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Transporter-mediated GABA responses in horizontal and bipolar cells of zebrafish retina

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Abstract

GABA-mediated interactions between horizontal cells (HCs) and bipolar cells (BCs) transform signals within the image-processing circuitry of distal retina. To further understand this process we have studied the GABA-driven membrane responses from isolated retinal neurons. Papain-dissociated retinal cells from adult zebrafish were exposed to GABAergic ligands while transmembrane potentials were monitored with a fluorescent voltage-sensitive dye (oxonol, DiBaC₄(5)). In HCs hyperpolarizing, ionotropic GABA responses were almost never seen, nor were responses to baclofen or glycine. A GABA-induced depolarization followed by after hyperpolarization (dep/AHP) occurred in 38% of HCs. The median fluorescence increase (dep component) was 0.17 log units, about 22 mV. HC dep/AHP was not blocked by bicuculline or picrotoxin. Muscimol rarely evoked dep/AHP responses. In BCs picrotoxin sensitive, hyperpolarizing, ionotropic GABA and muscimol responses occurred in most cells. A picrotoxin insensitive dep/AHP response was seen in about 5% of BCs. The median fluorescence increase (dep component) was 0.18 log units, about 23 mV. Some BCs expressed both muscimol-induced hyperpolarizations and GABA-induced dep/AHP responses. For all cells the pooled Hill fit to median dep amplitudes, in response to treatments with a GABA concentration series, gave an apparent k of 0.61 μ M and an n of 1.1. The dep/AHP responses of all cells required both extracellular Na⁺ and Cl⁻, as dep/AHP was blocked reversibly by Li⁺ substituted for Na⁺ and irreversibly by isethionate substituted for Cl⁻. All cells with dep/AHP responses in zebrafish have the membrane physiology of neurons expressing GABA transporters. These cells likely accumulate GABA, a characteristic of GABAergic neurons. We suggest Na⁺ drives GABA into these cells, depolarizing the plasma membrane and triggering Na⁺, K⁺-dependent ATPase. The ATPase activity generates AHP. In addition to a GABA clearance function, these large-amplitude transporter responses may provide an outer plexiform layer GABA sensor mechanism.

Keywords

Outer plexiform layer; ATPase; GABA transporter; baclofen; glycine; horizontal cell; bipolar cell

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Introduction

The actions of GABA on distal retinal neurons modify and transform visual signals as they propagate forward to the inner retina and brain. GABA is a neurotransmitter candidate for at least some types of vertebrate retinal horizontal cells. In goldfish, H1 type horizontal cells, but not other types, take up and release GABA (Marc *et al.*, 1978). A perhaps analogous type of horizontal cell in zebrafish displays a GABAergic molecular signature (Sandell *et al.*, 1994; Connaughton *et al.*, 1999; Marc & Cameron, 2001; Yazulla & Studholme, 2001). There are at least three morphological cone horizontal cell types in zebrafish (Connaughton *et al.*, 2004; Song *et al.*, 2008), and recently 5 spectral types have been described (Nelson & Connaughton, 2007). At least one of these types may take up and release GABA in synaptic or extra-synaptic communication with bipolar cells, photoreceptors, or other horizontal cells.

Vertebrate retinal horizontal cells may, or may not express one or more of the following GABA-related membrane functions: GABA_A receptors, GABA_C receptors, electrogenic GABA transporters (Malchow & Ripps, 1990; Qian & Dowling, 1993; Dong *et al.*, 1994; Blanco *et al.*, 1996; Verweij *et al.*, 1998), either of the GABA synthetic enzymes GAD65 or GAD67 (Vardi *et al.*, 1994), or high GABA content in the cytoplasm (Wassle & Chun, 1989; Grunert & Wassle, 1990; Connaughton *et al.*, 1999). Some zebrafish horizontal cells stain for GABA (Sandell *et al.*, 1994; Connaughton *et al.*, 1999) and express mainly GAD67 (Connaughton *et al.*, 1999; Connaughton *et al.*, 2001; Yazulla & Studholme, 2001). The vesicular GABA transporter VGAT intensely labels the zebrafish horizontal cell layer (Yazulla & Studholme, 2001), and in mouse retina syntaxin-4 and SNAP-25 localize to horizontal-cell dendrites (Hirano *et al.*, 2007), all suggestive of release of vesicular GABA. In zebrafish horizontal cells GABA membrane physiology remains to be explored.

Horizontal cells may release GABA to influence bipolar cells. The dendrites of vertebrate bipolar cells are commonly reported to express GABA_A and GABA_C receptors (Qian & Dowling, 1995; Vaquero & de la Villa, 1999; Du & Yang, 2000; Billups & Attwell, 2002; Varela *et al.*, 2005). While the greatest density of GABA receptors is localized on bipolar cell axon terminals (Tachibana & Kaneko, 1988), the relatively weaker GABA responses in the soma-dendritic region can not be neglected in theories of retinal information processing. These distal receptors provide a potential target for GABA released from horizontal cells. Horizontal cells are a conduit for wide-field stimuli that modulate the responses of the narrower-field bipolar cells. The major circuit for this interaction appears to be indirect, through the cones (Baylor *et al.*, 1971; Stell & Lightfoot, 1975; Lasansky, 1981; Kamermans *et al.*, 2001). However, horizontal cells have long been known to synapse directly on bipolar cell dendrites. As an example, axon terminals of salamander horizontal cells synapse on the dendrites of salamander bipolar cells (Lasansky, 1973, , 1980).

The physiological role of horizontal-cell GABA on bipolar-cell dendrites has been perplexing, both from the perspective of actual evidence, and from the perspective of theoretical use. Bipolar cell surround interactions would appear to require different signaling systems for ON and OFF type bipolar cells, whereas GABA is only one molecule. Vardi (Vardi *et al.*, 2000) proposed that GABA might acquire two different actions if the postsynaptic bipolar cells expressed chloride transporters that either normally extruded (KCC2) or accumulated chloride (NKCC), thus providing, relative to membrane potential, either depolarizing or hyperpolarizing gradients for the GABA Cl⁻ ionotropic channel. While antibody localizations confirmed that in fact ON type bipolar cells expressed NKCC transporters and OFF type bipolar cells expressed KCC2 transporters (Vardi *et al.*, 2000), electrophysiological measurements have not provided a uniform picture. Depolarizing dendritic chloride gradients were found in isolated mouse rod bipolar cells (Varela *et al.*, 2005), and some mouse bipolar cell types studied in slice

(Duebel *et al.*, 2006), but not in either isolated rat bipolar cells (Nelson *et al.*, 1999) or rat bipolar cells studied in slice (Billups & Attwell, 2002).

This study examines depolarizing GABA voltage responses in dissociated zebrafish retinal neurons. The common, hyperpolarizing, GABA_{A,C} responses of zebrafish bipolar cells, that are found on both dendrites and axon terminals, are described in a companion paper (Connaughton *et al.*, in preparation). The focus of this study is on the less common GABA-induced depolarizations followed by after-hyperpolarizations (dep/AHP responses). Dep/AHP responses occurred in about 1/3 of isolated zebrafish horizontal cells and about 1/20 of isolated bipolar cells. These responses have the characteristics of a GABA membrane transporter. Ionotropic GABA receptors were not found on isolated zebrafish horizontal cells. We have used the voltage sensitive dye oxonol (DiBaC₄(5)) instead of microelectrode recordings in order to minimally disrupt normal membrane potential, as well as chloride and other electrochemical gradients contributing to the various types of GABA response.

Materials and methods

Preparation of isolated cells

Adult zebrafish (*Danio rerio*, EK strain) were dark-adapted overnight then decapitated according to an Animal Study Protocol approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke, NIH, in accordance with National Research Council Guidelines and Public Health Service Policy on Humane Care and Use of Laboratory Animals. The isolation and recording procedures follow those previously described (Nelson *et al.*, 2003). Dark adapted eyes were removed; and cornea, iris, and lens dissected away. Retinas were teased free, incubated with 33 U·ml⁻¹ papain (Worthington Biochemical Corp, Lakewood, NJ), 0.19 U·ml⁻¹ dispase (Sigma Chemical Co., St. Louis MO) in 70% L-15 culture media (Life Technologies (Gibco BRL), Grand Island, NY), dissociated by trituration (Connaughton & Dowling, 1998), plated on poly-D-lysine coated plastic culture dishes (2.1·μg cm⁻², Collaborative Biomedical Products, Bedford, MA; Nalge Nunc International, Rochester, NY), and studied acutely, within 2-6 hr of dissociation. Dissociated horizontal and bipolar cells were identified morphologically using Hoffman optics in an upright microscope. Horizontal cell bodies appeared large and flattened as compared to bipolar cells, and were polymorphic. As this study investigates potential horizontal-cell-to-bipolar-cell interactions in the outer plexiform layer of zebrafish, axotomized bipolar cells were of particular interest. BCs without axons could be readily identified by a flask-shaped soma with dendritic tufts emerging from the top. For oxonol photometry, area of interest maps (AOI's) were generated on Hoffman images and later applied to fluorescence time-series micrographs as seen through the same objective. In some cases fluorescence (FL) measurements were collected from cell body, dendrites and/or axon terminal regions of individual bipolar cells using separate AOI's. For completeness, the GABA physiology gathered from the AOI's of all cells in the microscope field was analyzed.

Recording solutions

The recording medium is the same as used in previous patch recordings from zebrafish retinal slice (Connaughton & Nelson, 2000; Connaughton *et al.*, in preparation) and in voltage probe recordings of glutamate responses in dissociated cells (Nelson *et al.*, 2003). This was composed of (in mM): 120 NaCl (120 LiCl for Na⁺ substitution, 120 Na⁺ isethionate for Cl⁻ substitution), 2 KCl, 1 MgCl₂, 3 CaCl₂, 3 D-glucose, 4 HEPES (pH adjusted to 7.4-7.5), and 80 nM DiBaC₄(5) oxonol (Invitrogen/Molecular Probes, Eugene, OR), the voltage probe. A primary 2 mM oxonol stock was dissolved in ethanol; from this a secondary 20 μM aqueous stock was prepared each experimental day to be further diluted to 80 nM for experimental use (ethanol carrier was diluted by 25,000 in the process). GABA agonists, antagonists and other ligands

were added to this recording medium in concentrations ranging from 0.1 to 100 μM . Reagents employed included GABA, muscimol, picrotoxin, glycine and glutamate (Sigma Chemical Co., St. Louis, MO); R(+)-baclofen, (-)-bicuculline methbromide (Research Biochemicals International, Natick, MA); gramicidin and recording-medium salts (Sigma Chemical Co., St. Louis, MO).

Voltage probe recording and data analysis

Plated cells were superfused under cover glass ($140 \mu\text{l}\cdot\text{min}^{-1}$; mean fluid velocity, $2600 \mu\text{m}\cdot\text{s}^{-1}$) with extracellular medium containing the fluorescent, voltage-sensitive dye oxonol ($\text{DiBaC}_4(5)$, 80nM). With this voltage probe, membrane depolarization causes fluorescence (FL) increase as the negatively charged probe equilibrates across the membrane (Waggoner, 1976). After initial equilibration with the voltage probe (10-20 min), FL responses to GABAergic ligands were obtained in parallel for each cell in the selected microscope field using intensified fluorescence video microscopy (V/ICCD GENIV, Princeton Instruments Inc., Trenton NJ), with images captured at 30s intervals. This rate is 3-4 times faster than the limiting median equilibration time constants for oxonol (see below). Data were acquired in a darkened room using 1s exposures (Texas red filter set, Chroma Technology Corporation, Brattleboro, VT) as the probe is readily bleached. Integrated FL from pixels within each AOI were summed and FL of equivalent background areas subtracted before conversion to $\log(\text{FL})$. The data acquisition process and software were developed in house by Walton et al (Walton *et al.*, 1993) and further refined by the authors for the present application.

$\log(\text{FL})$ time series were adjusted in two further ways to correct for drift. First, as previously described (Nelson *et al.*, 2003), the mean $\log(\text{FL})$ time series for cells unresponsive to treatments was calculated and this signal was subtracted from the $\log(\text{FL})$ signals of responsive cells. The amount of this subtracted drift signal was optimized so that net fluorescence variance over time in the responsive cells was minimized. This compensates in part for systematic factors such as initial oxonol loading, bleaching, focus drift, source or camera variations. Further residual linear drift ($<0.01 \log\cdot\text{min}^{-1}$) was eliminated by adding or subtracting linear segments across the record so as to bring $\log(\text{FL})$ values before the beginning of each treatment to the same level. In cells with prolonged after-hyperpolarizing (AHP) responses that encroached on the following treatment, this latter approach was not applied.

For counting purposes, responses to drug treatments were simply scored as responsive (i.e., noticeable change in FL), or not. While the threshold for scoring a response depended on factors such as cell noise level and the appearance of a recognized waveform, in general the peak to peak change needed to exceed 0.05 $\log(\text{FL})$ units to be counted as a response. For treatments with antagonists, individual cell responses were categorized as blocked, not blocked, partially blocked, or enhanced. "Blocked" responses were those with less than 20% of control amplitude remaining. "Not blocked" responses retained 80% to 120% of the control response amplitude. With "enhanced" responses amplitudes increased beyond 120% of control. Responses categorized as a "partial block" had between 20% and 80% of the control response remaining.

The various aspects of cellular responses and images were organized and compiled in Filemaker Pro v. 6 (Santa Clara, CA). Data plotting, adjusting and statistics were performed in Origin v. 7 (OriginLab Corporation, Northampton MA). Images were measured in Irfanview 3.8 (www.irfanview.com). Because the distributions of oxonol fluorescence responses are generally skewed and typically bounded by '0', we found medians were more representative of histogram peaks than means, and more appropriate in representing population data. Two-tailed Mann-Whitney U tests were employed to study the significance of differences in these medians.

Membrane potentials, calibrations and time constants

Only cells depolarized by gramicidin (cells with negative resting potential) were considered in this study. Gramicidin makes cells permeable to monovalent cations, establishing a transmembrane potential of '0' mV (Maric *et al.*, 2000). The median resting potential for horizontal cells measured this way was -0.39 log units. (about -50 mV, N=78). The median log(FL) equilibration time constant for gramicidin treatment in horizontal cells was 2.10 min (N=76). Measures for the simultaneously recorded isolated bipolar cells were -0.53 log units (about -68 mV, N=278: all regions), and 1.65 min, (N=262, all regions). Horizontal cell resting potentials were significantly less hyperpolarized than bipolar cell resting potentials, and equilibration time constants were significantly slower ($p < 0.001$, Mann Whitney U test). The calibrations of Nelson *et al* (1999) are used to estimate membrane potential from log(FL) changes. These calibrations suggested a curved transform with a conversion constant of $129 \text{ mV} \cdot \log^{-1}$ for depolarizing excursions, and $72 \text{ mV} \cdot \log^{-1}$ for hyperpolarizing excursions. Fluorescence changes of about $100 \text{ mV} \cdot \log^{-1}$ have elsewhere been reported for the oxonol probe (Dall'Asta *et al.*, 1997; Langheinrich & Daut, 1997). Horizontal cells with depolarized resting potentials, as previously reported (Nelson *et al.*, 2003), were excluded from the current study unless glutamate was one of the treatment protocols. Glutamate stimulates ATPase activity and generates resting potential in these depolarized neurons. Without glutamate, they can not be distinguished from unresponsive cells.

Results

Morphology of dissociated cells

Viewed in Hoffman optics, horizontal cells were larger in size, more flattened in appearance than bipolar cells and other retinal neurons which surrounded them. Unlike in situ stains (Connaughton *et al.*, 2004; Song *et al.*, 2008), axons were not found and may have been sheared off. In zebrafish different L- or C-type physiologies can not as yet be associated with morphological features in culture, so we have followed the previous convention of dividing plated cells into two size types (McMahon, 1994; Connaughton & Dowling, 1998). There are the somewhat smaller round radiate types with 5-7 dendrites (H_A), and larger types with irregularly shaped cell bodies and 2 or 3 stout dendrites (H_B) (Connaughton & Dowling, 1998). The cell bodies of H_A types (28/80, 35%) were $11.4 \pm 2.2 \times 9.4 \pm 1.6 \mu\text{m}$ diameter (major and minor axes). Cell bodies of the more numerous H_B type cells (52/80, 65%) were $14.5 \pm 2.4 \times 11.2 \pm 1.5 \mu\text{m}$ diameter. These dimensions are similar to those previously reported (Connaughton & Dowling, 1998). Horizontal cells represented only a small fraction of dissociated cells. Even in our selected fields only about 3% (80/2396) were horizontal cells.

Bipolar cell bodies were flask shaped with short dendrites emerging from the top. Some (42 / 223, 19%) retained axons with terminal boutons. Bipolar cell bodies, where measured, were $8.2 \pm 1.3 \times 7.1 \pm 1.0 \mu\text{m}$ (N=155), smaller than horizontal cell bodies. Bipolar cell bodies were convex and appeared to protrude farther into the medium than the mesa-like horizontal cell counterparts. Morphologically identifiable bipolar cells were 12% (298/2396) of plated and studied cells.

The remaining plated cells were rounded in appearance, and similar to bipolar cells in size. With diolistic staining of zebrafish slices, a shotgun approach without known bias, 48% of stains were bipolar cells (Connaughton *et al.*, 2004). While the type-specific efficiencies of cell dissociation and diolistic staining may differ somewhat, still it seems reasonable to suppose that a substantial fraction the remaining dissociated cells were also bipolar cells.

GABA and glutamate after-hyperpolarizations in horizontal cells

In previous studies (Nelson *et al.*, 2003), horizontal cells responded to glutamate treatment with waveforms composed of depolarizations and/or after-hyperpolarizations (dep/AHP). The AHP component was traced to glutamate stimulation of $\text{Na}^+ \text{K}^+$ ATPase activity. GABA elicits a similar pattern of response. In Fig. 1, a horizontal cell, because of depolarized initial resting potential, does not react immediately to glutamate application, but hyperpolarizes on withdrawal of glutamate (AHP). This AHP response restores membrane potential. When GABA is then applied to the same cell a GABA-induced depolarization is seen. With GABA removal, a second, weaker AHP occurs after a short delay. In a third treatment in the same cell, the combination of GABA and glutamate produces an even more robust depolarization followed by AHP. GABA does not act to antagonize the glutamate response, as might be expected of an inhibitory neurotransmitter, but rather appears synergistic. In a 4th treatment on this same cell glutamate alone evokes another dep/AHP response. In the 5th and final treatment, gramicidin is applied. This irreversibly permeabilizes the membrane to cations, depolarizing the cell to '0' membrane potential. In a total of 27 horizontal cells found with dep/AHP responses to glutamate, 13 dep/AHP responses were also evoked by GABA. No GABA-induced hyperpolarizations were seen in this group.

GABA and muscimol responses in horizontal and bipolar cells

Horizontal and bipolar cells that are depolarized by GABA are less affected by muscimol. In Fig. 2A is an example of a bipolar-cell subtype that responds to GABA with a dep/AHP response. Muscimol (1st treatment), an agonist for $\text{GABA}_{\text{A,C}}$ ionotropic receptors, evokes a smaller dep/AHP response from this cell than GABA (2nd treatment). More typically, however, bipolar cells hyperpolarize in response to either of these agonists (Fig. 2B, 1st and 2nd treatments). This latter pattern suggests $\text{GABA}_{\text{A,C}}$ receptors are expressed on bipolar cell dendrites and caused hyperpolarization. This common, hyperpolarizing, bipolar-cell GABA response is further developed in a companion paper (Connaughton *et al.*, in preparation).

The probability of GABA or muscimol evoking hyperpolarizing or dep/AHP responses in horizontal and bipolar cells is summarized in Fig. 3. About 38% of horizontal cells responded to GABA with dep/AHP responses (27 /71; Fig. 3A), about evenly distributed between H_A (12) and H_B (15) types. Depolarizing components were found in 18 of these H_A or H_B types, while the remainder only responded with the AHP component. For cells with depolarizing components, the median amplitude (both H_A and H_B) was 0.17 log units, about 22 mV. GABA-evoked hyperpolarizations in horizontal cells (3%, 2 /71) were almost non existent as compared to dep/AHP responses, or as compared to bipolar cell hyperpolarizations (67%, 194/288). A large group of horizontal cells did not respond to GABA at all (59%, 42/71). Nonetheless these latter cells responded robustly to gramicidin, indicating an intact and functional plasma membrane.

With muscimol treatments only 8% of horizontal cells responded with dep/AHP (3/40), while ~90% did not respond. Muscimol was less effective than GABA in evoking dep/AHP in horizontal cells ($p < 0.001$, Chi Square test). In horizontal cells there was no pattern of responsiveness either to baclofen (a GABA_B agonist) or glycine, with 86% (12/14) not responding to baclofen, and 93% (14/15) not responding to glycine (data not shown).

Bipolar cell patterns of response to GABA and muscimol differed from those of horizontal cells (Fig. 3B). For GABA, 194 of 288 bipolar cell regions (67%) hyperpolarized. Similarly for muscimol 56 of 91 bipolar cell regions (62%) hyperpolarized. GABA-evoked dep/AHP responses were seen in 5% of bipolar cell regions (13/288), much less common than bipolar-cell hyperpolarizations, or horizontal-cell dep/AHP responses ($p < 0.001$, Chi Square test). Nonetheless the 13 examples of this response in bipolar cells are sufficient to group as a subtype

and to characterize. Eight of the 13 GABA-excited bipolar cell regions were recorded from axotomized cells. This suggests, at least, a soma-dendritic origin for bipolar cell dep/AHP responses, as is also true for hyperpolarizing responses (Connaughton *et al.*, in preparation). Muscimol evoked dep/AHP responses occurred in 2% of bipolar cells (2/91). While this fraction was not significantly less than for GABA ($p \geq 0.05$, Chi Square test), it is clear from individual records (Fig. 2A) that for GABA-excited bipolar cells, as for horizontal cells, the actions of GABA and muscimol are indeed distinct, and that muscimol was the weaker agonist for the dep/AHP response.

Actions of ionotropic GABA antagonists

Antagonists of ionotropic GABA receptors did not block GABA-induced dep/AHP responses in any type of dissociated zebrafish retinal neuron. This suggests the dep/AHP mechanism does not involve ionotropic GABA receptors. For bipolar cells, the combination of bicuculline and picrotoxin failed to block a GABA-induced dep/AHP response (Fig. 2A, 3rd treatment). In the same microscope field, these same antagonists effectively blocked a GABA-induced bipolar-cell hyperpolarization (Fig. 2B, 3rd treatment). In Fig. 4 a horizontal cell depolarizes in response to GABA, and an AHP response is seen (1st and 2nd treatments). Although the AHP component is reduced, in the presence of the bicuculline and picrotoxin mixture (2nd treatment), the depolarizing component persists.

Bicuculline and picrotoxin insensitivity of the dep/AHP response was the rule not only for horizontal cells and bipolar cells, but for all dissociated cells where this response was seen. Taken as a group, which includes 3 horizontal cells and 3 bipolar cells, 31 of 32 dep/AHP responses to GABA (10 or 20 μM) failed to be either fully or partially blocked by picrotoxin (25 or 50 μM , data not shown). In the dep/AHP group as a whole, some depolarizing responses (38%, 12/32) were enhanced by picrotoxin, but no enhancements occurred among horizontal cells (0/3). Among cells with measured initial GABA depolarizing components in the picrotoxin-treated group, the median control and recovery response was 0.14 log units ($n = 58$). The median picrotoxin treated GABA response was 0.21 log units ($n = 29$). The increase is significant ($p < 0.01$, Mann Whitney U test). This suggests an enhancement of depolarizing GABA responses by picrotoxin in some retinal neurons.

Bicuculline (25 or 50 μM) was not an effective antagonist of horizontal cell dep/AHP. Of 6 such responses seen in horizontal cells only 1 was partially blocked and none of 3 bipolar cell dep/AHP was blocked (data not shown). Enhancement was seen in 1 of the 6 horizontal cell dep/AHP responses. Bicuculline was ineffective at blocking dep/AHP responses in other dissociated retinal neurons. Six of 51 responses (12%) were blocked or partially blocked. Response enhancement occurred in 41% of cases (21/51). Among bicuculline treated cells with initial GABA depolarizations, the median control and recovery response was 0.10 log units ($n = 92$). The median bicuculline treated GABA depolarization was 0.19 log unit ($n = 46$, data not shown). This increase was significant ($p < 0.01$, Mann Whitney U test). As with picrotoxin, the change was not in the direction of response blockade, but of response enhancement.

Together with weak muscimol sensitivity (Figs. 2 & 3), the ineffectiveness of ionotropic GABA antagonists, applied either alone or in combination, suggests that *depolarizing* GABA responses in dissociated zebrafish retinal neurons, including some horizontal cells and a subtype of bipolar cells, do not result from the actions of ionotropic GABA receptors. In the same experiments, however, the *hyperpolarizing* responses evoked by GABA in bipolar cells were blocked by picrotoxin (but not bicuculline) with a complete or partial block observed in all cases (Fig. 2; see also Connaughton *et al.*, in preparation).

Dose dependence of GABA dep/AHP responses

GABA-induced dep/AHP responses were evoked by treatments using less than 1 μM GABA (Fig. 5). Five concentrations were applied to each cell, each increasing by a factor of 2, as seen in Fig. 5A. While the initial concentration in Fig. 5A was 0.25 μM GABA, initial concentrations in other experiments varied from 0.125 to 1.0 μM GABA. The half-amplitude concentration was estimated by eye for 25 cells. This median was 0.5 μM . This group included 6 identified horizontal cells with a median half-amplitude concentration of 1.5 μM . The horizontal cell half amplitudes did not differ from the group as a whole ($p \geq 0.05$, Mann Whitney U test). No bipolar cells were identified in this treatment group.

The *dep* components of the dep/AHP responses were then measured and pooled so that median responses could be calculated for each concentration tested in this set of experiments. The pooled data were fit to a Hill model (Fig. 5B). An apparent k value (half saturation) of 0.61 μM was found with an n value of 1.15.

Sodium and chloride requirement of GABA dep/AHP responses

The sodium and chloride requirements of dep/AHP responses were investigated by lowering ionic concentrations in the perfusate. In Fig. 6A GABA induces a dep/AHP response in a dissociated neuron (1st treatment). With replacement of NaCl by LiCl in the extracellular medium, both the depolarization and the AHP components were lost (2nd treatment). This blockade was reversible. In 15 cells with dep/AHP responses, all were blocked by lithium for sodium substitution. In 8 cases a small hyperpolarizing GABA response was revealed during the substitution. The median depolarization in control and recovery treatments was 0.15 log units ($n = 30$). The median response in the absence of $[\text{Na}]_o$ was -0.025 log units ($n = 15$). The change was significant ($p < 0.001$, Mann Whitney U test). One of the 15 cells in this group was an identified bipolar cell.

Lithium substitution did not block GABA-induced hyperpolarizations, but did reduce response amplitudes (data not shown). The median hyperpolarization in control and recovery was -0.155 log units ($n = 48$). The median hyperpolarization in the $[\text{Na}]_o$ free medium was -0.095 ($n = 24$). The amplitude reduction was significant ($p < 0.01$, Mann Whitney U test). This group included 7 identified bipolar-cell AOI's.

Lowering extracellular chloride was achieved by substituting sodium isethionate for sodium chloride. This reduced $[\text{Cl}^-]_o$ from 130 mM to 10 mM. As seen in Fig. 6B, this treatment abolished both depolarizing and AHP components of the dep/AHP response. In this example the depolarizing GABA response does not recover, but the AHP component is restored. Fourteen dissociated neurons with GABA-induced dep/AHP responses were treated with low $[\text{Cl}^-]_o$. In 13 cases the dep/AHP response was blocked or partially blocked. In one case the response was enhanced. The result is significant in that a GABA depolarization resulting from opening ionotropic channels in cells with high $[\text{Cl}^-]_i$ might be expected to increase in amplitude when $[\text{Cl}^-]_o$ was lowered. The opposite was observed. The median control depolarization was 0.13 log units. In low $[\text{Cl}^-]_o$ the median response changed to 0.00 log units. The change is significant ($p < 0.001$, Mann Whitney U test). The group included one identified horizontal cell and one bipolar cell.

Association of GABA and muscimol responses

Dep/AHP response enhancement, sometimes caused by GABA antagonists, might result from blockade of an underlying but hidden, hyperpolarizing, ionotropic GABA mechanism mixed together with the dep/AHP mechanism. Indeed lithium blockade of the dep/AHP mechanism left a small, residual, median GABA hyperpolarization. This could be evidence for two mechanisms on the same neuron: a dep/AHP mechanism and an ionotropic GABA mechanism.

In one example of a bipolar type with dep/AHP response to GABA (Fig. 7, 1st treatment), muscimol induced a hyperpolarizing response (2nd treatment). Glycine also evoked hyperpolarization in this cell (4th treatment). In 3 of 5 bipolar cell AOIs depolarized by GABA, a subsequent dose of muscimol evoked a hyperpolarizing response. In 8 horizontal cells depolarized by GABA, no muscimol-evoked hyperpolarizing responses were seen. Neither of these results appears to be preferential association of the two mechanisms as, in any event, ~60% of bipolar cells are hyperpolarized by muscimol (Fig. 3B), while virtually no horizontal cells are (Fig. 3A). In 187 other cells with GABA evoked dep/AHP, there were 54 cases (29%) of muscimol inducing a hyperpolarizing response. This is 7 short of the 61 that might have been expected by independent association (based on the frequency of each response in this population), but the difference is not significant ($p \geq 0.05$, Chi Square test). Taken together these results suggest that depolarizing GABA responses and hyperpolarizing muscimol responses on bipolar cells and other retinal neurons are separate mechanisms, and can associate independently. The exception to this pattern appears to be horizontal cells, where hyperpolarizing, ionotropic, GABA receptors are excluded, but the dep/AHP mechanism is preferentially expressed.

Muscimol induced dep/AHP was much less common than GABA induced dep/AHP, nonetheless, where present, the mechanism appears to be shared. In the present study there were 900 dissociated cells, excluding identified horizontal and bipolar types, where GABA treatment was followed by muscimol treatment. GABA dep/AHP responses were found in 187 of these. Among these 187, muscimol dep/AHP occurred in 27 cases. But only 9 cases were expected by independent association based on the separate frequency of occurrence of GABA and muscimol dep/AHP responses in this population. The excess is significant ($p < 0.01$, Chi square test), suggesting that in the general population of retinal neurons there is an increased probability of muscimol dep/AHP responses among cells with a GABA dep/AHP response, albeit the frequency was very low.

Discussion

GABA depolarizes and excites certain populations of zebrafish retinal neurons. The response combines depolarization with after hyperpolarization (dep/AHP) and occurred in 14% of viable, dissociated zebrafish retinal neurons (335 of 2396), as studied by voltage probe fluorescence. Many dep/AHP responses originate with cells whose native circuitry lies in the outer plexiform layer. The dep/AHP response is the only GABA response of zebrafish horizontal cells. It occurred in 38% of cases. This included both the smaller H_A and larger H_B types. Hyperpolarizing (ionotropic) GABA responses were seldom found in zebrafish horizontal cells. An infrequent bipolar-cell subtype (5%) also displayed the dep/AHP response, in some cases mixed with the ionotropic hyperpolarizing mechanism that is nearly universal to bipolar cells. The latter mechanism was only revealed using muscimol as an agonist rather than GABA. Bipolar cells both with and without axons displayed GABA dep/AHP, suggesting that this GABA response mechanism often resided in the soma and dendrites. While bipolar cells might display the dep/AHP GABA response, it was at a lower frequency than in dissociated retinal neurons in general.

GABA depolarizations are transporter mediated

GABA excitation in dissociated zebrafish retinal neurons has the physiological properties of a GABA_A plasma membrane transporter. It is poorly evoked by muscimol, an agonist of ionotropic GABA_{A,C} receptors (Qian & Dowling, 1993) or by baclofen, a GABA_B agonist. It is not blocked by picrotoxin, a GABA_A and GABA_C antagonist (Qian & Dowling, 1993), by bicuculline, a GABA_A antagonist, or by the combination. It is suppressed by removal of either sodium or chloride ions from the extracellular medium. These are just the properties described

for GABA transporters in horizontal cells (Malchow & Ripps, 1990). The transporter response, as seen in voltage probe recordings, has the additional feature of evoking AHP responses. This may result from $\text{Na}^+ \text{K}^+$ -ATPase activation, as occurs with glutamate stimulation of zebrafish horizontal cells (Nelson *et al.*, 2003). GABA transport through the plasma membrane is powered by the sodium gradient and causes sodium to enter the cytoplasm. This may stimulate $\text{Na}^+ \text{K}^+$ -ATPase, whose net ionic transfers are electrogenic and restore hyperpolarized membrane potential. On this hypothesis GABA and glutamate would have very similar actions on zebrafish horizontal cells, except that a greater depolarization might be evoked by GABA than glutamate, as the GABA transporter is sodium selective, while glutamate, through ionotropic AMPA receptors, evokes mixed cation permeability. In bipolar cells, and perhaps some other retinal neurons, the depolarizing action of the GABA transporter is tempered by the hyperpolarizing action of ionotropic GABA receptors. In these cells the presence together of GABA receptors and GABA transporters would help to make transport more efficient by allowing Cl^- inflow to neutralize charge buildup from transporter Na^+ inflow.

One difference from previous results is that the apparent k value of $0.61 \mu\text{M}$ found with voltage probe recordings is much lower than typically found in voltage clamp experiments. These values range from 10 to $100 \mu\text{M}$ (Malchow & Ripps, 1990; Takahashi *et al.*, 1995; Krause & Schwarz, 2005). Voltage saturation may be the cause. In this case uptake may also be limited by membrane potential.

Muscimol transport

Muscimol is sometimes transported by GABAergic neurons. Radioactive muscimol has been used as a marker for select populations of GABAergic amacrine cells in retina (Yazulla & Brecha, 1980; Pourcho & Goebel, 1983). It is not effectively transported by the GABAergic H1 horizontal cell of goldfish, however, even though this cell takes up GABA so avariciously, as to deprive other GABAergic neurons of marker (Yazulla & Brecha, 1980; Yazulla, 1991). The molecular basis for muscimol transport in some GABAergic neurons but not others is not known. What is clear in the present results is that, similar to other teleost retinas, zebrafish horizontal cells do not transport muscimol nearly as effectively as GABA.

In the present study both GABA and muscimol were applied to a large population of cells that excluded morphologically identified bipolar and horizontal cells. Among cells found to have GABA dep/AHP responses, the likelihood of a muscimol dep/AHP response was preferentially enhanced. This suggests that the two response mechanisms are tightly linked, and that the GABA plasma membrane transporter can, in some cases, also transport muscimol. The likelihood was low, however, as only 1 of 7 cells with the GABA transporter activity also gave evidence of muscimol transport.

Depolarizing chloride gradients were rare

Vardi et al (2000) have suggested that GABA might depolarize retinal neurons, in particular ON type bipolar cells, through the action of the chloride accumulating transporter NKCC. On the whole this appears not to be the case in dissociated zebrafish retinal neurons. The action of the picrotoxin sensitive ionotropic GABA receptors was universally hyperpolarizing, as seen particularly in the dominant GABA hyperpolarizing response of zebrafish ON bipolar cells, using either dissociated cells or the retinal slice (Connaughton *et al.*, in preparation). GABA induced depolarizations were seen in some bipolar cells, but those appear to result from a GABA membrane transporter. Voltage probe studies of isolated rat bipolar cells also revealed a dominant GABA hyperpolarizing response (Nelson *et al.*, 1999), a result also obtained with microelectrode recording (Yamashita & Wassle, 1991; Billups & Attwell, 2002). GABAergic depolarizations were found in other neurons dissociated from rat retina with the voltage-probe technique, but this response was not further characterized (Nelson *et al.*, 1999).

The GABA transporter as part of distal neural circuitry

The present findings remind us that there is yet another GABA sensor in distal retina: the GABA transporter. Depolarizations through this mechanism were substantial. In bipolar cells containing both GABA receptors and GABA transporters, transporter-mediated excitation was sometimes stronger than receptor mediated hyperpolarization, at least in the long term, as demonstrated in voltage probe recordings. The median GABA evoked depolarization in horizontal cells was 22 mV. The half-maximal response for such excitation was evoked by about $\sim 1 \mu\text{M}$ GABA, suggesting high sensitivity. GABAergic horizontal cells might depolarize each other, not only through gap junctions, but also through the GABA transporter.

Kamermans and Werblin (1992) found that GABA-mediated positive feedback between horizontal cells slowed kinetics of the light response. Ionotropic GABA receptors gating reversed chloride gradients, in addition to GABA transporter action, were included in this model, but the analysis may still apply in the absence of GABA receptors, as found in zebrafish. Delay of horizontal cell light responses by GABA has also been reported in rabbit A-type horizontal cells. These appear to express GABA_A type receptors, but no transporter (Blanco & de la Villa, 1999). Neither GABA, picrotoxin, nor bicuculline methyl bromide affect the kinetics of horizontal cell light responses in cat, however delayed kinetics and depolarization were seen with delta-amino-valeric-acid, a GABA analogue (Frumkes & Nelson, 1995; Frumkes *et al.*, 1995). Modulation of response kinetics may be one role for GABA in distal retina, and depolarizing, transporter-mediated GABAergic interactions between horizontal cells may contribute to this.

GABAergic horizontal cells

In prescient work, Marc *et al* (1978) concluded that only one of 4 horizontal cell types in goldfish retina, the H1 type, was GABAergic, taking up and releasing GABA, a result that may apply also to zebrafish (Marc & Cameron, 2001). While we can not as yet provide a morphological correlate for the GABAergic type, present results suggest ~ 1 in 3 dissociated zebrafish horizontal cells express a GABA transporter, as measured by a depolarizing GABA response. The unique feature of zebrafish horizontal cells is that no ionotropic GABA receptor is co-expressed. Clearly across species, horizontal cells are very heterogeneous for GABAergic mechanisms. Rod horizontal cells of white perch express only GABA_C receptors (Qian & Dowling, 1993). A-type horizontal cells of rabbit retina express only GABA_A receptors (Blanco *et al.*, 1996). Cone horizontal cells of catfish express GABA_A receptors, GABA_C receptors and GABA transporters (Dong *et al.*, 1994; Takahashi *et al.*, 1994). On the other hand horizontal cells in skate retina are insensitive to the GABA_{A/C} agonist muscimol, don't express any GABA receptors, but do express a GABA transporter (Malchow & Ripps, 1990). Zebrafish horizontal cells appear to belong to this latter type. This lack of GABA receptors is made particularly striking as neighboring bipolar cells dissociated on the same culture plate are robustly hyperpolarized by GABA_{A/C} agonists.

GABAergic bipolar cells

The identification of a bipolar-cell GABA transporter marks a potentially GABAergic neuron. In studies of bipolar cell GABA responses in zebrafish, hyperpolarizing GABA responses were found associated with the soma and dendrites of ON-type bipolar cells, and depolarizing GABA responses were found associated with the dendrites of OFF type bipolar cells (Connaughton *et al.*, in preparation). It now appears likely that such depolarizations reflect the action not of a GABA receptor, but of a GABA transporter, and therefore some OFF-type bipolar cells in zebrafish may in fact be GABAergic.

In the whole, retinal bipolar cells are purely glutamatergic, but in cat retina, two OFF bipolar types which express both GAD65 and VGAT have been identified (Kao *et al.*, 2004). These

are markers for GABA synthesis and release. Such cells express VGLUT as well, making them also glutamatergic. A few GABA containing bipolar cells had been previously noted in cat and monkey retinas (Wassle & Chun, 1989; Grunert & Wassle, 1990). GAD, GABA and GAT have also been found in a subpopulation (12%) of salamander bipolar cells (Yang & Yazulla, 1994; Yang *et al.*, 1997). These cells are also glutamatergic and were found among both morphologically ON and OFF types (Yang, 1997). Bipolar-cell mediated inhibitory responses have been seen in ganglion-cell recordings from this species (Yang & Wang, 1999), and an unusual CNQX-insensitive, light-evoked current, that could originate with a GABAergic ON bipolar, is also seen in some bipolar cells of salamander (Gao *et al.*, 2000).

Two groups of GABA containing bipolar cells were identified in zebrafish by Marc and Cameron(2001), type BC1 and BC6. The latter, with a weak GABA signal, was thought analogous to type Bb3 in goldfish (Ishida *et al.*, 1980) and has the molecular and morphological signature of a glutamatergic ON type. The former, uniquely, has the molecular signature of a GABAergic inhibitory neuron, similar to H1 horizontal cells. The 5% of GABAergic bipolar cells in the present study might represent the BC1 type.

VGAT in the outer plexiform layer

In zebrafish (Yazulla & Studholme, 2001), as in cat (Kao *et al.*, 2004), the vesicular GABA transporter VGAT is the most intensely expressed GABA transporter in the outer plexiform layer. As synaptic vesicles are largely absent in bipolar-cell and horizontal-cell dendritic processes in this layer, Kao et al (2004) have suggested that VGAT may be localized instead in the plasma membrane and mediate non vesicular GABA release (Schwartz, 1982; Yazulla & Kleinschmidt, 1983; Schwartz, 1987). Current results do not confirm this. VGAT is powered by pH and electrochemical gradients (McIntire *et al.*, 1997), whereas the transporters tested in this study were driven by sodium and chloride gradients, more typical of plasma membrane transporters. Further, more recent molecular studies have confirmed that other proteins associated with vesicle release, such as syntaxin-4 and SNAP-25, are localized to horizontal cell dendritic tips (Hirano *et al.*, 2007). This suggests that some horizontal cells contain the full complement of uptake and release mechanisms normally associated with GABAergic neurons. Such a model of horizontal cells might more readily explain data such as GABA-mediated GABA release (Schwartz, 1982), as a GABA transporter acting as GABA sensor would depolarize horizontal cells triggering vesicular release. The notion of transporters as sensors has been recently buttressed by the decomposition of the transporter response into a stoichiometric (1 GABA:2 Na⁺:1 Cl⁻) component, and a nonstoichiometric, Na⁺ channel, transmitter component of 3 to 5 times greater magnitude(Krause & Schwarz, 2005). This latter component might certainly serve a signaling role.

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Fig. 1.

GABA, glutamate (glut) and the combination evoke depolarization/after-hyperpolarization (dep/AHP) in a dissociated horizontal cell. Arrows point to AHP components, which follow stimulation by any of these three treatments. The asterisk marks the peak of a depolarizing response evoked by GABA alone. The cell is an H_A type. In this and following figures dashed bars are drug treatments with concentrations given in μM and the log(FL) '0' level (dotted line) set to the peak value attained with gramicidin (grami) permeabilization (last treatment). This FL value corresponds to '0' mV membrane potential.

Fig. 2.

Bipolar cells can be either depolarized or hyperpolarized by GABA. *A.* A bipolar type with dep/AHP response to GABA responds with much lower amplitude to muscimol (Musc). The arrow points to the GABA AHP component. A combination of bicuculline (Bic) and picrotoxin (Picro) fails to block the GABA dep/AHP response. *B.* Another bipolar cell type is hyperpolarized in response to GABA and muscimol. The bicuculline/picrotoxin combination blocks most of the hyperpolarization. Both bipolar cells were recorded simultaneously on the same plate and are both axotomized types revealing different soma-dendritic GABA responses. Records are from the soma regions.

Fig. 3.

Statistics for GABA and muscimol response types in horizontal and bipolar cells. *A*. GABA, but not muscimol, evokes dep/AHP responses in horizontal cells. *B*. GABA and muscimol evoke hyperpolarizing responses in most bipolar cells, but GABA evokes dep/AHP responses in about 5% of bipolar cells. Responses were categorized as hyperpolarizing (hyp), dep/AHP, or no response (no resp). Most typically dep/AHP included both a log(FL) increase during GABA treatment (*dep*) followed by decrease below pretreatment levels after GABA removal (*AHP*). However some examples lacked one or the other component. GABA doses tested, 2-20 μ M; muscimol dose, 10 μ M.

Fig. 4.

Picrotoxin and bicuculline do not block GABA-induced depolarizations in horizontal cells. GABA depolarizes this horizontal cell and induces AHP (arrow) producing a dep/AHP response. The depolarization persists in the presence of bicuculline (Bic) and picrotoxin (Picro). The horizontal cell morphology is H_A type.

Fig. 5.

Dose-response properties of dep/AHP responses. *A*. GABA treatments, increasing from 0.25 μM to 4.00 μM, evoke increasing amplitudes of depolarization. The half amplitude dose is about 0.5 μM for this H_A type horizontal cell. *B*. A Hill function is fit to pooled data. The *dep* components of dep/AHP responses were measured for different GABA concentration series spanning an overall range of 0.125 to 16 μM (all steps differed by a factor of 2). Individual cells received only 5 of these doses. Median response amplitudes for all cells are plotted against concentration and fit to a Hill model using N weighting for each point.

Fig. 6.

Ionic dependence of dep/AHP responses. *A.* GABA evoked dep/AHP response is blocked by substituting lithium chloride for sodium chloride (LiCl for NaCl) in the extracellular perfusate. The effect was reversible. *B.* GABA evoked a dep/AHP response is blocked when sodium chloride is replaced by sodium isethionate in the extracellular medium. $[Cl^-]_o$ was lowered from 130 mM to 10mM (LoCl) by this treatment. Both the depolarization and the AHP response are blocked. The depolarizing response does not recover. Arrows point to AHP response components. In each case responses were recorded from isolated cell bodies of bipolar-cell-like dimensions.

Fig. 7.

Mixed GABA mechanisms in a bipolar cell depolarized by GABA. A bipolar cell depolarized by GABA is hyperpolarized by muscimol (Musc). Glycine (Gly) also hyperpolarizes this cell. Baclofen (Bac) has little effect. The bipolar cell image revealed a long axon. The recording is from the cell body. This dual response was identified in 3 of 5 bipolar cells depolarized by GABA, and tested with both compounds.