Spectral Responses in Zebrafish Horizontal Cells Include a Tetrachro- 
matic Response and a Novel UV-Dominated Triphasic Response

Victoria P. Connaughtont and Ralph Nelson2

1Department of Biology, American University, Washington, DC 20016; and 2Neural Circuitry Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Rockville, Maryland

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Connaughton VP, Nelson R. Spectral responses in zebrafish horizontal cells include a tetraphasic response and a novel UV-dominated triphasic response. J Neurophysiol 104: 2407–2422, 2010. First published July 7, 2010; doi:10.1152/jn.00644.2009. Zebrafish are tetra-chromats with red (R, 570 nm), green (G, 480 nm), blue (B, 415 nm), and UV (U, 362 nm) cones. Although neurons in other cyprinid retinas are rich in color processing neural circuitry, spectral responses of individual neurons in zebrafish retina, a genetic model for vertebrate color vision, are yet to be studied. Using dye-filled sharp microelectrodes, horizontal cell voltage responses to light stimuli of different wavelengths and irradiances were recorded in a superfused eyecup. Spectral properties were assessed both qualitatively and quantitatively. Six spectral classes of horizontal cell were distinguished. Two monophasic response types (L1 and L2) hyperpolarized at all wavelengths. L1 sensitivities peaked at 493 nm near the G cone absorbance maximum. Modeled spectra suggest equally weighted inputs from both R and G cones and, in addition, a “hidden opponency” from blue cones. These were classified as R−/−G−/G−(b+). L2 sensitivities were maximal at 563 nm near the R cone absorbance peak; modeled spectra were dominated by R cones, with lesser G cone contributions. B and UV cone signals were small or absent. These are R−/−G−/−. Four chromatic (C-type) horizontal cells were either depolarized (+) or hyperpolarized (−) depending on stimulus wavelength. These types are biphasic (R+/+G−/B−−) with peak excitation at 467 nm, between G and B cone absorbance peaks, UV trichromatic (r−/−G+/U−−) with peak excitation at 362 nm similar to UV cones, and blue triphasic (r−/−G+/B−/U−−) and blue tetraphasic (r−/−G+/B−/U−−), with peak excitation at 409 and 411 nm, respectively, similar to B cones. UV trichromatic and blue tetraphasic horizontal cell spectral responses are unique and were not anticipated in previous models of distal color circuitry in cyprinids.

INTRODUCTION

Tetrachromatic vision is common in lower vertebrates (fish and turtles) and birds. In these species, an ultraviolet (UV or U) sensitive cone photoreceptor is present in addition to cones sensitive to red, blue, and green light. Zebrafish, an animal model rich in genetic manipulations, is a tetrachromat. This study identifies the impact of tetrachromacy on spectral properties of zebrafish horizontal cells and the distal retinal circuitry that processes this spectral information.

Horizontal cells contact cones directly, and their light responses reflect selective input from different combinations of spectral cone types. In other species, luminosity (monochromatic or L-type) horizontal cells are hyperpolarized after stimulation with all wavelengths, with sensitivity paralleling the absorbance spectrum of red cones. The response of chromaticity (or C-type) horizontal cells changes polarity depending on stimulating wavelength. C-type biphasic cells depolarize for red cone selective stimuli but hyperpolarize for stimuli maximally absorbed by green or blue cones. In trichromats, C-type triphasic responses depolarize for midspectral stimuli selective for green cones. The depolarization is flanked by long and short wavelength hyperpolarizations arising from red and blue cones (Djamgoz 1984; Djamgoz and Ruddock 1979; Fukurotani and Hashimoto 1984; Gottesman and Burkhardt 1987; Hashimoto and Inokuchi 1981; Hashimoto et al. 1988; Norton et al. 1968; Ohtsuka and Kouyama 1986; Yazulla 1976).

The different spectral responses of horizontal cells are attributed to different patterns of cone contacts found among different morphological types. In goldfish, cone horizontal cells are classified as H1, H2, and H3 types, roughly in order of dendritic diameter. H1 cells are monophasic, H2 cells are biphasic, and H3 cells are triphasic (De Aguiar et al. 2006; Downing and Djamgoz 1989; Stell et al. 1975; for reviews, see Kamermans and Spekreijse 1995; Twig et al. 2003). Selective cone contacts combine with feedback circuits between horizontal cells and cones (Kamermans et al. 1991, 1989a,b; Stell and Lightfoot 1975) to comprise the underlying circuitry generating multiphasic horizontal cell responses to color.

Although horizontal cell processing of red, green, and blue cone inputs is well characterized, the role of the UV cone and its postsynaptic connections is less studied. In turtle, all horizontal cells are hyperpolarized by UV light stimulation (Ammermuller et al. 1998; Ventura et al. 1999), but only the triphasic cells receive direct UV cone excitation (Ventura et al. 2001; Zana et al. 2001). In fish, triphasic horizontal cells also receive inputs from UV cones (Hashimoto et al. 1988) and, in some species of cyprinids, a tetrachromatic response—hyperpolarized to red, depolarized to green, hyperpolarized to blue, depolarized to UV—has been reported (De Aguiar et al. 2006; Fukurotani and Hashimoto 1984; Harosi and Fukurotani 1986; Hashimoto et al. 1988).

We identify six spectral types of horizontal cell in the zebrafish retina using sharp electrode recording techniques in perfused retina-eyecup wholemounts. Of particular interest is the identification of two horizontal cell types that process UV cone signals: a novel triphasic type that is selectively hyperpolarized by UV stimulation and a tetrachromatic type with light responses similar to blue triphasic responses but also with UV depolarizations. In microelectrode stains, both these UV signaling cell types were wide in dendritic field, analogous to the H3 morphological type. There is also a blue triphasic type of similar morphology. Monophasic and biphasic responses were also identified and these responses were generated in types
morphologically similar to H1/2 cells. These data add sophisticated tetrachromatic spectral processing to previous descriptions of distal retinal circuitry and suggest that zebrafish is an exceptionally interesting model of vertebrate color vision.

**METHODS**

**Preparation and perfusion of zebrafish retina eyecups**

Light-adapted adult zebrafish (*Danio rerio*, EK strain) were decapitated according to an Animal Study Protocol approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke, National Institutes of Health, in accordance with National Research Council Guidelines and Public Health Service Policy on Humane Care and Use of Laboratory Animals. Eyes were removed and mounted upright on 10-mm squares of black nitrocellulose filter paper (Millipore, Billerica, MA). Under a dissecting microscope (MZ12-5, Leica, Bannockburn, IL), the anterior segment was removed. Short radial cuts were made in the limbus, allowing the eyecups to flatten for microelectrode access.

MEM (glutamine-, HEPES-, and pyruvate-free, Invitrogen, Carlsbad, CA, catalog number 11090–099) was the perfusion solution used for zebrafish eyecups. The medium was equilibrated with bubbled 95% O2–5% CO2. MEM is an Earles salts, bicarbonate-buffered, cell culture medium containing amino acid and vitamin supplementation. Stable ERG b-wave responses are recorded from zebrafish eyecups for periods in excess of 10 h using this medium (Nelson and Singla 2009).

Flattened retina eyecups were rinsed for 10 min in bubbled MEM and transferred to a stimulation, recording, and perfusion chamber mounted on a microscope stage (Olympus BX51, Olympus America, Center Valley, PA). Submerged eyecups were superfused with MEM from the vitreal side (0.3 ml/min) through a 3.25-in gel-loader pipettor tip (Thomas, Swedesboro, NJ).

**Electrodes, recording, and staining**

Sharp microelectrodes were pulled to a tip resistance of ∼300 MΩ (3 M KCl) on a Flaming/Brown microelectrode puller (Model P-87, Sutter Instrument, Novato, CA). For microelectrode staining, tips (∼1,000 MΩ) were filled with Alexa 594 dye dissolved in 200 mM KCl (Invitrogen). The remainder of the microelectrode was filled with 300 mM KCl. Stain was injected electrophoretically (∼300 nA, 15 s).

All recordings were made during the 14-h day cycle of the zebrafish habitat in a dark recording cage without imposed background illumination. Cell penetration was signaled by appearance of membrane voltages and responses to 570 nm test stimuli (600 ms duration, 3 s interval). Raw data traces were acquired with pCLAMP (ver. 8.3) software and an Axopatch 200B amplifier. Traces were imported into Origin (v. 8.0, ORIGINLab, Northampton MA) to display them graphically, to measure response amplitudes, and to fit spectral functions to response data sets using a model and nonlinear-least-squares fits (NLSF). The mean change in membrane potential over the first 300 ms of stimulation was used as the measure of response amplitude and scaled by a constant (∼1.4) to determine the peak amplitude. Taking the mean of many signal measurements throughout the maintained light response of horizontal cells reduced the impact of noise. In comparison, the direct measure of peak amplitude looks at only a single point in time. This mean was adjusted to the peak amplitude by a fixed factor for each cell. The mean of these factors was 1.36 ± 0.10 (n = 50).

z-axis image stacks of stained cells were obtained manually (40× water, Olympus America; RS170 cooled CCD video camera, Dage MTI, Michigan City, IN). Out-of-focus light was removed in Adobe Photoshop (v 7.5) before z-axis projections, and look up table (LUT) transformations were made in ImageJ. Morphological measurements were made in Origin 7.5 or ImageJ.

**Optical source and calibration**

Stimuli were delivered through the microscope epifluorescence illumination port and a 4 × UV plan-apo objective. A Xenon source focused onto a liquid light guide through intervening filter wheels (Lambda 10–2) provided the excitation light. Interference filters provided wavelengths from 330 to 650 nm at 40 nm intervals (20 nm half-band, Chroma Technology, Brattleboro, VT). Metallic ND filters provided stimulus attenuations from 0.5 to 7.5 log units (Andover, Salem, NH). Monochromatic illumination was quantified with a calibrated silicon detector (Newport, Irvine, CA). Maximum intensities ranged from 6.3 log(hv·μm−2·s−1) at 330 nm to 8.0 log(hv·μm−2·s−1) at 490 nm. To stimulate horizontal cell spectral responses, four different fixed sequences containing 9–27 broad field stimuli with wavelengths ranging from 650 (red) to 330 nm (UV) and irradiances ranging from 0.5 to 7 log(hv·μm−2·s−1) were delivered.

**Spectral model**

We model horizontal cell light responses as resulting from a sum of four cone signals. Each cone signal is represented as an independently saturable Hill function (Nelson and Singla 2009). The model does not differentiate synaptic pathways of individual cone inputs but says simply that, whatever the pathway, the signals arising from different cone colors are separately saturable. All cells included herein were modeled. To be modeled, a series of stable responses to stimulus sequences of different wavelengths and irradiances were required. The minimum stimulus number was 9, because this is the number used in the cell staining sequence, which covers all wavelengths with a single fixed irradiance. The largest number of stable responses recorded from an individual cell was 166. To achieve this number, multiple stimulus sequences were delivered, with repetitions, so that a greater range of wavelengths and irradiances were studied. The model appears in Eq. 1

\[
V = V_{g}I/(I + k_{gb}/A_{g}(\lambda)) + V_{b}I/(I + k_{gb}/A_{b}(\lambda)) + V_{u}I/(I + k_{gb}/A_{u}(\lambda)) + V_{u}I/(I + k_{gb}/A_{u}(\lambda)) \quad (1)
\]

Peak horizontal cell response amplitudes (V) for stimuli of different wavelengths (λ) and irradiances (I) are fit by this three-dimensional model to yield amplitudes and sensitivities for component cone color signals. The parameters \(V_{g}, V_{g}, V_{b}, V_{u}\) and \(V_{u}\) are the saturated voltages for individual red, green, blue, and UV cone signals that combine to make up the horizontal cell response. The parameters \(k_{gb}\) and \(k_{ub}\) are the mean, half-saturating-irradiance values for red, green, blue, and UV cones; for these functions, we used published absorption nomograms. For red, green, and blue cones, the nomogram of Hughes et al. (1998) was used. The Palacios nomogram (Palacios et al. 1998) was used for the UV cone. The coefficients of the order 8 polynomials used to calculate the absorption nomograms can be found in Nelson and Singla (2009). A nonlinear least squares method (NLSM, Origin 8.0, OriginLab, North Hampton, MA) fit horizontal cell data sets to the model (Eq. 1).

Two variants of the model (Eq. 1) were tested. In the first (K5), \(k_{g}\) is set equal to \(k_{gb}\). In the second (KSU), \(k_{g}\) is set equal to \(k_{gb}/10\). In other words, for KSU, the UV cone signal is preset to be 1 log unit more sensitive than red, green, or blue cones, as judged by half-saturation. This adjustment was spurred by the finding that UV triphasic horizontal cells were found to be ∼1 log unit more sensitive than other horizontal cell types. Based on values of the coefficient of...
Spectral responses of zebrafish horizontal cells were modeled to identify and quantify inputs from the different cone photoreceptors. Resultant fit parameters of the model, \( V_s, V_g, \) and \( V_u \) represent the different saturated amplitudes from the contributing R, G, B, and UV cones, respectively, and are presented as mean \( \pm SD \) for each horizontal cell spectral type. Positive values indicate depolarizing (sign-inverting) input to horizontal cells; negative values indicate sign-conserving (hyperpolarizing) input. Each mean value was calculated from a mean of 22–35 responses per cell, and parameters were averaged from at least five modeled cells of each spectral type. As a result of the model, a color code was assigned to each spectral type, with capital letters identifying major cone inputs, lowercase letters representing minor cone inputs, and \( r^2 \) reflects the fit of the model to the data.

determination (\( r^2 \)), the K5 model was better suited to monophasic and biphasic response patterns, and the K5U model was better suited to triphasic and tetraphasic response patterns. The use of models with different \( k_u \) values does not imply multiple sensitivity types for UV cones. The K5 model is applied only to physiological types with no UV cone signals (\( V_u = 0 \)).

The purpose of these two models is to predict single-wavelength irradiance response functions, to generate spectral sensitivity curves for UV cones. In the K5 model, these values are the same; in the K5U model values indicate sign-conserving (hyperpolarizing) input. Each mean value was calculated from a mean of 22–35 responses per cell, and parameters were averaged from at least five modeled cells of each spectral type. As a result of the model, a color code was assigned to each spectral type, with capital letters identifying major cone inputs, lowercase letters representing minor cone inputs, and \( r^2 \) reflects the fit of the model to the data.

**Results**

**Terminology**

Light responses in zebrafish horizontal cells were identified as either monophasic (or L-type, 66%) or multiphasic chromatic (C-type; 34%). Two monophasic and four chromatic patterns were found. Monophasic responses included a spectral type peaking near the green cone absorbance maximum (L1), in addition to the more commonly described type with a spectral peak similar to red cones (L2). Multiphasic cells included a unique triphasic UV-prefering cell and a blue-prefering tetraphasic response, in addition to the more conventional triphasic blue-prefering and biphasic blue-green preferring responses. Overall, responses from 149 horizontal cells were recorded, modeled, and classified. A subset of recorded cells (24) was intracellularly labeled, allowing us to correlate cell morphology with spectral response.

**Tetraphasic cells**

In seven cells, stimulation with different wavelengths resulted in a tetraphasic response (Fig. 1), characterized by a hyperpolarization to red-yellow stimulation (650–570 nm), a depolarization to green and blue-green (530 and 490 nm), a hyperpolarization to blue and violet (450, 410, and/or 370 nm), and a depolarization to UV (330 nm). At threshold, the mean null points were 539, 447, and 377 nm (Table 2). Response waveforms were rectangular and sustained at all wavelengths except the UV, where multiphasic waveforms were observed (Fig. 1). Mean peak amplitude for either depolarizing or hyperpolarizing phases was 8–9 mV (Table 2), with a sensitivity peak for hyperpolarization at 411 nm and a sensitivity peak for depolarization at 485 nm. In tetraphasic cells, increasing stimulus irradiance changed the spectral properties. In particular, the response to UV stimulation reversed from depolarizing to hyperpolarizing as stimulus irradiance increased (Figs. 5C and 6C). This eliminated the UV-null point and converted the response to one similar to triphasic blue-prefering cells. At 490 nm, responses also changed from depolarizing to hyperpolarizing with increasing irradiance (Fig. 5C). This change, however,
TABLE 2. Physiological properties of horizontal cell spectral types  

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Color Code</th>
<th>Spectral Model</th>
<th>Max Hyp &amp; Dep Amplitudes</th>
<th>Mean Wavelengths</th>
<th>Maximal Sensitivity</th>
<th>Null Points</th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td>Tetraphasic</td>
<td>r−/G+/B−/u+</td>
<td>K5U</td>
<td>−7.51 ± 2.23mV</td>
<td>411 nm (hyperpolarization)</td>
<td>539 nm, 447 nm, 377 nm</td>
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<tr>
<td>triphasic Blue</td>
<td>r−/G+/B−/u−</td>
<td>K5U</td>
<td>+8.87 ± 2.51mV</td>
<td>485 nm (depolarization)</td>
<td>539 nm, 450 nm</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>triphasic UV</td>
<td>r−/G+/U+</td>
<td>K5U</td>
<td>−4.86 ± 2.79mV</td>
<td>409 nm (hyperpolarization)</td>
<td>536 nm (hyperpolarization)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Biphasic</td>
<td>R+/G−/B−</td>
<td>K5</td>
<td>+2.31 ± 1.23mV</td>
<td>485 nm (depolarization)</td>
<td>472 nm (depolarization)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>R−/G−/(b+)</td>
<td>K5</td>
<td>−6.45 ± 1.06mV</td>
<td>467 nm (hyperpolarization)</td>
<td>524 nm</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>R−/g−</td>
<td>K5</td>
<td>−7.23 ± 2.20mV</td>
<td>493 nm</td>
<td>—</td>
<td>86</td>
<td></td>
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</tbody>
</table>

Amplitude values (mean ± SE) show the maxima of direct hyperpolarizing (hyp; −) and feedback depolarizing (dep; +) signals seen in the light responses from a spectral type (given as mean ± SE). The mean of spectral maxima (depolarization), minima (hyperpolarization), and null points were calculated from spectral model fits (Eq. 1; Table 1). Color code letter designations reflect the means of cone signals in each class (Table 1).

did not eliminate the blue null point, but accompanied it’s migration to longer wavelengths (Fig. 6C).

Blue and UV triphasic cells

As a group, triphasic horizontal cells (n = 15) hyperpolarized to red-yellow (650, 610, and/or 570 nm) but depolarized to green and near blue (530, 490, and/or 450 nm). For deep blue or UV stimulation, the response of blue and UV triphasic cells differed. UV triphasics (n = 10) hyperpolarized strongly at 370 and 330 nm (Fig. 2A), whereas blue triphasics (n = 5) responded weakly (Fig. 2B). Response waveforms were sustained. For blue triphasics, the mean sensitivity peak for hyperpolarization was 409 nm; for UV triphasics, it was 362 nm. The mean yellow and blue null points for blue triphasics and blue triphasic cells were similar, ~540 and 450 nm, respectively (Table 2). Mean yellow and blue null points for UV triphasics were distinct at 556 and 408 nm (Table 2).

Mean depolarizing and hyperpolarizing peak amplitudes ranged from 2 to 9 mV (Table 2). For triphasics, increasing stimulus irradiance changed the response waveform in two cells. In one, the hyperpolarizing response to long wavelengths became depolarizing, and an atypical green-blue biphasic response was observed. In the other, the depolarizing response to middle wavelengths became hyperpolarizing, and a monophasic (L-type) response was seen. In the remaining cells, the triphasic response was unaltered by changes in stimulus irradiance (Fig. 6, B and F).

Morphologically, triphasic responses were recorded from cells with large dendritic fields (Fig. 4). Primary dendrites of these cells were long and extended away from the cell body. Dendritic field diameters of UV-preferring cells averaged 42 μm along the major axis and 32 μm along the minor axis (n = 4; Table 3). Axons are seen on these cells, with an average length of 176 μm. The single blue-preferring triphasic cell that was labeled was similar in field size to UV cells, measuring 40 μm

![FIG. 2. C-type triphasic response patterns. A: UV-triphasic cell (r−/G+/U−) is hyperpolarized by red and yellow (650 and 570 nm), depolarized by blue-green (490 nm), hyperpolarized by the brighter irradiance of blue-violet (410 nm), and strongly hyperpolarized by both dim and bright UV (330 nm) irradiances. B: blue-triphasic cell (r−/G+/B−/u−) is hyperpolarized by red (650 nm), depolarized by yellow (570 nm), depolarized at threshold by blue-green (490 nm), strongly hyperpolarized by violet (410 nm) for both dim and bright irradiances, and hyperpolarized only for the brighter of the UV (370 nm) stimuli. Stimulus irradiances, given near the end of each trace, delivered approximately equal quanta at each wavelength, except that more energy is required at 650 nm to show the response. Units are log(hv·μm⁻²·s⁻¹).]
along the major axis and 27 μm along the minor axis. No axon was seen on this cell.

**Biphasic and monophasic cells**

Three other spectral response patterns were recorded (Fig. 3). In 28 cells, a blue biphasic response was identified (Fig. 3A). These cells depolarized for long wavelengths (570 and 650 nm) and hyperpolarized for middle, short, and UV wavelengths (≤530 nm). The mean null point at threshold occurred at 524 nm, and the maximal and most sensitive hyperpolarizing response (∼6mV) was recorded in the blue (mean 467 nm; Table 2). In the great majority of cells, increasing stimulus irradiance did not change the biphasic response pattern (i.e., Fig. 6A), although, in four cells, increasing stimulus irradiance changed the response polarity at longer wavelengths resulting in an atypical monophasic response. In general, the response waveform was sustained, although transients at light on or off were sometimes observed, particularly for wavelengths near the null point.

In the remaining cells (n = 99), monophasic responses were recorded. These cells hyperpolarized at all wavelengths and irradiances tested. Amplitudes monotonically increased with stimulus irradiance (Figs. 3, B and C, 5, D and E, and 6, D and

<table>
<thead>
<tr>
<th>HC Physiology</th>
<th>Color Code</th>
<th>Cells Stained</th>
<th>HC Morphology</th>
<th>Cell Body Major Diameter, μm</th>
<th>Dendritic Field Major Diameter, μm</th>
<th>Dendritic Area, μm²</th>
<th>Axon Length, μm</th>
<th>Axons Stained</th>
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<tbody>
<tr>
<td>L1 R~/G~/~(b+)</td>
<td>1</td>
<td>H1/H2</td>
<td>13 × 9</td>
<td>28 × 28</td>
<td>621</td>
<td>68</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>L2 R~/g~</td>
<td>13</td>
<td>H1/H2</td>
<td>10 ± 3 × 7 ± 3</td>
<td>26 ± 5 × 20 ± 6</td>
<td>416 ± 193</td>
<td>231 ± 97</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

**Monophasic (L-types)**

**Biphasic, triphasic, tetraphasic (C-types)**

| Biphasic | R~/G~/~B~— | 5      | H1/H2 | 11 ± 3 × 8 ± 3 | 26 ± 6 × 20 ± 5 | 422 ± 162 | 277 ± 52 | 3 |
| UV triphasic | r~/G~/~U— | 4      | H3    | 11 ± 2 × 8 ± 1| 42 ± 6 × 32 ± 4 | 1048 ± 269 | 176 ± 99 | 4 |
| BL triphasic | r~/G~/~B~/~u— | 1     | H5    | 8 × 7    | 40 × 27          | 856           | —      | 0 |
| BL tetraphasic | r~/G~/~B~/~u+ | 0     | —      | —       | —                 | —              | —      | — |

For each spectral class of horizontal cell, measurements of cell body diameter, dendritic field diameter, dendritic area, and axon length were made from cells intracellularly stained with Alexa 594 after intracellular recordings of light responses. Values are means ± SE. Color code letter designations reflect the means of cone signals in each class, as determined from the spectral model (Eq. 1; Table 1).

![Fig. 3](http://jn.physiology.org/)

**FIG. 3.** Biphasic C-type and monophasic L-type responses do not involve UV cones. A: C-type biphasic cell (R~/G~/~B~—) is depolarized by red and yellow (650 and 570 nm) but hyperpolarized by blue-green, violet, and UV (490, 410, and 330 nm). B: L1 type monophasic cell [R~/G~/~(b+)~] is hyperpolarized by all wavelengths. Red and green cones contribute about equally to the response, but hidden opponency from blue cones reduces response amplitudes for 410 nm stimulation. C: L2 type monophasic cell (R~/g~—) is hyperpolarized by all wavelengths. Peak voltage response occurs with yellow (570 nm) stimulation, at the red cone absorbance peak. For all traces, responses to 2 different stimulus irradiances are presented with the irradiances given near the end of each trace. These stimuli deliver in parallel approximately equal quanta at each wavelength, except that more energy is required at 650 nm to show red responses. Units are log(hv·μm²·s⁻¹).
E). Sensitivity was maximal for red and/or green light stimulation. Based on modeled spectral properties (below), monophasic cells were classified as either red-preferring L2-types (peak sensitivity >530 nm, mean 563 nm, n = 86) or red/green L1-units (peak sensitivity ≤530 nm, mean 493 nm, n = 13), following the classification of monophasic cells in the literature (Hashimoto and Inokuchi 1981; Hashimoto et al. 1988; Ruddock and Svaietichin 1975).

The response waveform of L1 cells was square and sustained (Fig. 3B). No on- and/or off-transients were observed after initial stimulation with long wavelength (570 nm) light; however, in some L1 cells, transients occurred at stimulus onset and offset with shorter wavelengths (i.e., blue or UV; Fig. 8). Peak amplitudes of L1 cells were larger than L2 cells, averaging 7 mV (Table 2). Maximal L2 responses averaged ~4 mV (Table 2). The response waveform of L2 cells (Fig. 3C) was sustained, sometimes with a transient on and/or transient off component.

Morphologies (Fig. 4B) and measurements (Table 3) of L1-type, L2-type, and biphasic cells were similar. The diameter of the dendritic field measured 28 μm (tip to tip) along the major and minor axes for L1 cells and averaged 26 μm along the major axis and 20 μm along the minor axis for L2 units (n = 13). Axons were found in 11 of 13 L2 cells, with a mean length of 231 μm, and on the single stained L1 unit, the axon measured 68 μm. Biphasic C-type dendritic fields measured 26 μm along the major axis and 20 μm along the minor axis (n = 5). Axons were found in three of five cases, with a mean length of 277 μm. For all three spectral types, dendritic processes were short and did not extend far from, and were partially obscured by, the cell body.

Spectral model

To determine the contributions of cone signals underlying different horizontal-cell spectral responses, we modeled the amplitudes, in response to stimuli of different wavelengths and irradiances, as being composed of a summation of independently saturable signals arising from each cone type (Eq. 1). The model sums four irradiance- and wavelength-dependent Hill functions, each with a separate saturation amplitude for each cone signal. The mean of modeled parameters, and the range and median r^2 for model fits for all horizontal cell types, appear in Table 1. Irradiance-response curves for individual examples of each of the six spectral types of horizontal cell appear in Fig. 5. The curves in each panel represent predictions of the model for one particular wavelength (either 650, 490 or 390 nm) that are based on the full stimulus set for all wavelengths and not just the data points at that particular wavelength. In Fig. 6, the same model is used to interpolate amplitudes of responses at three different levels of equal quantal irradiance across the spectrum. The irradiance levels are chosen to evoke 20, 50, and 80% of red, blue, or green cone saturation amplitude, at their respective absorbance maxima, for that particular cell. In both Figs. 5 and 6, the modeled saturation voltages and half-saturation irradiances appear on the figure panels. These fit parameters were used to generate the curves in Figs. 5 and 6. V_{max} parameters for red, green, blue, and UV cone signals may be of either sign. In this way, monophasic, biphasic, triphasic, and tetraphasic spectral patterns, as well as sign-reversing irradiance-response curves, can all be represented with one or the other of the two models.

Horizontal cell spectral responses are typically coded using an R±/G±/B± nomenclature, where letters indicate the cone signals present, and ± indicates the sign of the signal. For zebrafish, we extend this to an R±/B±/G±/UV± system. Furthermore, we use capital and lowercase letters to represent the magnitude of the amplitude contributions. Hidden opponent signals are enclosed in parentheses. V_r, V_g, V_b, and V_u parameters are easily translated into this nomenclature (Table 1). Table 1 also shows the mean values of fit parameters for the six horizontal cell spectral types, together with the naming scheme derived from these parameters. The mean of threshold spectral peak and null points are also predicted from the cell models and appear in Table 2.

Modeled properties of monophasic cells

Dominant input to L2 cells was from red cones. L2 cells also received a lesser input from green cones (Table 1). For all L2 cells, 71% of net, absolute, saturated cone signal amplitude (i.e., the sum of the absolute values of V_r, V_g, V_b, and V_u) arose from red cones and 21% arose from green cones. Blue and UV cone influence was negligible. Accordingly, L2 cells are denoted R−/g− (Table 1).

Responses of L2 monophasic cells (47 of 86) were best fit by the K5 model, which considers all cones equal in sensitivity. The result should not be taken to imply that the UV cone signal is equal in sensitivity to red, green, and blue cones for monophasic cells. In the mean, there is no significant UV signal in this group (Table 1). For cells without a UV cone signal, the K5 model is appropriate. The median K5 r^2 for this group was 0.94. The model shows spectral peaks for this type migrating toward the green as irradiance levels increase (Fig. 6D). The magnitude of the move corresponded to the percentage of green cone input and occurred in 74 of 86 L2 monophasic cells. The maximum green shift observed occurred from 563 (peak at threshold, Table 2) to ~525 nm.

L1 units received nearly equal hyperpolarizing inputs from both red and green cones (Table 1; Fig. 6D), with these cones providing 46 and 42%, respectively, of net saturated input. The remaining significant signal was a hidden depolarizing influence of blue cones (13%). This was only rarely large enough to be seen as a blue depolarizing response (Fig. 8), but rather acted to reduce hyperpolarizing amplitudes in the blue. L1 cells are R−/G−/b+. These types (8 of 13) were also best fit by the K5 model. The median K5 r^2 for this group was 0.93. The spectrum of L1 cells was notably broad, often with a double-peaked appearance as seen in the low irradiance curve of Fig. 6D. In contrast to L2 cells, spectral peaks migrated from green to red as irradiance levels increased (13 of 13 cells), but this migration was smaller, going no further than 520 nm from a 493 nm threshold value (Table 2).

Modeled properties of biphasic cells

Biphasic cells were depolarized by red cones, whereas blue and green cones provided hyperpolarizing inputs. In the mean, blue and green cone hyperpolarizations provided 29 and 41%, respectively, of the summed absolute V_{max} inputs, whereas red cone depolarization provided 28%. As a group, input to bipha-
FIG. 4. Zebrafish horizontal cells generating different spectral responses can be distinguished morphologically. In general, the cell bodies of zebrafish horizontal cells are small (8–12 μm diam), as are the dendritic fields (20–40 μm diam). Long axons (200–400 μm) project from these cells to terminate in a long cylindrical varicosity or axon terminal, typical of cyprinids. The shown cells were filled with Alexa 594 by electrophoresis from the microelectrode (note recording electrode tip at bottom of E) after recording of spectral responses. Cells differed in soma size and dendritic extent (Table 3). Triphasic UV-preferring cells (B) and triphasic blue-preferring cells (A) had morphology similar to H3 type horizontal cells (Li et al. 2009; Song et al. 2008). Biphasic (C), monophasic L1-type (D), and monophasic L2-type cells (E) were morphologically similar to H1/H2 horizontal cells (Li et al. 2009; Song et al. 2008). C’, D’, and E’ are higher-magnification images of the cells in C, D, and E. Each cell was stained in a separate eyecup preparation and is a projection of 1 or more z-axis stacks. Fluorescence intensity was color coded using ImageJ fire LUT. Scale bars = 20 μm.
FIG. 5. Irradiance-response relations for different spectral classes of zebrafish horizontal cells. Each graph shows 3 wavelengths of irradiance-response data and model predictions for an individual cell from each spectral class. These are 3 of the 9 wavelengths that were studied in each of these cells. The curves are predictions based on modeled data for all 9 wavelengths. A: C-type biphasic cell is depolarized monotonically by red (650 nm) (●), but hyperpolarized monotonically by blue-green (490 nm) (*) and UV (370 nm) (▲) stimuli for all irradiances. The K5 model is applied. B: UV triphasic cell is maximally hyperpolarized by 370 nm UV stimuli, slightly hyperpolarized by red 650 nm stimuli, and depolarized by blue-green 490 nm stimuli. UV (370 nm) half-saturation is 1 log unit more sensitive than the green (490 nm) half-saturation, even though both wavelengths are close to pigment peaks. The K5U model is applied. C: the response sign of the blue tetraphasic cell depends not only on wavelength but on stimulus irradiance. UV stimuli (370 nm) are slightly depolarizing near threshold, but hyperpolarizing for brighter stimuli. Red (650 nm) stimuli hyperpolarize the cell regardless of brightness. Threshold depolarization for blue-green (490 nm) stimuli reverses to hyperpolarization at brighter irradiances. The K5U model is applied. D: L1 monophasic cell hyperpolarizes for all wavelengths, but saturation amplitudes are greater in the blue-green (490 nm) and UV (370 nm) than in the red (650 nm). The K5 model is applied. E: irradiance-response characteristics for L2 monophasic cells are similar to the L1 cell (D). The K5 model is applied. F: a blue triphasic cell is similar in irradiance-response characteristics the blue tetraphasic cell (C). In the cell shown, there seems to be a hidden UV opponency that is simply too small to overcome the hyperpolarizing influences of blue cone signals for UV stimulation. As with the blue tetraphasic type, reversal of response sign is seen with brighter irradiances for blue-green (490 nm) stimuli. The K5 model is applied. In A–F, inset values are model fit parameters (Eq. 1), “stim” is the number of stimulus-response datapoints modeled, and $r^2$ is the coefficient of determination for the fit. For K5 models, a single $\log(k)$ value is fit. For K5U models $\log(k)$ for UV cones ($\log(k_u)$) is set to 1 log unit more sensitive than $\log(k)$ for red, green, or blue cones ($\log(k_{rgb})$). The $\log(k)$ values are in units of $\log(\text{hv·μm}^{-1} \cdot \text{s}^{-1})$ and are the mean half-saturating values of irradiance at the absorbance maxima for contributing cones. Saturated cone signal amplitudes are given in $V_r$, $V_g$, $V_b$, and $V_u$. Positive values indicate depolarizing (sign-inverting) input to horizontal cells; negative values indicate hyperpolarizing (sign-conserving) input.
FIG. 6. Modeled spectral patterns of zebrafish horizontal cell types. Graphs of response amplitude vs. stimulating wavelength were generated from the spectral model (Eq. 1). Three plots at different equal quanta irradiances across all wavelengths are provided for each cell type. The irradiances correspond to 20, 50, and 80% saturation stimuli for red, green, or blue cones at their absorbance peaks. The cells and models are the same as given in the legend of Fig. 5.

A: C-type biphasic cell is excited by blue and green cones but inhibited by red cones. Red, depolarizing peaks migrate to longer wavelengths with brighter stimuli.

B: C-type UV triphasic cell is excited mainly by UV cones, inhibited by green cones, and excited to lesser extents by both blue and red cones. Quantal levels that are semisaturating for green, and red cones saturate the UV signal.

C: tetraphasic cell has 4 spectral zones of different response polarity. In the far UV (330 nm), small depolarizations are evoked for dim stimuli. Blue and near-UV stimuli evoke hyperpolarizations, as do red and yellow stimuli, but to a lesser extent. Green and blue-green stimuli strongly depolarize the cell.

D: L1-type monophasic cell has excitation peaks for both red and green cone signals at low irradiance. The green peak migrates toward the red for brighter excitation levels.

E: L2-type monophasic cells are predominantly red cone driven, but a small green cone shoulder drives spectral peaking toward the green for brighter stimuli.

F: C-type blue triphasic cell hyperpolarizes for blue stimuli, depolarizes for green and blue-green stimuli, and exhibits a lesser hyperpolarization for red and yellow stimuli. In A–F, numerical values on each trace are modeled wavelengths for maxima and minima. These cells are the same as modeled in Fig. 5, and the insets giving model parameters are explained in the legend of Fig. 5.
sic horizontal cells from UV cones (2% depolarization) was negligible. Biphasic cells are R+/G−/B−. Biphasic cells (14 of 28) were best fit by the K5 model with a median K5 $r^2$ of 0.93, as appropriate to a cell type without UV cone signals. Although red cone depolarization was never reversed by increases in illumination, irradiance response curves at this wavelength were characterized by a signature peak and decline, because green cone hyperpolarization encroached on saturated red cone depolarization (Fig. 5A). This action resulted in a progression of depolarizing spectral peaks from red toward the far red as irradiance levels increased (Fig. 6A), sometimes shifting peaks as far as 600 nm.

**Modeled properties of blue triphasic and tetraphasic cells**

These cells receive dominant hyperpolarizing input from blue cones (Figs. 5, C and F, and 6, C and F). They also receive hyperpolarizing inputs from red cones and depolarizing inputs from green cones. The mean spectral peak for hyperpolarization for both types was ~410 nm (Table 2), near the 415 nm blue cone $\lambda_{\text{max}}$ (Robinson et al. 1993). The mean spectral peak for depolarization was 485 nm (Table 2), near the 480 nm green cone $\lambda_{\text{max}}$. The major difference between these types is the response to UV stimulation: UV responses in triphasic blue cells are hyperpolarizing, whereas, near threshold, tetraphasic cells are depolarized by UV stimulation (Figs. 1, 2B, and 6, C and F, Table 1). When five triphasic blue-prefering responses were modeled (Table 1), 49% of $V_{\text{max}}$ input to these cells was blue cone hyperpolarization, 33% was green cone depolarization, and 7% was red cone hyperpolarization. On average, the UV cone contribution was 11% hyperpolarization. Blue-prefering triphasic cells are, as a group, $r$=−/G+/B−/u−. The models of seven tetraphasic responses (Table 1) similarly assign 44% of saturated input as blue cone hyperpolarization, 38% as green cone depolarization, and 13% as red cone hyperpolarization, but with 5% UV cone depolarization. This UV opponent signal combines with depolarization from the green cone spectral beta peak (Figs. 5C and 6C) to cause UV depolarization for dim UV stimuli. For brighter stimuli, the dominant blue cone hyperpolarization prevails, converting the tetraphasic response into a triphasic response. Tetrapsis cells are $r$=−/G+/B−/u+. These types (9 of 12) were best fit by the K5U model with a resulting median K5U $r^2$ of 0.90.

The tetraphasic shift from UV depolarization to UV hyperpolarization is seen in the K5U model (Fig. 6C; 330 nm). A further depolarizing to hyperpolarizing shift is modeled for both blue triphasic and tetraphasic types. This occurs near the green cone absorbance maximum (490 nm; Fig. 5, C and F). Response sign reverses with irradiance, as depolarization by green cones was overtaken by hyperpolarization from spectrally adjacent blue and red cones. This was accompanied by a red-directed migration of the yellow null point and of the green and red spectral peaks (Fig. 6, C and F), rather than a loss of the midspectral depolarizing component.

**Modeled properties of UV triphasic types**

UV-prefering triphasic units receive major hyperpolarizing inputs from UV cones, as well as smaller depolarizing inputs from green and blue cones and hyperpolarizing inputs from red cones (Figs. 5B and 6B; Table 1). The mean spectral peak for hyperpolarization occurred at 362 nm (Table 2), exactly matching the 362 nm UV cone $\lambda_{\text{max}}$ (Robinson et al. 1993). In this group, the mean spectral peak for depolarization occurred at 472 nm (Table 2), between green and blue cone $\lambda_{\text{max}}$. As a group, modeled UV-triphasic responses (Table 1) show UV cone hyperpolarization contributes 56% of the summed cone input; blue cone input, on average, provided hyperpolarizing signals of 2%, and green cone depolarizing inputs provided 34%. A small (8%) hyperpolarization was contributed by red cones. UV-prefering triphasic cells are $r$=−/G+/U−. These types (6 of 10) were best fit by the K5U model with a calculated median K5U $r^2$ of 0.95.

UV signals in triphasic cells were especially sensitive. The greater sensitivity of UV as opposed to green cone signals is easily seen in Fig. 5B, where the irradiance-response curve and points at 370 nm (close to the UV cone peak) are left shifted by about 1 log unit compared with the irradiance-response curve and points at 490 nm (close to the green cone peak). This dataset shows directly the appropriateness of the K5U model, with its more sensitive UV cone signal, for the UV triphasic cells.

Because of the cone sensitivity disparity in these cells, this second model (K5U) was introduced to accommodate spectral types with substantial UV cone signals. In the K5U model, the UV cone signal is set 1 log unit more sensitive than the other cone types, resulting in a better fit for all triphasic and tetraphasic responses. A factor of 20 times greater sensitivity for UV, compared with red cone signals, has been reported in aspartate-isolated cone-PIII signals from zebrafish retina (Nelson and Singla 2009).

**Discussion**

**Spectral classification of zebrafish horizontal cells**

There are four cone types in zebrafish retina (Engstrom 1960; Robinson et al. 1993) and three morphological types of cone-connected horizontal cell (Connaughton et al. 2004; Li et al. 2009; Song et al. 2008). The different cone types are arranged in an ordered mosaic and, whereas eight cone opsin are expressed in zebrafish (Chinen et al. 2003) each cone contains a single dominant pigment, maximally sensitive to UV (362 nm), blue-violet (415 nm), blue-green (480 nm), or yellow (570 nm) (Robinson et al. 1993). These are the UV-, short-, middle-, and long-wavelength cones, respectively, here referred to as UV (or U), blue (B), green (G), and red (R) cone types. To pursue the question of how zebrafish horizontal cells connect with and integrate multiple cone signals, we generated a model to quantify the contributions of the different cone inputs. In total, six horizontal cell spectral types were distinguished and named, including two monophasic and four multiphasic chromatic types. All types responded to all stimulus wavelengths, including far red (650 nm). For this to be true, all six spectral types must receive signals from at least red cones (the farthest red of the cone spectra). However, only L2 monophasic types were dominated by red cones; the other types were dominated by one to three cones of other spectral
signatures. Thus red and/or green cone excitation was largest in L-type monophasic cells, blue cone signals dominated blue-prefering triphasic and tetraphasic cells, and UV cone signals dominated UV-prefering triphasic cells. No horizontal cell type was found to receive an exclusively dominant, hyperpolarizing input from green cones, but green cone signals were prominent in the depolarizing, spectral opponent responses of several types and in the hyperpolarizing responses of both L1-type monophasic cells and biphasic cells.

**UV light responses**

Two responses displayed prominent UV cone input. The first is a novel triphasic response with maximum sensitivity and response amplitudes for UV light stimulation. This “UV-prefering” horizontal cell is hyperpolarized by UV wavelengths, depolarized by short and middle wavelengths, and hyperpolarized by long wavelengths. It is a uniquely sensitive, low-threshold type. The second UV response is a tetraphasic light response, characterized by hyperpolarizations to both red and deep blue stimuli and depolarizations to both blue-green and low-level UV stimuli. UV-sensitive cones exist in a variety of species, including fish (goldfish, carp, zebrafish, salmon), turtles, rodents, insects, and birds. Behavioral responses to UV light are found in turtle (Arnold and Neumeyer 1987), roach (fish) (Douglas 1986), and birds (Hart 2001; Smith et al. 2002; reviewed in Goldsmith 1994) and zebrafish (Risner et al. 2006). ERG recordings identify UV cone contributions in zebrafish (Bilotta et al. 2005; Hughes et al. 1998; Nelson and Singla 2009) and juvenile goldfish (Chen and Stark 1994). Horizontal cell hyperpolarizations to UV light have been reported in turtle (Ammermuller et al. 1998; Ventura et al. 1999; Zana et al. 2001) and fish (Hashimoto et al. 1988). Tetraphasic light responses were first described in the 1980s in fish (Fukurotani and Hashimoto 1984; Harosi and Fukurotani 1986; Hashimoto et al. 1988); however, with the exception of Japanese dace (Hashimoto et al. 1988), we found no reports describing UV-prefering triphasic responses in horizontal cells in any of the above species.

**Non-UV light responses**

Mono-, bi-, and triphasic light responses also occur in zebrafish horizontal cells. Monophasic responses have been consistently identified in all vertebrates and are characterized by hyperpolarizing responses to all stimulating wavelengths. Most monophasic

![FIG. 7. Distribution of cone excitation for different spectral classes of cone horizontal cells. Distribution of dominant excitatory (hyperpolarizing) cone signals among (A) monophasic, (B) biphasic, and (C) tri-/tetraphasic horizontal cells in zebrafish retina. These types are proposed to originate with H1, H2, and H3 morphological types (Li et al. 2009). For each cell type, the fractional input provided by 1 of 2 dominant excitatory cones is scaled to a number of cone contacts in a model cell with 10 total cone inputs from 2 dominant types: red and green cone inputs for monophasic cells (A), green and blue cone inputs for biphasic cells (B), and blue and ultraviolet cone inputs for tri- and tetraphasic cells (C). In each case, the number of cells with each of the 11 potential fractional cone signal combinations is compared with a binomial model, where P represents the probability that a cone contacts the dominant type (R for monophasics, G for biphasics, UV for tri and tetraphasics). In no case is a binomial model shown appropriate. The vertical dashed line in A–C is the cone fraction seen in the zebrafish cone mosaic for the pair of cones providing excitation. In A, red and green cones are equal in number; in B, there are 2 green cones for every blue cone; and in C, the blue and UV cones are equal in number. A: L1 monophasic cells are represented by green bars in the leftmost peaks (fewer contacts with R cones); the remainder (red bars) are L2 monophasic cells (majority of contacts with R cones). B: biphasic cells receive a distribution of excitation from green and blue contacts. The input is predominantly green, although not to the extent seen in the photoreceptor mosaic. C: UV triphasic cells are represented in the rightmost peaks (purple bars), and tri/tetraphasic blue cells are represented by the leftmost peaks (blue bars). The fraction of cone contacts is computed in each case as $10 \times \frac{V_{max}}{V_{max1} + V_{max2}}$, where $V_{max}$ comes from the dominant cone (i.e., R cones for monophasic cells, G cones for biphasic cells, UV cones for tri- and tetraphasic cells), and $V_{max2}$ represents the other cone type (i.e., g cones, b cones, or b cones, respectively). In particular cases where a dominant or nondominant cone is found to provide a depolarizing influence, its hyperpolarizing $V_{max}$ was set to 0."

J Neurophysiol • VOL 104 • NOVEMBER 2010 • www.jn.org
types are dominated by red cone inputs (De Aguiar et al. 2006; Djamgoz 1984; Djamgoz and Ruddock 1979; Djamgoz et al. 1985; Ohtsuka and Kouyama 1986; Sakai et al. 1997; Ventura et al. 1999, 2001; Yang et al. 1983; Yazzulla 1976). However, monophasic cells receiving dominant or nondominant inputs from more than one photoreceptor type, such as red and green (Dacey et al. 1996, 2000; De Aguiar et al. 2006; Downing and Djamgoz 1989; Hashimoto and Inokuchi 1981; Hashimoto et al. 1988; Kamermans and Spekreijse 1995; Kamermans et al. 1989b; Sakai et al. 1997; Twig et al. 2003; Yang et al. 1983; Yazzulla 1976), red and blue (Nelson 1985), green and blue (Djamgoz 1984; Djamgoz and Ruddock 1979; Djamgoz et al. 1985; Yazzulla 1976), or red, green, and blue (Asi and Perlman 1998; Dacey et al. 1996; Twig et al. 2003; Yazzulla 1976) also exist.

In this study, monophasic responses were the most frequent type recorded. These responses were separated into two types based on the wavelength of maximum sensitivity (Hashimoto and Inokuchi 1981; Hashimoto et al. 1988; Ruddock and Svaetichin 1975). Most (87%) were L2-types maximally stimulated by red cones, with spectral peaks slightly to the green side of the 570 nm red cone λ\text{max}. The remaining L1 cells received approximately equal inputs from both red and green cones, resulting in a broad spectral plateau with maximal sensitivity slightly to the red side of the 480 nm green cone λ\text{max}. L1 and L2 monophasic types can be considered as either distinct or two limbs of a single distribution (Fig. 7A). In either case, it is useful to maintain L1 and L2 nomenclature, because the properties of these two limbs differ, and it provides for correspondence with use for other cyprinids, such as dace (Hashimoto and Inokuchi 1981; Hashimoto et al. 1988), where green cone (L1) and red cone (L2) dominated monophasic types are described.

In a study of red and green cone signals for H1 horizontal cells of old world primates, Dacey et al. (2000) found a peaked distribution ranging from 2 to 9 red cone inputs of a possible 10 total red plus green. This distribution includes both red-sensitive and green-sensitive horizontal cells. They concluded that, whereas H1 monophasic cells in primate are selective for red and green cones and reject blue cones, they do not discriminate red from green, and that the variability in red versus green signals reflects the variable local density of red and green cones. Similar to primate H1 cells, zebrafish monophasic horizontal cells vary in respect to red and green cone mixing, with a distribution of ratios ranging from 5 to 10 red cone inputs out of a possible 10 red plus green (Fig. 7A). This is unlikely to reflect local variations in the density of zebrafish red and green cones, however, because zebrafish cones are in a mosaic with precise cone ratios (Engstrom 1960). The red and green cone ratio is 1:1 because these are, respectively, the pigments in the principal and accessory members of the double cone. The variability of red and green cone signals for zebrafish monophasic horizontal cells reflects variability in horizontal cell excitatory synaptic contacts. This might arise either in a single cell type or in multiple cell types with an overlapping set of connectivity patterns. Differences in the numbers of inputs from red and green cones onto individual H1/H2 horizontal cells was originally suggested by Li et al. (2009) in their morphological classification of zebrafish horizontal cells. In that study, H1/H2 cells contacted 15–20 cones of three spectral types. Like primate H1 cells, zebrafish monophasic cells reject input from SWS1, which is the UV cone in the case of zebrafish or the blue cone in the case of old world primates.

The L2 limb for zebrafish L-type cells (Fig. 7A) is truly monophasic. On the other hand, along with the increasing green cone signals found in the L1 limb, increasing antagonism from blue cones is also found. In L2 types, the blue cone opponent signal is only 8% as large as the red cone signal. In L1 types, it is 27% as large (Table 2). In rare cases, this blue cone signal actually evokes L1 threshold spectral antagonism with blue-green stimulation (Fig. 8). Unlike L2, L1 is a monophasic opponent type, with typically hidden short wavelength depolarizing influences.

Biphasic units in zebrafish showed a mean threshold null point at 523 nm, with depolarizing responses observed at longer wavelengths and hyperpolarizing responses at shorter wavelengths. This “classic” biphasic response has been reported extensively in the literature. In zebrafish, these cells receive dominant hyperpolarizing inputs from blue and green cones and opponent depolarizing input from red cones. In the analogue turtle R/G biphasic horizontal cells, there is variability in the opponency between red and green cones. Twig and Perlman (2004) identify four factors. 1) Null points migrate to longer wavelengths with brighter stimuli. This is seen in current data (Fig. 6A) and is a natural outcome of the model. 2) Null points migrate with chromatic adaptation. 3) Even in a single cell, the null point depends on the size of the stimulated area. 4) There is null point variability between animals, and, by inference, variability in the ratios of cone types contacted by R/G cells. Relating to points 3 and 4, Twig and Pearlman (2004) invoked regional and interanimal variability in cone distributions, and in addition, the general variability in horizontal cell connectivity with the preferred cone types. In zebrafish (Fig. 7B), the green cone fraction of excitation for R+/G−/B− biphasic cells varies from ~3 of 10 to almost 10 of 10 blue plus green cones exciting the biphasic type. Because of the regular cone mosaic in zebrafish, the ratio of green to blue cones is fixed at 2:1 or ~7 of 10 green plus blue cones. Compared with the density ratio for blue and green cones, excitation of zebrafish biphasic horizontal cells appears slightly to favor blue cones. Although differences in the properties of blue and green cones might explain a general bias toward one
cone type or the other, the spread of excitation ratios tends to suggest a lack of rigidity in synaptic contact patterns for excitation by blue or green cones in biphasic cells. Like monophasic horizontal cell types, biphasic types selectively reject excitation by UV cones (Table 1). Triphasic responses are also widely reported, with most hyperpolarizing to long wavelengths, depolarizing to middle wavelengths, and hyperpolarizing to short wavelengths (Djamgoz 1984; Djamgoz and Ruddock 1979; Gottesman and Burkhardt 1987; Hashimoto and Inokuchi 1981; Ohtsuka and Koyama 1986, 1985; Ventura et al. 1999; Yazulla 1976). The blue triphasic responses found in zebrafish is similar, with maximal hyperpolarizations at blue wavelengths (409 nm) and maximal sensitivity for depolarization in the blue-green (485 nm). These cells receive dominant hyperpolarizing inputs from blue cones, dominant opponent input from green cones, and in the mean, a small hyperpolarizing influence (−1.4 mV) from UV cones (Table 1). A synergistic excitation by both blue and UV cones has also been noted in the analogous Y/B spectrally opponent responses seen in the turtle retina (Zana et al. 2001). In the main, blue triphasic types are excited by blue and, to a lesser extent, UV cones. In some cases, however, there is no UV excitation, but UV opponent influences are too small to offset blue cone excitation. The distribution of excitatory blue and UV inputs for blue triphasic, UV triphasic, and blue tetraphasic types is grouped in Fig. 7C. The grouping is based on the likelihood that all these spectral types may originate with the anatomically blue and UV cone contacting H3 cells of zebrafish (Li et al. 2009; Song et al. 2008). Unlike monophasic and biphasic types, there is little evidence of a continuous distribution of blue and UV excitation in this group; rather, peaks emerge at the UV and blue cone dominated ends of the distribution. Because there are equal numbers of blue and UV cones within the zebrafish cone mosaic, the distribution (Fig. 7C) is quite different from what might be expected of a nonpreferential connection pattern for H3 cells. In terms of synaptic affinities, H3 seems to encompass multiple cell types.

Morphology

In goldfish (Stell and Lightfoot 1975) and carp (Weiler 1978), there are four morphological types of horizontal cells. H1, H2, and H3 cells contact cones; H4 cells contact rods. Of the cone-contacting types, H1 cells are smallest in dendritic field, whereas H3 cells are largest (Stell and Lightfoot 1975). Goldfish H1 cells contact all cone types indiscriminately and generate L-type responses. H2 cells contact green and blue cones and generate biphasic light responses; H3 types contact blue cones and generate triphasic responses (Stell and Lightfoot 1975; Stell et al. 1975, 1982). Miniature (presumably UV; Hisatomi et al. 1996) cones contact all three HC types (Kamermans and Spekreijse 1995; Stell and Lightfoot 1975). Recent studies in zebrafish (Li et al. 2009; Song et al. 2008) have identified analogous morphological types; however, the connectivity patterns are somewhat different. H1 cells contact red, green, and blue cones but not UV cones, H2 cells contact green, blue, and UV cones but not red, and H3 cells contact blue and UV cones only.

In zebrafish, six categories of spectral response arise from the H1, H2, and H3 morphologies. Based on our microelectrode stains, monophasic (both L1 and L2) and biphasic responses seem to be generated by horizontal cells of similar morphology (Fig. 4B; Table 3). The dendritic fields of these cells are narrow in extent and similar in size to the zebrafish H1/2 horizontal cells seen in anatomical studies (Li et al. 2009; Song et al. 2008). Li et al. (2009) reported a small round cell body for H1 types and a large irregular cell body for H2 types and suggested the former generate monophasic responses and the latter are biphasic. However, our microelectrode stains do not suggest an easy distinction between monophasic and biphasic cells based on dendritic and cell body morphology alone (see circuitry discussion below).

Both the blue-prefering and UV-prefering triphasic responses were recorded from horizontal cells with wide dendritic fields. These zebrafish H3 cells (Song et al. 2008) contact both blue and UV cones (Li et al. 2009). Although the rare tetrathaphasic horizontal cell (~5% of all horizontal cells) is yet to be stained, the physiologically similar blue-prefering triphasic cell is a likely template for its circuitry and morphology. Thus the H3 morphology may support three physiologically distinct spectral patterns. The ability for one cell type to generate more than one spectral response suggests that only some of the contacts represent direct cone excitation, whereas others are presynaptic feedback contacts. Only the hyperpolarizing signals that we have modeled are potentially direct excitatory input from cones and that is why only the distribution of these signals is modeled as a potential count of cone contacts in Fig. 7.

Circuitry underlying horizontal cell spectral responses

The circuitry underlying the spectral responses of bi- and triphasic horizontal cells was originally studied in goldfish by Stell and Lightfoot (Stell and Lightfoot 1975; Stell et al. 1975). They proposed that each morphological type of horizontal cell received excitatory inputs from a single cone type: H1 cells received input from red-sensitive cones, H2 cells received input from green-sensitive cones, and H3 cells received input from blue-sensitive cones (Stell and Lightfoot 1975; Stell et al. 1975). A cascading feedback model from each horizontal cell to cones of the next shorter wavelength accounted for the spectral polarities observed in biphasic and triphasic responses. This was later modified (Kamermans et al. 1991, 1989a,b; Kraaij et al. 1998) to reflect more flexible horizontal cell to photoreceptor connections, with each horizontal cell receiving variable input from, and providing feedback to, all contacted cone types. In this latter model, the differences in the strengths of the feedforward and feedback inputs determine horizontal cell responses.

To better describe the spectral circuits in zebrafish horizontal cells, the different cone signals contributing to the spectral responses were extracted (Eq. 1), and the results suggest potential anatomical connections. Our model, with five adjustable parameters, says that spectral and irradiance responses can be accounted for by the sum of four independently saturable cone signals. In stable datasets, this model fits ~95% of data set variance (r²). It also accurately predicts observed data and reproduces findings from other models (Kamermans and Spekreijse 1995), such as the migration of spectral peaks in L2 monophasic cells toward the green with increasing stimulus irradiance (Fig. 6E) and the larger amplitude of responses for green simulation as compared with red (Fig. 5, D and E). These
are natural consequences of our model even though it does not include co-saturation based on competing synaptic conductances of red and green cones within the horizontal cell, as in the Kamermans model.

Our data predict monophasic cells should receive excitatory inputs from red and green cones. In particular, L2 (R−/g−) cells should receive excitation mainly by red cone contacts, and L1 [R−/G−/(b+)] cells should receive excitation equally from red and green cones. Analyzing photoreceptor contacts of DiI-stained H1/2 horizontal cells in a UV cone–labeled zebrafish transgenic, Li et al. (2009) concluded that L1- and L2-type responses arise from red, green, and blue cone-connected H1 cells and suggested that the L1/L2 distinction originates from variability in the number of connections to each photoreceptor type. The presence of red and green cone excitation, and the lack of UV signals, in L-types identified in this study fit the proposed H1 connectivity, as do modeling the fractional input of different cone types (Fig. 7A). The blue cone contacts of H1 are presumptive sites of horizontal cell output through feedback, although as previously noted, some of the red and green cone contacts may also be feedback sites (Downing and Djamgoz 1989; Kamermans and Spekreijse 1995). Hyperpolarizations for blue and UV stimulation in H1 monophasic cells originate only via excitation of red and green opsins and not by excitatory contacts with blue and UV cones.

Biphasic cells in zebrafish are designated R+/G−/B−. These responses could originate in the green, blue, and UV cone-contacting H2 cells (Li et al. 2009). In this scheme, excitatory hyperpolarizations by light originate in green and blue cone contacts, whereas the red cone depolarizing signal arises through feedback onto blue cones from monophasic horizontal cells (although some feedback onto green cones is not excluded). Because UV signals are not seen in biphasic cells, the UV cone contacts are putative feedback output sites. Hyperpolarizations in biphasic cells are typically generated by green cone excitation, with lesser contributions from red and blue cone excitation (Kamermans and Spekreijse 1995). In zebrafish, however, although the proportion of blue and green signals varies among cells, green and blue cones contribute about equally, on average, to biphasic responses (Table 1), resulting in the large hyperpolarizations observed in response to stimulation with both middle and short wavelengths.

UV-preferring trichaphasic cells in zebrafish are denoted r−/G+/U−. Microelectrode stains show H3 morphology, known to contact both blue and UV cones (Li et al. 2009). In this circuitry, excitatory hyperpolarizations by light originate in the UV cone contacts, whereas the remaining contacts provide presumptive feedback output as UV horizontal cells feed back to blue cones. Hyperpolarizing signals from red cones and depolarizing signals from green and blue cones are accommodated by feedback contacts onto UV cones from biphasic horizontal cells, thus creating a trichaphic UV horizontal cell.

Tetraphasic cells in zebrafish are r−/G+/B−/u+. In this case, excitatory hyperpolarizations by light originate with the blue cone contacts, whereas the UV contacts provide feedback to UV cones, perhaps supplementing the blue cone feedback signal also received from biphasic H2 cells. No horizontal cell physiology exactly mimics the r−/G+ component of the tetraphasic response. Nonetheless, it must be assumed that this also arises through the documented feedback contacts from biphasic cells. It would appear that, in the antagonism between a blue cone feedback signal arising through biphasic input, and the direct hyperpolarization of the blue cone synapse, the direct phototransduction signal prevails. The blue-prefering triphasic response is modeled as r−/G+/B−/u−. A wide dendritic field suggests that this cell is also one of the blue and UV cone-contacting H3 cells of Li et al. (2009). While both triphasic and tetraphasic types are dominated by blue cone excitation, in the mean, the triphasic type is hyperpolarized by UV cones, whereas the tetraphasic type is depolarized. Perhaps there is some synaptic jitter here also, so that some tetraphasic cells make an infrequent excitatory contact with a UV cone, thus canceling the impact of UV cone feedback depolarization seen in the blue cone excitation pathway and yielding a blue trichaphic response pattern.

We have based the above spectral circuits in zebrafish on the pattern of ideas established by Stell and colleagues in goldfish (Stell and Lightfoot 1975; Stell et al. 1975, 1982), together with recently completed anatomical studies and analysis in zebrafish (Li et al. 2009). The combined results indicate horizontal cell circuits in zebrafish have properties that deviate from the original cascade model. For example, the tetrachromatic span of zebrafish cones stretches the original trichromatic circuitry, particularly in the mid-spectrum. Furthermore, there is an additional L-type cell in zebrafish that covers both red and green cones, and zebrafish monophasic L-type cells seem to extend feedback circuitry beyond green cones to blue cones. Similarly, biphasic cells seem to extend their reach from blue cones to UV cones. A unique UV horizontal cell in zebrafish directs feedback signals toward blue cones, thereby inhibiting intrinsic blue pigment sensitivity not only on the long wavelength limb through L-type monophasic cells, but on the short wavelength limb through UV-preferring triphasic cells. The resultant tetrachromatic horizontal cell response, with a signal flow from short toward longer wavelengths, like the UV-preferring triphasic response, was not envisioned in the original cascade model (Stell and Lightfoot 1975), and extrapolation of this model to include UV cones (see Fig. 7 of Stell et al. 1982) would, in fact, suggest a UV-hyperpolarizing tetraphasic response (r+/G−/B+/u−), which is different from observed. Given these differences, it is possible that spectral responses in zebrafish horizontal cells can be better described by extending the modified model of Kamermans and colleagues (Kamermans et al. 1991, 1989a,b; Kraaij et al. 1998).

As with cone oil droplet filtering, horizontal cell feedback can make sensitivity spectra narrower than absorbance spectra. An example is the tetraphasic cell (Fig. 6C), where horizontal cell circuitry seems to pinch and narrow the blue opsin absorbance spectra from both long and short wavelength sides to further hone the accuracy of the color sense. Avian oil droplets perform a similar function (Bowmaker 1980; Hart 2001; Vorobyev 2003; Vorobyev et al. 1998) if only from the short wavelength limb. In the case of zebrafish L1 and biphasic responses, which have less feedback and more synergistic integration of excitatory cone signals, broader spectral peaks were generated (Fig. 6, A, D, and E). Thus at the level of cone
output synapses, horizontal cell circuitry creates and projects forward a modified and refined color sense.

**Role of UV vision**

Zebrafish are a tropical freshwater species native to India. In the aquatic environment, UV light is attenuated by dissolved organic matter that is present (Kelly et al. 2001). Thus for zebrafish to respond to UV light in their natural habitat, they must be active in daytime in either clear water or at the surface, situations suggested for other cyprinids with UV sensitivity (Fukurotani and Hashimoto 1984; Hashimoto et al. 1988). In carp, UV and blue systems are color opponent (Hawryshyn and Harosi 1991), with opponency potentially occurring in proximal retina, similar to findings in turtle (Ventura et al. 2001). Whatever the specific purpose, it is clear that spectral response processing in the distal zebrafish retina is complex and includes a specific circuit for UV light that is responsible for generating triphasic and tetraphasic responses.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

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