

Loss of Melanopsin-Expressing Retinal Ganglion Cells in Patients With Diabetic Retinopathy

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PURPOSE. Photo-entrainment of the circadian clock is mediated by melanopsin-expressing retinal ganglion cells (mRGCs) located in the retina. Patients suffering from diabetic retinopathy (DR) show impairment of light regulated circadian activity such as sleep disorders, altered blood pressure, and abnormal melatonin secretion. The aim of this study was to assess the impact of DR on the expression of mRGCs in the human retina.

METHODS. The expression of mRGCs and RGCs was determined in eye sections containing retinal tissue from patients with DR ($n = 6$) and respective age-matched controls ($n = 8$) using immunohistochemistry by costaining with antibodies against RNA binding protein with multiple splicing (RBPMS), which identified RGCs and melanopsin which identified the mRGCs.

RESULTS. The expression of RGCs in the retina from patients with severe DR was significantly reduced to a density of 146 ± 76 cells/mm² compared with controls at 1280 ± 249 cells/mm². The density of mRGCs was also significantly reduced from 3.12 ± 0.54 cells/mm² in controls to 0.72 ± 0.18 cells/mm² in patients with DR, with significant loss of 73.5% and 81.9% in mRGC density in the ganglion cell layer (GCL) and inner nuclear layer (INL), respectively.

CONCLUSIONS. Our findings show that DR affects the expression of mRGCs in the human retina and could explain the abnormal circadian activity observed in patients with DR.

Keywords: diabetic retinopathy, melanopsin, neurodegeneration

Diabetic retinopathy (DR) is the most common complication of diabetes.¹ Ophthalmoscopically, it presents with microaneurisms, hard exudates, neovascularization, retinal or macular oedema, vitreous hemorrhage, and retinal detachments causing eventual legal blindness in adults on working age.^{2,3} The notion that DR is asymptomatic until severe damage has occurred, has long been overturned by the finding that neurodegeneration of retinal ganglion cells (RGCs) occurs early in DR pathophysiology.^{4–6} Recent studies have also shown abnormal regulation of melatonin secretion and diurnal blood pressure, poor sleep quality, and daytime sleepiness in patients with DR, suggesting disruption of the circadian rhythm.^{7–9}

The circadian rhythm is driven by the brain's biological clock located in the suprachiasmatic nucleus (SCN).¹⁰ These rhythms are adjusted by light (photo-entrained) daily, through input from a small population of intrinsically photosensitive RGCs expressing the photopigment melanopsin (mRGCs).¹¹ The mRGCs expressed in the human retina, comprise less than 1% of RGCs, they project primarily to the SCN and olivary pretectal nucleus (OPN) aiding the nonimage forming (NIF) functions (photo-entrainment, pupillary light reflex [PLR], and sleep/wake cycles).^{12–14}

Previous studies have shown that mRGCs are more robust and resistant to damage incurred due to optic nerve damage, glaucoma, inherited optic neuropathies, and glutamate-induced toxicity compared with non-melanopsin expressing RGCs.^{13,15,16} In patients with DR, recent studies have shown

impairment of mRGCs function, proposing the testing of mRGCs function as a diagnostic tool for early detection of DR.¹⁷ However, currently there is no understanding of the histologic changes of mRGCs in patients with DR. Considering the prominent neurodegeneration of RGCs, we aimed to determine if mRGCs expression is affected in DR progression in the human retina.

MATERIALS AND METHODS

Patient Samples

Paraffin-embedded, human eyeball sections were obtained from the Department of Ophthalmology at Rigshospitalet (Copenhagen, Denmark) of patients with DR and respective age-matched controls in accordance with the Declaration of Helsinki for research involving human tissue. The DR group consisted of six patients (mean \pm SD, 60 ± 22 ; range, 29–84; 5 males, 1 female) where enucleation was carried out due to retinal damage due to DR. Evaluation by an ophthalmologist, using the International Clinical Diabetic Retinopathy Severity Scale outlined by the American Academy of Ophthalmology revealed that all patients in the DR group were classified to be in advanced stages of DR.¹⁸ Four patients were diagnosed with proliferative diabetic retinopathy due to the presence of advanced neovascularization in the retina and the other two patients showed signs of macular edema as well as advanced DR.¹⁸ Patients with visible retinal



detachment were not considered in this study (a patient showed secondary hemorrhagic glaucoma as a result of DR progression). The control group consisted of eight patients (mean \pm SD, 60 \pm 14; range, 33–75; 3 males, 5 females) whose eye had been enucleated due to extraocular (orbital) cancer treatment. These eyes were normal with normal retinas. Six of the patients in the control group were used in a previous study.¹⁹

Immunohistochemistry

Immunohistochemistry was performed from patients with DR as described previously.¹⁹ Briefly, each of every fifth of 5- μ m thick tissue sections from a series of 30 horizontal retinal sections of the entire retina containing the central and peripheral retina, ranging from the nasal retina to the temporal retina with a visible the optic nerve head was stained after sections were deparaffinated, rehydrated, and processed for antigen retrieval. Immunohistochemistry was performed using a previously described protocol.¹⁹ The antibodies used in this study was an in-house primary antibody against melanopsin (code no:5J68; RRID: AB_2629473), characterized previously.¹³ This antibody recognizes the C-terminal part of human, but not rodent melanopsin and shows identical staining as an anti-melanopsin antibody directed against the N-terminal part of melanopsin.^{13,20} A primary antibody against RNA binding protein with multiple splicing (RBPMS; code no:1832, RRID: AB_2492225; PhosphoSolutions, Aurora, CO, USA), characterized by Rodriguez et al.,²¹ was used to identify RGCs in the retina. Control experiments were performed by preabsorption of primary antibodies with their respective antigen or by eliminating the primary antibody, which abolished all staining.

Cell Counting and Imaging Analysis

In accordance with previous studies, we counted mRGCs (melanopsin and RBPMS expressing cells) and RGCs (RBPMS expressing cells) expressed in either the GCL and INL using widefield microscopy to estimate the density of these cells in the human retina.^{19,22,23} As every fifth section of 5- μ m thickness was analyzed (section separation), each section was assumed to represent a 25- μ m diameter, and any cells within this diameter was likely to be observed, reducing the possibility of double counting and over estimation.²² The derived cell count for each patient was divided by the derived retinal length multiplied by the determined constant thickness of 0.125 mm (5 \times 25 μ m) to calculate the average density of each cell type in the entire retina.

Cell density

$$= \frac{\text{total cell count}}{(\text{retinal length (mm)} \times \text{retinal thickness (0.125 mm)})}. \quad (1)$$

Double staining of slides from patients with DR and age-matched controls allowed for comparative analysis of both RGCs and mRGCs densities.

Images of both mRGCs and RGCs were obtained using an IMIC confocal microscope system equipped with filter settings for 4',6-diamidino-2-phenylindole (DAPI), Alexa488, and Alexa594 (IMIC; FEI, Munich, Germany) and acquired using LA software (Live-Acquisition v2.2.0; FEI, Munich, Germany).¹⁹ The images were then stitched together and saved as TIFF files using Fiji software (Fiji Image-J v1.49; Madison, WI, USA).

Statistics

For statistical analysis, differences in group means were assessed by unpaired, two-tailed Mann-Whitney *U* test as the data was not assumed to be normally distributed. Results are

presented as mean \pm SEM. All statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

RESULTS

Immunohistochemistry was used to assess the expression of mRGCs and RGCs in the retina of patients with DR and controls. For each patient the retinal length was derived in millimeters as the length of the peripheral and central retina (Fig. 1A). In all sections with RGCs present, the RBPMS-positive RGCs presented with positive cytosolic RBPMS staining of the cell (Figs. 1B, 2A–E), while the mRGCs presented with significant melanopsin staining in the membrane of the cell soma and in the dendrites and coimmunostaining for RBPMS (Figs. 1B, 2D). The control retinas showed an organized retina with RGCs predominantly present in the GCL with fewer cells located in the INL (Fig. 1B). Melanopsin-expressing RGCs were present in both the GCL and INL (Fig. 1B) with melanopsin-positive dendritic fibers in two layers corresponding to S1 and S5 of the IPL (Fig. 1B, indicated double arrows and by arrows, respectively). In comparison, assessment of the retinal sections from patients with DR showed areas with glial proliferation causing disorganization of cells in the different layers of the retina, with corresponding areas of more preserved organized retinal structure, more similar to that observed in controls (Fig. 2). In all retinas with DR, a marked reduction of RGCs expressing RBPMS and mRGCs was observed, and accordingly, much fewer melanopsin containing processes were observed, primarily located in what seem to be the a disorganized S1 layer (Figs. 2A–E).

Cell counting revealed that, although RBPMS-positive RGCs were observed in retinal sections from both the control group and the DR group, a significant reduction of 88.6% in the average density of RBPMS-positive RGCs was observed (1280 \pm 249 cells/mm² in controls compared with 146 \pm 76 cells/mm² in group with DR; *P* = 0.003; Fig. 3A). A significant loss of 76.9% in average density of mRGCs was also observed in the retina of patients with DR compared with the controls (3.12 \pm 0.54 cells/mm² for controls and 0.72 \pm 0.18 cells/mm² for patients with DR; *P* = 0.005; Fig. 3B). The relative expression of both RGCs and mRGCs in the retina from patients with DR estimated by the mRGCs/RGCs percentage was 0.49% compared with controls of 0.24%.

The distribution of mRGCs in the controls, were found to be 60% of mRGCs in the GCL and 40% in the INL, while in the retina of patients with DR, 68% of mRGCs were located in the GCL and 32% in the INL. Analysis of the average mRGCs density in the GCL and INL showed a significant loss of 73.5% in the GCL (1.86 \pm 0.27 cells/mm² for controls to 0.50 \pm 0.09 cells/mm² for the diabetic group; *P* = 0.003) and 81.9% in the INL compared with controls (1.26 \pm 0.31 cells/mm² to 0.23 \pm 0.14 cells/mm²; *P* = 0.005; Figs. 3C, 3D).

DISCUSSION

During the last two decades, the importance of mRGCs in maintaining NIF functions has become evident.¹¹ In the present study, we show, for the first time, extensive loss of mRGCs in patients with severe DR. Our findings suggest that not only symptoms of visual impairment but also the NIF functions regulated by the retina are affected causing circadian misalignment, pupillary defects, and sleep disorder. In accordance, studies carried out in humans have shown impaired melatonin secretion, abnormal post-illumination pupil response (PIPR), and sleep-wake disorders.^{7,8,17} An

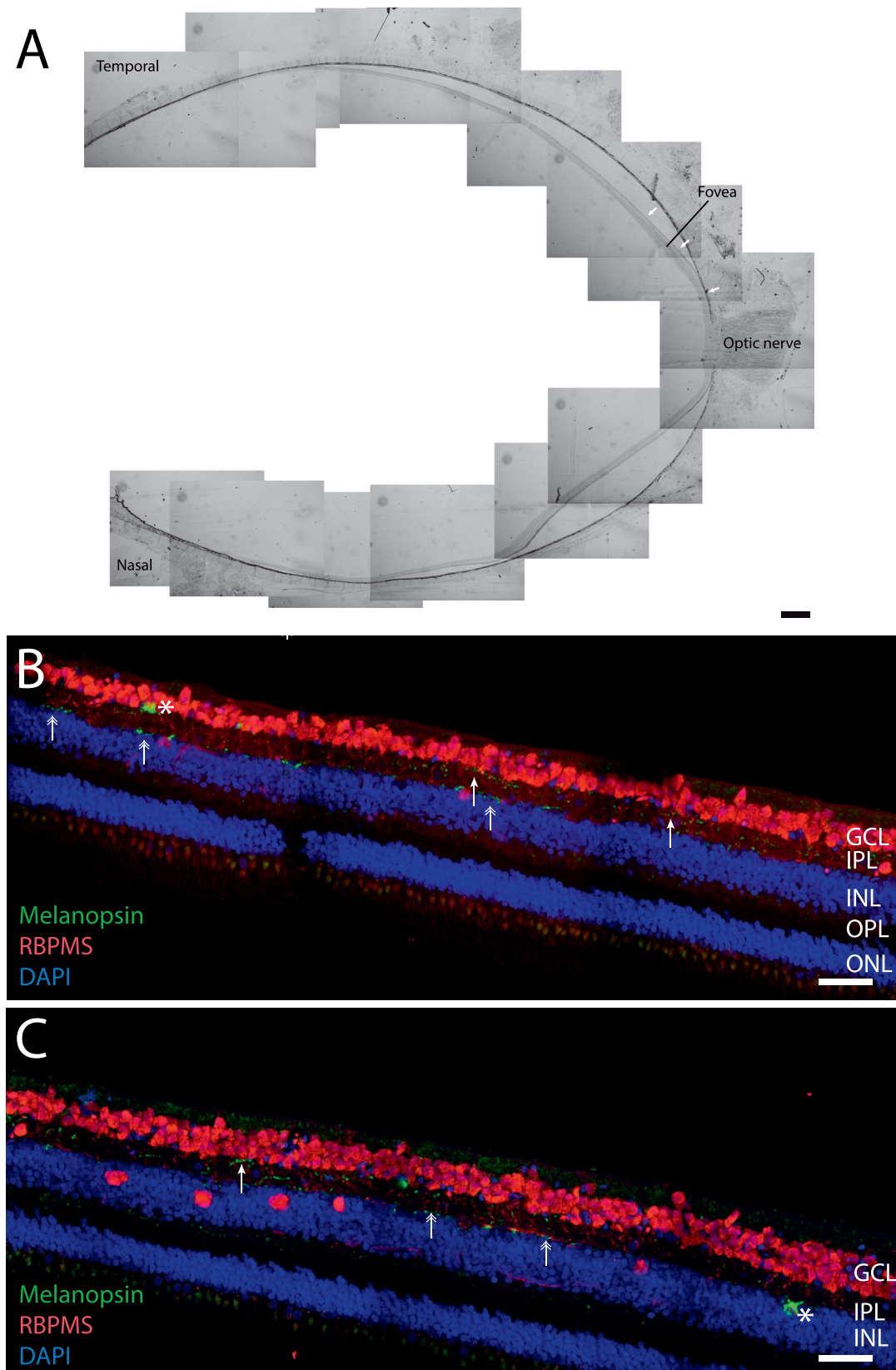


FIGURE 1. (A) Whitefield microscopic image of paraffin-embedded control human eye tissue section. Example of horizontal section containing the entire retina with indication of the temporal, foveal, and nasal part used for cell counting (see Methods section). (B) Confocal image showing all RGCs (*red*) using the RGC marker RBPMS (*red*), and melanopsin-immunoreactive RGCs (*green*) and nuclear DAPI (*blue*) in the central retina of a control. RNA binding protein with multiple splicing-positive RGCs are found primarily in the GCL, and also in displaced RGCs located in the INL. Melanopsin-expressing RGCs (*asterisk*) are found costored with RBPMS in the GCL (B) and displaced in the INL (C). Melanopsin-immunoreactive fibers are observed in the S1 (*double arrow*) and in the S5 (*arrow*) layers of the IPL. Scale bars: (A) 2 mm, (B, C) 50 μ m.

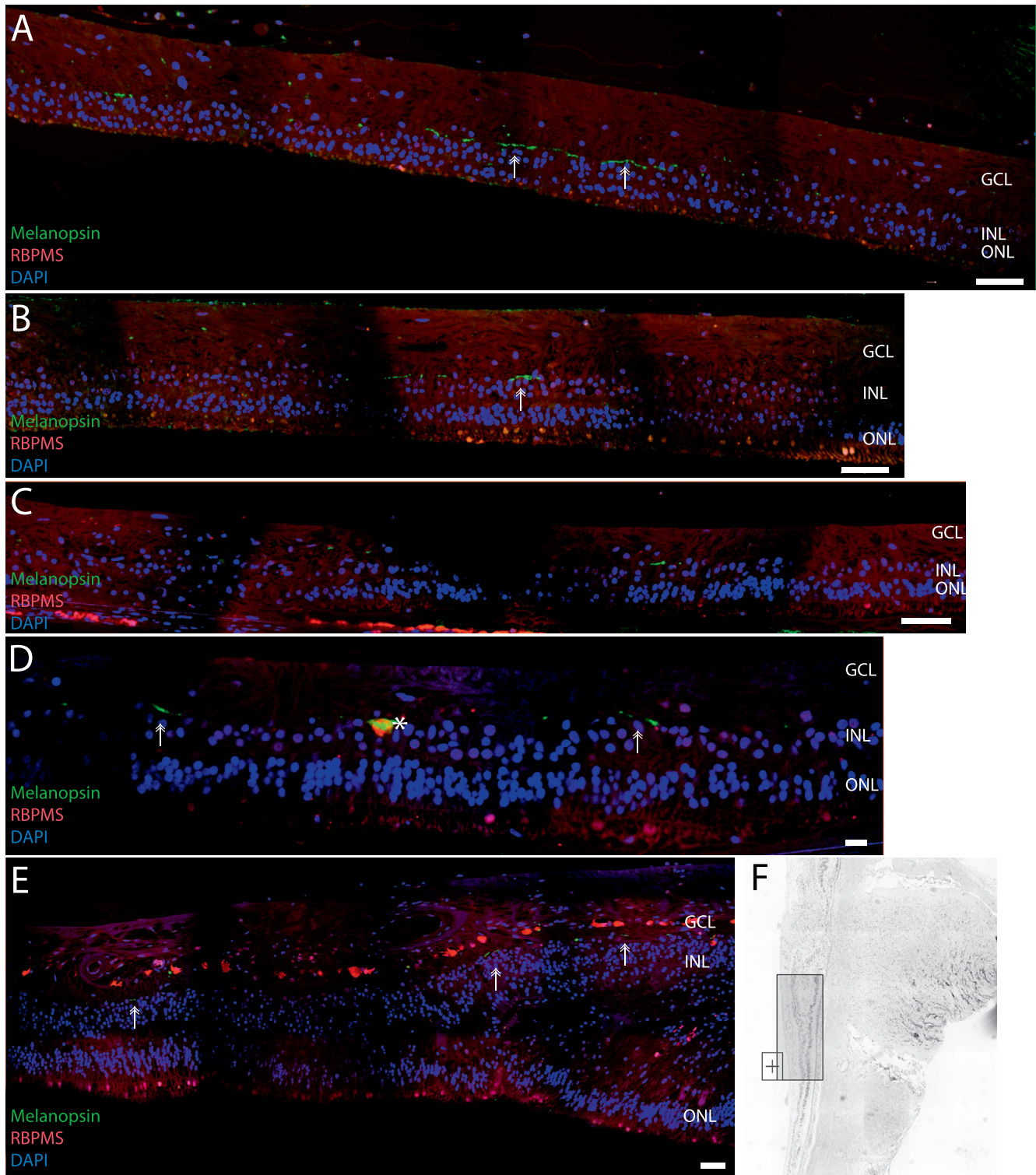


FIGURE 2. Retinal ganglion cells immunostaining for RBPMS (red) and melanopsin (green) and nuclei staining (DAPI) for structural anatomy in peripheral (A–D) and central retina (E) of a patient with DR. (F) Shows a wide-field overview of the DR retina with the frame indicating the images in (E). Arrows in (A–E) indicate melanopsin-immunoreactive dendrites located in what seem to be the S1 of the IPL. Note the mRGCs (asterisk) costoring RBPMS in the INL in panel D and note the remaining RBPMS containing RGCs in the central retina in panel E. Scale bars: (A–C) 50 μ m, (D–E) 25 μ m.

animal study conducted by Su et al.²⁴ also showed impairment of the circadian rhythm prior to development of DR in mice similar to the findings of Feigl et al.,¹⁷ in humans proposing that mRGCs loss might occur prior to neurodegeneration of conventional RGCs.

In contrast to our findings, some studies using experimental models of DR in mice have shown preservation of mRGCs. Fernandez et al.²⁵ showed preservation of mRGCs even in late stages of DR, despite significant loss of RGCs using a streptozotocin (STZ)-induced diabetic rat model. Using a

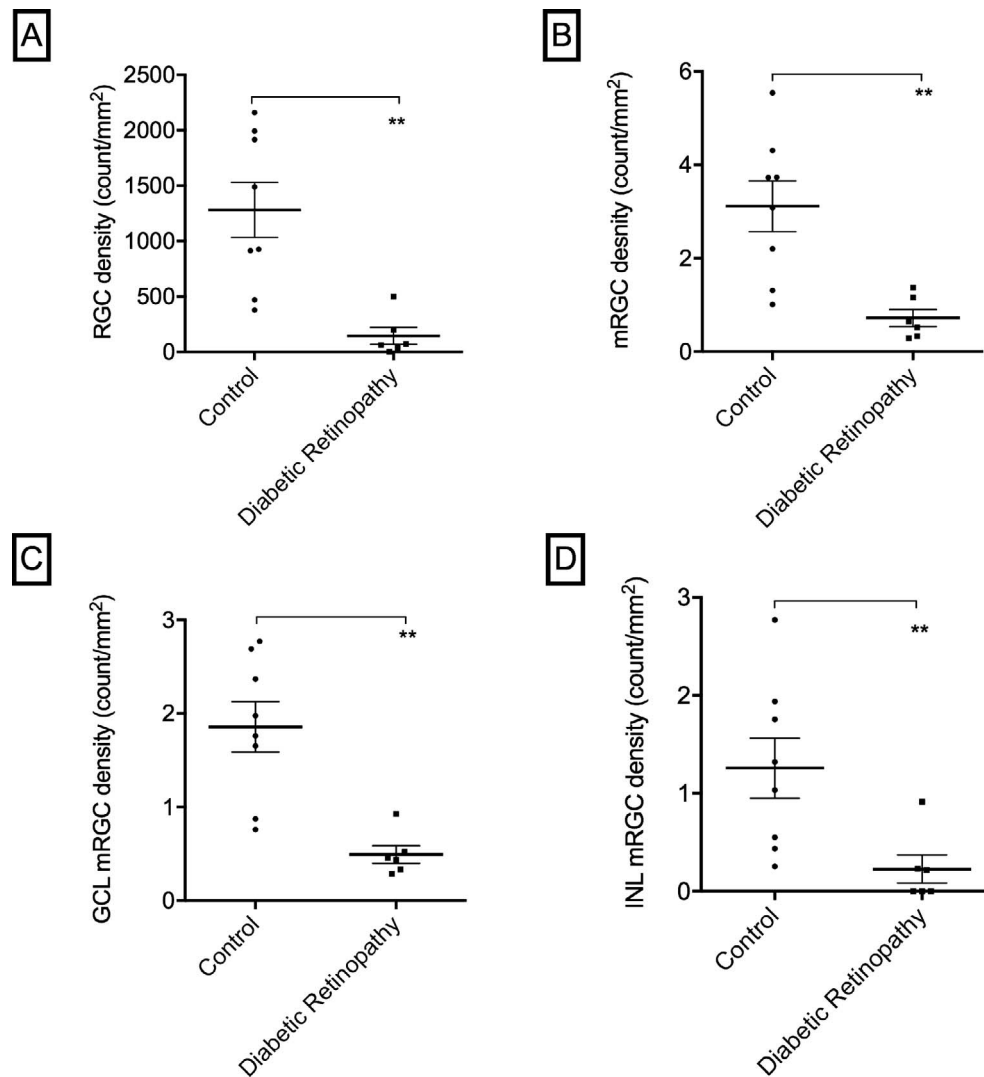


FIGURE 3. Retinal density of RGCs and melanopsin cells for controls compared with patients with DR expressed as cell counts per millimeter squared. (A) Significant loss of average total RGCs in the retina from patients with DR compared with controls ($P = 0.0027$). (B) Quantification of average mRGC density showing a significant loss of mRGCs in the retina from patients with DR ($P = 0.0047$). (C, D) Analysis of the distribution of mRGCs between the GCL and INL ($P = 0.0027$) and INL ($P = 0.0047$) shows a significant loss of mRGCs in both layers in the retina from patients with DR compared with the controls.

similar model, Kumar et al.²⁶ showed increase in expression of melanopsin in mice as well as an increase in mRGCs controlled PLR function. Studies using both *Ins2^{Akita}* and STZ-induced mice model have shown preservation of morphologically abnormal mRGCs with abnormal function.^{27,28} The distinction in results between these animal studies and our study could be due to differences in the underlying mechanisms activating DR.²⁹

In a previous study, we conducted in patients with varying stages of glaucoma, we observed a significant loss of mRGCs in the retina of patients with glaucoma, who often develop circadian disruption.¹⁹ In the patients with glaucoma, we also showed a discrepancy in the reduction of mRGCs density between the GCL and INL with significant loss primarily in the GCL and sparing in the INL.¹⁹ In the present study, patients with DR showed significant loss of mRGCs both in the GCL and INL. The resulting percentage of mRGCs in relation to RGCs in the retina of patients with DR is in range with the estimates shown for a normal retina at less than 1%, while the patients with glaucoma had a ratio of 14%.^{13,19} Both glaucoma and DR caused marked neurodegeneration of both RGCs and mRGCs,

though differences in pathophysiology between glaucoma and DR could have a bearing on the impact to mRGCs, as we have observed that mRGCs are not lost early in glaucoma progression.¹⁹ More studies are required to identify the underlying factors causing neurodegeneration of mRGCs in the retina of patients with DR, some studies propose the involvement of increased inflammation, glutamate excitotoxicity, and oxidative stress.⁴

The growing incidence of diabetes mandates novel strategies for diagnosing the onset of DR.^{30,31} The asymptomatic progression of DR until vision is compromised poses a major problem for diagnosis and treatment³²; the results of this study supports testing of mRGCs function as a novel diagnostic biomarker perhaps through measurement of the PIPR or light-regulated melatonin suppression in clinical evaluation of DR.^{7,11,17}

In conclusion, for the first time, we demonstrate that mRGCs are lost in severe stages of DR compared with controls. Further studies are required to elucidate if possible, the progressive loss of mRGCs in DR pathophysiology.

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