A color-coding amacrine cell may provide a blue-Off signal in a mammalian retina

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Retinal amacrine cells are thought to lack chromatic or color-selective light responses and have only a minor role in color processing. We found that a type of mammalian (Ictidomys tridecemlineatus) amacrine cell selectively carries a blue-On signal, which is received from a blue or short wavelength–sensitive (S) cone on bipolar cell. This glycinergic inhibitory S-cone amacrine cell is ideally placed for driving blue-Off responses in downstream ganglion cells.

Blue-green dichromacy is the fundamental color mechanism in mammals and provides advantages relative to monochromacy with respect to color discrimination and circadian entrainment. Two blue-green opponent responses have been recorded in retinal ganglion cells: blue-On/short green-Off and blue-Off/long green-On, signaling blue light On and Off, respectively. A distinctive S-cone On bipolar cell (SCB) exclusively receives S-cone input and relays blue-On signals to the small bistratified ganglion cell, which also receives yellow-Off signals from Off cone bipolar cells (that contact both medium wavelength–sensitive and long wavelength–sensitive cones). In contrast, the retinal pathway that mediates the blue-Off response has not yet been identified. Typically, retinal Off signals originate from Off cone bipolar cells. However, an S-cone–selective Off bipolar cell has not been identified. Alternatively, an inhibitory amacrine cell can invert blue-On signals, obviating the need for an S-cone Off bipolar cell. However, amacrine cells are not usually considered to have a direct role in color vision insofar as color-selective amacrine cell responses have not been recorded.

We recorded from amacrine cells in slices from the cone-dominant ground squirrel to identify those receiving S-cone–selective inputs (Online Methods). To search for S-cone–mediated amacrine cell signals, we used a silent substitution technique that selectively stimulated S cones (Supplementary Fig. 1). Most amacrine cells either lacked substantial S-cone input or received nonselective cone inputs (Fig. 1a and Supplementary Fig. 2). A small number, however, demonstrated selective responses to the S-cone isolating stimulus (SCIS; Fig. 1b and Supplementary Fig. 2), and were thus referred to as S-cone amacrine cells (SCAs, n = 12). SCA voltage responses followed blue-light modulation, whereas green light alone elicited little response, indicating prominent chromatic selectivity (Fig. 1b). SCAs exhibited abundant spontaneous synaptic currents that were presumably triggered by frequent excitatory inputs (excitatory postsynaptic currents) from bipolar cells (Fig. 1c). The SCA response to a 1-s blue-light pulse was a relatively sustained depolarization with a hyperpolarizing overshoot at pulse offset (Fig. 1c), which may temporarily pause the SCAs synaptic output. Assuming that the SCA output is inhibitory, suppression of inhibition can provide an excitatory drive to downstream neurons.

Tracer-filled SCAs (Fig. 2) had a narrow dendritic field (60.5 ± 5.2 µm, n = 10) and a two-tier ramification near the inner and outer inner plexiform layer (IPL) boundaries (Fig. 2a). Dendrites descended through the IPL, bestowing a bell-shaped profile in vertical sections (Fig. 2a,b,f,g). The upper dendritic tier was slender and sparse compared with the lower tier, which contained large varicosities (Fig. 2a,c,f,g). The varicosities stratified at the same level as SCB axon terminals (Fig. 2e,g). Antibody to the glycine transporter labeled SCAs (n = 4; Fig. 2d), a characteristic of glycinergic amacrine cells, which suggests that SCAs can inhibit downstream neurons.

Consistent with its proposed position in the S-cone pathway (Fig. 3a), SCA responses to the SCIS were abolished by 4 µM (2S)-2-amino-4-phosphonobutyric acid (l-AP4), which blocks synaptic transmission between S cones and SCBs (10.0 ± 1.9% of control, n = 3; Fig. 3a). SCA responses to the SCIS were reversibly abolished.

Figure 1 SCA light responses. (a) An amacrine cell was unresponsive to SCIS (upper), but was responsive to green LED (lower); one-tier ramification (right). Scale bar represents 10 µm. (b) Sample SCA was unresponsive to green LED stimulus (upper), but was responsive to SCIS (lower). (c) Sample SCA current (upper) and voltage (lower) responses to a 1-s blue-light pulse. Inset, spontaneous excitatory postsynaptic currents. GCL, ganglion cell layer; INL, inner nuclear layer.
by 5 µM of the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), which should block signaling at the bipolar-to-amacrine cell synapse (8.7 ± 3.5% of control, n = 5; Fig. 3a). In addition, SCB axon terminals were physically opposed to puncta that were labeled by antibody to the AMPA-type glutamate receptor subunit GluR2/3 (Fig. 3a).

The presumed role of SCAs in the S-cone pathway mirrored that of AII amacrine cells in the rod pathway (Fig. 3b), which form gap junctions with SCBs and may carry S-cone signals14. Although the block of SCIS input to SCAs by NBQX suggested otherwise, we wanted to further rule out the possibility that the SCAs in the S-cone pathway were AII cells. SCA responses to the SCIS were unaffected by 100 µM meclofenamic acid (MFA), a gap junction blocker (95.0 ± 6.6% of the control, n = 3; Fig. 3b), which blocked gap junctional conductances between cones in the same retinal slice (data not shown). We conclude that SCAs are not AII cells, but are instead unique chromatic-selective amacrine cells.

Could SCAs supply a blue-Off signal to blue-Off/green-On ganglion cells? Anatomical descriptions of blue-Off ganglion cells suggest that they are monostratified cells with dendrites ramifying near the inner border of the IPL7,15, matching the stratification of the lower tier of SCA dendrites. Thus, it is possible that SCA varicosities form synapses with dendrites of the blue-Off cell.
ganglion cell (Supplementary Fig. 3). Alternatively, SCAs may inhibit green-On bipolar cell terminals, which are presynaptic to the ganglion cell (Supplementary Fig. 3).

Another potential target of SCAs is the intrinsically photosensitive retinal ganglion cell (ipRGC)\(^2,3\) that, in the primate retina, had blue-Off/yellow-On cone responses in addition to intrinsic photosensitivity\(^3\). Dendrites of ipRGCs ramify at the inner and outer borders of the IPL, co-stratifying with SCA dendrites. If SCAs provide blue-Off inputs to ipRGCs, they could function in the ancient blue-yellow color system that synchronizes the biological clock with the environment by signaling dawn-dusk spectral shifts\(^1-3\). In conclusion, our findings support the existence of a color-coding amacrine cell that is well-positioned to provide blue-Off signals in the color pathway of the mammalian retina.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.C. and W.L. conceived the project and designed the experiments. S.C. performed the experiments. S.C. and W.L. analyzed the data. W.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Preparation and electrophysiology. All animal procedures were approved by the Animal Care and Use Committee of the National Eye Institute. The procedures for making ground squirrel retinal slices have been described\textsuperscript{16,17}. During recording, we superfused the tissue with bicarbonate-buffered Ames’s media (Sigma-Aldrich). The pipette solution contained (in mM): 99 mM potassium gluconate, 32 mM KCl, 4.5 mM MgCl\textsubscript{2}, 10 mM HEPES, 5 mM tetrapotassium BAPTA (Molecular Probes), 3 mM ATP, 1 mM GTP and 0.1 mM Neurobiotin (Vector Laboratories). We adjusted the pH of the media to 7.4 with KOH. We mounted slices on a Zeiss Examiner 1D microscope and superfused continuously at 31–33 °C. Slices were stimulated with full-field light generated from green (574 nm) and blue (465 nm) LEDs mounted on a microscope video port. We modulated the intensities of the green and blue LEDs with pulse-width modulation controlled by custom-made software. According to the response-intensity curve of the M cones\textsuperscript{16}, we set the mean intensity of the green LED to be close to the half saturating level (2.5 × 10\textsuperscript{4} photons per μm\textsuperscript{2}) in most experiments. Recordings were obtained with Axopatch 200B amplifiers (Molecular Devices), and currents were low-pass filtered at 1 or 5 kHz using the four-pole Bessel filter on the amplifier. Data were digitized at 10 kHz using an ITC-18 interface (HEKA) controlled by a Dell computer running IgorPro 6.0 (WaveMetrics). We analyze data with custom-made software (IgorPro 6.0). In voltage-clamp experiments, we maintained cone, bipolar and amacrine cell membrane voltages at –70 mV unless otherwise indicated. NBQX (1-AP4 from Tocris, and MFA from Sigma-Aldrich). All other chemicals were from Sigma-Aldrich.

Live calibration of the SCIS. To identify amacrine cells that carry S-cone signals, we needed a light stimulus that exclusively stimulated S-cones. Owing to their broad spectral sensitivity profile, M cones in the ground squirrel retina also respond to short wavelength stimuli\textsuperscript{19}. To overcome this problem, we adopted an SCIS obtained using the silent substitution method\textsuperscript{20}. We performed whole-cell recordings from M cones in the ground squirrel retinal slices and stimulated the retina with full-field light generated from green (574 nm) and blue (465 nm) LEDs. The intensities of the green and blue LEDs were modulated by 1- or 2-Hz sinusoidal waves (180° out of phase). We then adjusted the intensity of the blue (465 nm) LED, so as to render it equal to the green LED in its efficacy to elicit M-cone responses. Consequently, the membrane potential of M cones was not modulated during the SCIS and any responses recorded from downstream retinal neurons were, in all likelihood, derived from S cones (Supplementary Fig. 1a). Indeed, S cones responded vigorously to such a stimulus (Supplementary Fig. 1b). The contribution of rod input is minimal because the ground squirrel retina is cone dominant, with fewer than 15% rods\textsuperscript{21}. Furthermore, in most experiments, the average green LED intensity was set close to half-saturation of M cones, a level at which rods are largely saturated (data not shown).

To validate the SCIS, we recorded from a type of Off cone bipolar cell (b7) that has been shown to lack S-cone inputs by means of direct electrical stimulation of S cones\textsuperscript{18}. As expected, although the b7 cell responded strongly to a green LED stimulus, it did not respond to our SCIS (Supplementary Fig. 1c). Anatomical analysis of the recorded b7 cell confirmed that its dendrites avoided S cones, even the ones well within its dendritic field (Supplementary Fig. 1d–f). Similar results were obtained from two additional cells. Thus, the SCIS reliably prevents false-positive results.

S-cone response index as a function of stratification. Voltage responses to the SCIS were recorded from a total of 157 ground squirrel amacrine cells. Separately, their voltage responses to green LED light were recorded, comprising their respective M-cone stimulus responses. Then, SCIS response amplitudes were divided by the sum of the SCIS response amplitudes and the M-cone stimulus response amplitudes. This result (B/(B + G)) was termed each amacrine cell’s S-cone response index. For 55 amacrine cells with post-recording images, their S-cone response index was then plotted as a function of that cell’s axon terminal stratification in the retina’s IPL (Supplementary Fig. 2). Location in the IPL was calculated as percentage of IPL depth, measured from its outermost margin (adjacent to the inner nuclear layer, 0% depth) to its innermost margin (adjacent to the ganglion cell layer, 100% depth). Amacrine cells with an S-cone response index above 0.8 were viewed as having a strong S-cone-selective response and were referred to as SCAs. All of these SCAs stratified in the innermost IPL stratum, roughly corresponding to the area in which an amacrine cell may receive input directly from S-cone bipolar cell axon terminals (Supplementary Fig. 2).

Immunocytochemistry. After recording or injection, slices were fixed and processed for immunocytochemistry as previously described\textsuperscript{2}. We used antibodies to GluR2/3 (1:100, Millipore #07-598), GluR4 (1:100, Millipore #AB1508), GluR5 (1:50, Santa Cruz Biotechnology #SC7616), glycine transporter 1 (1:1,000, Millipore #AB1770) and glycine (a kind gift from D. Pow, the University of Queensland). Slices were mounted and imaged in the vertical or flat-mount orientation. Images were acquired with a LSM-510 confocal microscope (Zeiss) and edited with Zeiss Zen software and Photoshop CS4 (Adobe Systems). An α-Plan-Apochromat 63×/1.40 oil immersion lens was used. Zeiss Zen software was used to generate three-dimensional rendering of cells.

Statistics. The paired \( t \) test was used for statistical analysis. Difference was considered statistically significant when \( P < 0.01 \). The data are represented as mean ± s.d.

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