Loss of Melanopsin-Expressing Retinal Ganglion Cells in Severely Staged Glaucoma Patients

Elisabeth Anne Obara,1 Jens Hannibal,1 Steffen Heegaard,2,3 and Jan Fahrenkrug1

1Department of Clinical Biochemistry, Bispebjerg Hospital, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
2Department of Pathology, Rigshospitalet, Eye Pathology Section, University of Copenhagen, Copenhagen, Denmark
3Department of Ophthalmology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

PURPOSE. Multiple studies have shown overwhelming evidence supporting the impairment of melanopsin function due to glaucoma. However, few studies have been carried out in humans analyzing the histology of melanopsin-expressing retinal ganglion cells (mRGCs) in retinas with glaucoma. The aim of this study was to analyze the pattern of expression of mRGCs relative to RGCs in the normal retina and retinas harboring varying stages of glaucoma.

METHODS. Paraﬁn-embedded human donor eyes with glaucoma (n = 11) and age-matched controls (n = 10) were obtained from Department of Pathology at Rigshospital (Copenhagen, Denmark) for detection of RNA binding protein with multiple splicing (RBPMS) and melanopsin by immunohistochemistry. The density of RBPMS-expressing RGCs and mRGCs in each retina was estimated as the total cell count in the total retinal area analyzed (cell counts/mm2).

RESULTS. No signiﬁcant difference was observed in mRGC expression in the normal retinas and mild-staged retinas with glaucoma; the densities of mRGCs were 3.08 ± 0.47 and 3.00 ± 0.13 cell counts/mm2, respectively. However, the severely staged retinas with glaucoma showed a signiﬁcant loss in mRGCs density, 1.09 ± 0.35 cell counts/mm2, with 75% of all retained mRGCs occurring in the inner nuclear layer.

CONCLUSIONS. This is the ﬁrst report illustrating histologic evidence for reduced mRGC density in the ganglion cell layer of retinas with severely staged glaucoma compared with age-matched controls. This result proposes evaluation of mRGCs integrity as a basis for assessing the pathophysiologic disease progression of glaucoma.

Keywords: melanopsin, immunohistochemistry, RBPMS
Melanopsin Expression in Glaucoma Patients

TABLE. Overview of Glaucoma Patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis (Mild/Severe)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS</td>
<td>50</td>
<td>Female</td>
<td>Secondary glaucoma (severe)</td>
</tr>
<tr>
<td>HA</td>
<td>51</td>
<td>Male</td>
<td>Secondary glaucoma (severe)</td>
</tr>
<tr>
<td>JG</td>
<td>60</td>
<td>Female</td>
<td>Primary glaucoma (mild)</td>
</tr>
<tr>
<td>IT</td>
<td>62</td>
<td>Male</td>
<td>Secondary glaucoma (severe)</td>
</tr>
<tr>
<td>WA</td>
<td>63</td>
<td>Female</td>
<td>Secondary glaucoma (severe)</td>
</tr>
<tr>
<td>BM</td>
<td>66</td>
<td>Female</td>
<td>Primary glaucoma (severe)</td>
</tr>
<tr>
<td>KM</td>
<td>68</td>
<td>Female</td>
<td>Angle closure glaucoma (severe)</td>
</tr>
<tr>
<td>AI</td>
<td>73</td>
<td>Female</td>
<td>Secondary glaucoma (severe)</td>
</tr>
<tr>
<td>ME</td>
<td>74</td>
<td>Male</td>
<td>Primary glaucoma (mild)</td>
</tr>
<tr>
<td>PM</td>
<td>81</td>
<td>Male</td>
<td>Open angle glaucoma (severe)</td>
</tr>
<tr>
<td>KM</td>
<td>88</td>
<td>Female</td>
<td>Secondary glaucoma (severe)</td>
</tr>
</tbody>
</table>

* Based on clinical and eye pathologist journal data.

immunohistochemistry of mRGCs and RGCs in the Human Retina

Immunohistochemistry (IHC) was performed on the paraffin-embedded human retinal sections after antigen retrieval (code no. S2031; ChemMat DAKO, Glostrup, Denmark) at pH 6.0 as previously described by La Morgia et al. Following antigen retrieval, washing, and treatment with 1% hydrogen peroxide (H₂O₂), the sections were blocked in 5% donkey normal serum and incubated in a mixture of the following two primary antibodies overnight at 4°C: Guinea pig anti-human RNA binding protein with multiple splicing (RBPM51) polyclonal antibody, (code no. 1832; PhosphoGlycoproteins, Aurora, CO, USA) was diluted 1:500. This antibody was raised against the N terminus of the RBPM51 polypeptide in humans as characterized by Rodríguez et al. The in-house raised rabbit anti-human melanopsin (C-terminal) polyclonal antibody (code no. 5J68; inhouse produced, polyclonal antibody; characterized by Hannibal et al.) was used in a dilution of 1:20,000. The following day, sections were washed and incubated with the secondary antibodies: Envision goat anti rabbit kit (EnVision+ System HRP; code no. K4002; ChemMat Dako) diluted 1:25 and Alexa 594 Donkey anti Guinea pig (code no. 706-585-148; Jackson Immunolab, West Grove, PA, USA) diluted 1:200 overnight at 4°C. On the third day, the sections were washed and incubated for 1 hour with an Alexa 488 tyramide (code no. T20922; Molecular Probes, Paisley, UK) diluted 1:250 for visualization of melanopsin, after which the sections were washed and mounted with coverslips using mounting media (glycerol; code no. G9012; Sigma Aldrich Corp., St Louis, MO, USA) in PBS, diluted 1:1 containing 4',6-diamidino-2-phenylindole (DAPI) (code no. D1306; Molecular Probes).

Cell Counting and Imaging Analysis of Human Retina

Each retina was analyzed for density of both mRGCs and RGCs. From every five sections in the set of the 30 sections, both cell types were objectively analyzed with regard to the following inclusion criteria: the mRGCs had to display immunostaining of cytoplasmic membrane and a visible round nucleus (central profile counting) and positivity for RBPM51. Retinal ganglion cells were also counted in a similar manner, as only those positive for RBPM51 with a prominent nucleus were counted. Localization of mRGCs in either the GCL or displaced to the INL were quantified separately to analyze the distribution of these cells in the retina. All counts derived were verified by a second independent observer to confirm the counting guidelines outlined were followed. Images were obtained using an IMC confocal microscope system equipped with filter settings for DAPI, Alexa 488, and Alexa 594 (IMC, FEI, Munich, Germany) to estimate the retinal length and acquire images for each retina. As every fifth section of 5-μm thickness was analyzed (section separation), each section was assumed to represent a 25-μm diameter, and any cells within this diameter were likely to be observed, reducing the possibility of double counting and overestimation. The derived count for each patient was divided by the derived retinal length multiplied by the determined constant thickness of 0.125 mm (125 μm) to calculate the average density of each cell type in the entire retina. Double staining of slides from glaucoma patients and age-matched controls allowed for comparative analysis of both RGCs and mRGCs densities.

Statistics

For statistical analysis, differences in group means were assessed by unpaired, two-tailed Student’s *t*-test, and *P* <
0.05 was considered significant. Results are presented as mean ± SEM. All statistical analyses were carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

RESULTS

Using IHC, all tissues were analyzed for the expression of RGCs and mRGCs in the normal and diseased human retina (Fig. 1). The majority of cells in the GCL of both the control and glaucoma groups are RGCs, affirmed by the characteristic cytoplasmic staining of RBPMS (Figs. 1B, 1F, 1J). In the control human retina, the GCL constituted a sparsely populated, single layer of cell bodies in the peripheral regions of the retina, with a gradual increase in thickness and cell density toward the central retina (Figs. 1A, 1B, 2A, 2B). Similar to the control retina, the mildly staged retinas with glaucoma showed a disparity in density of cells located in the central and periphery of the retina (Figs. 1E, 1F, 2E, 2F). In contrast, the severely staged retinas with glaucoma showed a reduced expression of RBPMS expressing RGCs and loss of cell bodies, accompanied with overall loss of retinal tissue in the GCL layer (Figs. 1I-J and 2I-J).

A heterogeneous population of RGCs in the GCL of the retina of both control and glaucoma patients was shown to express melanopsin located in the soma and dendrites of the cell (Figs. 1C, 1G, 1K, 2C, 2G, 2K). The mRGCs were not restricted to the GCL as approximately 45% were also observed displaced to the INL of the control human retina, with fibers visible in two layers located in the inner and outer the borders of the IPL (Figs. 1C, 1D, 2C, 2D). The staining pattern of mRGCs in the mild and severely staged retinas with glaucoma resembled the control retina with the mRGCs located in the INL surrounded by a dense network of cell bodies, rendered visible by DAPI staining (Figs. 1I–L and 2E–H). In the severely staged retinas with glaucoma, however, approximately 75% of all mRGCs counted were located in the INL. The ONL was similar to the INL densely populated by cell bodies rendered visible by DAPI staining, showing no expression of RBPMS or melanopsin (Fig. 1).

To quantify the density of mRGCs and RGCs in the entire retina, the retinal length was measured per patient. The control and glaucoma group had an average retinal length of 33.93 ± 2.29 and 36.14 ± 2.06 mm, respectively, which was not significantly different.

In the severely staged glaucoma group showed significant disruption of primarily the GCL layer with a 200-fold loss of RGCs compared with the control group (Fig. 3A). In contrast, the mildly staged glaucoma group showed no significant disruption of the integrity of the GCL or the cells in this layer.

**Figure 1.** Photomicrographs of peripheral (thin) retinas of (A–D) control, (E–H) mild glaucoma, and (I–L) severe glaucoma. (A) DAPI, control human retina showing the GCL, IPL, INL, outer plexiform layer (OPL), and ONL, with cell bodies in the GCL, INL, and ONL. (B) RNA binding protein with multiple splicing, image showing RBPMS positive cells, RGCs (arrowhead) located in the GCL with non-RBMPs cells interspersed between the RGCs. (C) Melanopsin, two melanopsin-positive cells in the GCL with its dendrite spanning IPL. (D) Merged, image combining all filters. Arrow pointing to non-RBMPs cell in GCL. (E) DAPI, mildly staged retina with glaucoma showing organized retina with GCL, IPL, INL, OPL, and ONL present. (F) RNA binding protein with multiple splicing staining showing preserved RGCs in the GCL (arrowhead). (G) Melanopsin, arrowhead pointing to positive cell in the GCL and fibers on the border of the IPL and INL. (H) Merged image combining all filters, also showing cells in the GCL not positive for RBPMs (arrow). (I) DAPI, severely staged retina with glaucoma showing no cells in GCL, but cells present in the INL and ONL. (J) RNA binding protein with multiple splicing, no positive cells in the GCL but a single cell present in the INL (arrowhead) (K) Melanopsin, a positive mRGC in the INL (arrowhead). (L) Merge, combination of all channels. Scale bars: 25 μm.
compared with the control group (Fig. 3A). The same trend was observed in mRGCs, as only the severely staged retinas with glaucoma showed a significant reduction in mRGCs density of 2.8-fold in comparison to the control group (Fig. 3B). In the severely staged glaucoma group, the population of mRGCs located in the GCL of the retina showed a significant loss of 6-fold in comparison to the control group (Fig. 3C), whereas no significant difference was shown in density of mRGCs occurring in the INL (Fig. 3D). Two of the severely staged retinas with glaucoma were completely devoid of both mRGCs and RBPMS cells. These results did not affect the significant decrease in mRGCs expression primarily in the GCL (Fig. 3C); therefore, these patients were not excluded from the analysis.

In the glaucoma group, the ratio of mRGCs/RGCs expressed in percent seemed to increase with disease severity. Patients with mild glaucoma had a percentage of 0.29 ± 0.07%, which did not differ significantly from the controls, whereas severe cases of glaucoma showed a significant increase of 48-fold. The same trend was observed when only analyzing mRGCs and RGCs in the GCL, as mRGC/RGC percentage in severe cases of glaucoma increased by 21-fold compared with controls.

The increase in mRGCs/RGCs percentage was accounted for by the changes in the distribution of both cell types in the retina. In the control group, the distribution of mRGCs was 54% in the GCL and 46% in the INL, whereas in severe cases of glaucoma, 75% of mRGCs were located in the INL. The major disparity between the severely damaged retinas with glaucoma and the normal retina with regard to mRGCs is in the sparing of mRGCs occurring in the INL and the massive loss of mRGCs in the GCL.

**DISCUSSION**

In this study we aimed at assessing the expression of mRGCs in patients with varying degrees of glaucoma. The notion whether mRGCs are spared or lost due to glaucoma is one of great speculation as there is a lack of human studies investigating the fate of mRGCs in the retina after damage due to glaucoma. Several animal studies using various experimental glaucoma models based on an increase in IOP have been used to elucidate the effect of glaucoma on mRGCs; however, conflicting reports of both sparing and loss have been shown. The complex pathology of glaucoma in humans is unparalleled to that observed in animal models, further complicating the direct adoption of the results obtained from animal studies to the human retina with glaucoma.

Glia as characterized as a progressive disease whereby disease severity is correlated to visual field defects, which is a direct measure of RGC density. The loss of RGCs has been
used as an important indicator of glaucomatous damage. RGC density has previously been accounted for based on axonal counts in the optic nerve. Here we assessed each case for RBPMS-expressing RGCs with a visible soma and counted every positive cell in a stereologic manner. As expected, the controls and mild cases of glaucoma had significantly higher RGC densities compared with severe cases, justifying the specificity of RBPMS for viable RGCs in the human retina. The melanopsin expressing RGCs were counted in a similar manner, showing for the first time, significant loss of mRGCs primarily in the GCL, as well as sparing of mRGCs in the INL of severely staged glaucoma patients. The preserved expression of mRGCs located in the INL supports the results derived from studies assessing mRGCs function in humans, which have shown impairment not elimination of both retinohypothalamic tract- and retinotectal tract-mediated response in patients with severely staged glaucoma. A recent study conducted by Perez-Rico et al. showed lowered suppression of melatonin in response to light primarily in severe cases of glaucoma in comparison to normal patients, proposing hampered mRGCs function primarily in severe cases. Studies conducted by Feigl et al. and Kankipati et al. showed a hampered PIPR in response to blue light in patients with severely staged glaucoma, but no dysfunction in patients with mild glaucoma.

In accordance with the results of La Morgia et al., our results show mRGCs on average are equally distributed in the control human retina between the GCL and INL. The dendrites of this small subset of cells have been shown to stratify in either the inner or outer IPL, with mRGCs located in the INL stratifying primarily in the outer layer of the IPL. Retrograde tracing studies in primates have shown that there is a consistent projection of inner and outer stratifying mRGCs.

**Figure 3.** Quantitative study of cells from control, mild glaucoma, and severe glaucoma groups. (A) Density of RGCs was expressed as RGC counts/mm². There was a significant loss of RGCs in the severely staged glaucoma group compared with the control group (****P < 0.001). No significant difference was observed between the control and mildly staged glaucoma group. (B) Density of mRGCs was expressed as mRGCs counts/mm². Significant loss of mRGCs was found in the severely staged glaucoma group but not in the mildly staged glaucoma group. Density of mRGCs in the (C) GCL and (D) INL of the retina. No significant difference in mRGCs density in the INL was found between all three groups. The severely staged glaucoma group showed a significant decrease in density of mRGCs localized in the GCL (**P < 0.05).
to the suprachiasmatic nucleus, pretectal, and lateral geniculate nucleus of the brain hence vital for both NIF and IF. It is likely that the remaining mRGCs in the INL of the retinas with glaucoma are responsible for the abnormal mRGCs response of lowered suppression of melatonin in response to light and hampered pupillomotor PPR.4,12,14,30,51,45 Animal studies investigating the role of mRGCs in the retina have shown that complete ablation of these cells resulted in lack of mRGCs function, and a loss of 80% is required for mRGCs function to be significantly impaired.24 46 In diseases such as Leber's hereditary optic neuropathy, where visual acuity is severely impaired, studies have shown the sparing of mRGCs projecting to the pretectum responsible for the pupillary reflex.47

The use of archived human tissue did not affect the presentation of both RGCs and mRGCs in the retina. Despite the small cohort size, the control group after analysis had an average mRGCs/RGCs percentage of 0.35%, which is in agreement with previous studies using freshly resected whole mount retinas showing a percentage of 0.2%–0.8%.12–14 The increase in average mRGCs/RGCs percentage to 14% observed in the severely staged retinas with glaucoma, with a maximum of up to 40%, can be adopted as the actual trend in the severely staged retinas with glaucoma. This increase was also observed when only cells in the GCL were analyzed. Two glaucoma patients appeared to be devoid of any RGCs and mRGCs in the entire retina due to end-stage disease. Despite the severe loss, the final statistical difference between controls and the glaucoma group was not dependent on these two patients’ results. It remains to be known if the loss of RGCs in the retina with glaucoma directly affects the integrity of mRGCs, as the mRGCs located primarily in the GCL are primarily affected. The localization of the spared mRGCs in the INL, which is less densely populated by RGCs, could factor in the sparing of these mRGCs. The underlying mechanisms aiding the preservation of mRGCs in the retina with glaucoma are yet to be fully outlined due to the complex nature of mRGCs. The sparing of mRGCs in Leber’s hereditary optic neuropathy has been attributed to their resistance, unlike classical RGCs, to both mitochondrial dysfunction, and cell death.40 47 An animal study by Li et al.38 showed resistance of mRGCs to damage by ocular hypertensive stress, despite loss of classical RGCs. Another study by the same group proposed that expression of phospho-Akt in mRGCs promotes cell survival via the phosphatidylinositol-3 kinase/Akt signaling pathway after optic nerve transaction.48 Studies have also touched on the coexpression of the neuropeptide pituitary adenylate cyclase-activating polypeptide in mRGCs as well as terminating in the IPL.50 It is possible that this may protect the displaced mRGCs from optic neuropathy seen in patients with glaucoma. Such information and further investigation of the human retina with glaucoma elucidate the mechanisms used by mRGCs aiding their survival and promote the development of strategies to prevent their damage in the human retina.

Human studies on mRGCs in the retina with glaucoma have been limited to functional studies probably due to the limitations in obtaining human retinal tissue. The present finding that mRGCs density decreases with progression of glaucoma to severe stages is in agreement with previous functional studies assessing mRGCs function in glaucoma patients. In conclusion, despite loss of IF functions in the retina with glaucoma, mRGCs are preserved to some degree and are vital for maintaining certain NIF functions.

Acknowledgments

The authors thank Anita Hansen for skillful technical assistance. Supported by the Danish Biotechnology Centre for Cellular Communication.

Disclosure: E.A. Obara, None; J. Hannibal, None; S. Heegaard, None; J. Fahrenkrug, None

References


