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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 MT₁ 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 melatoninergeric modulation of ipRGCs.

Keywords

Melatonin; retina; retinal ganglion cell; circadian rhythm; melanopsin; light response

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AUTHOR CONTRIBUTIONS

D.D.H. conducted pilot experiments and made the recordings shown in Fig. 5A; W.P. generated and analyzed all other electrophysiological data; K.Y.W. conceived the project, designed the research, performed the immunohistochemistry and confocal imaging, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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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 MT₁ and MT₂ 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 recording with relative ease (Estevez et al., 2012, Schmidt et al., 2014, Reifler et al., 2015), thereby obviating the need for fluorescent labelling (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 recording 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% O₂ 5% CO₂, 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 recording 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 H₂O. 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 PBS 4 times and incubated for 2 hrs 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-MT₁ (Alomone Labs AMR-031; 1:200; Israel) or rabbit anti-MT₂ (Alomone Labs AMR-032; 1:50 or 1:200). After 4 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 5 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-MT₁ 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 melatonin secretion is low (Cahill and Besharse, 1992, Tosini and Menaker, 1996). In normal Ames' medium, M4 cells had a resting membrane potential of -63.1 ± 1.2 mV and spiked spontaneously at 36.7 ± 6.2 Hz. Bath application of 10 nM melatonin had no significant effects on either of these resting properties (Fig. 1B, C). By contrast, extrinsic photoresponses were significantly altered. In response to a 1 s stimulus evoking only extrinsic responses, all cells generated a sustained depolarization accompanied by a spiking increase, and then hyperpolarized transiently after stimulus offset (Fig. 1A). 10 nM melatonin significantly increased both the duration and peak amplitude of the light-evoked depolarization (Fig. 1D, E). Paradoxically, the light-evoked increase in spike rate was reduced by melatonin, from 63.4 ± 9.0 Hz to 36.7 ± 8.0 Hz (Fig. 1F). All three effects could be reversed upon washout of melatonin (Fig. 1D – F).

Since the melatonin stock solution was made using DMSO as vehicle, adding this solution to Ames' medium introduced not only melatonin but also DMSO with a final concentration of 0.01% (v/v). Thus, we performed two control experiments to verify that the effects shown in Fig. 1 were due to melatonin; these controls were done during both daytime and nighttime. In the first control, 0.01% DMSO was added to Ames' medium and was found to have no significant effects on the resting membrane potential, spontaneous spike rate, extrinsic photoresponse duration, extrinsic photoresponse amplitude, or light-evoked spiking increase; example recordings from one cell are shown in Fig. 2A. In the second control, 10 nM melatonin was applied to Ames' medium containing the melatonin receptor antagonist luzindole (2 µM), and no significant changes were observed for any of the parameters measured (Fig. 2B – G). These controls confirmed that the changes seen in Fig. 1D – F were attributable to melatonin.

Because retinal dopamine content is high during subjective day (Doyle et al., 2002) and melatonin inhibits endogenous dopamine release (Dubocovich, 1983), our daytime melatonin application could conceivably have exerted its effects by suppressing dopaminergic modulation. To test this possibility, we applied 10 nM melatonin in the presence of 5 μ M SCH 23390 and 5 μ M spiperone, which are antagonists for D1- and D2-class dopamine receptors respectively. Although spiperone is also an antagonist for serotonin receptors (Hoyer et al., 1994), the rat retina contains insignificant levels of serotonin (Brunken et al., 1993, Han et al., 2007) and so for the purpose of this experiment, spiperone can be assumed to specifically target D2-class dopamine receptors. In the presence of these antagonists, M4 cells had a relatively depolarized resting potential of -48.1 ± 1.8 mV, and action potentials became very small and were often difficult to distinguish from spontaneous synaptic potentials. Thus, we were not able to analyze spontaneous spike rates or light-evoked spiking increases. Applying melatonin on top of the dopamine receptor antagonists still significantly increased both the duration and peak amplitude of M4 cells' extrinsic photoresponses (Fig. 3C, D), suggesting that melatonin can modulate these responses independent of dopaminergic signaling. Interestingly, melatonin also hyperpolarized the resting potential (Fig. 3B), an effect not seen when melatonin was added to normal Ames' medium (Fig. 1B).

Nocturnal endogenous melatonin modulates extrinsic light responses

To test for modulation by endogenous melatonin, we recorded from M4 cells during the animals' subjective night when melatonin release is high (Cahill and Besharse, 1992, Tosini and Menaker, 1996), and applied 2 μ M luzindole to disrupt endogenous melatonin signaling. Resting potentials and spontaneous spike rates were not altered (Fig. 4B, C), consistent with the lack of effects of exogenous melatonin on these properties (Fig. 1B, C). On the other hand, the duration of the extrinsic photoresponse was significantly reduced, while the light-evoked spiking increase was dramatically enhanced from 9.4 ± 9.7 Hz to 43.2 ± 14.2 Hz (Fig. 4D, F). The peak amplitude of the light-evoked depolarization appeared somewhat reduced, although this reduction did not reach statistical significance ($p = 0.175$; Fig. 4E). These effects on extrinsic photoresponses were the opposite of those caused by exogenous melatonin (Fig. 1D – F), and thus confirmed that M4 cells are modulated by endogenous melatonin.

Modulation of intrinsic light responses

All the light responses examined so far were synaptically driven. To study intrinsic melanopsin-based photoresponses, L-AP4, DNQX and D-AP5 were included in Ames' medium to block rod/cone signaling (Fig. 5A), and light steps with an intensity exceeding the melanopsin activation threshold were used. Unlike the extrinsic light responses which showed remarkable stability over time (Fig. 2), the intrinsic photoresponses of virtually all recorded cells exhibited a gradual rundown in peak amplitude, probably partly reflecting light adaptation (Wong et al., 2005) and partly due to intracellular dialysis. To circumvent this issue, we tested whether the addition of 10 nM melatonin reduced or accelerated the rate of rundown, which would indicate an amplification or attenuation of the intrinsic light response respectively. The rate of rundown seemed slightly reduced during melatonin application, although none of the data points deviated significantly from the first-order

decay fit of the rundown (Fig. 5B). The time to peak of these photoresponses, which was more stable than their peak amplitude, showed little change in the presence of melatonin (Fig. 5C). The duration of light-evoked spiking was also relatively stable initially, and was dramatically reduced upon melatonin application (Fig. 5D). However, spiking duration continued to drop during melatonin washout, making it unclear whether this decrease was real or merely a general rundown similar to that seen for the light-evoked depolarization (Fig. 5B).

M4 cells express MT₁ melatonin receptors

In light of melatonin's modulatory effects, we next performed immunohistochemistry to assess whether M4 cells express melatonin receptors. Because there are no known specific markers for this ipRGC type, we used SMI-32 to stain both ON and OFF alpha cells, and the ON-stratifying immunopositive cells would correspond to M4 cells (Schmidt et al., 2014). We found that all SMI-32-immunopositive somas ($n > 80$ cells) were also stained by an antibody against the MT₁ melatonin receptor, including those whose dendrites stratified near the retinal surface and were thus presumed M4 cells (Fig. 6A, *arrows*). MT₁ antibody staining was restricted to the cytoplasm, because this antibody preferentially recognizes non-membrane-bound MT₁ receptors (Sheng et al., 2015). MT₁ immunostaining disappeared when the primary antibody was pre-adsorbed with the blocking peptide (Fig. 6B) or omitted (Fig. 6C), confirming the specificity of the staining. We also tried an antibody for the MT₂ receptor but the staining pattern was variable and thus inconclusive (data not shown).

DISCUSSION

To date, only two studies have investigated neuromodulation of ipRGC physiology: dopaminergic modulation of M1 cells (Van Hook et al., 2012), and adenosinergic modulation of unclassified ipRGCs (Sodhi and Hartwick, 2014). The present work focused on the M4 type because it is relatively easy to identify for whole-cell recording. Considering that M4 cells contribute to image-forming as well as non-image-forming vision (Estevez et al., 2012, Schmidt et al., 2014), our results suggest that melatonin may modulate both forms of vision. However, this modulation is unlikely to affect circadian photoentrainment because M4 cells do not project to the central circadian pacemaker in the suprachiasmatic nucleus (SCN). Nevertheless, modulation of circadian photoentrainment by retinal melatonin remains possible because melatonin receptors are present in M1 cells (Sengupta et al., 2011, Sheng et al., 2015), which mediate most if not all retinal input to the SCN (Ecker et al., 2010). Melatonin might not modulate M1 and M4 cells similarly, however, because neuromodulators often affect different ganglion cells differently (Glickman et al., 1982, Zalutsky and Miller, 1990, Downing and Djamgoz, 1993). Indeed, dopamine probably has opposite effects on M1 versus M4 cells, since dopaminergic activation depolarizes the former (Van Hook et al., 2012) whereas we found that blocking dopamine receptors depolarized the latter (compare Figs. 1B and 3B), suggesting that receptor activation would hyperpolarize M4 cells.

Potential loci of melatonergic modulation

Because M4 cells express MT₁ receptors, melatonin could induce the observed effects by acting directly on these ipRGCs. While MT₁ receptor expression in mammalian retinal ganglion cells has been documented extensively (Fujieda et al., 1999, Fujieda et al., 2000, Meyer et al., 2002, Savaskan et al., 2002, Scher et al., 2002, 2003, Baba et al., 2009, Sengupta et al., 2011, Baba et al., 2013, Sheng et al., 2015), only three papers have investigated its expression in specific ganglion cell types, namely, M1 ipRGCs (Sengupta et al., 2011, Sheng et al., 2015) and M4 ipRGCs (the present study). M4 cells might also contain MT₂ receptors because even though we were unable to examine MT₂ immunoreactivity in these cells, widespread expression of this receptor has been observed among mammalian ganglion cells (Savaskan et al., 2007, Zhao et al., 2010, Sheng et al., 2015), although it is absent in mouse ganglion cells (Baba et al., 2013).

Melatonin could also modulate M4 cells by acting presynaptically, since the outer retinal photoreceptors and many interneurons express melatonin receptors (Huang et al., 2013, Wiechmann and Sherry, 2013). Melatonin serves as a dark-adaptive signal and has been shown to potentiate rod signaling but attenuate cone signaling. Specifically, it increases rods' light responses (Wiechmann et al., 2003, Baba et al., 2009), rod input to ON bipolar cells (Ping et al., 2008, Baba et al., 2009) and horizontal cells (Ribelayga et al., 2004), and the excitability of rod bipolar cells (Yang et al., 2011). Moreover, melatonin decreases horizontal cells' cone-driven light responses (Ribelayga et al., 2004, Huang et al., 2005), which could reduce these interneurons' feedback to cones. Since this feedback contributes to the inhibitory surrounds of bipolar and ganglion cells' receptive fields, a reduction in its strength should augment these cells' responses to full-field light. Furthermore, melatonin potentiates the response of AMPA receptors to glutamate (Huang et al., 2005). Because M4 cells receive both rod- and cone-driven excitatory inputs (Zhao et al., 2014) and have antagonistic center/surround receptive fields (Estevez et al., 2012, Zhao et al., 2014), any of the above effects could contribute to the observed augmentation and lengthening of the extrinsic photoresponse (Fig. 1D, E).

Besides acting directly on neurons containing melatonin receptors, melatonin could indirectly affect neurons that lack these receptors by suppressing dopamine release and hence dopaminergic signaling (Dubocovich, 1983, Ribelayga et al., 2004). Dopaminergic signaling is not required for melatonin's modulation of M4 cells' extrinsic light responses, since such modulation persisted even when dopamine receptors were blocked (Fig. 3C, D). Unexpectedly, adding melatonin during dopamine receptor blockade significantly altered M4 cells' resting potential, an effect not seen when melatonin was added alone (compare Figs. 1B and 3B). Presumably, this effect was masked when dopaminergic signaling was intact, suggesting that at least some aspects of M4 cells' physiology are under dopaminergic as well as melatonergic modulation. Consistent with this notion, the recordings made under normal conditions (Fig. 1B, D, E, "Ames' medium") and during dopamine receptor block (Fig. 3B–D, "DA antag") showed dramatically different ($p < 0.05$, Student's t test) resting potentials, photoresponse durations, and photoresponse amplitudes.

Because M4 cells probably receive inhibitory glycinergic input (Koulen et al., 1996, Pang et al., 2003, Majumdar et al., 2007, van Wyk et al., 2009) and melatonin augments glycine-induced currents in ganglion cells via MT₂ receptors (Zhang et al., 2007, Zhao et al., 2010), melatonin might be expected to attenuate these ipRGCs' extrinsic light response. But we observed an enhancement instead (Fig. 1E), suggesting that either M4 cells lack MT₂ receptors, or melatonin potentiates excitatory inputs more than the glycinergic input.

Though melatonin enlarged the graded extrinsic light response of M4 cells, it paradoxically lessened the associated elevation in spike rate (compare Figs. 1E and 1F). This may partly reflect spike block by excessive depolarization (Hu et al., 2013), since spiking was interrupted at the peak of the light-evoked depolarization (Fig. 1A, *bottom recording*). But this brief spike block cannot fully account for the rather substantial reduction in the total number of spikes evoked during the 1 s light step (Fig. 1F), suggesting that melatonin probably also modulates voltage-gated ion channels that regulate spike rate. Melatonergic enhancement of delayed-rectifier K⁺ currents in spiking neurons has been reported (Huan et al., 2001, Liu et al., 2007).

It is reasonable to expect melatonin to modulate melanopsin-based photoresponses, because: 1) protein kinase A (PKA) can phosphorylate melanopsin (Blasic et al., 2012); 2) both MT₁ and MT₂ receptors can modulate PKA activity (Huang et al., 2013); 3) manipulating the cAMP/PKA pathway alters the duration of rat ipRGCs' intrinsic light responses (Sodhi and Hartwick, 2014); and 4) rat M4 cells contain melatonin receptors (Fig. 6A). Indeed, melatonin has been shown to downregulate melanopsin mRNA levels in *Xenopus* dermal melanophores (Bluhm et al., 2012). However, exogenous melatonin did not significantly alter the amplitude of M4 cells' intrinsic light responses (Fig. 5B). While it did shorten the duration of the spiking response, such shortening could not be reversed upon melatonin washout and thus could reflect a general rundown (Fig. 5D). This insignificant amplitude change and gradual rundown could have been caused by intracellular dialysis of second messengers such as cAMP, and so our results cannot rule out or support melatonergic modulation of the intrinsic light response.

Circadian modulation of ipRGC physiology

Pronounced differences were noted between our daytime and nighttime data, with the cells recorded at night having a more positive resting potential, a lower spontaneous spike rate, longer-lasting extrinsic light responses, and smaller extrinsic photoresponses in terms of both peak amplitude and spiking increase (Fig. 7). These differences cannot be explained by a difference in the animals' prior dark adaptation (14 hrs for the daytime experiments vs. 23 hrs for the nighttime experiments) because the dark adaptation of both rods and cones is complete within 5 hrs (Perlman, 1978, Behn et al., 2003) and so they should have been fully dark-adapted in both animal groups. Even if they needed more than 14 hrs to fully dark-adapt, the longer dark adaptation preceding the nighttime experiments would have resulted in larger photoresponses at night, the opposite of that observed (Fig. 7D, E). Thus, these day-versus-night differences likely reflect circadian changes. Since exogenous melatonin also increased photoresponse duration and decreased light-evoked spiking (Fig. 1D, F) while applying luzindole at night had the opposite effects (Fig. 4D, F), the nocturnal increase in

melatonin secretion could underlie the circadian variation in these two properties. By contrast, the nighttime depolarization of the resting potential, decrease in spontaneous spike rate and decrease in photoreponse amplitude were not expected from our melatonin or luzindole results (Figs. 1B, C, E and 4B, C, E). It is possible that endogenous melatonin has long-term effects different from the acute effects we studied. Indeed, different durations of dopaminergic activation have been reported to exert seemingly opposite effects on ipRGCs: it attenuated intrinsic light responses within minutes (Van Hook et al., 2012) but promoted melanopsin expression over several hours (Sakamoto et al., 2005). Another possibility is that certain aspects of circadian modulation of M4 cells are mediated by neuromodulators besides melatonin that show circadian variation, for example dopamine (Wirz-Justice et al., 1984) and adenosine (Ribelayga and Mangel, 2005).

Though ipRGCs are well-known to be crucial for photoentrainment of the central circadian clock (Goz et al., 2008, Guler et al., 2008, Hatori et al., 2008), little work has been done to investigate circadian modulation of ipRGC physiology or ipRGC-driven visual behavior. IpRGCs' intrinsic photoreponses increase slightly during early subjective night (Weng et al., 2009), and circadian rhythms in the human pupillary reflex have also been demonstrated (Zelev et al., 2011). Consistent with the nighttime reduction in light-evoked spiking (Fig. 7E), Zelev and colleagues showed that the cone-driven post-illumination pupil response (which is thought to reflect ipRGCs' extrinsic photoreponse) was significantly weaker during subjective night. This weakening preceded the SCN-driven rise in melatonin by ~4 hrs (Zelev et al., 2011), suggesting it may be caused by circadian modulation within the retina.

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ABBREVIATIONS

D-AP5	D-(–)-2-amino-5-phosphonopentanoic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
IPL	inner plexiform layer
ipRGC	intrinsically photosensitive retinal ganglion cell
L-AP4	L-(+)-2-amino-4-phosphonobutyric acid
MT₁	melatonin receptor type 1
MT₂	melatonin receptor type 2
PBS	phosphate buffered saline
PKA	protein kinase A
SCN	suprachiasmatic nucleus

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HIGHLIGHTS

- Intrinsically photosensitive retinal ganglion cells (ipRGCs) are novel photoreceptors.
- We tested if M4-type ipRGCs are modulated by melatonin, a hormone secreted at night.
- Both exogenous and endogenous melatonin altered M4 cells' light-evoked responses.
- M4 cells express the MT₁ receptor, suggesting melatonin might act directly on them.
- We also detected circadian variations in M4 cells' electrophysiology.

Figure 1. Daytime application of exogenous melatonin modulated M4 cells' extrinsic light response

A, example recordings from one cell. B – F, averaged group data quantifying the effects of exogenous melatonin on M4 cells' resting membrane potential (B), spontaneous spike rate (C), duration of light-evoked depolarization (D), peak amplitude of light-evoked depolarization (E), and light-evoked elevation in spike rate (F). $N = 53$ cells. **, $p < 0.01$.

Figure 2. Control experiments confirmed that exogenous melatonin acted through melatonin receptors

In *A*, DMSO (the vehicle for dissolving melatonin) was applied to normal Ames' medium with a final concentration of 0.01% ($n = 12$ cells) whereas in *B – G*, 10 nM melatonin (dissolved in DMSO) was applied to Ames' medium containing 2 μ M luzindole, a melatonin receptor antagonist ($n = 19$ cells). Neither treatment significantly affected M4 cells' resting membrane potential, spontaneous spike rate, duration of light-evoked depolarization, peak amplitude of light-evoked depolarization, or light-evoked increase in spike rate, and population data (*C – G*) are shown for the luzindole experiment. These control experiments were done during both daytime and nighttime.

Figure 3. The effects of exogenous melatonin did not require dopaminergic signalling

10 nM melatonin was applied in the presence of 5 μ M SCH 23390 (D1-class dopamine receptor antagonist) and 5 μ M spiperone (D2-class dopamine receptor antagonist) during daytime. *A*, example recordings from one cell. *B – D*, averaged group data quantifying how adding melatonin on top of dopamine receptor antagonists affected M4 cells' resting membrane potential (*B*), duration of light-evoked depolarization (*C*), and peak amplitude of light-evoked depolarization (*D*). $N = 26$ cells. **, $p < 0.01$.

Figure 4. Nighttime blockade of endogenous melatonin signaling had effects opposite from those of daytime melatonin application

A, example recordings from one cell. B – F, averaged group data summarizing the effects of nighttime luzindole (2 μ M) application on M4 cells' resting membrane potential (B), spontaneous spike rate (C), duration of light-evoked depolarization (D), peak amplitude of light-evoked depolarization (E), and light-evoked increase in spike rate (F). $N = 22$ cells. **, $p < 0.01$.

Figure 5. The effects of daytime melatonin application on M4 cells' intrinsic light responses

A, example recordings from one cell to illustrate the use of the rod/cone signaling blockers L-AP4 (10 μM), DNQX (20 μM) and D-AP5 (5 μM) to isolate the intrinsic melanopsin-based light response. **B**, the peak amplitude of the intrinsic light response is plotted against time to illustrate the gradual rundown of this response and the statistically insignificant reduction of this rundown during melatonin (10 nM) application. **C**, applying 10 nM melatonin had no effect on the time to peak of the intrinsic light response. **D**, 10 nM melatonin seemed to dramatically reduce the duration of the light-evoked spiking response, although this reduction was not reversible. In **B** – **D**, light intensity was 14.4 log photons $\text{cm}^{-2} \text{s}^{-1}$ and stimulus duration was 1 s. $N = 36$ cells.

Figure 6. M4 cells express MT₁ receptors

A, all ganglion cells immunopositive for the relatively specific alpha cell marker SMI-32 (magenta) also showed MT₁ antibody staining within their cytoplasm (green). The cells highlighted with arrows had dendrites stratifying near the retinal surface and were probably M4 cells. B, C, control experiments showed that MT₁ antibody staining disappeared when the anti-MT₁ antibody was pre-adsorbed with the antigen (B) or omitted (C), while SMI-32 immunostaining remained.

Figure 7. Evidence for circadian variations in M4 cell physiology

The “Ames’ medium” data from Figs. 1B–F (daytime recordings) and 4B–F (nighttime recordings) are reproduced here to illustrate day/night differences in resting membrane potential (A), spontaneous spike rate (B), photoreponse duration (C), photoreponse amplitude (D), and light-evoked spiking increase (E). *, $p < 0.05$; ***, $p < 0.001$.