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Electrophysiological evidence of $GABA_A$ and $GABA_C$ receptors on zebrafish retinal bipolar cells

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Abstract

To refine inhibitory circuitry models for ON and OFF pathways in zebrafish retina, GABAergic properties of zebrafish bipolar cells were studied with two techniques: whole cell patch responses to GABA puffs in retinal slice, and voltage probe responses in isolated cells. Retinal slices documented predominantly axon terminal responses; isolated cells revealed mainly soma-dendritic responses. In the slice, GABA elicited a conductance increase, GABA responses were more robust at axon terminals than dendrites, and E_{rev} varied with $[Cl^-]_{in}$. Axon terminals of ON- and OFF-type cells were similarly sensitive to GABA (30–40pA peak current); axotomized cells were unresponsive. Bicuculline-sensitive, picrotoxin-sensitive, and picrotoxin-insensitive components were identified. Muscimol was as effective as GABA; baclofen was ineffective. Isolated bipolar cells were either intact or axotomized. Even in cells without an axon, GABA or muscimol (but not baclofen) hyperpolarized dendritic and somatic regions, suggesting significant distal expression. Median fluorescence change for GABA was −0.22 log units (~−16mV); median half-amplitude dose was 0.4μM. Reduced $[Cl^-]_{out}$ blocked GABA responses. GABA hyperpolarized isolated ON-bipolar cells, OFF-cells were either unresponsive or depolarized. Hyperpolarizing GABA responses in isolated cells were bicuculline and TPMPA insensitive, but blocked or partially blocked by picrotoxin or zinc. In summary, axon terminals contain bicuculline-sensitive $GABA_A$ receptors and both picrotoxin-sensitive and insensitive $GABA_C$ receptors. Dendritic processes express zinc- and picrotoxin-sensitive $GABA_C$ receptors.

Keywords

retina; bipolar cells; GABA; zebrafish

Introduction

In retina, immunocytochemical studies of neural circuitry show that the dendritic arbors and axon terminals of bipolar cells are surrounded by GABAergic processes. In the outer plexiform layer, processes of GABAergic horizontal cells surround bipolar cell dendrites; while in the inner plexiform layer, processes of GABAergic amacrine cells surround bipolar

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axon terminals. Physiological studies report localization of GABA sensitivity. GABA-evoked currents are greatest at bipolar terminals (i.e., Tachibana & Kaneko, 1987; Karschin & Wassle, 1990; Lukasiewicz *et al.*, 1994; Tachibana & Kaneko, 1998) with smaller amplitude currents typically elicited from the soma and/or dendritic arbor. In some species large dendritic currents also occur (Qian & Dowling, 1995). Overall GABAergic inhibitory feedback from neighboring amacrine cells is dominant (Tachibana & Kaneko, 1987, 1998), and occurs through direct GABAergic input onto bipolar cell terminals.

In many species, signals from GABAergic horizontal cells may arrive both directly and indirectly onto bipolar cell dendrites. The indirect route is through a feedback synapse involving photoreceptors (Burkhardt, 1977; Kondo & Toyoda, 1983; Wu, 1986; Wu & Maple, 1998). Direct morphological contacts between GABAergic horizontal cells and bipolar cells have also been seen, albeit infrequently (Dowling & Werblin, 1969; Lasansky, 1973; Fisher & Boycott, 1974; Kolb & Jones, 1984; Linberg & Fisher, 1988). In zebrafish at least one class of horizontal cell is GABAergic and is a candidate presynaptic element (Connaughton *et al.*, 1999; Marc & Cameron, 2001; Yazulla & Studholme, 2001; Nelson *et al.*, 2006). The horizontal cell layer of zebrafish stains richly for VGAT, vesicular transporters for GABA, and the outer plexiform layer stains for GABA receptors (Yazulla & Studholme, 2001). This suggests distal GABAergic synaptic activity. Although the postsynaptic elements have not been identified, bipolar cell dendrites are a likely target for horizontal cell GABA. GABAergic interplexiform cells also appear to synapse onto bipolar cell dendrites in some species (Kolb & West, 1977; Crooks & Kolb, 1992), though GABAergic interplexiform cells have not been reported in zebrafish (Connaughton *et al.*, 1999; Marc & Cameron, 2001; Yazulla & Studholme, 2001).

GABA application typically elicits a chloride current that hyperpolarizes bipolar cells. GABA-elicited currents have both transient and sustained components. The transient response is mediated by $GABA_A$ receptors; while the sustained component results from the activation of $GABA_C$ receptors (Tachibana & Kaneko, 1987; Qian & Dowling, 1993; Feigenspan & Bormann, 1994; Qian & Dowling, 1995; Lukasiewicz & Wong, 1997; Lukasiewicz & Shields, 1998; McGillem *et al.*, 2000). Baclofen-sensitive $GABA_B$ receptors have only been identified on salamander bipolar cells (Maguire *et al.*, 1989), though a different $GABA_B$-like receptor modulating Ca^{+2} currents has been identified in goldfish Mb bipolar terminals (Heidelberger & Matthews, 1991). Interestingly, though most bipolar cells appear to express both $GABA_A$ and $GABA_C$ receptors, it is the $GABA_C$ receptor that underlies the majority of GABA-elicited responses in these cells (Lukasiewicz & Wong, 1997; Lukasiewicz & Shields, 1998; Nelson *et al.*, 1999; Kaneda *et al.*, 2000; McGillem *et al.*, 2000). The combined expression of $GABA_A$ and $GABA_C$ receptors on bipolar cells (Zhang & Yang, 1997; Euler & Wassle, 1998; Du & Yang, 2000) allows these cells to respond to a range of GABA concentrations and time courses within the synaptic cleft, as these different receptor types display different sensitivities and kinetics to GABA and/or GABA agonists (Woodward *et al.*, 1993; Lukasiewicz & Wong, 1997; Lukasiewicz & Shields, 1998; Lukasiewicz *et al.*, 2004).

Microelectrode studies show $GABA_A$ and $GABA_C$ receptors may perform functionally distinct roles. $GABA_C$ receptors preferentially suppress ON responses of third order neurons

(Zhang & Slaughter, 1995); while $GABA_A$ activation suppresses the OFF pathway (Zhang & Yang, 1997). Further, GABAergic ligands affect the amplitude and time course of the ERG b-wave (Kapousta-Bruneau, 2000; Dong & Hare, 2002; Hanitzsch *et al.*, 2004; Lukasiewicz *et al.*, 2004). Pharmacological studies examining GABAergic inputs to mammalian rod bipolar cells have identified responses mediated predominantly by $GABA_C$ receptors (Feigenspan & Bormann, 1994; Vaquero & de la Villa, 1999; McGillem *et al.*, 2000; Frech & Backus, 2004). However, recent findings indicate recurrent inhibition of rod bipolar terminals by A17 amacrine cells is mediated only through $GABA_A$ type receptors (Singer & Diamond, 2003). The net effect of GABAergic inputs is to modulate calcium current activity in bipolar cell terminals (Maguire *et al.*, 1989; Matthews *et al.*, 1994; Wu & Zhu, 2000; Singer & Diamond, 2003; Frech & Backus, 2004) altering neurotransmitter release (Mack *et al.*, 2000; Singer & Diamond, 2003; Hull & von Gersdorff, 2004) and/or suppressing spontaneous EPSCs in postsynaptic ganglion cells (Freed *et al.*, 2003).

Zebrafish is a current model for vertebrate vision. The voltage-gated (Connaughton & Maguire, 1998) and glutamate-gated (Connaughton & Nelson, 2000) responses of bipolar cells have been documented and their morphology described (Connaughton *et al.*, 2004). However, the inhibitory circuitry modifying bipolar cell responses has not been determined in this species. This information is important, given the continued identification of zebrafish with visual system mutations localized to the retina. The present work is a study of inhibitory mechanisms on zebrafish bipolar cells. We have employed parallel techniques: the retinal slice with cell attached patch electrodes, and dissociated cell preparations using the oxonol fluorescent probe. Recordings in the slice best revealed GABAergic inputs to bipolar cell terminals; GABA responses at the dendrites were typically small and/or absent in this preparation. In contrast, recordings of inhibitory responses from dissociated cells appeared to originate predominantly in soma and dendritic regions. In both preparations, multiple GABA receptor types were identified. Parts of this work has appeared previously in abstract form (Connaughton *et al.*, 2000).

Methods

Care and maintenance of animals

Zebrafish (*Danio rerio*) were obtained from a local supplier (Petsmart, Inc.) and maintained in the laboratory in 40L aquaria at 28–29°C on a 14hr light:10hr dark photoperiod until needed for experiments. Each day, temperature and water level in the aquaria was checked and the fish were fed (Tetramin flakes). Animal care and use protocols were approved by the appropriate committees at both American University and the National Institutes of Health.

Preparation of retinal slices and patch clamp recordings

Retinal slices were prepared as described previously (Connaughton & Nelson, 2000; Connaughton, 2003). In brief, fish were removed from aquaria and decapitated with a dorsal-ventral incision. Following enucleation, the anterior segment was removed. The retina (with attached sclera) was placed vitreal-side down on a piece of Millipore filter paper (0.45μm pore diameter). The filter paper and retina were transferred into the recording chamber and anchored into place on Vaseline strips. Retinal slices (~100μm thick) were cut

with a fresh razor and each slice rotated 90° within the recording chamber to view the retina in cross section. Tissue was submerged in the standard Ringer's solution containing 120mM NaCl (Na-isethionate in low Cl^- solution), 2mM KCl, 1mM $MgCl_2$, 3mM $CaCl_2$, 4mM HEPES, and 3mM D-glucose brought to pH 7.4–7.5 with NaOH. In some experiments 5mM $CoCl_2$ was added to the bath solution, to block synaptic transmission.

Bipolar cells were initially identified by a soma position in the distal to mid inner nuclear layer (INL). Lucifer Yellow (1% solution), present in the patch pipette, stained recorded neurons and confirmed bipolar cell morphology. Ligand-gated currents were recorded from zebrafish bipolar cells using whole-cell voltage clamp techniques. A cesium-based intracellular solution blocked K^+ currents. This solution (Low Cl^-) contained 12mM CsCl, 104mM Cs-gluconate (116 mM CsCl in high Cl^-solution), 1mM EGTA, 4mM HEPES, and 0.1mM $CaCl_2$ brought to pH 7.4–7.5 with KOH. Calculated liquid junction potentials (bath pipette) for 3 combinations of pipette and bath solutions are as follows: High Cl^- internal solution and standard external solution (lacking cobalt) = 5.3mV; Low Cl^- internal solution and standard external solution (lacking cobalt) = 15.8mV; Low Cl^- solution and cobalt-containing external solution = 16.0mV (Kenyon, 2002). Text and figures are uncorrected. GABA agonists were applied using a second puffer pipette, positioned to stimulate processes in either the inner or the outer plexiform layer. Each GABA agonist was applied in a separate experiment. The GABA antagonists bicuculline (≤200μM) and picrotoxin (≤200μM) were applied in the bath solution.

Currents were elicited by focal puffs of GABA (100μM or 1mM), muscimol (200μM), or ± baclofen (200μM) onto bipolar cell dendrites or axonal boutons (50ms puff, 15psi). To determine reversal potentials of these currents, holding potentials (V_{hold}) were increased stepwise (20 mV increments) from either − 60mV or − 70mV to reach a maximum of +10mV or +20mV. Raw current traces were boxcar filtered in pCLAMP (9 data points on each side) prior to data analysis. Peak current amplitude at each holding potential was measured and plotted as a current-voltage relationship (Microcal Origin version 7.0). The reversal potential (E_{rev}) for each current was determined by a linear regression through the I-V plot (only fits with $r^2 \geq 0.7$ included).

Patch and puff pipettes of ~1μm tip diameter were pulled from thin-walled, filamented borosilicate glass (World Precision Instruments) using a Flaming-Brown P-80 pipette puller (Sutter Instruments, Novato, CA). Cells were visualized using an Olympus fixed-stage compound epifluorescence microscope fitted with a 40× water immersion lens and Hoffman modulation contrast optics. Data were collected using an Axopatch 1-C patch clamp amplifier and pCLAMP software (ver. 6.0 and 8.0).

Cell isolation and fluorescent probe measurements

Following established protocols (Connaughton & Dowling, 1998; Nelson *et al.*, 2003), zebrafish were dark adapted overnight and decapitated. The retinas were removed and cell adhesions were enzymatically broken in a solution consisting 33U/ml papain (Worthington), 70% Leibovitz (L-15) medium, 0.19U/ml dispase (Sigma Chemical Co.), and 0.05% DNAase (Sigma Chemical Co.). The pH was adjusted to neutral with 0.07M NaOH. The cells were physically separated by gentle trituration, and the resultant suspension plated on a

rectangular stripe of poly-D-lysine, coated across the center of plastic culture dishes. Cells were allowed to settle and adhere for at least 1.5 hours before the start of experiments.

Dissociated bipolar cells were identified morphologically, with a round cell body connected to a thin axon, or by a flask-shaped cell body with dendritic tufts. Plated cells were superfused under a raised cover glass (mean fluid velocity = 2600μm/s) with the above standard extracellular solution (lacking cobalt) and containing the fluorescent, voltage-sensitive dye oxonol (DiBaC4(5), 80nM). Cells were perfused for at least 10min for initial probe equilibration. Agonists and antagonists were applied as a stock solution diluted in the extracellular solution. Voltage-probe responses to GABA, GABA agonists, glycine, or glutamate were obtained in parallel for each cell in a microscope field using intensified fluorescence video microscopy, with images captured at 30s intervals. The integrated fluorescence of each cell (FL) was summed and the fluorescence of equivalent background areas subtracted before logarithmic conversion. Histograms of log(FL) responses to treatments revealed skewed distributions, so that (for positive going responses) means were greater than histogram peaks. Medians and histogram peaks agreed well, however, and so median responses are adopted here to characterize typical responses from within distributions. The significance of differences among treatments was evaluated using 2-tailed Mann-Whitney U tests.

Oxonol entry causes retinal cells to fluoresce with a brightness corresponding to internal concentration. A depolarization was indicated by an increase in fluorescence, while a hyperpolarization was indicated by a decrease in fluorescence as the negatively charged oxonol redistributed in Nernstian fashion across the cell membrane (Nelson *et al.*, 1999; Nelson *et al.*, 2003). Following treatments with inhibitory or excitatory ligands, 1μM gramicidin was administered to permeabilize the cells to monovalent cations. This caused the membrane potential to increase to 0mV (Nelson *et al.*, 2003). Only cells or cell regions depolarized by gramicidin are included in this study. The median fluorescence increase induced in bipolar cells by gramicidin was 0.52 (P25/75; 0.36/0.67) log units (n = 269). Nelson *et al.* (1999) found 1 log unit fluorescence increase from resting fluorescence corresponded to 129mV of Goldman potential, making the corresponding range in median membrane potential about −65 to −79 mV. The median gramicidin time constant at the 2600μm/min flow rate was 1.65 min for cell bodies (n = 197), 1.75min for dendrites (n = 60), and 1.95min for terminals (n = 5). These values were not significantly different ($p \geq 0.05$; Mann Whitney U test). Oxonol was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, Mo).

GABA responses observed using the oxonol probe were recorded in both axon bearing and axotomized cells (soma and dendrites only). Most dissociated bipolar cells lacked axons and terminals. The morphology of axonless bipolar neurons, however, was characteristic: a flask-shaped/elongate cell body with dendritic tufts. While round somata without processes were far more common in these dissociations (and a large fraction of these are likely to be bipolar neurons) the physiological patterns of such cells are not included here. Comparison of responses of either intact cells vs. axotomized cells, or between different cell regions (dendrites vs. soma) were found to be similar ($p > 0.05$; Mann-Whitney U test). Further, the likelihood of GABA-induced hyperpolarization did not differ between isolated axon-bearing

or axotomized cells (axon-bearing, n = 38 of 61 responders or 62%; axotomized, n = 94 of 150 responders or 63%). As a result, data collected all isolated bipolar cells were pooled for analysis, and they are referred to simply as isolated cells or regions within the text.

Results

In addition to documenting GABA responses in zebrafish bipolar cells, this study also compares dissociated and non-dissociated responses of these cells as almost all experiments were performed both within the retinal slice and using cultured cells. Exceptions to this include analysis of the effects of zinc and TPMPA, which were performed using isolated cells. In both preparations, GABA responses were correlated with B_{on} (ON-) and B_{off} (OFF-) type bipolar cells, either based on cell morphology (slice) or glutamate responses (isolated cells). Results from both preparations are discussed together, with examples presented from each.

Localization of GABA responses

The location of bipolar cell GABA receptors was determined by focally applying GABA (100μM or 1mM) onto either the dendritic arbor in the outer plexiform layer (OPL; Figure 1) or the axon terminals in different zones of the inner plexiform layer (IPL; Figure 1). High concentrations of GABA (i.e., 1mM) were more effective, as they were probably better able to overcome uptake and removal by *in vivo* transporter systems (Wu, 1986).

GABA currents in the slice were recorded using two different intracellular solutions and the cobalt-containing Ringer's solution. Initially, recordings were made with the Low Cl^- intracellular solution (E_{Cl} = −61mV). Using this solution, focal application of the lower GABA dose (100μM) to the dendritic arbor failed to elicit a response in all cells examined (n = 6 of 6). Similarly, application of 100μM GABA onto axonal boutons in the IPL elicited a response in only some (3 of 6) cells. These GABA-elicited currents were characterized by a conductance increase and an average reversal potential of −32.7 ± 8.47mV. Four of the six cells tested (two responding to GABA and two not responding) had identifiable axon terminals.

Experiments performed using 1mM GABA and the Low Cl^- pipette solution gave similar results, as the application of GABA to the IPL elicited robust responses in 10 of 20 cells. All 10 responders had identifiable axon terminals. Eight of the ten cells not responding to 1mM GABA lacked terminals. The reversal potential of these GABA-elicited responses averaged − 38.02 ± 5.6mV (n = 10; E_{Cl} = − 61mV). The fact that, overall, cells without axon terminal boutons typically lacked GABA responses suggests IPL GABA receptors are localized to this region, and are not found on the remaining axonal process. GABA responses were elicited from bipolar cells with terminals located throughout the IPL (Figure 1), including monostratified B_{off} cells with terminals in sublamina a (n = 3), monostratified B_{on} cells with terminals in sublamina b (n = 4), and multistratified B_{off} and B_{on} cells with terminals in both sublaminae (n = 2). Application of 1mM GABA onto the dendritic arbor elicited a response in a minority of cells (3 of 12 cells), in one case with and in two cases without cobalt in the extracellular solution.

GABA-elicited currents recorded following stimulation of axon terminals were typically 30–40pA. Peak current amplitude, recorded at a holding potential of − 60mV (High Cl^- intracellular solution) averaged 36 ± 16.5pA (n = 7) for 1mM GABA and 21.6 ± 14.7pA (n = 21 cells) for 100μM GABA. These currents were consistently more robust than currents elicited at the dendritic arbor (typically ~5pA) suggesting a greater density of GABA receptors on axonal boutons. Given the more robust and consistent responses recorded from GABA application onto bipolar terminals, the remaining experiments in the slice focused on these responses only.

GABA responses were accompanied by a conductance increase. The reversal potentials of GABA responses were sensitive to changes in E_{Cl} (Figures 2*A* and 2*B*) resulting from altering internal [Cl^-]. Using 1mM GABA, E_{rev} increased from − 38.02 ± 5.6mV (n = 10; Low Cl^- solution) to 0.39 ± 4.29mV (n = 6; High Cl^- solution). Similarly, with the lower GABA dose (100μM) E_{rev} increased from −32.7 ± 8.47mV (n = 3; Low Cl-) to − 0.25 ± 5.21mV (n = 20; High Cl^-). This data indicates GABA elicited a chloride-sensitive current. This change in reversal potential with altered internal chloride was observed in both B_{off} type cells (n = 12) and B_{on} type cells (n = 11) using either GABA concentration.

Chloride-sensitivity was also observed in isolated cells as low extracellular Cl^- eliminated GABA responses in these conditions (Figure 2*C*). In normal external chloride, GABA reduced voltage probe fluorescence, signaling membrane hyperpolarization. Reducing chloride in the extracellular medium greatly attenuated this response (Figure 2*C*), confirming that bipolar cell GABA responses are chloride mediated. In these experiments, 100% of bipolar cell hyperpolarizations (n = 16) were decreased by 80% or more following the reduction of $[Cl^-]_{out}$ to ~10μM. Quantitatively, the median control hyperpolarization of − 0.24 log units decreased to a median response of 0.0 log units in low chloride. The median recovery response was − 0.10 log units. As compared to combined control and recovery responses (median − 0.13 log units), the median GABA response in low chloride was significantly reduced ($p < 0.001$ Mann-Whitney U test).

Isolated zebrafish bipolar cells were highly sensitive to GABA, in contrast to the high GABA concentrations (1mM) required in the slice, but in agreement with published findings on isolated cells (i.e, Feigenspan & Bormann, 1994; Matthews *et al.*, 1994; Vaquero & de la Villa, 1999; Qian *et al.*, 2001). Sub-saturating GABA-induced hyperpolarizations were typically observed only for doses less than 1μM (Figure 3*A*). Examination of the concentration dependence of voltage probe GABA responses (Figure 3*B*) revealed a median half-amplitude point at 0.4μM (P25/75; 0.2/0.8μM). Combining all experiments, the median peak hyperpolarization induced by GABA was −0.22 log units (P25/75; − 0.37/− 0.15 log units; n = 156; GABA concentration of 10 or 20μM only), corresponding to a hyperpolarization of ~ −16mV (Nelson *et al.*, 1999). Few regions were either depolarized by GABA or responded with after hyperpolarizations (Nelson *et al.*, 2006).

As documented in the slice, GABA responses of both ON- and OFF-bipolar cells were identified in isolated cells. ON-type responses, identified by glutamate hyperpolarizations, were highly associated with GABA hyperpolarizations (Figure 4*A*), as 83% of isolated cells responded to both compounds. In contrast, OFF-type responses, identified by glutamate

depolarizations, were not associated with GABA hyperpolarizations. Rather, 38% of OFF-cells were depolarized by both glutamate and GABA application, and GABA did not evoke a change in membrane fluorescence in the remaining 62%. GABA insensitive and GABA depolarized OFF-type bipolar cells are illustrated in Figures 4*B* and 4*C*. Cells that were insensitive to glutamate could not be classified as ON- or OFF-types. GABA hyperpolarizations were characteristic of this group, and they were encountered at a frequency intermediate between identified ON- and OFF-types. In this glutamate-insensitive group, GABA hyperpolarizations were found in 7 of 12 regions. Overall, the positive association of GABA hyperpolarizations with ON-type bipolars, and the negative association with OFF-type bipolars were significant ($p < 0.001$; Chi Square test).

Though GABA application in the slice evoked only small responses at the dendritic arbor, isolated axotomized cells displayed robust decreases in membrane fluorescence following GABA application. This latter finding confirms GABA receptors are located on the dendritic arbor and soma of zebrafish bipolar cells and suggests that dendrites are capable of large GABAergic responses even in the absence of an axon terminal.

Multiple GABA receptor types are present on zebrafish bipolar cells

To examine whether $GABA_A$, $GABA_B$, or $GABA_C$ receptors were active, specific agonists and antagonists were applied. Within the slice, these experiments were performed using the cesium-based (High Cl^-) intracellular solution and 5mM cobalt in the bath. Agonists were puffed onto bipolar terminals and antagonists were dissolved in the bath solution. For fluorescent probe measurements, the different compounds were dissolved directly into the standard extracellular perfusate (lacking cobalt).

Application of baclofen (200μM), a $GABA_B$ receptor agonist, failed to elicit a response from bipolar cells examined in the retinal slice (Figure 5*A*, n = 6 of 6 cells). Similarly, in isolated cells, perfusion of baclofen (100μM) had little effect on membrane potential (Figure 5*C*). In 75% of isolated cells (n = 21 of 28), no changes were observed. In the remainder, small miscellaneous effects occurred including low amplitude hyperpolarizations (n = 3), a small depolarization (n = 1), and small after hyperpolarizations (n = 3). The distribution of responses, including depolarizing, hyperpolarizing and non-responses following baclofen application was significantly different from the strongly hyperpolarized distribution evoked by GABA ($p < 0.001$ Mann-Whitney U test).

In contrast, the $GABA_{A/C}$ receptor agonist muscimol elicited consistent responses (Figure 5*B*) both within the slice (200μM; n = 6 of 7 cells) and in voltage-probe recordings (Figure 5*C*). The currents evoked by puffing muscimol on bipolar cells recorded in retinal slices were similar in time course and amplitude to those elicited by GABA. The average peak current elicited by muscimol (lowCl^- pipette solution) at $V_{hold} = -60$mV was 6.2 ± 11.6pA (n = 4); while the average GABA current recorded under these conditions was 10.4 ± 3.29pA (n = 4). The calculated reversal potential of muscimol-elicited currents was − 45.4 ± 21.6mV (n = 4), similar to GABA-evoked reversal potentials recorded with the same solutions.

Muscimol was equally as effective as GABA in hyperpolarizing all regions of isolated bipolar cells, with 62% of isolated cells hyperpolarized by 10 or 20uM muscimol. Of 84 cells treated with both GABA and muscimol, 47 (or 56%) were hyperpolarized by each treatment, far more than the 33 (or 39%) predicted by independent association ($p < 0.001$, Chi-square test). GABA and muscimol responses were highly correlated.

Some (12 of 49) isolated bipolar cells were hyperpolarized by glycine, with a median amplitude of −0.16 log units. The bipolar cell of Figure 5*C*, however, does not have a significant response to 20μM glycine. Seventy-three percent of bipolar cells recorded with voltage-probe did not respond to glycine (n = 36 of 49). When found, the amplitude distribution of glycine hyperpolarizations was not significantly different than that found for GABA hyperpolarizations ($p > 0.05$, 2-tail Mann-Whitney U test). In 47 cells treated with both GABA and glycine, hyperpolarization by both inhibitory transmitters was seen in 6 cases, about the same number (7) expected based on independent association. GABA and glycine hyperpolarizations were not linked ($p > 0.05$, Chi-square), and probably reflect different receptor systems. Eight of the 12 glycine-induced hyperpolarizations occurred in isolated axotomized cells, suggestive of soma-dendritic glycine receptors. Within the retinal slice, puffs of glycine (1mM or 5mM) also elicited responses from ~30% of bipolar cells (Figure 5D). Following application in the OPL, cells responded with a conductance increase and a mean reversal potential of −49 ± 9mV (n = 8). Bipolar cell terminals were also sensitive to glycine. When puffed into the IPL, glycine again evoked a conductance increase, with an average reversal potential of −38 ± 10.8mV (n = 7). Though current amplitude recorded from individual cells was sometimes greater at the dendritic arbor than the axon terminal, the average amplitude of glycine-evoked currents from both regions was similar (6 ± 5.3pA at the dendrites; 5 ± 1.9pA at the terminals, V_{hold}= −70mV, low Cl^- intracellular solution). Thus, direct glycinergic inputs are found on both zebrafish bipolar dendrites and axon terminals, similar to findings for GABA. However, a much lower fraction of cells, less than half, are glycine sensitive.

The above results with selective GABAergic agonists reveal that ionotropic GABA receptors on zebrafish bipolar cells are directly activated by exogenous GABA, and that metabotropic receptors evoke little standing current. In order to discriminate between the different ionotropic GABA receptor types, selective antagonists were applied. In retinal slice, 100–200μM bicuculline, a $GABA_A$ antagonist, partially blocked responses elicited by 100μM GABA, with all cells tested displaying bicuculline sensitivity. On average, bicuculline blocked 39% (range = 21% − 49%; n = 4) of the GABA-elicited current in cell-attached patch recordings (Figure 6*B*). In isolated bipolar cells, bicuculline was also found to be an incomplete blocking agent (Figure 6*C*). Bicuculline partially blocked GABA-induced hyperpolarizations in only 32% (7 of 22 regions). (A complete block was identified in one cell.) Complete block was defined as < 20% of control response amplitude remaining; partial block was defined as >20% but < 80% of control response amplitude remaining. In the presence of bicuculline, the median amplitude of response for isolated cells was − 0.195 log units, the identical value obtained for both control and recovery responses. These response amplitudes in both treated and treatment-free groups were not significantly different ($p > 0.05$, Mann-Whitney-U test). Thus, in both slice and isolated cells partial or no

bicuculline sensitivity was the rule; with most isolated cell responses identified as bicuculline-insensitive.

In isolated cells, bicuculline partially blocked muscimol-induced hyperpolarizations in 20% of cases (Fig. 7*A*). No complete blocks were observed. In the regions studied, the median control and recovery muscimol response (−0.195 log units) was again not significantly different ($p > 0.05$, Mann-Whitney U-test) from responses recorded in the presence of bicuculline (−0.31 log units). Overall, in both the slice and isolated cell recordings, bicuculline application typically blocked < 50% of the GABA-elicited (or muscimol-elicited) response.

Picrotoxin is a mixed $GABA_A$ and $GABA_C$ receptor antagonist. Application of 100–200μM picrotoxin (with puffs of 100μM GABA) also resulted in a partial block of GABA-elicited currents recorded in the retinal slice, with all cells tested sensitive to picrotoxin. GABA was typically applied 2–4x after picrotoxin application, with changes in amplitude measured after the final application. In these conditions, a maximum 74% decrease (Figure 6*A*) in GABA-elicited current amplitude was observed (range = 10% to 74%; n = 7). In isolated cells, picrotoxin application (25 and 50μM) decreased GABA-induced hyperpolarizations, with 100% of cells tested displaying partial or complete picrotoxin sensitivity. Picrotoxin decreased GABA-evoked changes in membrane fluorescence an average of 81%, with a complete block observed in 65% of cells. In isolated cells, the median of control and recovery responses was − 0.175 log units. In the presence of picrotoxin the median response was significantly decreased to 0.0 log units (Figure 6*C*) ($p < 0.001$, Mann-Whitney U-test).

Further, in isolated cells, picrotoxin also completely or partially blocked muscimol-induced hyperpolarizations in 100% of regions tested (Figure 7*A*). In these regions, the median control and recovery amplitude of muscimol hyperpolarizations was − 0.195 log units. Picrotoxin significantly decreased this to − 0.05 log units ($p \leq 0.01$, Mann-Whitney U test). Overall, picrotoxin was a far more effective antagonist than bicuculline for the GABA-(and/or muscimol-) induced hyperpolarizations in isolated bipolar cells.

Co-application of bicuculline (200μM) and picrotoxin (100μM) resulted in the greatest block of currents elicited by 100μM GABA (65% average decrease; n = 3) in the retinal slice, and gave the most consistent reduction in current amplitude. Though there was no complete block of GABA-evoked responses in the slice, all cells tested were sensitive to the co-application of blockers. The effect of the combination of blockers on isolated cells was similar to that observed with picrotoxin alone, with 100% of cells tested sensitive to the co-application of antagonists and most showing a complete block of GABA-elicited responses. In this latter group the median hyperpolarizing response amplitude in control and recovery treatments was − 0.14 log units. With the antagonist combination, this was reduced to 0.0 log units, a significant effect ($p < 0.001$, Mann-Whitney U test).

GABA responses of isolated cells were sensitive to exogenous zinc application (Figure 7*B*). At low doses (20μM), 72% of GABA hyperpolarizations were significantly antagonized, with 3 being completely blocked, and 2 partially blocked. At higher doses (100μM), all regions displayed zinc sensitivity (n = 10 of 10, with 4 completely blocked, and 6 partially

blocked). The fraction blocked or partially blocked at the two doses was not significantly different ($p > 0.05$; Chi Square test). Overall, 88% of GABA hyperpolarizations in isolated cells were blocked or partly blocked by zinc. The median, GABA-induced hyperpolarization in control and recovery treatments was − 0.21 log units, whereas the median hyperpolarization with zinc treatment was significantly reduced to − 0.04 ($p < 0.001$, Mann-Whitney U test). Zinc-sensitive GABA receptors are present on zebrafish bipolar cells.

Surprisingly, TPMPA, a competitive antagonist at $GABA_C$ receptors, failed to block GABA hyperpolarizations in most (79%) isolated bipolar cells. Two agonist/antagonist ratios were used: 10μM GABA with 20μM TPMPA, and 2μM GABA with 20μM TPMPA. In experiments using the former concentrations (1-to-2 ratio), 1 of 11 hyperpolarizations blocked. With the latter concentrations (1-to-10 ratio), 2 of 13 hyperpolarizations were blocked or partially blocked. The results obtained using the different agonist/antagonist ratios were not significantly different ($p > 0.05$; Chi Square test). Combining all data ($n = 24$) shows the median control and recovery response was − 0.205 log units. This diminished to − 0.188 log units during TPMPA application. This difference is not significant ($p > 0.05$, Mann-Whitney U test). An example of TPMPA failing to block a GABA hyperpolarization, even at a 10 to 1 antagonist/agonist ratio, appears in figure 7*C*.

Discussion

GABA responses on retinal bipolar cells have been identified in many species. Invariably bipolar cells express a combination of GABA receptors, with the $GABA_A$ and/or $GABA_C$ types predominating. Our results showing GABA responses with the pharmacological properties of A- and C-type receptors on the processes of zebrafish bipolar cells are consistent with these previous immunocytochemical (Greferath *et al.*, 1993; Yazulla & Studholme, 2001; Zhang *et al.*, 2003; Klooster *et al.*, 2004) and physiological (i.e., Feigenspan *et al.*, 1993; Qian & Dowling, 1993, 1995; Qian *et al.*, 1997; Du & Yang, 2000; Qian *et al.*, 2001) findings. GABA responses on isolated zebrafish bipolar cell dendrites were largely insensitive to bicuculline, but very sensitive to picrotoxin, suggesting these processes express $GABA_C$-type receptors. On axon terminals, however, GABA-elicited currents were partially blocked by either bicuculline and/or picrotoxin, indicating both $GABA_A$ and $GABA_C$ receptors are expressed on synaptic boutons. The direct action of GABA on $GABA_B$ type receptors was not identified in either isolated cells or retinal slices.

GABA receptors on zebrafish bipolar cells have unique antagonist sensitivity

An interesting finding in our results is that antagonist pharmacology of the GABA receptors on zebrafish bipolar cells appears to be somewhat unique. In particular, our results suggest that there are multiple subtypes of $GABA_C$ receptors expressed on these cells. GABA currents recorded from axon terminal stimulation in the retinal slice were only partially blocked by picrotoxin, revealing an insensitive component. Even co-application of bicuculline and picrotoxin did not produce a complete block, suggesting a mixture of picrotoxin-sensitive and picrotoxin-insensitive $GABA_C$ receptors on axon terminals of zebrafish bipolar cells. Though picrotoxin was an effective antagonist of GABA responses in isolated cells, a GABA response component sometimes remained (see Figure 6C),

indicating an insensitive component could also be present on the dendrites. Though picrotoxin-insensitive $GABA_C$ receptors have been identified in rat bipolar cells (Feigenspan *et al.*, 1993; Feigenspan & Bormann, 1994), this finding is in contrast to reports from other teleost retinas (Tachibana & Kaneko, 1987; Qian & Dowling, 1993; Matthews *et al.*, 1994; Qian & Dowling, 1994, 1995; Han *et al.*, 1997).

In isolated cell voltage probe recordings, GABA responses sensitive to picrotoxin were also partially blocked by zinc, but not TPMPA. TPMPA completely blocks focally applied $GABA_C$-mediated bipolar cell responses in salamander (Gao *et al.*, 2000), rabbit (McGillem *et al.*, 2000) mouse (Frech & Backus, 2004; Lukasiewicz *et al.*, 2004) and goldfish (Hull *et al.*, 2006). TPMPA was also an effective antagonist of $GABA_C$-mediated light-evoked IPSCs in mouse rod bipolar cells (Eggers & Lukasiewicz, 2006). GABA responses of zebrafish bipolar cells appear to be TPMPA insensitive, even at high antagonist/agonist ratios. Zinc is also a reported antagonist of $GABA_C$ receptors (Qian & Dowling, 1995; Qian *et al.*, 1997; Qian *et al.*, 1998; Han & Yang, 1999; Kaneda *et al.*, 2000), while its effects on $GABA_A$ receptors vary and appear only at high concentrations. $GABA_A$ receptors on mouse and carp bipolar cells are inhibited by zinc (Han & Yang, 1999; Kaneda *et al.*, 2000) but only at higher doses or to a much lesser extent than $GABA_C$ receptors. $GABA_A$ responses on skate bipolar cells are enhanced (Qian *et al.*, 1997), and $GABA_A$ receptors on hybrid bass bipolar cells are insensitive to zinc (Qian & Dowling, 1995). In the present study, low zinc concentrations (20μM) blocked most GABA-elicited changes in membrane voltage in isolated bipolar cells. These findings are consistent with the effects of a similar concentration (10μM) on hybrid bass bipolar cells (Qian & Dowling, 1995) and suggest the zinc-sensitive receptors are $GABA_C$ types. We found neither further blockade, nor enhancement at the high (100μM) zinc dose. As zinc has been shown to either antagonize or enhance $GABA_A$ responses at high doses (Qian *et al.*, 1997; Kaneda *et al.*, 2000); this suggests a lack of zinc sensitive $GABA_A$ receptors in zebrafish bipolar cells.

The effects of zinc and TPMPA were examined at ratios in which the antagonist was either 2x or 10x more concentrated than GABA. In other preparations, much lower TPMPA/GABA (McGillem *et al.*, 2000; Zhou & Dacheux, 2005; Ivanova *et al.*, 2006) and zinc/GABA (Qian & Dowling, 1995; Qian *et al.*, 1996; Han & Yang, 1999; Kaneda *et al.*, 2000) ratios successfully blocked $GABA_C$ responses. Similarly, in the slice, bicuculline and picrotoxin were applied at concentrations of 100 or 200μM, with 100μM GABA. This GABA concentration reflects the concentration within the puffer pipette and is most likely an overestimate of the concentration actually seen by the receptors due to diffusion after puffing, uptake, and the proximity of the puffer pipette to the receptors (i.e., (Lukasiewicz & Wong, 1997; Lukasiewicz & Shields, 1998). Comparable antagonist/GABA ratios were effective in blocking GABA responses in other preparations (Qian & Dowling, 1993, 1995; Blanco *et al.*, 1996; Han *et al.*, 1997; Lukasiewicz & Shields, 1998; McGillem *et al.*, 2000; Pan, 2001; Zhou & Dacheux, 2005). This data suggests that the antagonist/GABA ratios used here should have been sufficient to block responses elicited through $GABA_C$ type receptors. Thus, the TPMPA- and picrotoxin-insensitivity observed in zebrafish bipolar cells is most likely due to the $GABA_C$ receptor subtypes expressed on these cells.

Overall, recordings made in retinal slices or using isolated cells indicate bicuculline-sensitive $GABA_A$ receptors are present. In addition, a diversity of $GABA_C$ receptor subtypes is expressed. Some $GABA_C$ receptors display "classic" pharmacology and are bicuculline-insensitive and picrotoxin-sensitive. These are found on both the dendrites and axon terminals. The dendritic $GABA_C$ receptors are also sensitive to zinc and insensitive to TPMPA. On axon terminals (and dendrites), some presumed $GABA_C$ receptors are picrotoxin-insensitive. Typically both picrotoxin-sensitive and insensitive subtypes are found on the same cell. This pharmacological pattern, while somewhat distinctive to zebrafish, displays characteristics similar to those found in other fish (Tachibana & Kaneko, 1987; Qian & Dowling, 1993; Matthews *et al.*, 1994; Qian & Dowling, 1994, 1995; Han *et al.*, 1997), though picrotoxin-insensitivity has been identified only in rat (Feigenspan *et al.*, 1993; Feigenspan & Bormann, 1994). TPMPA-insensitive $GABA_C$ receptors have not been previously reported, though a small GABA-evoked current remained in rabbit OFF-bipolar cells following co-application of bicuculline and TPMPA (Zhou & Dacheux, 2005).

Variation in the distribution of $GABA_A$ and $GABA_C$ receptors

The distribution of $GABA_A$ and $GABA_C$ responses on zebrafish bipolar cells appears to vary in two ways: (1) between the dendritic and terminal processes of individual cells and (2) among the different types of bipolar cells. Overall, $GABA_C$ responses are more abundant than $GABA_A$ responses on all cells. $GABA_C$ responses are found on both dendrites and terminals; while $GABA_A$ responses are more prominent on the terminals. Similar to our findings, immunocytochemical data (Yazulla & Studholme, 2001) shows positive label for $GABA_A$ and $GABA_C$ receptor subunits in both plexiform layers, but with the $GABA_C\rho$ subunit labeling particularly intense in the OPL. $GABA_A$ subunits were also selectively distributed. Only OFF-bipolar cells and the large B_{on}-s6 bipolar cells expressed the $\alpha 3$ subunit of the $GABA_A$ receptor. Dendritic label of $\alpha 3$ OFF bipolar cells was particularly intense. This may be related to the difference in GABA physiology seen between OFF- and ON-type dendritic regions in the present study. The GABA $\alpha 1$ subunit was more broadly distributed.

Variability in GABA receptor expression may also occur among the different classes of bipolar cells, as found in rat (Euler & Wassle, 1998), bullfrog (Du & Yang, 2000), and skate (Qian *et al.*, 2001). Physiological studies indicate bullfrog OFF-bipolars have a greater density of $GABA_A$ receptors at their dendrites and more $GABA_C$ receptors at their terminals. ON-bipolar cells have more $GABA_A$ receptors than $GABA_C$ receptors on both sets of processes (Du & Yang, 2000). Similarly, salamander OFF-bipolar cells express $\alpha 1$, $\beta 1$, $\beta 2/3$, and $\gamma 2$ subunits of $GABA_A$ receptors (Zhang *et al.*, 2003) and ON- and OFF-bipolar cells in rat are labeled with an antibody to the $\rho 1$ subunit of $GABA_C$ receptor (Klooster *et al.*, 2004). We have previously identified multiple subtypes of ON- and OFF-bipolar cells in zebrafish (Connaughton *et al.*, 2004). Our voltage probe recordings from isolated cells suggest these ON- and OFF- types express different combinations of receptors. GABA-induced soma-dendritic hyperpolarizations were observed in ON-type cells that were also hyperpolarized by glutamate, but not in OFF-type cells that were depolarized by glutamate. In the slice, however, GABA responses were identified on dendritic and axonal processes of both ON- and OFF- cell types. While we do not know why there is a difference

in the GABA response of OFF-bipolar cells recorded here, we speculate that the lack of a hyperpolarizing response of the isolated cells to externally applied GABA may reflect a difference in GABA receptor expression among OFF-bipolar subtypes.

Differential antagonist sensitivities may reflect differences in subunit composition of the different GABA receptors

Zinc, TPMPA, and picrotoxin sensitivities suggest bipolar cell GABA subunit compositions. Zinc sensitivity of $GABA_C$ receptors can be determined by the ρ subunits (Wang *et al.*, 1995; Zhang *et al.*, 2001; Qian & Pan, 2002), with the different homomeric receptors having differential sensitivities to zinc (Qian *et al.*, 1998). White perch homomeric ρ2A receptors displayed the greatest zinc sensitivity of the four ρ subunits (Qian *et al.*, 1998). Further, zinc sensitivity of the perch ρ1B subunit is enhanced if it is co-expressed with the γ2 subunit in *Xenopus* oocytes (Qian & Ripps, 1999; Qian & Pan, 2002). Given this information we hypothesize that $GABA_C$ receptors on zebrafish bipolar cells are most likely either homomers composed of one ρ subunit (such as ρ2A) or heteromers composed of different combinations subunits (such as ρ1B and γ2). If this were the case, zebrafish receptors assembled from ρ2A subunits or ρ1Bγ2 would have the greatest sensitivity to zinc. The other subunit combinations would also be sensitive to zinc, but to a lesser extent. $GABA_A$ receptors would be insensitive to zinc, due to the presence of the γ subunit. In agreement with this, both plexiform layers in the zebrafish retina were labeled with antibodies to ρ, as well as β2/3, receptor subunits (Yazulla & Studholme, 2001).

The hypothesis that ρ1B subunits form $GABA_C$ receptors on some zebrafish bipolar cells is further supported by the results of the TPMPA experiments. TPMPA-insensitivity observed here contradicts most reports within the literature. However, TPMPA-insensitivity has been observed in expression systems (Pan *et al.*, 2005) and suggests possible GABA receptor subunit composition in zebrafish. The different ρ subunits (i.e., ρ1A, ρ1B, ρ2A, and ρ2B) display differential sensitivities to TPMPA: A-type receptors are highly sensitive to TPMPA; B-type receptors are less sensitive (Pan *et al.*, 2005). As the GABA responses in most isolated bipolar cells were insensitive to TPMPA, these receptors are probably assembled from ρ1B and/or ρ2B subunits. These two subunits are also differentially sensitive to picrotoxin (Qian *et al.*, 1998), with picrotoxin insensitivity in rat determined by the rρ2 subunit (Zhang *et al.*, 1995). The varied expression of the ρ2 and ρ1 subunits among zebrafish bipolar cells could account for the range of picrotoxin sensitivities observed in the slice and in isolated cells. Focusing on the ρ2B subunits, one might speculate that picrotoxin sensitive or insensitive assemblies might be formed depending on whether the γ2 subunit happened to be incorporated (Qian & Ripps, 1999).

Glycine sensitivity

Glycine-evoked currents (in slices) or membrane hyperpolarizations (voltage probe recordings) were observed in zebrafish bipolar cells. Responses were obtained from both dendritic and axonal processes. These results are unique as glycine-evoked currents have not been previously reported in fish bipolar cells (Kaneko *et al.*, 1991; Shen *et al.*, 2005). However, bipolar cells in mammals (Cui *et al.*, 2003; Frech & Backus, 2004; Zhou & Dacheux, 2005; Eggers & Lukasiewicz, 2006; Ivanova *et al.*, 2006) and other lower

vertebrates (Maple & Wu, 1998; Qian *et al.*, 2001; Du & Yang, 2002a, 2002b) are glycine-sensitive.

Not all zebrafish bipolar cells were sensitive to glycine, with responses observed in ~30% of either isolated cells or cells within the retinal slice. This suggests that glycinergic innervation might be type specific, whereas GABAergic innervation might be universal. The fact that zebrafish bipolar cells receive direct glycinergic inputs agrees with the immunocytochemical localization of glycine-positive processes (Connaughton *et al.*, 1999) and glycine receptor subunits (Yazulla & Studholme, 2001) in both plexiform layers of the zebrafish retina. Glycinergic inputs to bipolar cell dendrites most likely arise from the population of glycine-containing interplexiform cells present in zebrafish (Connaughton *et al.*, 1999; Marc & Cameron, 2001); while presynaptic glycine-containing amacrine cells most likely provide inputs to bipolar terminals.

Glycine application evokes a current with a negative E_{rev}, similar to GABA, suggesting a chloride current. Overall, currents evoked by glycine were similar in amplitude to GABA-evoked responses at the dendritic arbor (5–10 pA), as were changes in membrane fluorescence; however, at the axon terminal, glycine-evoked currents were smaller than GABA-evoked responses. This suggests that GABA receptor activation mediates most of the inhibition onto axon terminals, whereas, inhibitory inputs to the dendritic arbor occur equally through the activation of both GABA and glycine receptors. Immunocytochemistical localization of GABA-and glycine-containing processes in the zebrafish retina again supports these findings. Glycinergic amacrine cells are half as numerous as GABAergic amacrine cells (Marc & Cameron, 2001) GABA-containing processes are located throughout both plexiform layers. Glycine heavily labels the middle of the IPL, while more sparse label is seen throughout the IPL and the OPL (Connaughton *et al.*, 1999).

Isolated cell vs. slice recordings

As two different methods were used to explore the localization of bipolar cell GABA sensitivity, it is worth discussing potential biases of these different approaches. For example, the protocols used in voltage-probe recordings probably favored $GABA_C$-type responses. Compounds were applied over 4 minutes, a time period that might result in greater desensitization of $GABA_A$ receptors, allowing us, in effect, to examine the effects of zinc and TPMPA on relatively isolated $GABA_C$ responses. In contrast, focal puffs of GABA into retinal slices would allow both $GABA_A$ and $GABA_C$ responses to be examined. This difference may account for the insignificant change in membrane fluorescence following bicuculline application seen in isolated cells. Another issue is the availability of dendritic GABA receptors, which may differ between the two preparations. In the slice, dendritic receptors may be located deep within the slice, rather than on the surface. Some exogenously applied compounds may be quickly removed by transporter activity (i.e., Wu, 1986). Thus, within the tissue, sparse dendritic receptors may not be easily accessed by externally applied GABA. Isolating cells removes uptake/removal systems and exposes dendritic receptors. Further, the voltage-probe recording method may increase sensitivity. Semi-saturating GABA concentrations were more than a decade lower in zebrafish bipolar cells with probe recording (0.4μM) than reported in hybrid bass bipolar cells by whole cell

currents (12.5μM; Qian & Ripps, 1999). Such increased sensitivities might arise if voltage saturation preceded current saturation. For example, approximating the membrane of an isolated bipolar cell as an 8μm sphere with a resistivity of $10^4\Omega\cdot cm^2$, it would have a conductance of 200pS, in which case the 16mV median hyperpolarization would require only 3pA, far less than the 30pA maximal amplitude commonly recorded from zebrafish bipolar cells in slice preparations. For both these reasons, the voltage probe studies of isolated cells may unmask the actions of a sparse population of dendritic GABA receptors.

Summary

Taken together, our results suggest a combination of $GABA_A$ and $GABA_C$ receptors are present on zebrafish bipolar cell processes, with the bulk of the response amplitudes, whether measured by whole-cell currents or voltage probe fluorescent changes, arising from $GABA_C$-like receptors. Glycine receptors are also present on both sets of processes, but not on all cells. Smaller $GABA_A$ response components were also seen that were selectively sensitive to bicuculline. $GABA_C$ receptors displayed a somewhat unique pharmacology as they were insensitive to TPMPA, and were only partially blocked by picrotoxin. Sensitivity to zinc and insensitivity to bicuculline follows a more conventional pattern.

GABAergic inputs to zebrafish bipolar cell dendrites most likely come from horizontal cells in the outer retina. There are at least three horizontal cell types in zebrafish (Connaughton *et al.*, 2004) and at least one of these types contains GABA (Connaughton *et al.*, 1999; Marc & Cameron, 2001; Yazulla & Studholme, 2001). The submicromolar sensitivity of isolated bipolar cells with only dendritic tops suggests that even extrasynaptic GABA might be an effective regulator of bipolar cell function. Inputs to axon terminals (either GABAergic or glycinergic) in the inner retina likely come from inhibitory amacrine cells (i.e., Tachibana & Kaneko, 1987, 1998). GABA receptors expressed on zebrafish bipolar cell terminals are similar in activity and general pharmacology to those described in other species. As wide field GABAergic amacrines are the presynaptic sources of GABA in all species thus far studied (Marc *et al.*, 1978; Pourcho & Goebel, 1983; Nelson & Kolb, 1985; Freed *et al.*, 1987; Yazulla *et al.*, 1987), it is likely that in zebrafish also GABAergic signal-processing roles in the inner retina are similar to those found in other vertebrates.

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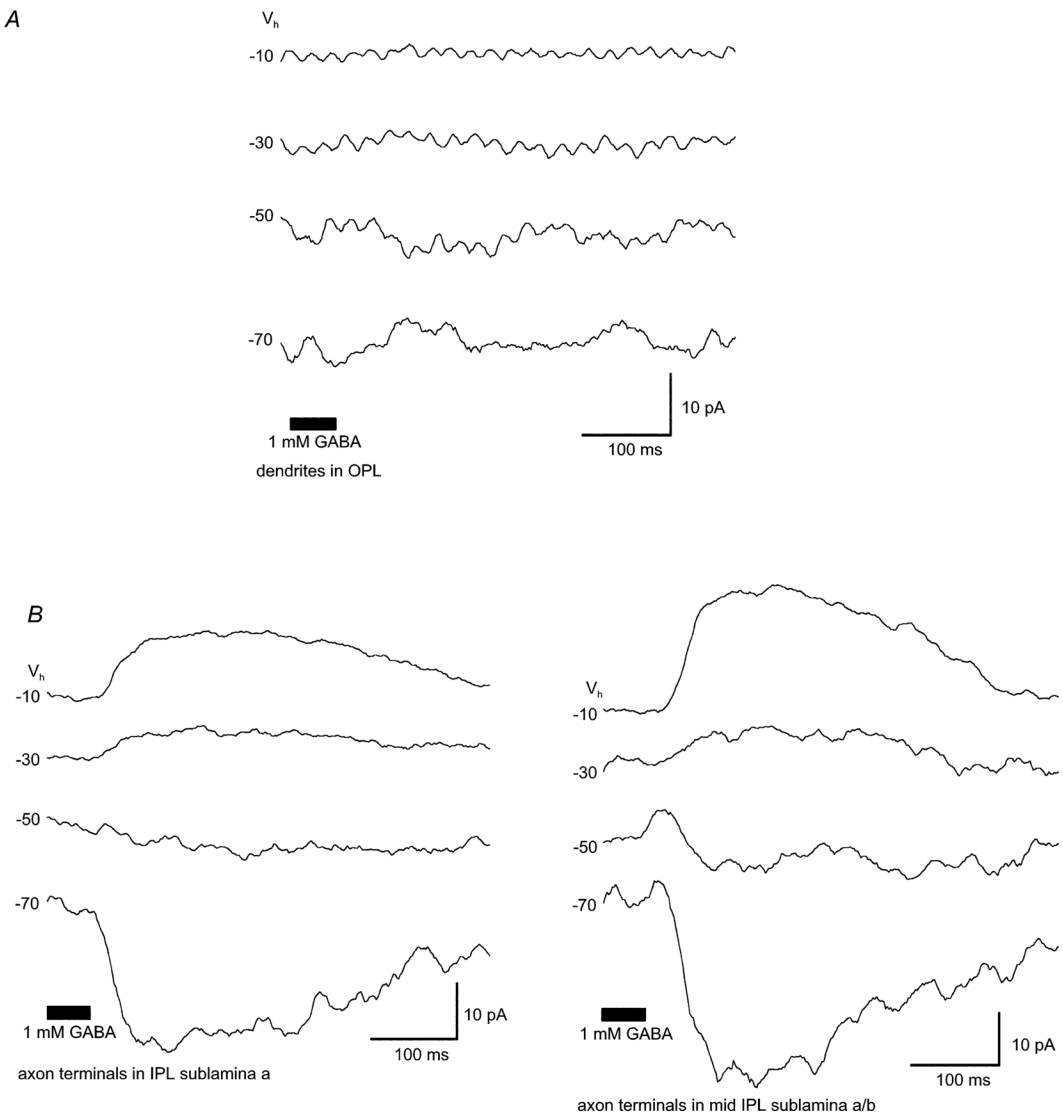

Figure 1. In the retinal slice preparation, GABA-evoked currents were more robust in the axon terminal than in the dendritic arbor

In the retinal slice preparation, focal application of GABA onto (*A*) the dendritic arbor (OPL) or (*B*) axonal bouton(s) of bipolar cells (IPL) evoked different amplitudes of whole-cell currents. GABA responses elicited from axon terminals (~30 to 40pA) could be recorded from all levels of the IPL (IPL sublamina a, mid IPL sublamina a/b) and were typically more robust than those elicited from the dendritic arbor (≤5pA). Only a minority of cells responded to GABA application in the OPL (n = 3 of 12 cells; 1mM GABA puff). All traces shown were recorded from the same cell. Values to the left of each trace are the holding potentials, the bar at the bottom denotes the duration of GABA application. Pipette solution is low Cl^- (E_{Cl} = −61mV).

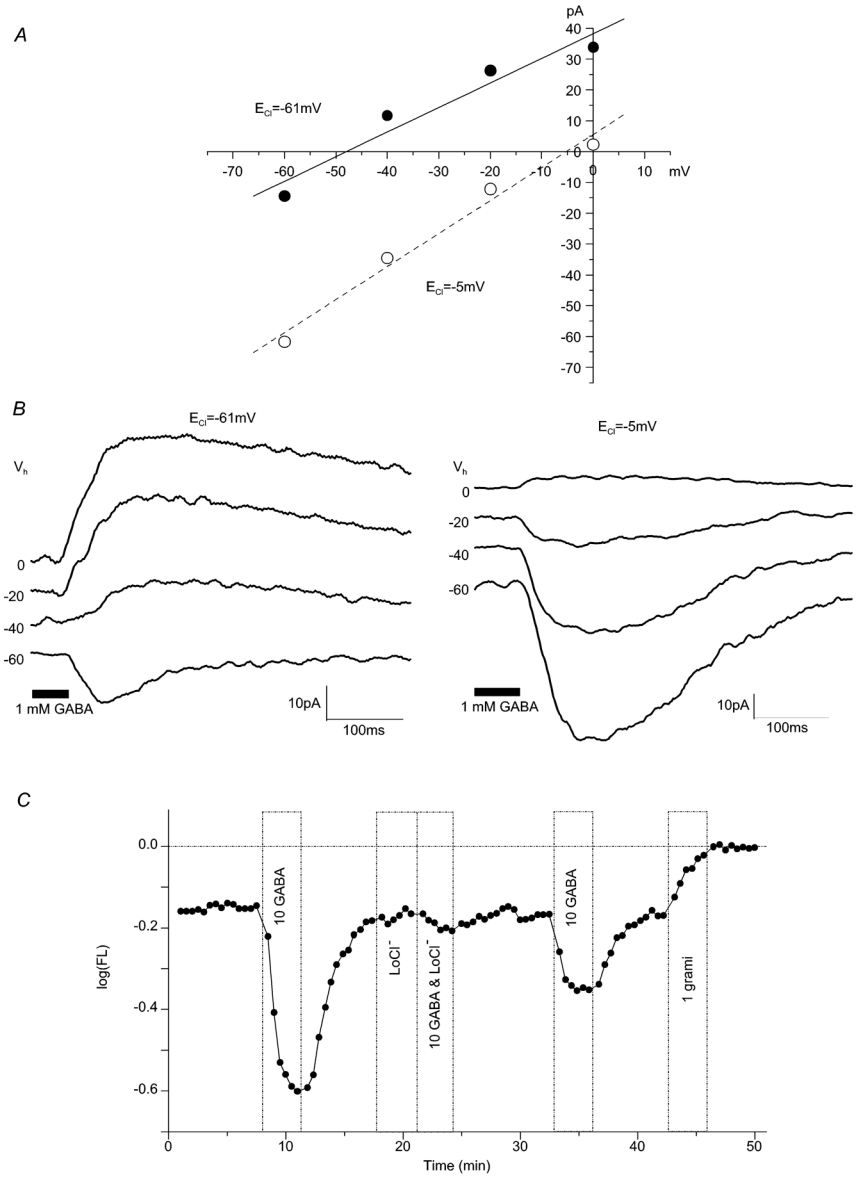

Figure 2. GABA elicits a chloride current in zebrafish bipolar cells

(*A*) GABA-evoked current-voltage relationships and (*B*) the accompanying whole-cell current traces change with high and low intracellular chloride concentrations. For $[Cl^-]_i$ of 12mM (E_{Cl} = − 61mV) the mean E_{rev} was − 38.02 ± 5.6mV (n = 10; 1mM GABA); for $[Cl^-]_i$ of 116mM (E_{Cl} = −5mV) the mean E_{rev} was 0.39 ± 4.29mV (n = 6; 1mM GABA). V_{hold} values (V_h) are given to the left of each trace (*B*). (*C*) Hyperpolarizing GABA responses recorded with a voltage probe in an isolated bipolar cell are blocked by removal of $[Cl^-]_o$ "LoCl" (Na isethionate substituted for NaCl in the extracellular media, $[Cl^-]_o$ = 10mM). In this and following voltage-probe figures, the voltage probe is 80nm oxonol, added to all solutions and log(FL) refers to background-subtracted oxonol fluorescence. Increases in log(FL) correspond to depolarization, and decreases to hyperpolarization. The '0' level of log(FL) is set by the final 1μM gramicidin treatment ("1 grami"), that forces cell

membrane potential to zero. 1 log of FL decrease corresponds to − 72mV, 1 log of FL increase corresponds to 129mV (Nelson *et al*., 1999). Treatments are shown by the bars, with concentrations given in μM.

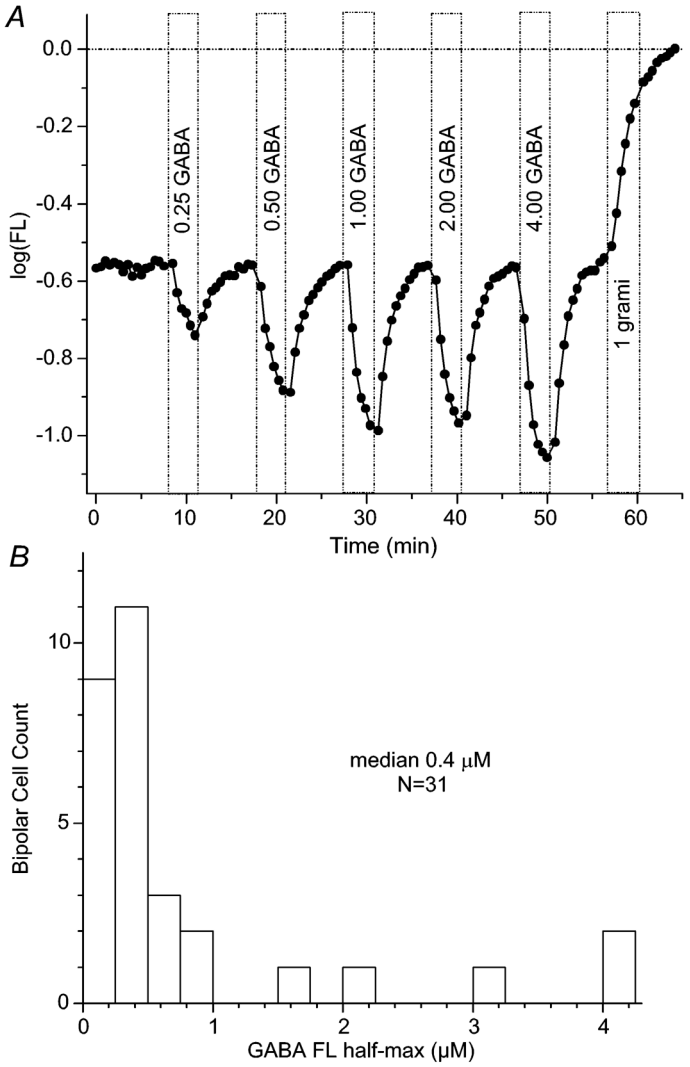

Figure 3. Isolated zebrafish bipolar cells are sensitive to a range of GABA concentrations

(*A*) Responses to different GABA concentrations in an isolated bipolar cell. GABA concentrations ranging from 0.25–4μM (*bars*) elicited hyperpolarizing responses from isolated bipolar neurons. In this example half-maximum response (half-max) occurred at ~0.5μM. (*B*) Histogram of half-maximum GABA concentrations for responses from 31 isolated bipolar cells. The median is 0.4μM. Figure conventions for voltage probe recordings are the same as described in Figure 2.

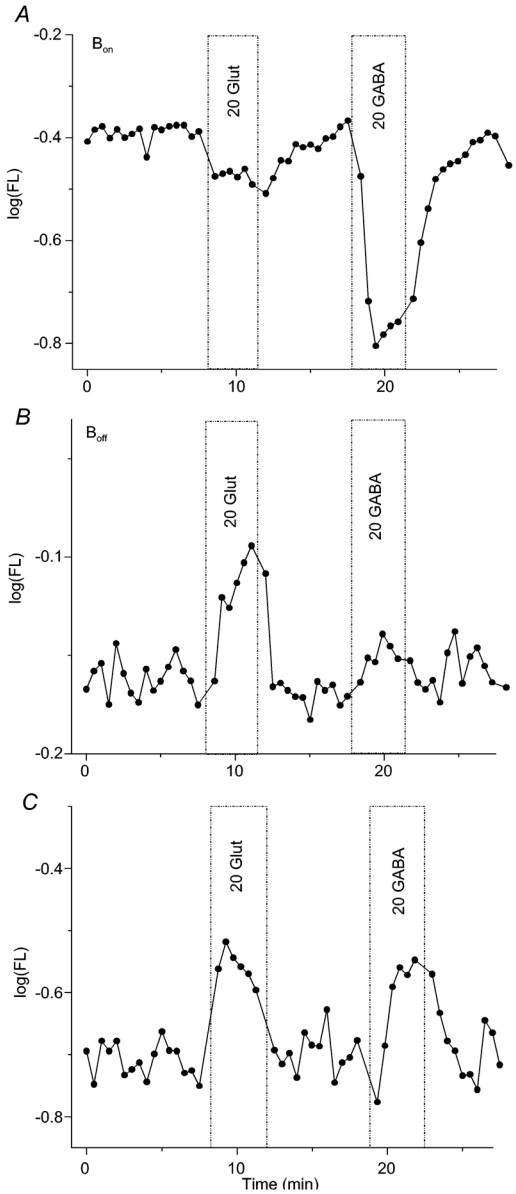

Figure 4. GABA responses of isolated soma-dendritic regions of B_{on} and B_{off} bipolar cells

(*A*) B_{on} type cell, characterized by glutamate hyperpolarization ("20 glut"), also hyperpolarizes robustly to GABA ("20 GABA"). (*B*) B_{off} type unit, characterized by glutamate depolarization, does not respond to GABA. (*C*) B_{off} type unit, characterized by glutamate depolarization, is depolarized by GABA. Figure conventions for voltage probe recordings are the same as described in Figure 2.

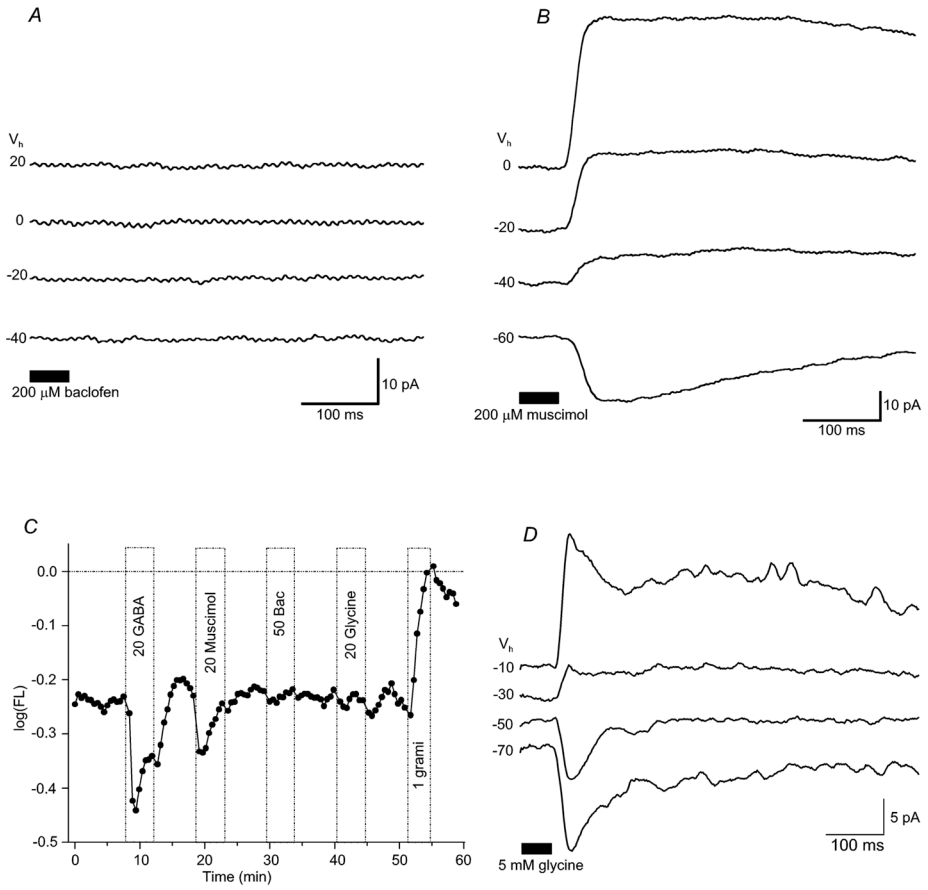

Figure 5. Muscimol, not baclofen, is an effective agonist of GABA receptors on bipolar cell axon terminals

Whole-cell current traces show responses to (*A*) 200μM ± baclofen or (*B*) 200μM muscimol applied onto axon terminals. E_{rev} of muscimol-elicited currents averaged −45.4 ± 21.6mV (n = 4). Baclofen failed to elicit responses. Each drug was tested in separate experiments. (*C*) Voltage-probe records from a dissociated bipolar cell reveal both GABA- (“20 GABA”) and muscimol-(“20 Muscimol”) induced hyperpolarizations. Baclofen (“50 Bac”) and glycine (“20 gly”) were not effective in this cell, though glycine induced hyperpolarizations in others. (*D*) Whole-cell current traces show responses to 5 mM glycine applied onto bipolar cell dendrites. E_{rev} of dendritic glycine-elicited currents averaged − 49 ± 9mV (n = 8). Bipolar cells were also sensitive to glycine applied to axonal boutons (not shown). Figure conventions for voltage-probe records are the same as described in Figure 2.

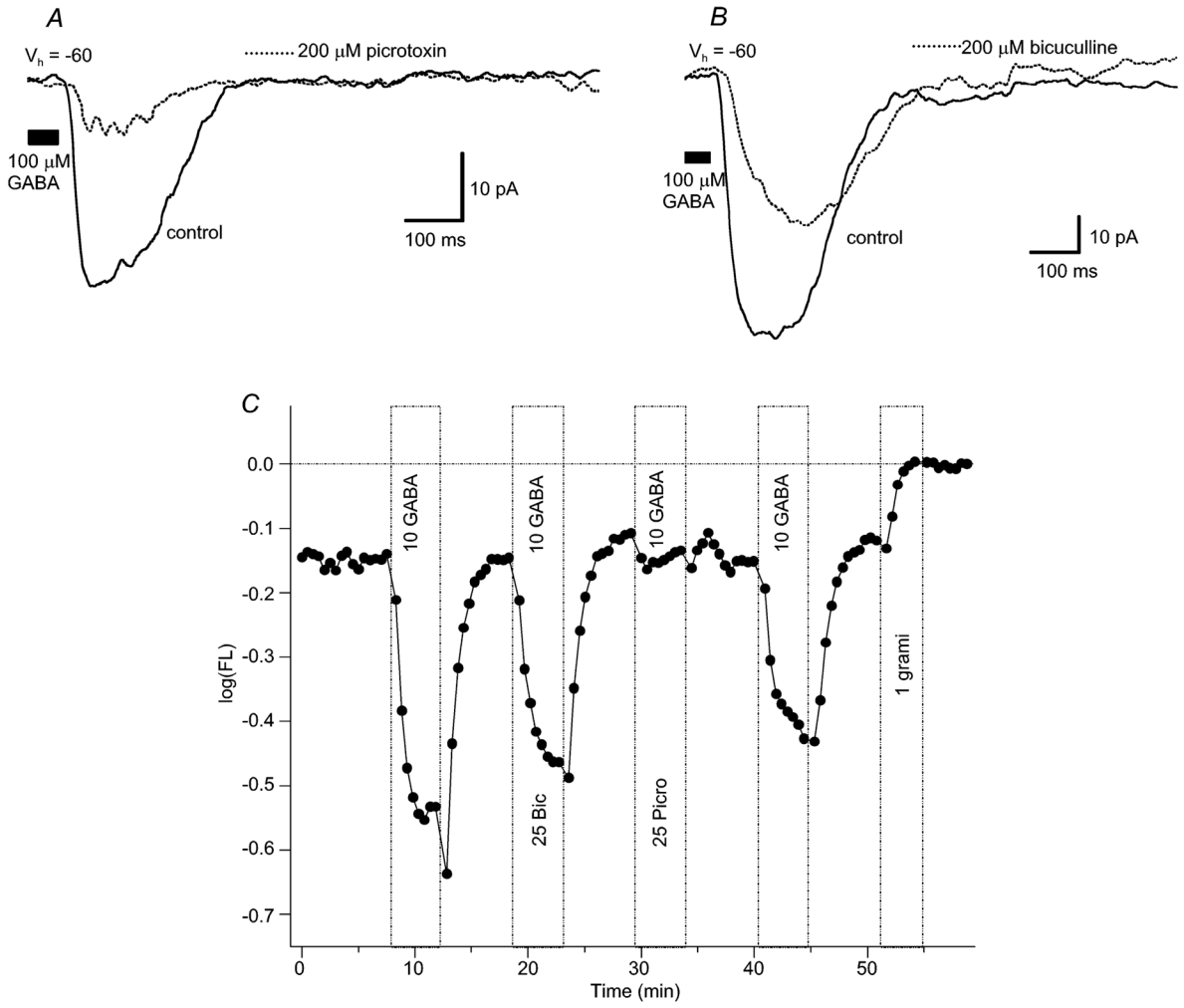

Figure 6. Picrotoxin is more effective than bicuculline at blocking GABA-evoked currents elicited at bipolar cell axon terminals or isolated soma-dendritic regions

(*A*) Whole-cell current traces show a decrease in GABA-gated currents observed after bath application of 200μM picrotoxin. (*B*) Whole cell current traces show lesser decrease in current after bath application of 200μM bicuculline. V_{hold}= −60mV for *(A)* and *(B)*. (*C*) Dissociated bipolar cells displayed similar antagonist sensitivity: picrotoxin decreased GABA-evoked fluorescence responses by ~80%, while bicuculline only partially antagonized the response. Figure conventions for voltage-probe records are the same as described in Figure 2.

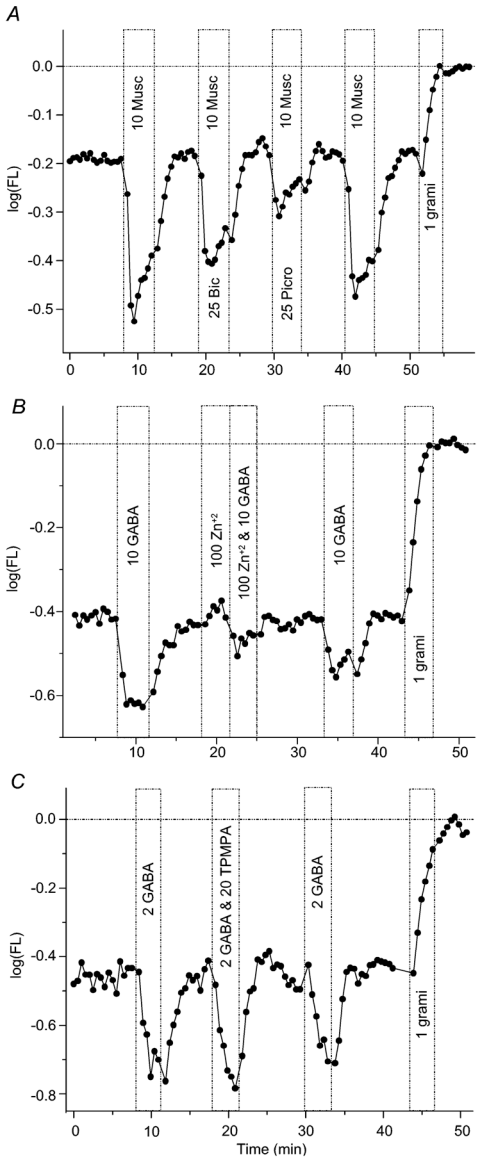

Figure 7. In isolated bipolar cells, zinc, but not TPMPA, antagonizes GABA-responses, muscimol responses are not blocked by bicuculline

(*A*) In an isolated bipolar cell, muscimol ("10 Musc"), like GABA, evokes a transient hyperpolarizing response. This muscimol response is only partially blocked by bicuculline, suggesting muscimol does not selectively activate $GABA_A$ receptors. The picrotoxin block is more complete, suggesting $GABA_C$ receptors are activated by muscimol and at least partially blocked by picrotoxin. (*B*) The co-application of zinc (100μM) together with GABA (10μM) reversibly abolished GABA-elicited hyperpolarization in an isolated bipolar cell. These findings, together with bicuculline insensitivity and high GABA sensitivity, suggest $GABA_C$ receptors are expressed on zebrafish bipolar cells. (*C*) TPMPA (20μM), a $GABA_C$ selective antagonist, fails to abolish hyperpolarizations evoked by GABA (2μM) in

an isolated bipolar cell, even in 10x excess. Figure conventions for voltage-probe records are the same as described in Figure 2.