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MELATONIN MODULATES M4-TYPE GANGLION-CELL PHOTORECEPTORS

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Abstract—In the retina, melatonin is secreted at night by rod/-cone photoreceptors and serves as a dark-adaptive signal. Melatonin receptors have been found in many retinal neurons including melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs), suggesting it could modulate the physiology of these inner retinal photoreceptors. Here, we investigated whether melatonin modulates the alpha-like M4-type ipRGCs, which are believed to mediate image-forming vision as well as non-image-forming photoresponses. Applying melatonin during daytime (when endogenous melatonin secretion is low) caused whole-cell-recorded M4 cells' rod/cone-driven depolarizing photoresponses to become broader and larger, whereas the associated elevation in spike rate was reduced. Melanopsin-based light responses were not affected significantly. Nighttime application of the melatonin receptor antagonist luzindole also altered M4 cells' rod/cone-driven light responses but in the opposite ways: the duration and amplitude of the graded depolarization were reduced, whereas the accompanying spiking increase was enhanced. These luzindole-induced changes confirmed that M4 cells are modulated by endogenous melatonin. Melatonin could induce the above effects by acting directly on M4 cells because immunohistochemistry detected MT_1 receptors in these cells, although it could also act presynaptically. Interestingly, the daytime and nighttime recordings showed significant differences in resting membrane potential, spontaneous spike rate and rod/cone-driven light responses, suggesting that M4 cells are under circadian control. This is the first report of a circadian variation in ipRGCs' resting properties and synaptic input, and of melatoninergic modulation of ipRGCs.

Key words: melatonin, retina, retinal ganglion cell, circadian rhythm, melanopsin, light response.

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Abbreviations: D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ipRGC, intrinsically photosensitive retinal ganglion cell; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; MT_1, melatonin receptor type 1; MT_2, melatonin receptor type 2; PBS, phosphate-buffered saline; PKA, protein kinase A; SCN, suprachiasmatic nucleus.

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INTRODUCTION

Melatonin is a hormone synthesized and secreted by the pineal gland and other tissues including the retina (Lerner et al., 1958; Cardinali and Rosner, 1971; Bubenik et al., 1974; Tosini and Menaker, 1996; Stefulj et al., 2001). Although the biochemistry and physiological actions of melatonin have been investigated extensively, its roles in the retina remain poorly understood. Melatonin is secreted at night by rod/cone photoreceptors and the melatonin receptors MT_1 and MT_2 have been detected in all five classes of retinal neurons including rod/cone photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells, suggesting this hormone performs diverse neuromodulatory functions (Huang et al., 2013; Wiechmann and Sherry, 2013).

Two recent studies reported the expression of melatonin receptors in intrinsically photosensitive retinal ganglion cells (ipRGCs) in mice and rats (Sengupta et al., 2011; Sheng et al., 2015). IpRGCs are inner retinal photoreceptors that contain the photopigment melanopsin and mediate not only pattern vision but also non-image-forming visual responses such as pupillary constriction and circadian photoentrainment (Do and Yau, 2010; Ecker et al., 2010; Schmidt et al., 2014). Though ipRGCs are directly light-sensitive, they nevertheless receive rod/cone-driven synaptic input through bipolar and amacrine cells (Wong et al., 2007; Zhao et al., 2014). Thus, melatonin could modulate ipRGCs by acting either directly on them or on their presynaptic circuits, or both. Here, we tested this hypothesis by focusing on the alpha-like M4-type ipRGCs, whose unusually large somas enable them to be identified for whole-cell recordings with relative ease (Estevez et al., 2012; Schmidt et al., 2014; Reifler et al., 2015), thereby obviating the need for fluorescent labeling (Berson et al., 2002; Ecker et al., 2010). We studied the effects of exogenous and endogenous melatonin on M4 cells' resting membrane potential, spontaneous spike rate, rod/cone-driven ("extrinsic") light responses, and melanopsin-mediated ("intrinsic") light responses. We also looked for the expression of melatonin receptors in these neurons.

EXPERIMENTAL PROCEDURES

Ethical approval

All experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan, and were carried out in accordance with the National Institutes of Health Guide

for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). All efforts were made to minimize the number of animals used and their suffering.

Animals

A total of ~150 male and female Sprague–Dawley rats aged 2–4 months were used. The animals were maintained in a 12-h light, 12-h dark cycle with lights on from 6 AM to 6 PM, and experiments were performed during the light phase between 9 AM and 4 PM unless stated otherwise.

Electrophysiological recording and chemicals

The procedures for euthanasia, eyecup generation, whole-cell recordings and light stimulation have been described in a recent publication (Reifler et al., 2015). To summarize, a rat was dark-adapted the night before each experiment beginning around 6 PM. The following day, it was euthanized around either 8 AM for the daytime recordings (9 AM–4 PM) or 5 PM for the nighttime recordings (6 PM–11 PM). Eyecups were generated in room-temperature Ames' medium gassed with 95% O_2 5% CO_2, and cut into quadrants. A quadrant was flattened on the recording chamber, superfused by 32 °C gassed Ames' medium at 2–3 mL min^{-1}, and kept in darkness except during stimulus presentation. The ganglion cell layer was visualized under a fixed-stage upright microscope (Eclipse FN1; Nikon Instruments, Melville, NY, USA) through infrared transillumination, and whole-cell recordings obtained from the largest somas visible using an internal solution containing (in mM) 120 K-gluconate; 5 NaCl; 4 KCl; 10 Hepes; 2 EGTA; 4 Mg-ATP; 0.3 Na-GTP; 7 Tris-phosphocreatine; and KOH to set pH at 7.3. These large cells included not only ON alpha (i.e. M4) cells but also delta and OFF alpha cells (Peichl, 1989), and M4 cells were identified by their ability to depolarize for the duration of a 10 s 480 nm light step with an intensity of 12.4 log photons cm^{-2} s^{-1} (Wong, 2012; Reifler et al., 2015).

All stimuli were full-field 480-nm lights presented from below the superfusion chamber, with intensity adjusted using neutral density filters. To selectively evoke extrinsic light responses, stimulus intensity was 10.5 photons cm^{-2} s^{-1}, below the threshold for melanopsin activation (Reifler et al., 2015). To induce intrinsic light responses in isolation, recordings were made in Ames' medium containing 10 μM L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 5 μM D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5), and stimulus intensity was 14.4 log photons cm^{-2} s^{-1}. All light stimuli were 1 s in duration unless stated otherwise.

L-AP4, D-AP5, DNQX, luzindole, SCH 23390 and spiperone were purchased from Tocris (Minneapolis, MN, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). Stock solutions were made by dissolving luzindole, melatonin and spiperone in DMSO, and all other chemicals in H_2O. Stock solutions were added directly to the superfusate reservoir instead of through a manifold system, because we have found that switching between solutions in a manifold often causes artifactual physiological changes.

Data analysis

All analyses were performed offline. Measurements of resting membrane potential, photoresponse duration and peak photoresponse amplitude were made after the recordings had been low-pass-filtered with a 10 Hz cutoff frequency. Photoresponse duration was measured as the time interval between stimulus onset and the most hyperpolarized point after stimulus offset (Fig. 1A). Peak photoresponse amplitude was measured relative to the pre-stimulus baseline. For analyzing spiking activity, CLAMPFIT software (Molecular Devices; Sunnyvale, CA, USA) was used to detect action potentials in unfiltered recordings. Light-evoked spiking increases were measured as the difference in the number of spikes detected during the 1 s light step versus during the 1 s immediately before stimulus onset. All statistical comparisons of two data groups (Figs. 2, 5B and 7) were made using the Student's *t*-test, whereas comparisons of three data groups (Figs. 1, 3 and 4) were made using one-way repeated-measures ANOVA followed by Holm-Šídák *post hoc* tests. In all statistical analyses, significance level was set at $p = 0.05$. Error estimates represent S.E.M.

Immunohistochemistry

Freshly isolated retinas were cut into quadrants, fixed for 30 min in 4% paraformaldehyde at room temperature, washed in phosphate-buffered saline (PBS) four times and incubated for 2 h in the primary block solution (PBS containing 10% normal donkey serum and 1–2% Triton X-100) at room temperature. The retinas were then incubated for 4 days at 4 °C in a primary block solution containing mouse anti-SMI-32 (BioLegend SMI-32P; 1:200; Dedham, MA, USA) and either rabbit anti-MT_1 (Alomone Labs AMR-031; 1:200; Israel) or rabbit anti-MT_2 (Alomone Labs AMR-032; 1:50 or 1:200). After four rinses in PBS, the retinas were incubated overnight at 4 °C in PBS containing 5% normal donkey serum, 0.25–0.5% Triton X-100, Cy5 donkey anti-mouse (Jackson ImmunoResearch 715-175-151; 1:200; West Grove, PA, USA), and FITC donkey anti-rabbit (Jackson ImmunoResearch 711–095-152; 1:200). After five rinses in PBS, each piece of retina was mounted on a slide, covered with VECTASHIELD (Vector Labs, Burlingame, CA, USA), and imaged at 0.38 μm z-stack steps using a confocal microscope (Leica SP5; Buffalo Grove, IL, USA). In the control experiments (Fig. 6B, C), the entire procedure was identical except that the anti-MT_1 antibody was either pre-adsorbed with the immunizing peptide or omitted.

RESULTS

Daytime melatonin application modulates M4 cells' extrinsic light responses

We examined the effects of exogenous melatonin during the animals' subjective day, when endogenous