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Controlling the number of melanopsin-containing retinal ganglion cells by early light exposure

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ABSTRACT

A small percentage of retinal ganglion cells (RGCs) express melanopsin and are intrinsically photosensitive (ipRGCs). Whether light can affect the development of ipRGCs is not clear. In the rat retina, we found constant light exposure during the first postnatal week significantly increased the number of melanopsin immunopositive ipRGCs. This increase was durable and specific for melanopsin immunopositive ipRGCs. BrdU labeling showed no proliferation of the melanopsin immunopositive ipRGCs during constant light exposure. Retrograde labeling from the superior colliculus showed that no other types of RGCs were induced to express melanopsin. Light exposure was effective in increasing melanopsin immunopositive ipRGCs only when it coincided with the apoptotic phase of RGC development. However, daily intravitreous injection of tetrodotoxin, blocking action potentials, abolished the light induced increase of melanopsin immunopositive ipRGCs. These findings indicate that early light exposure can increase the number of melanopsin immunopositive ipRGCs through a process dependent on intrinsic photosensitive spiking activity. Furthermore, the increase of melanopsin immunopositive ipRGCs is potentially induced by apoptosis suppression in ipRGCs or enhanced expression of melanopsin.

1. Introduction

The discovery of a subtype of retinal ganglion cells (RGCs) utilizing a novel opsin, melanopsin, as a photopigment to generate intrinsic light responses has allowed for rapid progress in the past decade toward understanding the non-image forming visual system (Provencio et al., 1998; Provencio et al., 2000; Berson et al., 2002; Hannibal et al., 2002; Hattar et al., 2002; Panda et al., 2002; Provencio et al., 2002; Hattar et al., 2003; Chen et al., 2011). It has been reported that constant light exposure suppresses melanopsin expression in the adult retina (Hannibal, 2006; Hannibal et al., 2007). Melanopsin-containing RGCs exhibit light responses at birth and therefore are the earliest light responsive neurons in the retina (Sekaran et al., 2005; Tu et al., 2005). Similar to many other types of RGCs, ipRGCs are overproduced and the population decreases in early development. We postulated that light affects the development of ipRGCs and investigated the effects of constant light exposure on developing and mature ipRGCs.

2. Methods

2.1. Animals and retina preparation

Albino Sprague Dawley rats obtained from the animal care facility of the Institute of Biophysics, Chinese Academy of Sciences, were used in this study. Use and handling of animals were strictly in accordance with the institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. During constant light exposure (LL) treatments, rats were exposed to a continual luminance of 400 lux. Control rats were maintained on a 12 h light/dark cycle (LD) with the same (400 lux) luminance during the day.

Animals were deeply anaesthetized with an i.p. injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and perfused with warm saline transcardially. Eyes were enucleated, marked at the nasal pole, and fixed for 10 min in 4% paraformaldehyde in 0.01 M

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PBS. Then retinas were isolated from the pigment epithelium while maintaining the reference at the nasal pole and were post-fixed for 1 h at room temperature.

2.2. Immunohistochemistry

After rinsing with 0.01 M PBS, retinas were incubated with primary antibodies in medium containing 0.01 M PBS with 1% BSA and 0.5% Triton-X 100 for 2 days at 4 °C. Then retinas were rinsed with 0.01 M PBS and incubated with secondary antibodies in the same medium with 0.05% DAPI overnight at 4 °C. Retinas were rinsed and whole-mounted with Vectashield (Laboratories, H-1000, Vector, Burlingame, CA). Images were acquired on an Olympus FV500 confocal microscope with a 10×objective (UPlanApo, N.A. = 0.40).

For retinal sections, retinas were cryoprotected in 30% sucrose in 0.01 M PBS overnight and frozen in OCT. Tissue was sectioned to a thickness of 18 μm using a cryostat (Leica CM, 1950). The sections were incubated with primary antibodies overnight, rinsed three times and incubated with secondary antibodies and 0.05% DAPI for 2 h at room temperature. After rinsing, the sections were coverslipped with Vectashield. Images were acquired on an Olympus FV500 confocal microscope with a 60 × oil-immersion objective (PlanApo, N.A. = 1.40).

2.3. Antibodies

Primary antibodies used in this study were: rabbit anti-melanopsin (1:400, PA1-781, Affinity BioReagents, Golden, CO), rabbit anti-PKCα (1:10,000, P4334, Sigma–Aldrich, St Louis, MO), mouse anti-neurofilament (1:200, N1042, Sigma–Aldrich, St Louis, MO) and mouse anti-BrdU (1:200, B8434, Sigma–Aldrich, St Louis, MO).

Secondary antibodies were all obtained from Jackson ImmunoResearch Laboratories (West Grove, PA): donkey anti-rabbit FITC (1:200, 711-095-152), donkey anti-mouse TRITC (1:200, 711-025-151) and donkey anti-rabbit Cy5 (1:200, 711-175-152).

2.4. In situ hybridization

A 539 bp rat PACAP fragment was amplified by RT-PCR (307 bp to 845 bp, primers: 5′-GTAGCGGAGCAAGGTTGGCCC-3′ and 5′-ACGGTAGCTGGCAACTCATCGCT-3′) from rat retinal total RNA and cloned into pGM-T (VT202-01, Tiangen, Beijing, China). Digoxigenin-labeled riboprobes were prepared by *in vitro* transcription reactions (11277073910, Roche, Mannheim, Germany) with linearized vector DNA as the template using SP6 (for antisense probe) or T7 (for sense probe) polymerase (10810274001 and 10881767001, Roche, Mannheim, Germany) following the manufacturer's instructions.

In situ hybridization of the whole mount retina was performed as described by Z Bao et al.(Bao and Cepko, 1997). Briefly, after fixation overnight in 4% PFA in 0.01 M PBS at 4 °C, rat retinas were rinsed and treated with 10 μg/ml proteinase K (1092766, Roche, Mannheim, Germany) for 15 min at room temperature. After 1 h pre-hybridization at 70 °C, the retinas were hybridized with PACAP riboprobes (0.2 μg/ml) overnight at 70 °C. Then retinas were washed and incubated overnight at 4 °C with sheep anti-digoxigenin-AP (1:10,000, 11093274910, Roche, Mannheim, Germany). After rinsing, the retinas were incubated with NBT/BCIP reagents (11383213001 and 11383221001, Roche, Mannheim, Germany) for color detection following product instructions. Images were acquired by a Nikon E800 microscope using a 4×• (Plan Fluor, N.A. = 0.13) or 10×• (Plan Apo, N.A. = 0.45) objective.

2.5. BrdU labeling

Rats reared in LL condition were injected i.p. daily with 50 mg/g body weight BrdU (B5002, Sigma–Aldrich, St Louis, MO) from P0 to P7 under hypothermia. Rats were returned to normal condition from P8 and sacrificed at P20. The retinas were prepared and cryosectioned. Sections were rinsed with 0.01 M PBS, incubated in 4 N HCl for 15 min at room temperature, stained with mouse anti-BrdU antibody (1:200, B8434, Sigma–Aldrich, St Louis, MO) and counterstained with rabbit anti-melanopsin antibody and rabbit anti-PKCα antibody.

2.6. Intraocular injection

From P0 to P7, rats were anaesthetized by hypothermia, the eyelid was opened with a small incision, and 0.5 μl tetrodotoxin (0.4 mM, T8024, Sigma–Aldrich, St Louis, MO) in 3.5 mM citrate buffer (pH = 4.8) was injected into the vitreous body with a glass micropipette daily. Rats in the control group were injected with citrate buffer only.

2.7. TUNEL

Whole-mount retinas were fixed for 20 min in 4% paraformaldehyde in 0.01 M PBS at room temperature. After incubation in 0.01 M PBS containing 2% Triton X-100 at 37 °C for 1 h, the retinas were stained with TUNEL reaction mixture at 37 °C for 1 h following the manufacturer's instructions (Roche, *In Situ* Cell Death Detection Kit, 12156792001, Boehringer Mannheim, Mannheim). After TUNEL staining, whole-mount retinas were counterstained with antibodies against melanopsin.

2.8. Retrograde labeling

Animals were anesthetized and placed in a stereotaxic device (Narishige Scientific Instruments, Tokyo). A craniotomy was preformed above the injection sites (SC: −6 and −7 mm AP, ±1.4 mm ML, 3.5 and 4.5 mm DV). Then 0.1 μl 4% FluoroGold (80014, Biotium, Hayward, CA) was injected at each point using a Hamilton syringe, which was kept in place for 10 min after injection. The animals recovered for 5 days before being sacrificed for examination of RGCs.

2.9. Quantification of the retinal ganglion cells

The micrographs of whole mount retinas were acquired by a Nikon E800 microscope with a 4×• objective (Plan Fluor, N.A. = 0.13) and individual images were stitched together to form a whole-retina montage. Cell counts were made by at least two individuals blind to the experimental condition and the average of the two observers was used.

To account for area difference, whole-mount retina was divided into four quadrants; micrographs were taken along the midline of each quadrant from the optic disc to the periphery at an interval of 500 μm. The density (cells/mm^2) of RGCs in each photograph was calculated and assigned to that site.

2.10. Statistical analysis

RGC density (cells/mm^2) or number is presented as mean ± standard error of the mean (SE), unless stated otherwise. One-way ANOVA (Origin 7.5) or Independent-samples *T* test (SPSS 11.5) was used for the analysis of the results. Statistical significance was set at $p < 0.025$ based on the Bonferroni correction for multiple comparisons when the *t*-test was applied or $p < 0.05$ for ANOVA analysis.