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Effects of light on the development of melanoposin containing retinal ganglion cells in rats

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Abstract. – OBJECTIVE: The present study was planned to evaluate the effect of light on the development of light-sensitive retinal ganglion cells.

MATERIALS AND METHODS: The Sprague-Dawley (SD) rats were segregated into 3 groups (n=18) which included routine feeding 10-day group 1, routine feeding 14-day group 2 and light-deprivation feeding 14-day group 3. The group 1 animals were routinely fed for 10 days in normal light conditions and were sacrificed for analyses on day 10. Similarly, group 2 animals were routinely fed for 14 days in normal light conditions and were sacrificed for analyses on day 14. The group 3 animals were kept were routinely fed for 7 days which was followed by their feeding in a light-deprived conditions and were sacrificed on day 14.

RESULTS: The expression of the opsin gene determined by real-time PCR in retinal tissues showed a significant decline in the light-deprived group 3 when compared to other two groups. Furthermore, the melanopsin protein also showed a significant decline in its protein expression in light-deprived group 3 as observed by immune-blot analyses. The immuno-fluorescence analyses also showed the similar trend confirming the effect of light on the development of retinal ganglion cells.

CONCLUSIONS: Light is essential for the proper development process of retinal ganglion cells as light directly affects regulatory opsin gene expression.

Key Words:
Melanopsin, Ganglion cells, Light, Sensitivity.

Introduction

The melatonin synthesis is under the continuous regulatory control of light. It is important as it affects normal physiological rhythms including sleep and behaviour[1]. Photoreception by virtue of eyes is crucial in mammals as it is also associated with other mental tasks like concentration, memory, mood, etc.[2]. The latest research advancements have revealed the importance of retinal ganglion cells (RGCs) that express the photo pigment melanopsin are themselves atypical photo-receptors[3,6]. The physiological centre associated with RGCs is the hypothalamic suprachiasmatic nuclei (SCN) comprised of the primary circadian pacemaker in mammals[3]. The retinohypothalamic tract (RHT) arises from a subset of retinal ganglion cells and conveys photic information to the SCN through the optic nerve[7].

The photosensitive retinal ganglion cells project to numerous brain regions, in addition to the SCN. In this way, they regulate non-image-forming functions, such as the suprachiasmatic nucleus (SCN) to photo entrain circadian rhythms and the olivary pretectal nucleus (OPN) to control the pupillary light reflex (PLR). Earlier studies on mice reported that loss of either the classical photoreceptors (rods and cones) or melanopsin photo pigment exhibited relatively normal circadian rhythms in response to ocular illumination[8-12]. However, the loss of both the classical photoreceptors and melanopsin resulted in the termination of all responses to light[13,14]. So, it is quite evident from the earlier studies the importance of RGCs. However, there is a paucity of information about the direct affects of light on the development of light-sensitive retinal ganglion cells. The present study is planned to check the effect of light on the development of this melatonin-containing retinal ganglion cells in rats.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats were procured from the Central Animal House, China. The animals were housed in polypropylene cages under hygienic conditions in the Departmental Animal House. The study was approved by Institutional Animal Ethics Committee.

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Experimental Design

The SD rats were segregated into 3 groups (n=18) which included routine feeding 10-day group1, routine feeding 14-day group 2 and light-deprivation feeding 14-day group 3. The group 1 animals were routinely fed for 10 days in normal light conditions and were sacrificed for analyses on day 10. Similarly, group 2 animals were routinely fed for 14 days in normal light conditions and were sacrificed for analyses on day 14. The group 3 animals were kept were routinely fed for 7 days which was followed by their feeding in a light-deprived condition and were sacrificed on day 14. Condition for light-deprivation: brightness of the feeding room ≤ 0.0004 cd/m^2

Retinal Tissue Sampling

Rats were injected intraperitoneally with 2% chloral hydrate (0.02 ml/g) for anesthesia which was followed by the sacrifice of the rats. Extracted their eyeballs, removed the crystalline lens and then took out the posterior eye cup. Then eye cups were cut into pieces and were placed in the Eppendorf tube for the next processing.

RNA Extraction

Embrittled the samples by liquid nitrogen which was followed by addition of 200 ml Trizol. Centrifuged for 3 min and again added 300 ml Trizol. Centrifuge again for another 2 min, before addition of 500 ml Trizol. Added 0.2 ml chloroform into every 1 ml Trizol reagent and vibrated the tube at room temperature for 5 min. Centrifuged under 4°C at the rate of 12000 × g for 5 min. After centrifuge, the mixed solutions were divided into two layers. The bottom layer was red phenol-chloroform phase; the middle and upper layer were a colorless aqueous phase. All RNA was distributed in the aqueous phase. 500 ml of the aqueous phase was, then, transferred into the new EP tubes. The same volume of isopropanol was then added to the aqueous phase in order to precipitate RNA. Blended and, then, incubated under 30°C for 10 minutes. The above tubes were then centrifuged at 4°C at the speed of 12000×g for 10 minutes. Purity and concentration determination was performed by using nucleic acid protein determinator.

Detection of Retina Melanopsin Protein

The protein retina melanopsin was detected by using polyacrylamide gel electrophoresis which was followed by western transfer. Exported the data in Tagged Image File Format (TIFF) and, then, analyzed the optical density of each band by Image J software.

Table I. Effect of light on the expression of gene opsin.

Groups	Mean Ct value
Group 1	24.90 ± 0.41
Group 2	24.45 ± 0.33
Group 3	23.54 ± 0.24[a]

Data are expressed in Mean ± SD (n=18); ap <0.05 by Least Significance Difference test when values are compared with group 1.Group 2

Retinal Melanopsin Protein Immunofluorescence Studies

Removed cornea, iris, and crystalline lens under the microscope after the sacrifice and reserved the eyecup. Dehydrated the eyecup by absolute ethyl alcohol-I and absolute ethyl alcohol-II for 30 min. Transparentized and embedded the eyeball under sagittal view at 12 o'clock position. Baked the 3 mm slices at 65°C for 6-12 hours. Packed into the box and preserved under normal temperature. Xylene dewaxing, gradient alcohol rehydration was performed. Added primary antibody by dripping followed by incubation at 4°C for a night. Added secondary antibody by dripping and incubation at 37°C for 60 min. Stained 4',6-diamidino-2-phenylindole (DAPI) nuclear under room temperature for 10 min. Sealed the sheet by glycerinum phosphate buffered saline (PBS), and observed under positive fluorescence microscope.

Statistical Analysis

The statistical significance of the data has been determined using one-way analysis of variance [ANOVA] followed a multiple post-hoc least significant difference test. The results are represented as Means ± SD. $p < 0.05$ was considered statistically significant.

Results

We observed the effect of light on the light sensitive opsin gene and observed light deprivation resulted in a significant decrease the expression of opsin gene in the light-deprived group 3 (Table I). However, the opsin gene did not show

Table II. Effect of light on the melanopsin protein expression.

Groups	Mean Ct value
Group 1	18245.92 ± 0.44
Group 2	17659.38 ± 0.39
Group 3	8377.78 ± 0.78[a]

Data are expressed in Mean ± SD (n=18); [a]p <0.05 by Least Significance Difference test when values are compared with group 1.

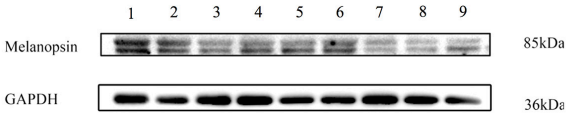


Figure 1. Protein expression of melanopsin.

any significant difference in rats when compared group 1 with group 2. Further, Western transfer results (Table II and Figure 1) also showed a significant decline of protein melanopsin in light-deprived group 3 which further confirmed the role of light in the production of melanopsin protein. Moreover, immune-fluorescence results followed the similar trend and are in line with above results. Group 1 animals (Figure 2 A,B), showed visually the expression of melanopsin protein with positive green coloration. Group 2 animals also showed the similar trend (Figure 2 C,D). However, light deprived group 3 animals showed a very light green coloration (Figure 2 E,F) indicative of a significant decline in the melanopsin protein concentration. Figure 2 G is the negative control.

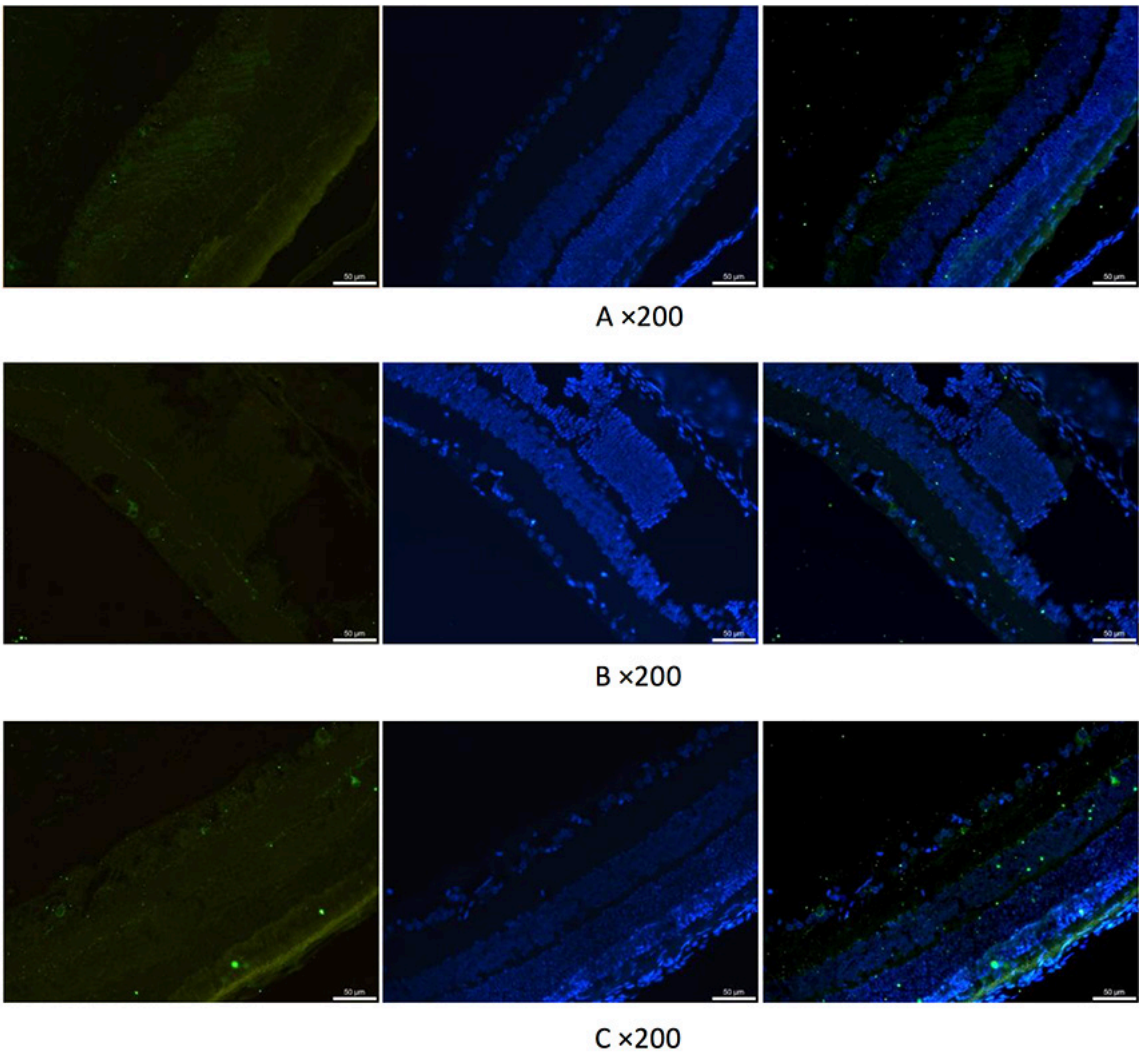
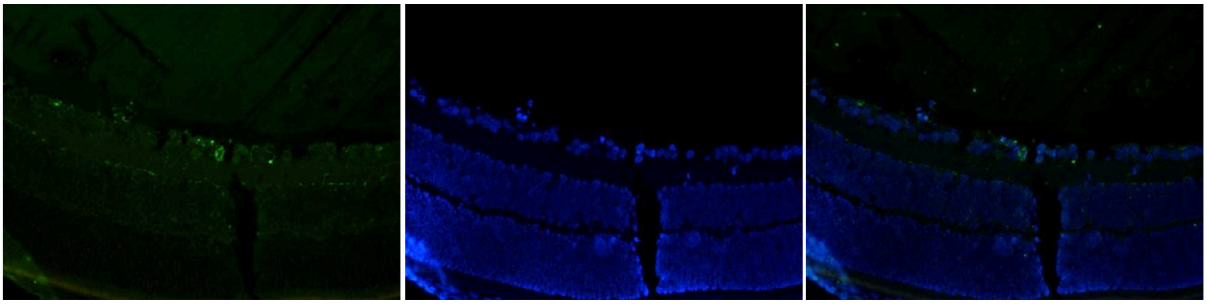


Figure 2. Immuno-fluorescence results for protein melanopsin (green coloration indicative of melanopsin protein).

Figure continued



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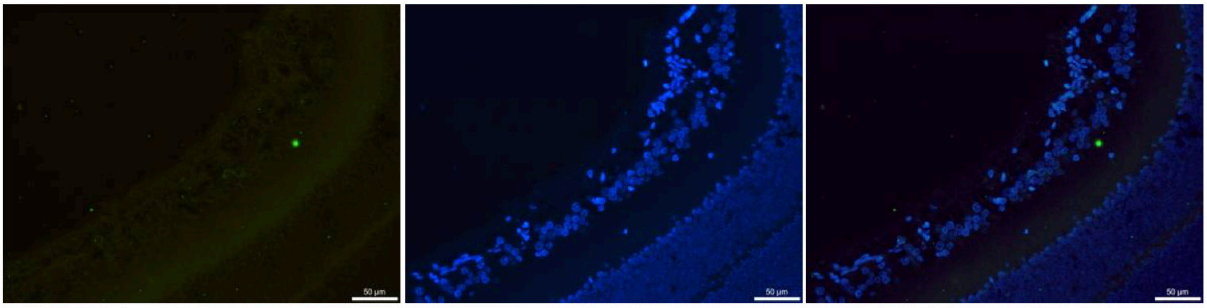

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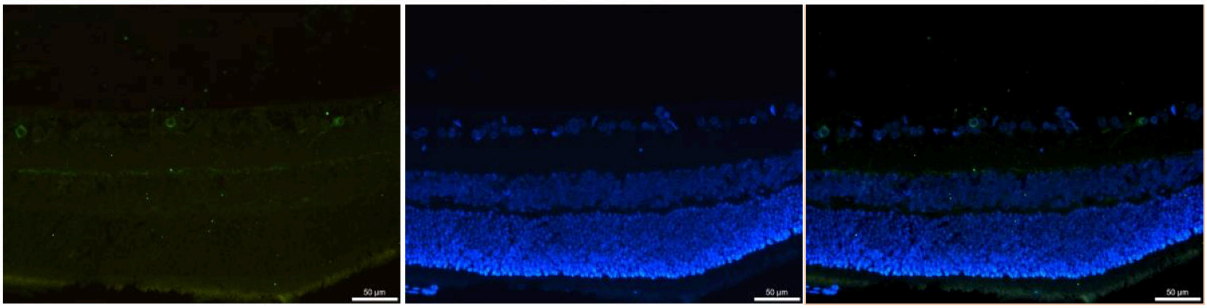
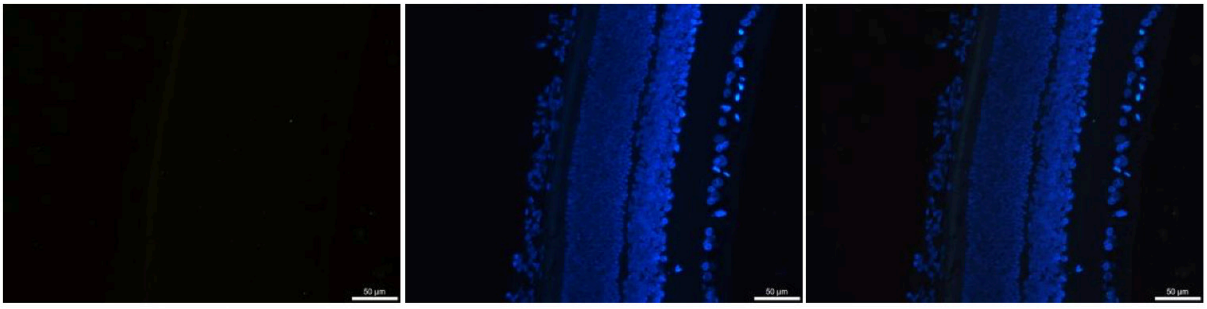


F ×200



G Negative control ×200

Figure 2. *(Continued).* Immuno-fluorescence results for protein melanopsin (green coloration indicative of melanopsin protein).

Discussion

The present study evaluated the effects of light deprivation on the melanopsin containing light-sensitive ganglion cells in newly born SD rats. The results clearly proved the importance of light for the proper development of photosensitive retinal ganglion cells. The photosensitivity in these cells is constituted by melanopsin protein[15]. Further, the melanopsin protein is the byproduct of gene opsin which regulates its physiological concentrations *in vivo*[16]. In the present study, we directly observed the effect of light on the gene opsin. Light deprivation resulted in the decline of its expression confirming its dependence on light for proper expression. The justification for the above observation could be the fact that the absorption of a photon of light results in the photoisomerization of the chromophore of opsin from the 11-*cis* to an all-*trans* conformation. The photoisomerization induces a conformational change in the opsin protein, causing the activation of the phototransduction cascade[17]. However, the opsin remains insensitive to light in the *trans* form. Furthermore, continuous supply of light is essential in order to replace light insensitive all-*trans* retinal by a newly synthesized 11-*cis*-retinal provided from the retinal epithelial cells. So, in the light deprivation conditions in the present work, the above process of regeneration might be affected which in turn resulted in the decline in the genetic expression of opsin.

Melanopsin protein is present intrinsically in photosensitive retinal ganglion cells (iPRGCs) in mammalian vertebrates and is encoded by gene opsin[18]. Melanopsin-containing ganglion cells, like rods and cones, exhibit both light and dark adaptation; they adjust their sensitivity according to the recent history of light exposure[19]. Evidence for melanopsin's physiological light detection has been tested in mice. A mouse cell line that is not normally photosensitive is rendered light-sensitive by the addition of human melanopsin[20]. In the present study, this protein also showed a significant decrease upon light derivation. This could be linked to the previous observation of decreased opsin gene expression. The decline in the expression of opsin gene might have contributed towards observed decrease in its protein expression in the form of melanopsin. Moreover, the above observation has been also confirmed biophysically by immune-fluorescence investigation.

The melanopsin gene gives green coloration upon staining with fluorescent tags in positive fluoresce microscopy analyses. The immuno-fluorescence results also correlated well with expression studies showing a decline in fluorescence in the light-deprived group animals. The biophysical confirmation of the decline in the concentration of melanopsin made it certain that light is essential for the proper development of ganglion cells as it directly stimulates the expression of regulator gene opsin.

Conclusions

The present study is first of its kind to relate the observation of light regulation of opsin gene by both molecular as well as biophysical assays. It is clear from above study that light is essential for the proper development of light-sensitive retinal ganglion cells. However, further studies are required in great detail to find exact molecular mechanism involved in this light-driven molecular process of physiological optics.

Acknowledgement

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Conflicts of interest

The authors declare no conflicts of interest.

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