subcellular distribution/localization of *Rbpms* differs between the injured retinal microglia and RGCs, consistent with that *Rbpms* localizes to cytoplasmic stress granules in other cell types^{24,25} and stress granules form in microglia activated in neurodegenerative diseases^{26–28}. Furthermore, genes ontology (GO) analysis of genes that are significantly enriched in *Rbpms*+ relative to *Rbpms*- microglia/macrophages identified “response to stress” amongst the topmost associated biological processes (Fig. 3J). Moreover, the genes enriched in the *Rbpms*+ microglia/macrophages included stress granule-associated genes (*Hspa1b*, *Hspa1a*, *Hspa5*, *Hsph1*, *Dnajb1*, *Egr1*, *Fos*, *Dusp1*, *Ppp1r15a*²⁹) along with *G3bp1*, which has been previously established to colocalize with *Rbpms* in stress granules²⁴ (Fig. 3K). Taken together, immunohistological and bioinformatics analyses suggests that, *Rbpms* protein localizes to stress granules in a subpopulation of retinal microglia/macrophages after optic nerve injury. Therefore, histological quantification of *Rbpms*+ RGCs by immunostaining after optic nerve injury should exclude cells in which *Rbpms* signal is restricted to subcellular granules and include only those cells in which the *Rbpms* signal is labeling cell soma diffusely.

DISCUSSION

We revealed herein the existence of an *Rbpms*+ subpopulation of retinal microglia/macrophages, some of which also migrate into the retina from the optic nerve after activation by ONC injury. *Rbpms*, *Anxa2*, and *Mpp1* are also expressed in microglia from other CNS regions, which were previously analyzed by bulkRNA-seq (see mouse microglia dataset at <https://www.brainrnaseq.org>)²³. *Rbpms* is an established pan-RGC marker^{8,9}, and *Spp1* is an established α RGC type and macrophage marker^{2,10,11,14–16}. Therefore, *Rbpms*+ and *Spp1*+ microglia/macrophages within retinal suspension could confound the identification of RGCs by scRNA-seq analysis, as occurred in a study by Li et al.⁵. However,

serendipitous discovery of the neuroprotective potential of *Anxa2* and *Mpp1* genes by Li et al.⁵ study has significant implications for developing neuroprotective therapies, regardless of that microglia/macrophages analysis assisted in their identification.

We demonstrate that in order to circumvent the potential pitfall of mistaking Rbpms+ microglia/macrophages for RGCs in scRNA-seq studies, detection of co-expression of pan-RGC markers (including Rbpms, *Slc17a6*, and *Sncg*) is necessary, the inclusion of neuronal-specific markers (e.g., *Tubb3* and *Syn1*) is preferred (*Tubb3* is also RGC-specific within the retina), and the inclusion of *Brn3A-C*, which between them cover all RGCs^{9,30,31}, should be considered. Similarly, in order to circumvent the potential pitfall of mistaking Spp1+ microglia/macrophages for α RGCs in scRNA-seq studies, it is important to first evaluate for co-expression of pan-RGC markers other than just Rbpms, and the inclusion of other pan- α RGC markers (e.g., *Nefh* and *Kcng4*) is preferred, with the caveat that *Kcng4* is downregulated after ONC injury unless pre-treated (with *Pten* KO in this case). Similar considerations need to be made when relying on α RGC subtype C42 marker *Fes*, as it is also expressed in a subpopulation of Rbpms+ microglia/macrophages. Furthermore, because our immunohistological and bioinformatics analyses demonstrated Rbpms protein localization to stress granules in a subpopulation of retinal microglia after optic nerve injury, histological quantification of Rbpms+ RGCs by immunostaining after optic nerve injury should exclude cells in which Rbpms signal is restricted to a subcellular granule, and include only those cells in which the Rbpms signal is labeling cell soma diffusely (as even if there is Rbpms in the injured RGCs' stress granules it would be masked by the diffuse Rbpms signal throughout RGC soma).

Microglia and macrophages are involved in retinal degenerative diseases that affect RGCs (e.g., glaucoma, traumatic optic neuropathy, ischemic optic neuropathy) and other retinal cell types^{32–36}. The application of scRNA-seq technologies for studying the cellular and molecular mechanisms of retinal degeneration resulting from diseases or injuries is becoming more widely used in the field. Therefore, the use of proper marker combinations we report, and consideration of the Rbpms+ subpopulation of microglia/macrophages we reveal, will assist future scRNA-seq studies of retinal degeneration and RGC biology. Furthermore, because the global transcriptomes of Rbpms+ and Rbpms–microglia/macrophages are overall similar, it remains to be investigated further whether the Rbpms+ subpopulation has unique functions. As stress granules are involved in other neurodegenerative diseases^{26–28}, our finding of Rbpms localization to stress granules in a subpopulation of microglia/macrophages after injury suggests that they may be involved in retinal degeneration as well.

MATERIALS AND METHODS

Animal use and surgeries.

All animal studies were performed at the University of Connecticut Health Center with approval of the Institutional Animal Care and Use Committee and of the Institutional Biosafety Committee, and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Mice were housed in the animal facility with a 12-h light/12-h dark cycle (lights on from 7:00 AM to 7:00 PM) and a maximum of five

adult mice per cage. The study used wild-type 129S1/SvImJ mice (The Jackson Laboratory). Optic nerve surgeries were carried out on mice of both sexes 8–12 weeks of age (average body weight 20–26 g) under general anesthesia, as described previously^{3,37–39}. Animals were sacrificed 2 weeks after ONC for histological analysis.

Immunohistological processing and analysis.

Standard histological procedures were used, as described previously^{3,37–40}. Briefly, anesthetized mice were transcardially perfused with isotonic saline followed by 4% paraformaldehyde (PFA) at 2 weeks after ONC, the eyes were dissected, postfixed 2 hours, flattened retinas were resected, after making 4 symmetrical slits embedded in OCT Tissue Tek Medium (Sakura Finetek), frozen, and cryosectioned at 14 μm horizontality (capturing the ganglion cell layer of the whole retina), and then immunostained and mounted on coated glass slides for imaging, as we described previously⁴¹. For immunostaining, the tissues were blocked with appropriate sera, incubated overnight at 4 °C with primary antibodies, Rbpms (1:500; guinea pig, 1832-RBPMS, PhosphoSolutions), Iba1 (1:600; rabbit, 019–19741, Wako), β III-Tubulin/Tuj1 (1:500; mouse, MMS-435P, BioLegend), and counterstained with DAPI (1:5000; Thermo Fisher Scientific) to label nuclei, then washed 3 times, incubated with appropriate fluorescent dye-conjugated secondary antibodies (1:500; all were IgG H+L; Alexa Fluor, Thermo Fisher Scientific) overnight at 4 °C, washed 3 times again, and mounted for imaging. Z-stacked images were acquired at 0.2 μm intervals using a confocal microscope (63x Oil; LSM800, Zeiss) and processed using ZEN software (Zeiss).

ScRNA-seq and data availability.

The cells identified as RGCs, which regenerated axons in response to Pten inhibition, were processed for scRNA-seq by plate-based SmartSeq2 in studies by Li et al.⁵ and Jacobi et al.⁶ and ourselves⁷. ScRNA-seq counts-matrix were assembled following alignment to the mouse genome and transcriptome using the Hisat2-Cufflinks pipeline. Counts-matrix for Li et al.⁵ (GSE206626) and Jacobi et al.⁶ (GSE202155) were obtained from Gene Expression Omnibus. RGCs were selected per criteria specified in the respective paper's methods sections. In scRNA-seq dataset from Li et al.⁵, 297 cells were initially identified as presumed RGCs (that regenerated axons), but only 17 cells were left after exclusion of macrophages/microglia in retrospect analysis reported herein (the other group of RGC-labeled cells in Li et al.⁵ study that survived but did not regenerate axons had just 8 RGCs and the remaining cells were microglia/macrophages when the appropriate markers were used). In the scRNA-seq dataset from Jacobi et al.⁶, 120 cells were identified as RGCs. In the scRNA-seq dataset we generated, 101 cells were identified as RGCs⁷ (GSE210137). 10x Genomics 3'-droplet based scRNA-seq dataset of adult uninjured and injured (2 weeks after ONC) RGCs, which included α RGCs (average of α subtypes C41, C42, C43, and C45), was obtained from Tran et al.¹¹ (GSE137400). We generated 10x Genomics 3'-droplet based scRNA-seq dataset of macrophage/microglia from adult (10 weeks old) uninjured and injured (2 weeks after ONC) retinas/optic nerves, using the methods we described previously^{10,41}. After demultiplexing and aligning to the mouse genome and transcriptome using the CellRanger pipeline, 1168 macrophage/microglia passed quality control (including filters of a minimum of 200 genes, 6500 maximum genes). Batches were merged, normalized and scaled using Seurat and batch correction/integration of data sets

was achieved using the R package harmony⁴². Comparative analysis of scRNA-seq datasets by Violin plots was performed using the R package Seurat v4.3.0. Correlation matrix was generated using base R software and heatmap with dendrogram was generated using the R package Superheat^{43,44}. The macrophage/microglia scRNA-seq data we generated, and the integrated data-frame from different cell types and conditions we assembled, are available through the NCBI GEO accession GSE228986. Seurat's FindMarkers function (with Wilcoxon rank sum test, and default parameters)⁴⁵ was used to identify significantly differentially expressed genes (DEGs; log₂ fold-change ≥ 0.25 , p -value = < 0.05) in Rbpms+ microglia/macrophage relative to the Rbpms- population. Gene ontology (GO) enrichment analysis was performed using the R package gprofiler2⁴⁶, with significantly upregulated DEGs as input and all genes expressed > 0.001 in the macrophage/microglia dataset as background. GO term enrichment plots and functional annotation and semantic clustering of GO terms was performed using the treeplot function from the Enrichplot R package along with the ggplot2 R package^{47,48}.

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ABBREVIATIONS

RGC	retinal ganglion cell
ipRGC	intrinsically photosensitive retinal ganglion cell
αRGC	α retinal ganglion cell
CNS	central nervous system
Mg/Mp	microglia/macrophage
scRNA-seq	single cell RNA sequencing
ONC	optic nerve crush
KO	knockout
KD	knockdown
FACS	fluorescence-activated cell sorting
GCL	ganglion cell layer

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Highlights

- Pan-retinal ganglion cell (RGC) marker Rbpms found in a subpopulation of retinal microglia/macrophages can confound identification of RGCs by scRNA-seq.
- Rbpms protein localizes to stress granules in retinal microglia/macrophages after optic nerve injury.
- Pan-RGC markers that are necessary for reliable RGC identification by scRNA-seq specified.

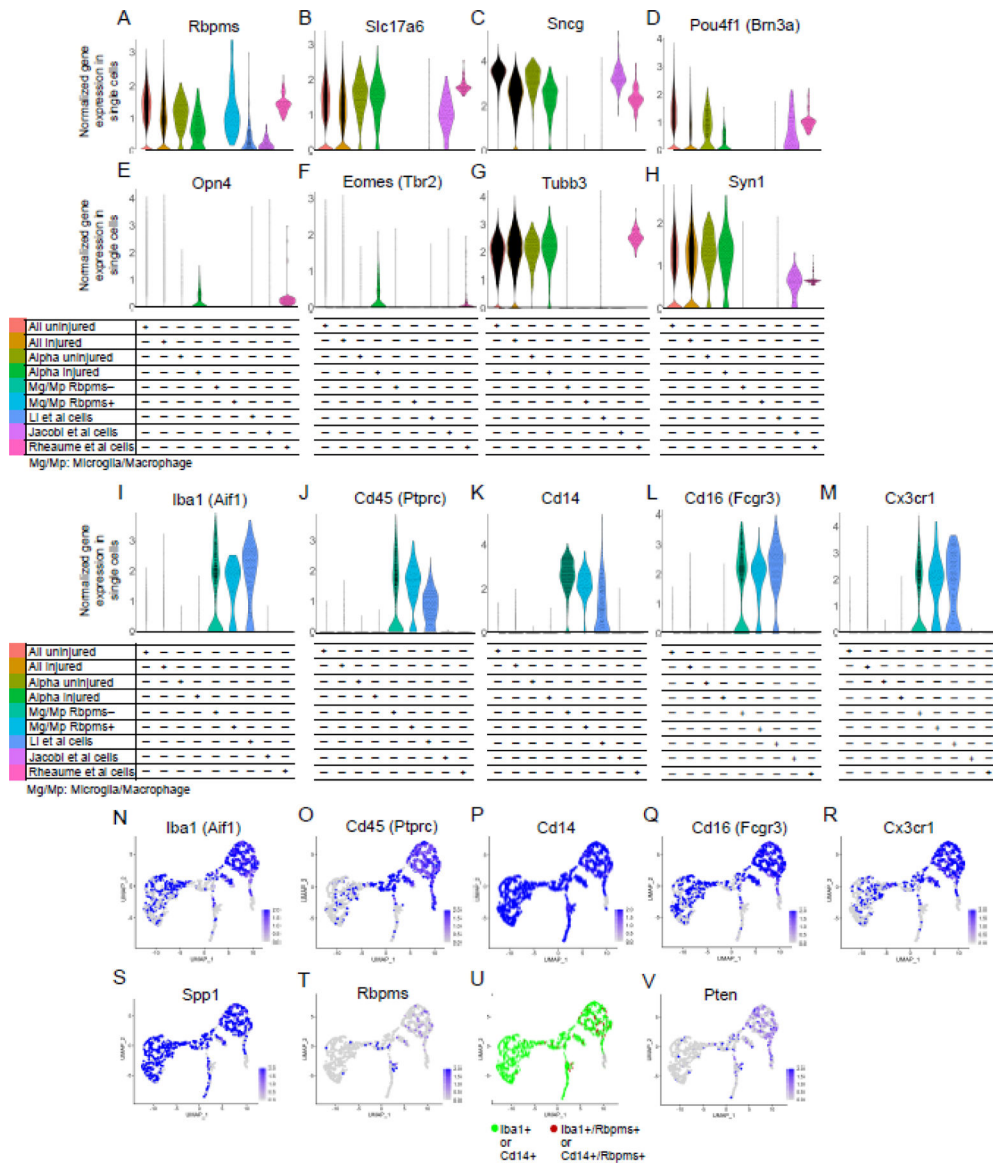


Figure 1. Analysis of pan-RGC, pan-neuronal, ipRGC, and microglia/macrophage gene markers in scRNA-seq datasets from different conditions and cell types

(A-H) Pan-RGC gene markers (A-D) are enriched in Tran et al. (2019) uninjured and injured RGCs, including in the α RGC subset, but with an exception for Rbpms (A) are not expressed in the microglia/macrophages (Mg/Mp). IpRGC markers (E-F) are not enriched in Li et al. (2022) or Jacobi et al. (2022) cells, but are expressed in non- α RGCs isolated by ourselves (Rheame et al., 2023). Neuronal-specific (G-H) gene markers are also enriched in axon-regenerating RGCs isolated by Jacobi et al. (2022) and ourselves (Rheame et al., 2023) but are absent from the majority of presumed RGC cells isolated by Li et al. (2022), with an exception for Rbpms (A) which is also enriched in the Rbpms+ subpopulation of microglia/macrophages. Tubb3 (G) is enriched in uninjured and injured untreated RGCs (Tran et al., 2019), and in axon-regenerating RGCs isolated by ourselves (Rheame et al., 2023).

(I-M) Established microglia/macrophage markers, Iba1 (*I*), Cd45 (*J*), Cd14 (*K*), Cd16 (*L*), and Cx3cr1 (*M*), are enriched in all microglia/macrophages (regardless of Rbpms expression) and in the presumed RGC cells isolated by Li et al. (2022), but not enriched in isolated RGCs from any other condition/group.

(N-S) UMAPs of scRNA-seq-profiled retinal microglia/macrophages after optic nerve injury show that, established microglia/macrophage markers, Iba1 (*N*), Cd45 (*O*), Cd14 (*P*), Cd16 (*Q*), Cx3cr1 (*R*), and Spp1 (*S*), between them are expressed in all (and co-expressed in most of) the microglia/macrophages we isolated.

(T-V) UMAPs of scRNA-seq-profiled retinal microglia/macrophages after optic nerve injury show that, Rbpms is expressed in a subpopulation of these cells (*T*), which co-express Iba1 and/or Cd14 microglia/macrophage markers (*U*), and nearly half of them do not express Pten (*V*).

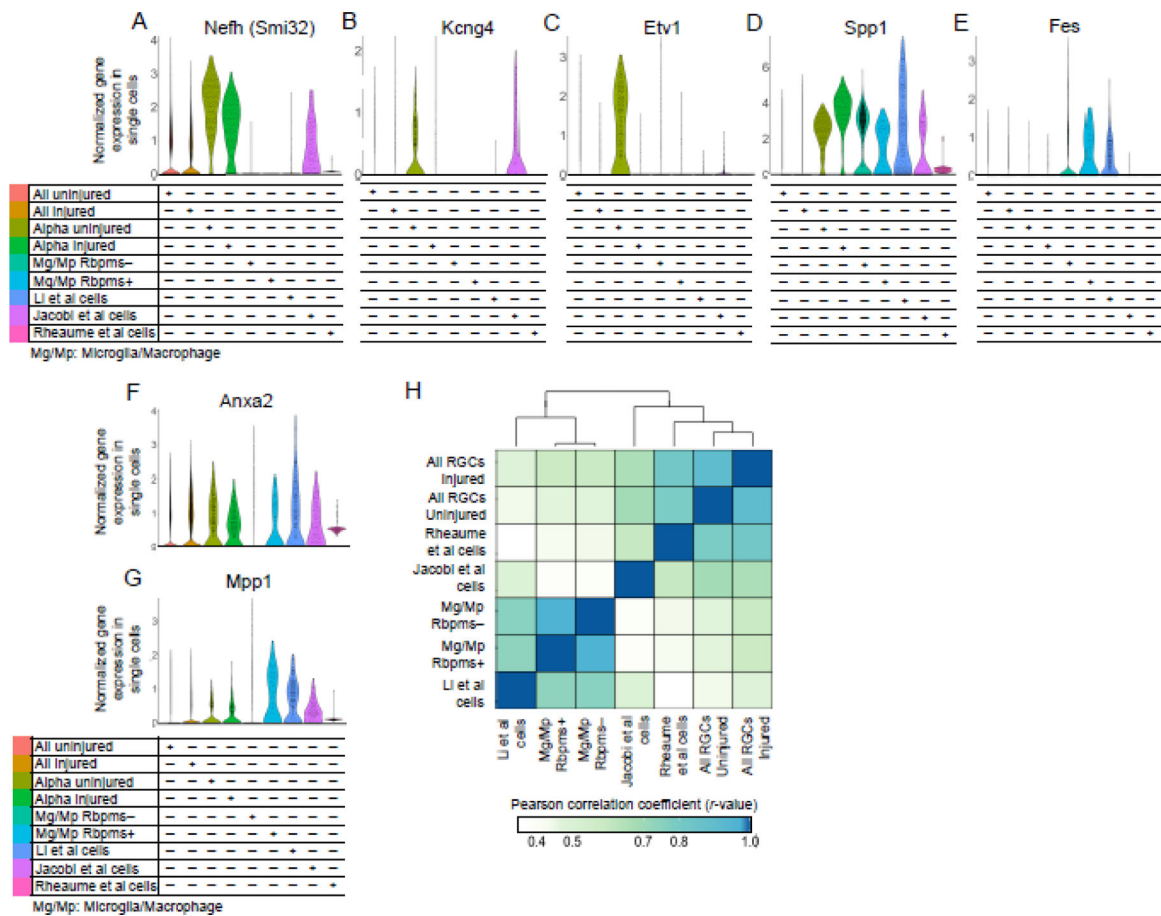


Figure 2. Analysis of α RGC gene markers and neuroprotective genes in, and global transcriptome correlation analysis of, scRNA-seq datasets from different conditions and cell types

(A-E) Pan- α RGC markers *Nefh* (A), *Kcng4* (B), and *Spp1* (D), are enriched in Tran et al. (2019) α RGC subset and in axon-regenerating RGCs isolated by Jacobi et al. (2022). *Spp1* (D) is enriched in the presumed RGC cells isolated by Li et al. (2022) more than in any other condition, but is also substantially enriched in the microglia/macrophages, regardless of Rbpms expression. *Kcng4* (B) and *Etv1* (C) α RGC markers are downregulated after ONC injury in Tran et al. (2019) α RGC subsets, but *Kcng4* downregulation is prevented by *Pten* KO in axon-regenerating RGCs isolated by Jacobi et al. (2022). *Fes* (E) was enriched only in the presumed RGC cells isolated by Li et al. (2022) and in the microglia/macrophages. No α RGC markers were meaningfully enriched in long-distance axon-regenerating RGCs isolated by ourselves (Rheume et al., 2023), except for low levels of *Spp1* (which is consistent with reports that *Pten* inhibition leads to *Spp1* upregulation).

(F-G) Neuroprotective genes found in the screen by Li et al. (2022), *Anxa2* (F) and *Mpp1* (G), are robustly expressed in the Rbpms+ microglia/macrophages, as well as in RGCs from every other condition/group.

(H) Analysis of the global transcriptomes clustered and correlated presumed RGC cells from the study by Li et al. (2022) more strongly with the microglia/macrophage datasets than with other RGC datasets, whereas Jacobi et al. (2022) cells and Rheume et al. (2023) cells

clustered and correlated more strongly with RGC datasets than with microglia/macrophage datasets.

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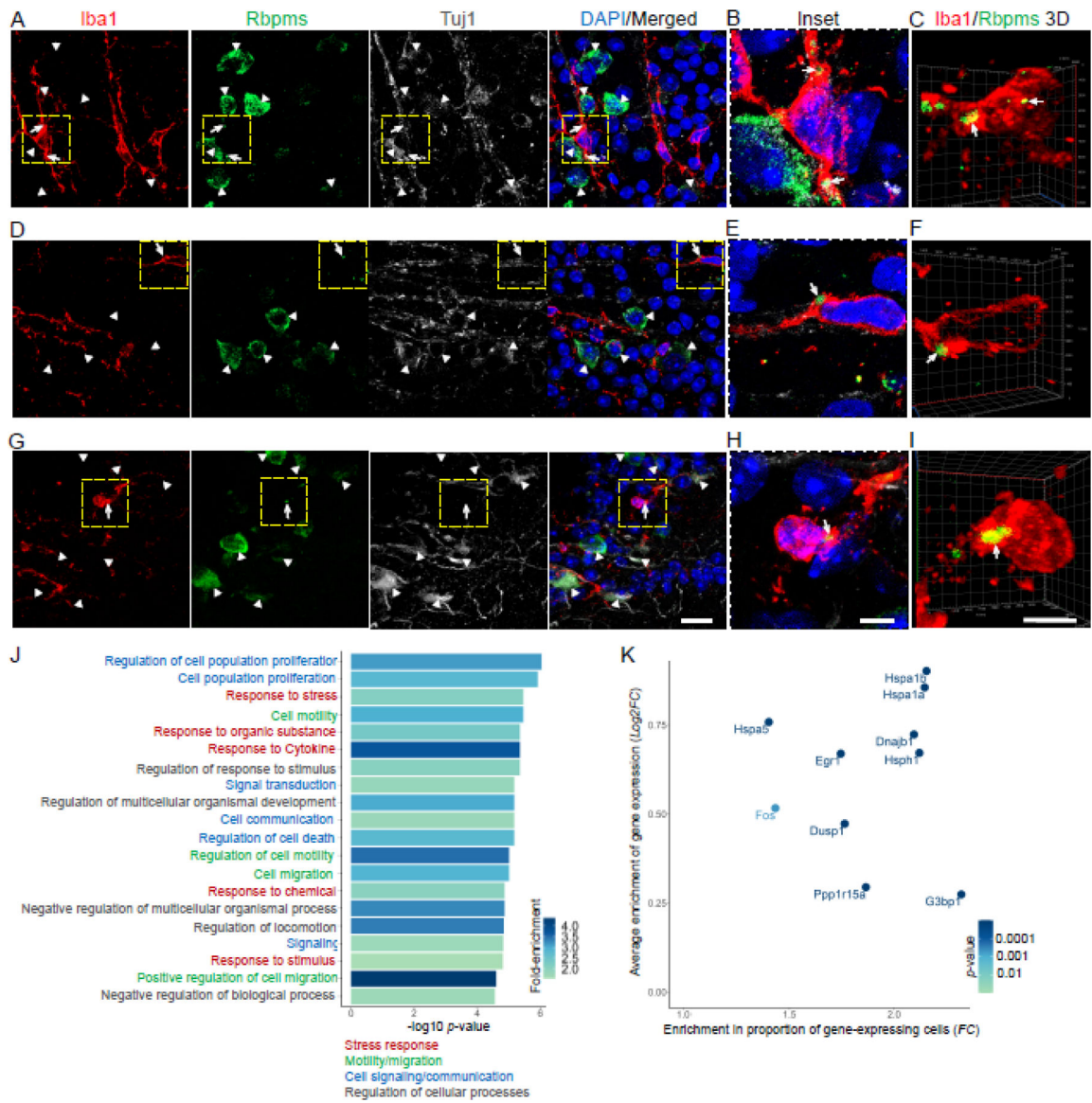


Figure 3. Rbpms protein localizes to stress granules in a subpopulation of retinal microglia/macrophages which are enriched for stress response genes after optic nerve injury

(A-I) Confocal images of the retinal flatmounts (at 2 weeks after ONC) co-immunostained for Rbpms, Iba1, Tuj1, and counterstained for DAPI (to label nuclei), show examples of Rbpms signal localization to granules in Iba1-labeled microglia/macrophages, as compared to diffuse cytoplasmic Rbpms signal in Tuj1-labeled RGCs, in the injured retinas' GCL. White arrowheads indicate Rbpms+/Tuj1+ RGCs. White arrows indicate Rbpms+ granules within the Iba1+ microglia/macrophages. Dashed yellow-line outlined regions in the main panels (A,D,G) are shown in insets (B,E,H) and in 3D renderings (C,F,I) at higher magnification for better visualization of the Rbpms+ granules' subcellular localization within the Iba1+ microglia/macrophages. Main panels scale bar: 20 μm; insets and 3D panels scale bars: 5 μm.

(J) Gene ontology (GO) analysis of genes enriched in Rbpms+ relative to Rbpms- microglia/macrophages identified GO biological processes (GO:BPs; 20 topmost significant

by adjusted p -value are shown) that included multiple cellular stress response-related terms. Further functional annotation and semantic similarity clustering of GO:Terms (using the EnrichPlot R package) identified 4 overarching enriched pathways, which included “stress response”. GO:BPs are color-coded according to overarching enriched pathways to which they belong, as marked. Color scale bar indicates GO:BP terms’ fold-change (FC) enrichment (see Methods).

(K) Enrichment of stress granule-associated genes in the Rbpms+ relative to Rbpms– microglia/macrophages is demonstrated by higher average expression of these genes (y -axis; \log_2 FC) and by higher proportion of Rbpms+ cells as compared to Rbpms– cells expressing these genes (x -axis; FC). Color scale bar indicates significance of average expression enrichment (adjusted p -value by Wilcoxon rank-sum test; see Methods).