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Synaptic inputs to retinal ganglion cells that set the circadian clock

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Abstract

Melanopsin-containing retinal ganglion cells (RGCs) project to the suprachiasmatic nuclei (SCN) and mediate photoentrainment of the circadian system. Melanopsin is a novel retinal-based photopigment that renders these cells intrinsically photosensitive (ip). Although genetic ablation of melanopsin abolishes the intrinsic light response, it has a surprisingly minor effect on circadian photoentrainment. This and other non-visual responses to light are lost only when the melanopsin deficiency is coupled with mutations that disable classical rod and cone photoreceptors, suggesting that melanopsin-containing RGCs also receive rod- and cone-driven synaptic inputs. Using whole-cell patch-clamp recording, we demonstrate that light triggers synaptic currents in ipRGCs via activation of ionotropic glutamate and γ -aminobutyric acid (GABA) receptors. Miniature postsynaptic currents (mPSCs) were clearly observed in ipRGCs, although they were less robust and were seen less frequently than those seen in non-ip cells. Pharmacological treatments revealed that the majority of ipRGCs receive excitatory glutamatergic inputs that were blocked by DNQX and/or kynurenic acid, as well as inhibitory GABAergic inputs that were blocked by bicuculline. Other ipRGCs received either glutamatergic or GABAergic inputs nearly exclusively. Although strychnine (Strych)-sensitive mPSCs were evident on many non-ipRGCs, indicating the presence of glycinergic inputs, we saw no evidence of Strych-sensitive events in ipRGCs. Based on these results, it is clear that SCN-projecting RGCs can respond to light both via an intrinsic melanopsin-based signaling cascade and via a synaptic pathway driven by classical rod and/or cone photoreceptors. It remains to be determined how the ipRGCs integrate these temporally distinct inputs to generate the signals that mediate circadian photoentrainment and other non-visual responses to light.

Keywords

circadian rhythms; intrinsic light response; melanopsin; photoentrainment; photoreceptor; postsynaptic current

Introduction

Known best for its role in vision, the retina also controls a number of non-visual responses to light, including photoentrainment of the circadian system and the pupillary light reflex. In mammals, classical rod and cone photoreceptors are not required for non-visual functions (Freedman *et al.*, 1999; Lucas *et al.*, 1999); instead, these functions are mediated by a small subset of retinal ganglion cells (RGCs) that express the novel photopigment melanopsin (Provencio *et al.*, 2000). Photoexcitation of melanopsin triggers an intracellular signaling

cascade that activates a depolarizing conductance, rendering these cells intrinsically sensitive to light (Berson *et al.*, 2002; Warren *et al.*, 2003). The axons of these intrinsically photosensitive (ip) RGCs form the retinohypothalamic tract (Gooley *et al.*, 2001; Hannibal *et al.*, 2002; Hattar *et al.*, 2002), a monosynaptic pathway that leads to the suprachiasmatic nuclei (SCN), two pear-shaped structures in the ventral hypothalamus that house the circadian pacemaker (Ralph *et al.*, 1990). Axonal branches also lead to the olivary pretectal nuclei that control the pupillary light reflex and brain regions that control the sleep–wake cycle (Gooley *et al.*, 2003; Morin *et al.*, 2003).

Melanopsin is a photopigment capable of activating a variety of G-proteins in a light-dependent manner (Newman *et al.*, 2003; Melyan *et al.*, 2005; Panda *et al.*, 2005; Qiu *et al.*, 2005), and genetic deletion of melanopsin has been shown to abolish the intrinsic light response in SCN-projecting RGCs. (Lucas *et al.*, 2003). Surprisingly, however, the behavioral responses, such as circadian photoentrainment and the pupillary light reflex, are only mildly impaired (Panda *et al.*, 2002; Ruby *et al.*, 2002; Lucas *et al.*, 2003). This apparent contradiction was resolved by the demonstration that all non-visual responses to light were abolished in melanopsin knock-out (KO) mice whose rod and cone photoreceptors were absent or disabled (Panda *et al.*, 2003; Hattar *et al.*, 2003). Although non-ipRGCs may also convey photic information to the SCN and olivary pretectal nucleus, it seems likely that melanopsin-containing ipRGCs can be stimulated by rod- and cone-driven synaptic input. In retrospect, this synaptic input might have been predicted from the fact that the dendritic processes of melanopsin-containing cells extend through the synapse-dense inner plexiform layer (IPL) for several hundred microns (Provencio *et al.*, 2002). Melanopsin-containing processes stratify both in the inner and outer laminae of the IPL, synaptic layers that contain the dendritic arbors of RGCs that depolarize (ON cells) or hyperpolarize (OFF cells) in response to light stimulation (Warren *et al.*, 2003). Ultrastructural studies have also revealed the presence of synaptic contacts onto the dendrites of ipRGCs (Belenky *et al.*, 2003).

Here, we briefly describe a light-driven depolarizing synaptic input that is mediated by AMPA-type glutamate receptors, as well as the spontaneous miniature postsynaptic currents (mPSCs) that arise by asynchronous release of presynaptic vesicles. Although they are neither as robust nor as common as those seen in the majority of RGCs mediating vision, mPSCs corresponding to both excitatory glutamatergic inputs and inhibitory γ -aminobutyric acid (GABA)ergic inputs were readily apparent in the ipRGCs.

Materials and methods

Tetramethylrhodamine-dextran (3000 MW; 10 mg/mL) was obtained from Molecular Probes (Eugene, OR, USA), and tetrodotoxin (TTX) from Sigma-Aldrich (St. Louis, MO, USA). Bicuculline (Bicuc), strychnine (Strych), kynurenic acid (Kyn) and DNQX were obtained from Tocris-Cookson (Ellisville, MO, USA).

Stereotaxic injection of fluorescent retrograde tracers

Surgical procedures were in compliance with NIH guidelines, and approved by the Institutional Animal Care and Use Committee of OHSU. To identify RGCs projecting to the circadian system, tetramethylrhodamine-dextran was injected into the SCN of Sprague–Dawley rats (120–170 g) using a stereotaxic apparatus (Cartesian Designs, Sandy, OR, USA), following anesthesia with a mixture of ketamine, xylazine and acepromazine. Between 2 and 10 days after injection, animals were killed in a dim room light by cervical dislocation following ether anesthesia.

Electrophysiology

The anterior chamber of each eye was removed under a Nikon dissecting scope (1.0×10^{-4} W/cm² white light), and the retinas were gently peeled from the eyecup and stored in oxygenated EMEM (HEPES modification) at RT. A small piece of retina was mounted with ganglion cell layer facing up on a poly-D-lysine-coated coverslip. The retinal flat mount and electrode were positioned using IR illumination (1.7×10^{-7} W/cm²), and SCN-projecting RGCs were identified by the presence of tetramethylrhodamine-dextran using epifluorescent illumination. Intrinsic light responses were verified for all cells categorized as ipRGCs.

Patch pipettes (5–10 MΩ) were fabricated from filamented borosilicate glass (O.D. 1.5 mm, I.D. 0.86 mm) using a P-97 electrode puller (Sutter Instruments, Novato, CA, USA). Recordings were made at 22 °C with a Multiclamp 700A amplifier controlled by pClamp9 software via a Digidata 1320 interface (Molecular Devices Corporation, Hayward, CA, USA). Conventional whole-cell recordings were typically made at a holding potential of –60 mV; series resistance (25–50 MΩ) was noted but uncompensated. Data were low-pass filtered at 2 kHz and digitized at 5–10 kHz. In most experiments, recording electrodes were filled with an intracellular solution containing (in mM): KCl, 121; MgCl₂, 1; HEPES, 10; EGTA, 10; Mg-ATP, 4; Na₃-GTP, 0.3; creatine phosphate (Na salt), 2; Lucifer yellow (0.1%) at pH 7.35 (290 mOsm). The bath solution typically contained (in mM): NaCl, 119; KCl, 2.5; MgCl₂, 1.3; CaCl₂, 2.5; NaHCO₃, 26.2; NaH₂PO₄, 1; Na-pyruvate, 2; D-glucose, 20; pH 7.4 (290–300 mOsm), and was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Internal and external solutions were varied slightly in some experiments with no discernable effect on the amplitude or frequency of synaptic events.

Light stimulation

Illumination was provided by a 250 W tungsten-halogen light source through the transmitted light path of an Olympus BX-51W microscope. Light intensities were determined using a radiometer (International Light, Newburyport, MA, USA). Recordings were conducted in a dimly lit room (5.0×10^{-7} W/cm² of background illumination). Intrinsic responses were elicited by exposure to 1.40×10^{-4} W/cm² white light.

Analysis of spontaneous postsynaptic currents

Events were selected and analysed with the assistance of Mini-Analysis software (6.0.1; Synaptosoft, Decatur, GA, USA), using a threshold of 4 pA (~ double the rms noise in most recordings). Only events that exhibited a rapid rise (< 4 ms time-to-peak) and a smooth decay to near baseline were selected for analysis. All PSCs were included for computation of mean amplitudes and frequencies; only events exhibiting a single peak were selected for determination of the decay time constant. At least 50 traces of each type of PSC were selected and fit with a monoexponential decay function. Peak amplitudes and decay time constants were subsequently averaged and analysed using Origin (5.0, Microcal Software, Northampton, MA, USA). As determined by linear regression analysis, there was no correlation between peak amplitude and decay time constant. Data are reported as means ± SD.

PSCs were separated by application of specific pharmacological agents, including Bicuc, Strych and Kyn or DNQX, which block GABA_A, glycine and ionotropic glutamate receptors, respectively. Histograms were constructed from equivalent time durations before and after application of the pharmacological agents indicated.

Results

Currents were recorded from SCN-projecting RGCs under conventional whole-cell voltage-clamp at –60 mV. In the absence of synaptic blockers, these cells responded to a step of bright

white light by generating a slowly activating inward current with a time-to-peak ranging from 1 to 2 s (Fig. 1A; left). This current persisted despite the presence of synaptic blockers, indicating that it was generated by an intracellular cascade. In addition to this intrinsic light response, a small percentage of ipRGCs (8 of ~ 170 cells) also exhibited a transient inward current with a latency of < 500 ms (Fig. 1A; left), reminiscent of the synaptically driven light responses of RGCs (Fig. 1A, right panel). These synaptic responses were, however, much more common in non-ipRGCs (41%; 21 of 52 cells). Under control conditions in TTX, the synaptic light response typically consisted of multiple current spikes in both the ip- and non-ipRGCs. For both cell types, application of the GABAergic antagonist, Bicuc (Fig. 1A; middle traces), inhibited the later peaks, thereby sharpening the light response. Subsequent application of the glutamatergic antagonists DNQX or Kyn, which inhibit AMPA receptors (Fig. 1A; bottom traces), blocked the remaining transient response. Application of Strych, which targets glycinergic synaptic transmission, had no effect on the synaptic transients in the ipRGCs; the small number of cells recorded, however, precludes a definitive conclusion.

To identify the types of synaptic inputs that impinge upon ipRGCs, we characterized the spontaneous PSCs manifest in these cells. Although most ipRGCs displayed synaptic events, only a small fraction exhibited enough for statistical analysis (Fig. 1B). Approximately 21% of ipRGCs (36 of 171) exhibited robust (> 5 events/s) or moderate (0.1–5 events/s) spontaneous synaptic activity. The majority of ipRGCs (135 of 171), however, were largely silent. In contrast, the vast majority of non-ipRGCs (118 of 129) displayed robust or moderate activity with large amplitude events (see below).

Most ipRGCs exhibited two types of synaptic events that were distinguished by amplitude and kinetics, as well as by pharmacology (Fig. 2). Under control conditions, the ipRGC shown in Fig. 2A exhibited events with amplitudes ranging from 5 to 30 pA. Two types of events were easily discernable – those with a relatively large amplitude and slow decay, and those with a small amplitude and rapid decay. Bath application of Bicuc, a blocker of GABA_A receptors, virtually eliminated all events with amplitudes greater than 12 pA (Fig. 2B). The remaining small-amplitude events were subsequently eliminated by application of Kyn, a broad-spectrum blocker of ionotropic glutamate receptors. When categorized by amplitude in the presence of TTX, events greater than 12 pA displayed an average amplitude of 13.7 ± 0.3 pA and decayed with a monoexponential fit τ of 20.8 ± 1.8 ms ($n = 174$). In contrast, the smaller events displayed an average amplitude of 7.9 ± 0.2 pA with a monoexponential fit decay τ of 4.4 ± 0.3 ms ($n = 102$). The superimposition of scaled average traces corroborated the presence of different types of PSC under each condition, and this allowed us to sort events as GABAergic or glutamatergic based on both peak amplitude and decay time constant criteria. Averaged traces from the two classes of events are displayed in Fig. 2C, which demonstrates that the small events (< 12 pA) under control conditions are virtually identical to those remaining after application of Bicuc. The histogram in Fig. 2D reveals that Bicuc selectively eliminated events > 12 pA in this cell, and increased the frequency of smaller events, probably by relieving a tonic inhibitory input on the presynaptic neuron. As shown in Fig. 2E, the PSCs remaining in the presence of Bicuc displayed much faster decay kinetics than the larger, Bicuc-sensitive events.

We found a striking difference between the amplitudes of GABAergic PSCs in ipRGCs and their non-ip counterparts. The average amplitude of GABAergic events in ipRGCs in the absence of TTX was 18.8 ± 9.9 pA ($n = 4622$ events from 5 cells), compared with 36.1 ± 23 pA in non-ipRGCs ($n = 7468$ events from 6 cells). Application of TTX had no measurable effect on the amplitude, decay time or frequency of PSCs in ipRGCs. In contrast to the GABAergic PSCs, there was no significant difference in the amplitude of glutamatergic events between ip- and non-ipRGCs (9.7 ± 3.86 pA; $n = 3708$ events from 7 cells vs. 10.7 ± 4.5 pA; $n = 2091$ events from 3 cells). Glutamatergic and GABAergic mPSCs could also be kinetically distinguished in both ip- and non-ipRGCs. The time constant of decay for the GABAergic

events was similar between ip- and non-ipRGCs, 14.9 ± 1 vs. 13.2 ± 1 ms, respectively. These decay time constants are substantially longer than those of glutamatergic events, which were 4.8 ± 0.4 ms and 3.16 ± 0.2 ms in ip- and non-ipRGCs, respectively.

Although most ipRGCs displayed both types of synaptic inputs, several cells exhibited primarily GABAergic (Fig. 3A–C) or glutamatergic (Fig. 3D and E) events. There was strong evidence of Bicuc-sensitive mPSCs in 63% of ipRGCs (5 of 8 cells). A slightly higher percentage of non-ipRGCs exhibited Bicuc-sensitive events (85%; 11 of 13 cells). Glutamatergic antagonists inhibited mPSCs in 3 of 4 ipRGCs cells (75%). Kyn inhibited synaptic events in 1 of 2 cells, while DNQX, a selective antagonist of AMPA receptors, successfully inhibited miniature synaptic events in 2 of 2 cells. Similarly, Kyn inhibited mPSCs in 4 of 6 non-ipRGCs cells (67%). The measured parameters for events in cells displaying only one predominant type of event were similar to the average values obtained by size-selection in other cells, buttressing the validity of our selection criteria. In the Bicuc-sensitive cell shown in Fig. 3A, the average event amplitude was 20.8 ± 0.2 pA with a mean decay time constant of 17.2 ms. For the glutamatergic cell depicted in Fig. 3D, the mean amplitude was 9.4 ± 0.1 pA with a decay time constant of 5.9 ms.

Strych was applied to 2 ipRGCs (see Fig. 3D), but we saw no evidence of Strych-sensitive events. Furthermore, application of Bicuc and Kyn abolished all synaptic activity in all cells tested. In contrast, Strych-sensitive events were readily detected in many non-ipRGCs ($n = 4$ of 7), indicating the presence of glycinergic inputs (data not shown). The limited number of cells in the current study, however, precludes any firm conclusions about the presence or absence of Strych-sensitive events in ipRGCs.

Discussion

Studies on melanopsin KO mice suggest that rod- and cone-driven synaptic input onto ipRGCs can compensate for the absence of an intrinsic light response, and partially restore non-visual responses to light. In this study, we present evidence that some ipRGCs do, in fact, receive light-evoked synaptic inputs. Light responses are comprised of both excitatory components, mediated by AMPA receptors, and inhibitory components, mediated by ionotropic GABA receptors. Similar results have been reported in abstract form by Dunn & Berson (2002). In both studies, however, only a small percentage of ipRGCs were reported to demonstrate light-evoked synaptic currents. In our study, the low response rate (5%) is likely attributable to the use of an isolated, bleached retina, which would severely impair rod and cone photoresponses; Dunn & Berson (2002), however, reported that only ~20% of ipRGCs exhibited a light-evoked synaptic response, even when recording from eye cup preparations, which should preserve rod and cone photosensitivity. In contrast to these results in rodents, melanopsin-containing RGCs in primates appear to receive robust input from both rods and cones (Dacey *et al.*, 2005).

Despite the fact that the synaptically mediated light responses are quite brief, unlike intrinsic light responses which can persist for minutes, they are remarkably efficient at driving the non-visual behavioral light responses in melanopsin KO animals. Loss of the intrinsic light response may cause enhancement of rod- and cone-driven synaptic inputs, but this hypothesis remains to be tested by comparing light-evoked and spontaneous synaptic events in wild-type and melanopsin KO animals.

Small spontaneous current deflections were indicative of asynchronous presynaptic release of neurotransmitter, and the application of pharmacological agents indicated that ipRGCs express both AMPA receptors and GABA_A receptors. On average, the GABAergic PSCs were approximately twice as small as those seen in non-ipRGCs, which were similar to those previously reported in rat retina (Protti *et al.*, 1997). The reason for this difference remains

unknown, but current knowledge suggests two possibilities. First, the dendritic arbors of ipRGCs are known to extend for up to 500 μm across the retina, and the synaptic inputs could be concentrated at the distal ends of these long, thin processes. This organization could lead to dendritic filtering with a consequent reduction in PSC amplitude measured at the soma; we saw no relationship, however, between the amplitude and decay time as would be predicted for this type of dendritic filtering. Second, the density, composition or geometry of postsynaptic receptors in ipRGCs could be different than those in non-ipRGCs.

Although most ipRGCs exhibited both excitatory and inhibitory inputs, there was a surprising heterogeneity, and some cells appeared to receive only one type of input. The dendritic arbors of melanopsin-containing ipRGCs have been reported to stratify primarily in the outermost lamina of the IPL, although the dendrites of some cells stratify exclusively in the innermost strata of the IPL or are bistratified (Warren *et al.*, 2003). RGCs whose processes stratify in the outer strata of the IPL are typically classified as 'OFF' cells because they depolarize in response to a reduction in light intensity. Although the anatomy of most melanopsin-containing RGCs is similar to that of an 'OFF' cell, they behave functionally as 'ON' cells that depolarize in response to light onset. This suggests that they must receive 'ON'-type synaptic input from bipolar cells on their proximal dendrites. Ultrastructural analysis of melanopsin-containing cells provides evidence for both excitatory and inhibitory synapses on both the proximal and distal dendrites, as well as excitatory synapses on the cell soma (Belenky *et al.*, 2003). In the future it will be interesting to compare the functional synaptic inputs in ipRGCs with differing anatomical stratification.

The function of the GABAergic inputs to ipRGCs remains unknown. In typical RGCs, GABAergic input from retinal amacrine cells is involved in lateral inhibition that refines the receptive field and contributes to motion and edge detection. Unlike most RGCs, however, ipRGCs rarely exhibit action potentials at their resting membrane potential. Perhaps this striking lack of spontaneous action potentials could be attributed to the GABAergic tone supplied by these inputs. Although it is clear that SCN-projecting RGCs can respond to light both through an intrinsic, intracellular cascade as well as rod- and cone-driven synaptic inputs, further work is required to elucidate how these two inputs are integrated to shape the light response in these cells.

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Abbreviations

Bicuc, bicuculline
 GABA, γ -aminobutyric acid
 ip, intrinsically photosensitive
 IPL, inner plexiform layer
 KO, knock-out
 Kyn, kynurenic acid
 mPSCs, miniature postsynaptic currents
 RGCs, retinal ganglion cells
 SCN, suprachiasmatic nuclei
 Strych, strychnine
 TTX, tetrodotoxin

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FIG. 1.

Synaptic inputs in intrinsically photosensitive (ip)- and non-ip retinal ganglion cells (RGCs). (A) Whole-cell currents were recorded from RGCs in a flat mount preparation. Retinas were bathed in control external solution at a holding potential of -60 mV (left). In an ipRGC, the light stimulus (bar) evoked a short-latency, transient inward current, followed by the long-lasting inward current of the intrinsic light response. The transient responses were reduced by application of bicuculline (Bicuc, 10 μ M), and abolished by subsequent application of DNQX (20 μ M). (right) In a non-ipRGC, a light stimulus evoked a burst of short-latency, transient inward currents. The later currents were blocked by application of Bicuc, and the initial spike was blocked by kynurenic acid (Kyn, 1.0 mM). (B) Comparison of spontaneous synaptic activity in non-ip and ipRGCs. Representative traces of robust (> 5 events/s), moderate (between 0.1 and 5 events/s) and silent (< 0.1 events/s) spontaneous synaptic activity in a non-ipRGC (left) and an ipRGC (right).

FIG. 2.

Excitatory and inhibitory miniature synaptic currents in an ipRGC. (A) Plot of time vs. amplitude of mPSCs. Pharmacological blockers were applied as indicated. Concentrations were as noted in Fig. 1. (B) Selected recordings obtained in the presence of the pharmacological agents indicated. Arrow indicates a GABAergic event, and asterisks indicate glutamatergic events. (C) Comparison of averaged traces of small (< 12 pA) and large (> 12 pA) events. (D) Histogram of mPSCs before and after application of bicuculline (Bicuc), a blocker of ionotropic GABA receptors. (E) Cumulative probability plot comparing decay time constants of events before and after application of Bicuc.

FIG. 3. Some ipRGCs exhibit predominantly GABAergic (A–C) or glutamatergic (D–F) mPSCs. (A and D) Time vs. amplitude plots. (B and E) Representative current traces. (C and F) Amplitude histograms in the presence of synaptic blockers.