Retinal Bipolar Cell Input Mechanisms in Giant Danio. III. ON-OFF Bipolar Cells and Their Color-Opponent Mechanisms

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Wong, Kwoon Y. and John E. Dowling. Retinal bipolar cell input mechanisms in giant danio. III. ON-OFF bipolar cells and their color-opponent mechanisms. J Neurophysiol 94: 265–272, 2005. First published March 9, 2005; doi:10.1152/jn.00271.2004. Whole cell patch recording was performed from morphologically identified cone-driven ON-OFF bipolar cells (Cabs) in giant danio retinal slices to study their glutamate receptors and light-evoked responses. Specific agonists were employed in the presence of cobalt, picrotoxin, and strychnine to characterize glutamate receptors on these cells. Most Cabs responded to both the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptor agonist kainate and the excitatory amino acid transporter (EAAT) substrate d-aspartate, and both responses were localized to the dendrites. Kainate generated depolarizations whereas d-aspartate had $E_{rev}$ close to $E_{Cl}$ and generated hyperpolarizations, indicating that AMPA/kainate receptors are sign-preserving, whereas the EAATs are sign-inverting. In response to white light, some Cabs gave ON bipolar cell-like responses whereas others gave OFF bipolar cell-like ones, but many cells’ responses had both ON and OFF bipolar cell properties. In response to appropriately colored stimuli, most Cabs responded to short and long wavelengths with opposite polarities and were thus double color-opponent. The depolarizing components of the responses to white or colored stimuli were suppressed by the EAAT blocker d-threo-benzoyloxyaspartate (TBOA), whereas the hyperpolarizing components were reduced by the AMPA/kainate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). These results are consistent with the hypothesis that both EAATs and AMPA/kainate receptors are involved in the generation of light-evoked responses in Cabs and that they confer these cells with ON and OFF bipolar cell properties, respectively. Cabs can generate double color-opponent center responses by receiving inputs from different cones through EAATs and from other cones through AMPA/kainate receptors.

INTRODUCTION

Color perception begins with the cone photoreceptors. Most vertebrates possess multiple types of cones, each of which is most sensitive to a certain range of wavelengths, e.g., ultraviolet, blue, green, and red. (Liebman and Granda 1971; Marc and Sperling 1976; Merbs and Nathans 1992; Tomita et al. 1967). However, the cones themselves cannot discriminate among wavelengths because many combinations of wavelength and intensity can lead to the same response in a cone. Color vision depends on the comparison of signals from two or more cone types by postreceptoral circuits. The neurophysiological basis for color comparison is color opponency, and neurons with this property respond to certain wavelengths with one polarity of voltage change but to others with the opposite polarity.

Substantial color processing takes place within the retina, and thus an understanding of retinal color-opponent mechanisms is crucial for understanding how the brain processes color information. Color-opponent retinal ganglion cells were first reported in goldfish, and subsequent experiments found similar cells in many other species, including primates (Daw 1968a,b; de Monasterio 1978a,b; Marchiafava and Wagner 1981; Wagner et al. 1960). In fish, construction of at least part of the ganglion cells’ color-opponent receptive fields begins in the outer retina because many horizontal cells and some bipolar cells are color-opponent (Kaneko 1973; Kaneko and Tachibana 1981, 1983; Svaetichin and MacNichol 1958; Tomita 1965). There are two main types of color-opponent bipolar cells. The single-opponent cells have centers that respond best to long wavelengths and surrounds that respond best to shorter wavelengths or vice versa. On the other hand, double-opponent cells have center and surround responses that change polarity when wavelength changes, e.g., the center depolarizes to long wavelengths but hyperpolarizes to shorter wavelengths, whereas the surround hyperpolarizes to long wavelengths but depolarizes to shorter wavelengths. In fish, most color-opponent bipolar cells are double opponent (Kaneko and Tachibana 1981, 1983; Shimbo et al. 2000). It is generally agreed that horizontal cells mediate the surround responses in bipolar cells, and mechanisms as to how color-opponent surround responses are generated have been proposed (Stell and Lightfoot 1975). However, the mechanisms as to how double-opponent center responses in bipolar cells are generated remain elusive (Shimbo et al. 2000).

In this paper, we investigated the glutamatergic input mechanisms and light-evoked responses of the ON-OFF bipolar cells in the giant danio (Danio aequipinnatus). These cells were morphologically identified by their having axon terminals in both sublaminae a and b of the inner plexiform layer (IPL). Such bistratified bipolar cells have also been identified in two other teleosts, namely goldfish (Sherry and Yazulla 1993) and zebrafish (Connaughton and Nelson 2000; Connaughton et al. 2004). These cells are cone-driven and thus are designated as Cabs (cone-driven bipolar cells with axon terminals in sublaminae a and b) (Sherry and Yazulla 1993), and the present study is the first attempt to characterize their light responses. We will show that many Cabs specialize in color processing, and our findings may shed light on the mechanisms generating center color opponency in fish bipolar cells.

Part of the present communication was previously reported in abstract form (Wong et al. 2003).
METHODS

Patch-clamp recording

The electrophysiological methods used were those described in Wong et al. (2005b); and all procedures were approved by the Institutional Animal Care and Use Committee at Harvard University. Briefly, dark-adapted adult giant danios (Segrest Farms, Gibson- ton, FL; EkkWill Waterlife Resources, Gibsonton, FL; Central Mass Aquatics, Worcester, MA) 1.5–3 in long were anesthetized in tricaine, decapitated, and their eyes removed under dim red light. The retinas were isolated from the pigment epithelium and placed photoreceptor side up on a piece of filter paper (type: 0.45 µm HA; Millipore, Billerica, MA). The retina-filter complex was cut into 200–µm slices using a chopper with Feather blades (Ted Pella, Redding, CA). The slices were incubated in a solution containing 25% (m/l) L-15 culture medium (Invitrogen, Carlsbad, CA) and 75% Ringer solution (see following text) with picrotoxin, strychnine, and D-(-)-2-amino-5-phosphonopentanoic acid (d-AP5) omitted at 8°C for 10 min to 8 h prior to recording.

A Dagan 8900 amplifier (Dagan, Minneapolis, MN) was used, and signals were low-pass filtered at 1 kHz for most experiments and at 10 kHz when the capacitative current was used to estimate the series resistance ($R_{\text{series}}$). The sampling rates were 2.02 and 20.4 kHz, respectively. After partial compensation, the remaining $R_{\text{series}}$ was 74.0 ± 3.2 (SE) MΩ ($n = 29$). Recordings were made from bipolar cells in the whole cell patch-clamp configuration using glass electrodes with 5- to 10-MΩ tip resistance under continuous superfusion with either control Ringer or drug solutions and under dim red light in the scotopic range. Data were acquired using PCLAMP software (Axon Instruments, Union City, CA).

Chemicals and solutions

The Ringer contained (in mM) 120 NaCl, 2 KCl, 1 MgCl$_2$, 2–3 CaCl$_2$, 4 HEPES, 4 d-glucose, 0.1–0.2 picrotoxin, 0.002–0.005 strychnine, and 0.03 D-AP5, and was set to pH 7.65–7.75 with NaOH. Picrotoxin and strychnine were included to eliminate the influence of amacrine cells on the bipolar cell responses. CaCl$_2$ in the Ringer was substituted with 3 mM CoCl$_2$ in experiments where synaptic release was blocked to ensure direct action of the puffed agonists on the bipolar cells being recorded. The N-methyl-D-aspartate (NMDA) receptor antagonist d-AP5 was also included because d-AP5, the agonist used to probe for the presence of excitatory amino acid transporters (EAATs) in Cabs, is also an agonist for NMDA receptors (Foster and Fagg 1987).

The intracellular solution contained (in mM) 104 K-glucuronate or Cs-methanesulfonate, 12 KCl or CsCl, 0.1 CaCl$_2$, 1 EGTA, 2 Na$_2$-ATP, 4 HEPES, 1 Na-GTP, 0.1–1 Na-cGMPS, and ~ 0.05% (g/ml) Lucifer yellow, and its pH was set to 7.4 with KOH or CsOH. Unless stated otherwise, the cesium-based intracellular solution was used in voltage-clamp experiments, whereas the potassium-based solution was used in current-clamp experiments. Reversal potentials were corrected for $R_{\text{series}}$, with Ohm’s law, $V_{\text{error}} = I_{\text{hold}}R_{\text{series}}$, where $V_{\text{error}}$ is the error in the reversal potential measurement, and $I_{\text{hold}}$ is the holding current at the reversal potential. All membrane potentials were corrected for the liquid junction potentials, which were 9.033 ± 0.076 mV ($n = 6$) and 13.43 ± 0.28 mV ($n = 6$) for cesium- and potassium-based internal solutions, respectively.

Chromatins were either bath-applied or puffed from multi-barrel pipettes (positioned near the dendrites except where noted). L(+)-2-amino-4-phosphonobutyric acid (L-AP4), D-AP5, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and d-(-)-3-benzoyloxyaspartate (TBOA) were purchased from Tocris (Ellisville, MO). Kainate was purchased from Ocean Produce International (Shelburne, Canada). All other chemicals were purchased from Sigma (St. Louis, MO).

Light stimulation

The light source was a 150-W tungsten-halogen bulb. Stimulus flash duration was controlled by an electromechanical shutter. The stimulus was presented through the camera port and focused onto the cell via the water-immersion objective lens. A slit was placed in the light path to restrict the light to an 80-µm-wide slit at the retina. The length of the slit extended from the photoreceptor outer segments to the ganglion cell layer, and the bipolar cell being recorded was positioned at the center of this slit. The white light stimulus had an intensity of ~68 µW cm$^{-2}$ at the retina. Light stimuli of other colors were obtained by inserting narrow-band interference filters into the light path. The wavelengths used and their intensities at the retina were 400 nm (0.26 µW cm$^{-2}$), 430 nm (2.8 µW cm$^{-2}$), 460 nm (3.8 µW cm$^{-2}$), 490 nm (1.8 µW cm$^{-2}$), 520 nm (14 µW cm$^{-2}$), 550 nm (10 µW cm$^{-2}$), 580 nm (4.4 µW cm$^{-2}$), 610 nm (6.6 µW cm$^{-2}$), and 640 nm (31 µW cm$^{-2}$). As the output of tungsten light sources is relatively weak in the ultraviolet (UV) region and the optical components of our setup were not designed to transmit UV wavelengths efficiently, UV stimuli were not tested. Light response data were either single responses or averages of two to six responses.

RESULTS

AMPA/kainate receptors and EAATs were found on most Cabs

Cabs were identified by the presence of one or more terminals in each of the two subliminae of the IPL (Fig. 1, top left). Using the voltage-ramp protocol described in Wong et al. (2005b) and/or the free-running current-clamp recording mode, 1.5–3 mM D-aspartate, 100 µM kainate, and 150–300 µM L-AP4 (agonists for EAATs, AMPA/kainate receptors, and group III metabotropic glutamate receptors, respectively) were puffed onto each of a total of 65 Cabs in the presence of the cobalt-based Ringer. In this Ringer, the mean input resistance of these cells with intracellular cesium was 1.94 ± 0.17 GΩ ($n = 27$). Of the 65 Cabs recorded, 48 responded to D-aspartate and kainate, 14 to D-aspartate, and 3 only to kainate; L-AP4 never elicited any response. The responses to D-aspartate could be reduced or abolished in the presence of the EAAT blocker TBOA (1–2 mM), confirming that D-aspartate acted specifically and directly on the EAATs of the Cabs recorded ($n = 15$; not shown).

The existence of three different pharmacological profiles may implicate functional subtypes within Cabs, although cells possessing both EAATs and AMPA/kainate receptors were by far the most common recorded. In voltage-clamp mode with intracellular cesium, both D-aspartate and kainate elicited conductance increases, with the reversal potentials being −46.0 ± 1.6 and 2.4 ± 2.3 mV, respectively ($n = 29$; Fig. 1, top right). Of the 48 Cabs responding to both D-aspartate and kainate, 25 were tested for agonist responses under current clamp. In the cobalt-containing Ringer, the resting potentials of these cells were −38.7 ± 2.0 and −33.3 ± 1.6 mV when measured with internal potassium ($n = 7$) and cesium ($n = 18$), respectively. Because these resting potentials were more positive than the $E_{\text{rev}}$ for D-aspartate but more negative than that for kainate, 22 of the 25 cells were hyperpolarized by D-aspartate (the remaining 3 gave no detectable voltage changes, though they gave current responses in the voltage ramp experiment), whereas all 25 cells were depolarized by kainate (Fig. 1, bottom).
Both receptor types were localized to the dendritic region. To investigate further the functions of the EAATs and the AMPA/kainate receptors on Cabs that had both receptor types, we examined their location on these cells. Even though the puffer pipette was placed at the outer plexiform layer (OPL) in the agonist response experiments described in the preceding text, the responses were not necessarily generated in the dendrites as the agonists could have spread to the cell body and axon terminals. To localize the origins of these responses, the puffer pipette was moved to four different positions around the cells, and brief puffs of D-aspartate or kainate applied at each location. For both D-aspartate and kainate, the biggest and fastest responses were obtained when they were puffed onto the OPL (Fig. 2). This suggests that both receptors are mainly on the dendrites. In addition, we encountered eight axonless cells with soma sizes similar to those of Cabs that responded to kainate with $E_{rev}$ close to zero and to D-aspartate with $E_{rev}$ between $-60$ and $-40$ mV (not shown), reinforcing the notion that both receptors are on the dendrites. Therefore both receptors might detect glutamate released from photoreceptors and mediate light responses. As these receptors trigger opposite membrane potential changes (Fig. 1, bottom), they would also induce light-evoked responses with opposite polarities. The sign-preserving AMPA/kainate receptors would generate hyperpolarizing light responses, whereas the sign-inverting EAATs would generate depolarizing ones. Evidence that both mediate light-evoked responses is provided in the following text.

Both AMPA/kainate receptors and EAATs mediate the light response of Cabs

To investigate light-evoked responses, the calcium-based Ringer was used. With internal potassium, the average dark resting potential was $-47.4 \pm 2.0$ mV ($n = 24$). Center-slit stimuli (see Methods) were used to minimize stimulation of the surround region of the receptive field. Responses to white light were obtained from 21 Cabs. These responses displayed a variety of waveforms, suggesting the presence of multiple subtypes of Cabs. In addition, the effects of the AMPA/kainate receptor antagonist DNQX and of the EAAT blocker TBOA suggested the participation of both receptor types in the generation of these responses.

Of the 21 Cabs tested with white light, 2 cells responded with mainly OFF bipolar cell-like properties (i.e., hyperpolarization at light on and depolarization at light off, e.g., Fig. 3, cell 1, left), whereas 11 others gave predominantly ON bipolar responses. The remaining 8 Cabs responded with mixed ON-OFF properties. OFF cells responded with $V_{hold} = -109$ mV and another cell to kainate ($V_{hold} = -69$ mV). The biggest response of each cell to each agonist was normalized to 1. The error bars represent SE.
cell-like responses (i.e., depolarization at light on and hyperpolarization at light off, e.g., Fig. 3, cell 2, left). The responses of the remaining eight cells had both off and on bipolar cell-like components, and two examples are illustrated in Fig. 3 (cells 3 and 4, “control” column). Cell 3 gave a transient depolarization shortly after the beginning of the stimulus, reminiscent of the response of an on bipolar. However, this transient depolarization was immediately followed by a hyperpolarization during the stimulus, and a transient depolarization was generated at light off, characteristics of the response of an off bipolar. Likewise, cell 4 responded to white light with both on and off bipolar cell-like components. It gave a depolarizing response that was sustained throughout the duration of the 1-s stimulus and a transient hyperpolarization at the termination of the stimulus, both characteristics of an on bipolar cell response. However, a transient depolarization immediately followed the hyperpolarization at light off, similar to the response of an off bipolar cell. In short, the responses of Cabs to center-slit white flashes displayed a variety of waveforms, perhaps indicating that multiple physiological subtypes of these bipolar cells exist. An explanation for how these diverse responses may be generated is presented in the Discussion.

To test whether the responses of Cabs to white light are generated by the AMPA/kainate receptors and/or the EAATs, the effects of 40 μM DNQX and 50–100 μM TBOA were tested. DNQX was tested on 11 Cabs, which included 5 on bipolar-like cells, 2 off bipolar-like cells, and 4 hybrid cells. With the AMPA/kainate receptors blocked by DNQX, the responses of the five on bipolar-like cells were largely intact (not shown). On the other hand, the hyperpolarizing responses of both off bipolar-like cells were blocked, revealing depolarizing responses (Fig. 3, cell 1). Similarly, the off bipolar-like features of all four hybrid cells were suppressed, and only the on bipolar-like components remained (Fig. 3, cell 3). Notice that in the presence of DNQX the dark resting membrane potentials of both cells 1 and 3 became more negative, confirming a reduction in the influx of cations through AMPA/kainate receptors.

The opposite result was obtained when TBOA was applied. A total of 15 Cabs were tested, including 10 on bipolar-like cells and 5 hybrid cells. With the EAATs blocked, on bipolar-like components in the responses were suppressed and replaced by off bipolar-like ones in a majority of cases (n = 11); two examples are shown in Fig. 3 (cells 2 and 4). Notice that the dark resting potentials of both cells 2 and 4 became more positive in the presence of TBOA, consistent with a reduction in chloride influx through EAATs. In the remaining four cells tested, all light responses were blocked by TBOA. When DNQX and TBOA were applied simultaneously, all Cab responses were nearly abolished (n = 4; not shown).

Taken together, these results suggest that the light response of most Cabs is mediated by both AMPA/kainate receptors and EAATs with the former generating off bipolar cell-like response components and the latter generating on bipolar cell-like components. Curiously, because the dark resting potential of Cabs (with intracellular potassium) in the calcium-based Ringer was –47.4 mV, whereas the Erev of Cabs’ response to D-aspartate measured with internal cesium was –46.0 mV (see preceding text), light-induced deactivation of EAATs was predicted to result in little voltage change or even slight hyperpolarizations. Thus we performed the aforementioned voltage-ramp experiment to determine the Erev of the D-aspartate response using internal potassium instead of cesium and obtained a value of –61.6 ± 1.7 mV (n = 18), which would enable the generation of depolarizing light responses through EAATs. This difference between the reversal potentials measured with the two internal solutions is not unexpected, because efflux of intracellular potassium through EAATs is involved in the transport of glutamate (Seal and Amara 1999).

As mentioned earlier, some Cabs (n = 4 of 15) lost all light responses in the presence of TBOA (but absence of DNQX). This is in agreement with the agonist response analysis presented above, which showed that some cells (n = 14 of 65) possess only EAATs but not AMPA/kainate receptors.

Many Cabs are double color-opponent

A likely function of bipolar cells using both AMPA/kainate receptors and EAATs was revealed when light responses were elicited by center-slit stimuli of various wavelengths. Blue (400–460 nm), green (490–550 nm), and red (580–640 nm) stimuli were presented to a total of 18 Cabs, 10 of which gave color-opponent responses. In all 10 cases, the responses to short wavelengths were dominated by voltage changes of one polarity and the responses to longer wavelengths by voltage
changes of the opposite polarity. In other words, they were double opponent. (Responses to UV light were not examined, and therefore the percentage of color-opponent cells could have been even higher.) Two examples are shown in Fig. 4, left. In the control Ringer, cell 1 depolarized to blue (430 nm) stimulus but hyperpolarized to a green (550 nm) stimulus, whereas cell 2 hyperpolarized to blue (460 nm) light but depolarized to red (640 nm) light. We did not encounter any triphasic responses (i.e., responses of 1 polarity to the shortest and the longest wavelengths and of the opposite polarity to the middle wavelengths) (Shimbo et al. 2000), but again, the exclusion of UV stimuli in our analysis could have caused any triphasic cells to be overlooked.

The role of AMPA/kainate receptors and/or EAATs in the generation of these color-opponent center responses were investigated pharmacologically. The effect of blocking AMPA/kainate receptors with DNQX was studied on two color-opponent Cabs. In control Ringer, one cell depolarized to blue, hyperpolarized to green and had undetectable response to red (B+G−), whereas the other cell depolarized to blue and green but hyperpolarized to red light (B+G+R−). In DNQX, both cells’ hyperpolarizing responses (to green and red stimuli, respectively) were blocked, although their depolarizing responses remained; thus both became nonopponent. One of these cells is shown in Fig. 4, cell 1. Similarly, when TBOA instead of DNQX was applied on four other color-opponent cells, all four cells lost their color opponency. In the control Ringer, these four cells were B+G−, B−G+, B−G+R+, and B+G−R−. With the EAATs blocked by TBOA, all four cells’ depolarizing responses (to blue, green and red, and blue stimuli, respectively) were either abolished or converted into hyperpolarizing responses, and an example is shown in Fig. 4, cell 2.

Therefore one of the likely functions of using both sign-preserving AMPA/kainate receptors and sign-inverting EAATs to mediate light responses is the generation of double color-opponent receptive field center responses. A Cab may receive inputs from shorter-wavelength cones through AMPA/kainate receptors (or EAATs) to generate hyperpolarizing (or depolarizing) responses to shorter wavelengths but from longer-wavelength cones through EAATs (or AMPA/kainate receptors) to generate depolarizing (or hyperpolarizing) responses to longer wavelengths.

**DISCUSSION**

**Prevalence of EAATs on teleost retinal bipolar cells**

EAATs in the CNS typically play supportive and modulatory roles, e.g., maintaining extracellular glutamate below neurotoxic levels, re-uptake of glutamate into the presynaptic terminal for the re-cycling of glutamate, reduction of synaptic release through negative feedback, prevention of neurotransmitter spillover, etc. (reviewed in Amara and Fontana 2002; Danbolt 2001). The mediation of light-evoked responses by EAATs on teleost retinal bipolar cells was the first demonstration that EAATs can function as glutamate receptors (Grant and Dowling 1995, 1996; Wong et al. 2005a,b). In the present communication, we have provided evidence that EAATs also directly mediate synaptic transmission from cone photoreceptors to another type of teleost retinal bipolar cell, namely the ON-OFF bipolar cells, further illustrating the importance of EAATs at the first synapse of the visual system in teleosts.

It remains to be tested whether the EAATs also play such a role in the OFF bipolar cells. Anatomical evidence for EAATs on OFF bipolar cell dendrites has been reported in various nonteleost species (Eliasof et al. 1998a,b; Euler and Wässle 1995; Fyk-Kolodziej et al. 2003, 2004; Gru¨nert et al. 1994; Rauter and Kanner 1994; Rauter et al. 1996; Reye et al. 2002). For teleosts, two anatomical studies of the distribution of GLT-1a (i.e., EAAT2A) and EAAC1 (i.e., EAAT3) in the goldfish retina showed that neither was expressed in the dendrites of OFF bipolar cells (Schultz and Stell 1996; Vandenberg et al. 2000). However, the expression of other EAATs in teleost OFF bipolar cells may not have been examined.

**ON-OFF bipolar cells in other species**

The present study has provided the first electrophysiological evidence that both sign-preserving and sign-inverting glutamate receptors can co-localize to the same teleost retinal bipolar cell and that both can mediate light responses. Consistent with our findings, electron microscopic studies have shown that some bipolar cells in carp and catfish retinas make both invaginating (presumably sign-inverting) (Stell et al. 1977) and noninvaginating (presumably sign-preserving) (Stell et al. 1977) synapses with photoreceptors (Hidaka et al. 1986; Saito et al. 1985).

In addition to fish, bistratified bipolar cells have been found in turtle and salamander retinas. In turtle, these cells hyperpolarize to white light (Ammermüller and Kolb 1995; Marchiafava and Weiler 1980; Weiler 1981), but in response to...
colored stimuli, some of them hyperpolarize to certain wave-
lengths and depolarize to others, i.e., they are color-opponent
(Ammermüller et al. 1995). A detailed electron microscopic
analysis of one such cell, which depolarized to red and hyper-
polarized to green stimuli, revealed that it made invaginating
and noninvaginating synapses with red and green cones, re-
spectively, raising the possibility that the color-opponent cen-
ter responses of these cells are generated by the utilization of
different types of glutamate receptors at these synapses
(Haverkamp et al. 1999). In another study, Pang et al. (2004)
measured light-induced current responses from the bistratified
bipolar cells in salamander and showed that these cells had two
 glutamate receptor mechanisms, one sign-inverting (mediated
by metabotropic glutamate receptor type 6, or mGluR6) and
one sign-preserving (mediated by AMPA/kainate receptors).
Both mechanisms were shown to mediate light-evoked re-
sponses, although responses to different wavelengths were not
investigated systematically to examine whether these cells
were color-opponent. Therefore bistratifying bipolar cells with
both sign-inverting and sign-preserving glutamate receptors
seem to be common across cold-blooded species, showing that
the classical division of bipolar cells into either depolarizing or
hyperpolarizing varieties is an oversimplification.

In contrast to our results, Connaughton and Nelson (2000)
did not encounter Cabs with both sign-preserving and sign-
inverting glutamate receptors in the zebrafish, a species closely
related to the giant danio (Meyer et al. 1993). The most likely
 explanation is that they used the general agonist L-glutamate
instead of the EAAT-specific D-aspartate to look for EAATs on
the Cabs. As L-glutamate activates not only the EAATs but
also the AMPA/kainate receptors, the reversal potentials for
the glutamate and the kainate responses might not have dif-
fered enough to reveal clearly the presence of EAATs, and the
differences were probably made even less obvious by the
20-mV incremental voltage steps used in that study.

Functions of Cabs

WIDENING THE RESPONSE DYNAMIC RANGE. In response to white
light stimulation, Cabs display three main types of response
waveforms, and two of these three types are illustrated in Fig.
3. The response of cell 1 resembled that of an OFF bipolar cell,
whereas the response of cell 2 was similar to that of an ON
bipolar. In the control Ringer (Fig. 3, left), both AMPA/kainate
receptors and EAATs were presumably functional, and cell 1
behaved like an OFF bipolar most likely because the AMPA/
kainate receptors dominated over the EAATs, whereas cell 2
responded like an ON bipolar because the EAATs dominated.
When the AMPA/kainate receptors on cell 1 were blocked with
DNQX, only the EAATs remained operational, thereby con-
verting the response to be ON bipolar cell-like. On the other
hand, the converse was true when the EAATs on cell 2 were
blocked by TBOA (Fig. 3). An obvious question is why both
sign-preserving and -inverting glutamate receptors are used to
generate these two response types. A plausible explanation is
that because AMPA/kainate receptors and EAATs induce volt-
age changes of opposite polarities, their responses partially
oppose each other and thereby prevent the light responses from
saturating easily. This results in a wider response dynamic
range than could be achieved with either receptor type alone,
and this may be the first function of Cabs.

CREATION OF NONLINEAR RECEPTIVE FIELDS. On the other hand,
in the third type of Cab response to white light, both OFF and ON
bipolar cell properties are manifested, as exemplified by Fig. 3,
cells 3 and 4. In these Cabs, neither glutamate receptor type
obscures the response of the other type, perhaps because the
AMPA/kainate receptors and the EAATs respond to presynap-
tic photoreceptors with different kinetics. For example, one of
these receptor molecules might be located farther away from
the glutamate release sites and thus responds to changes in
 glutamate concentration more slowly so that the depolarizing
and hyperpolarizing components are separated temporally. As
these Cabs depolarize at both light on and light off, their light
responses show rectification and frequency doubling. Rectifi-
cation is responsible for generating the nonlinear receptive
field of the Y-type ganglion cells originally discovered in the
cat retina (Enroth-Cugell and Robson 1966; Hochstein and
Shapley 1976). Y-type-like ganglion cells have been reported
in teleost retinas (Bilotta and Abramov 1989; Sakai and Naka
1995). Thus part of the nonlinear properties of these teleost
ganglion cells may originate at the dendrites of Cabs by mixing
ON and OFF bipolar cell properties in the same cell.

Half (n = 4 of 8) of the Cabs demonstrating hybrid ON and
OFF bipolar cell light response properties had very transient
responses (e.g., Fig. 3, cell 3). In contrast, all of the cone-
driven or mixed-input ON bipolar cells examined (which pos-
sess only sign-inverting glutamate receptors) were much more
sustained (Wong et al. 2005b). An appealing explanation is that
in Cabs, the sign-preserving AMPA/kainate receptor response
and the sign-inverting EAAT response act to truncate each
other, resulting in phasic voltage changes. However, this does
not appear to be the case because blocking one of the two
 glutamate receptors did not cause the remaining response to
become more sustained (n = 3 of 3; e.g., Fig. 3 cell 3). We
propose that the transient quality of these responses is mainly
due to activation and/or inactivation of voltage-gated channels.
A variety of voltage-gated potassium and calcium channels are
present in Cabs (Connaughton and Maguire 1998).

GENERATION OF COLOR OPPONENCY. Color-opponent retinal bi-
polar cells in fish were first reported by Kaneko (1973). The
underlying synaptic mechanisms were investigated in a more
recent study by Shimbo et al. (2000), using carp. Annuli and
center spots at various wavelengths were presented, and the
reversal potentials for the responses were estimated by inject-
ing depolarizing or hyperpolarizing currents. The authors con-
cluded that the color-opponent responses to the annuli could be
explained by the horizontal cell cascade model proposed by
Stell and Lightfoot (1975), which postulates that a network of
sign-preserving feedforward and sign-inverting feedback inter-
actions between cones and horizontal cells gives rise to color-
opponent responses in the horizontal cells. On the other hand,
Shimbo et al. found that the responses to center spots could not
be explained by this model and thus were most likely generated
by cells/mechanisms other than the horizontal cells. The center
responses to some wavelengths were found to reverse at
relatively positive membrane potentials; the authors proposed
this to be an indication of direct inputs from the cones. In
contrast, the responses to other wavelengths had a more nega-
tive $E_{rev}$ and were proposed to be mediated by inhibitory
transmitters released by amacrine cells onto these bipolar cells’
axon terminals.
By using a stimulus intended to activate mainly the center region of the giant danio Cabs, we found that just over half \((n = 10 \text{ of } 18)\) of these cells were color-opponent. Due to technical limitations, wavelengths in the UV region were not tested. In giant danio, there are UV cones with peak sensitivity at 358 nm (Palacios et al. 1996) that could conceivably elicit Cab responses with different polarities than those triggered by the other three cone types. Therefore the actual percentage of color-opponent Cabs in giant danio could be even higher. Due to the inclusion of picrotoxin and strychnine in our Ringer, the involvement of amacrine cells is unlikely, although we cannot rule out that under conditions where GABAergic and glycinergetic synaptic transmission is preserved, amacrine cells could participate in the generation of bipolar cell color opponency. With inputs from amacrine cells blocked and influences from the horizontal cell-mediated surround minimized, the photoreceptor-Cab synapse becomes the most likely site where the color-opponent responses we obtained were generated. Because Cabs have both sign-preserving AMPA/kainate receptors and sign-inverting EAATs on their dendrites, and pharmacologically blocking either of these receptor mechanisms abolished color opponency in these cells, we propose that Cabs receive inputs from certain cones through AMPA/kainate receptors to generate hyperpolarizing responses to certain wavelengths and from other cones through EAATs to generate depolarizing responses to other wavelengths. These two mechanisms are consistent with, respectively, the positively reversing and the negatively reversing mechanisms in the color-opponent bipolar cells described by Shimbo et al. 2000. As mentioned earlier, some bipolar cells in turtle may also use both sign-preserving and -inverting glutamate receptors to generate color opponency, although in that case the sign-inverting mechanism is presumably mGluR6 rather than EAATs (Haverkamp et al. 1999).

Glutamatergic input mechanisms generating bipolar cell responses: a summary

In this series of papers, we have shown by electoretinographic and patch-clamp recordings that EAATs, mGluR6, and AMPA/kainate receptors account for most if not all light-evoked responses of retinal bipolar cells in the giant danio. The various responses recorded reflect the types of glutamate receptors present. On bipolar cells, which depolarize to light, use sign-inverting EAATs and mGluR6 with the cone-driven cells using the former exclusively and the mixed-input cells using both mechanisms. EAATs and mGluR6 may confer cone and rod inputs with different properties, and interaction between these receptors in the mixed-input on bipolar may underlie rod-cone suppression in these cells (Wong et al. 2005b). Off bipolar cells, which hyperpolarize to light, use sign-preserving AMPA/kainate receptors (Wong et al. 2005a), although, as mentioned above, whether or not they also use EAATs remains to be tested. On-off bipolar cells, which display a variety of complex (including color-opponent) responses to light, use both sign-inverting EAATs and sign-preserving AMPA/kainate receptors, probably in various ratios and with varying response kinetics (the present paper).

Such a generalization, namely that the glutamate receptors determine the response polarities of on, off, and on-off bipolar cells, also appears to apply to species other than teleosts (McGille and Dacheux 2001; Pang et al. 2004; Thoreson and Witkovsky 1999). The only difference between teleosts and other species is that in the latter, only mGluR6 and AMPA/kainate receptors are believed to be used (Thoreson and Witkovsky 1999). However, EAATs are expressed on the dendrites of many bipolar cells in most non teleost species examined (e.g., salamander: Eliasof et al. 1998a,b; cat: Fyk-Kolodzie et al. 2004; rat: Rauen et al. 1996), and a review of the literature suggests that mGluR6 may not account for all on bipolar cell responses in many nonteleost species (Wong et al. 2005b). Thus the involvement of EAATs in photoreceptor-bipolar cell signaling in species besides teleosts has not been ruled out.

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