Pharmacological Analysis of the Activation and Receptor Properties of the Tonic GABA<sub>B</sub>R Current in Retinal Bipolar Cell Terminals

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Abstract

GABAergic inhibition in the central nervous system (CNS) can occur via rapid, transient postsynaptic currents and via a tonic increase in membrane conductance, mediated by synaptic and extrasynaptic GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) respectively. Retinal bipolar cells (BCs) exhibit a tonic current mediated by GABA<sub>A</sub>Rs in their axon terminal, in addition to synaptic GABA<sub>B</sub>R and GABA<sub>A</sub>-R currents, which strongly regulate BC output. The tonic GABA<sub>B</sub>-R current in BC terminals (BCTs) is not dependent on vesicular GABA release, but properties such as the alternative source of GABA and the identity of the GABA<sub>B</sub>-Rs remain unknown. Following a recent report that tonic GABA release from cerebellar glial cells is mediated by Bestrophin 1 anion channels, we have investigated their role in non-vesicular GABA release in the retina. Using patch-clamp recordings from BCTs in goldfish retinal slices, we find that the tonic GABA<sub>B</sub>-R current is not reduced by the anion channel inhibitors NPPB or flufenamic acid but is reduced by DIDS, which decreases the tonic current without directly affecting GABA<sub>B</sub>-Rs. All three drugs also exhibit non-specific effects including inhibition of GABA transporters. GABA<sub>B</sub>-R p subunits can form homomeric and heteromeric receptors that differ in their properties, but BC GABA<sub>B</sub>-Rs are thought to be p1-p2 heteromers. To investigate whether GABA<sub>B</sub>-Rs mediating tonic and synaptic currents may differ in their subunit composition, as is the case for GABA<sub>A</sub>-Rs, we have examined the effects of two antagonists that show partial p subunit selectivity: picrotoxin and cyclothiazide. Tonic and synaptic GABA<sub>B</sub>-R currents were differentially affected by both drugs, suggesting that a population of homomeric p1 receptors contributes to the tonic current. These results extend our understanding of the multiple forms of GABAergic inhibition that exist in the CNS and contribute to visual signal processing in the retina.

Introduction

GABA, the major inhibitory neurotransmitter in the CNS, evokes transient postsynaptic currents (IPSCs) via ionotropic GABA<sub>A</sub> and GABA<sub>C</sub> receptors, as well as slower synaptic responses via metabotropic GABA<sub>B</sub> receptors (GABABRs). In addition, there is increasing evidence that GABA evokes a tonic increase in membrane conductance by activating extrasynaptic GABA receptors, either as a result of spill-over from synapses or via a non-synaptic mechanism [1]. Tonic GABA<sub>B</sub> currents are mediated by GABA<sub>B</sub>-Rs in brain regions such as the hippocampus, cerebellum and thalamus, where they have a role in controlling neuronal excitability and network interactions [2,3]. In the retina, a GABA<sub>B</sub>-R-mediated tonic current occurs in the synaptic terminals of bipolar cells (BCs), which similarly regulates membrane excitability [4,5]. Bipolar cell terminals (BCTs) also exhibit rapid synaptic GABA<sub>B</sub>-R and GABA<sub>B</sub>-C currents that mediate feedback inhibition and limit BC glutamate release, thereby modulating the light responses of ganglion cells, the output cells of the retina [6].

We have found that the tonic GABA<sub>B</sub>-R current in BCTs, like some tonic GABA<sub>B</sub>-R currents [7–10], is not dependent on vesicular GABA release [11]. The alternative source of GABA is currently unknown but does not appear to involve reversal of GABA transporters or release via hemichannels or P2X<sub>7</sub> receptors [11]. It was recently shown that the tonic release of GABA from cerebellar glial cells can occur via Bestrophin 1 (Best1) Cl<sup>-</sup> channels [12], which have a significant permeability to large anions such as thiocyanate, glutamate and glycine [13,14]. In addition, volume-regulated anion channels (VRACs) have been implicated in the non-vesicular release of neurotransmitters [15]. Astrocytic or neuronal release via anion channels may therefore be a potential source of GABA for activating the tonic GABA<sub>B</sub>-R current in BCTs.

Tonic GABA<sub>B</sub>-R currents are mediated by receptors that differ in their subunit composition from synaptic GABA<sub>B</sub>-Rs, conferring distinct receptor properties that are suited to their localization and function, such as high GABA sensitivity and reduced desensitization [16,17]. GABA<sub>C</sub>-Rs are composed of p subunits which are highly expressed in the retina but are also localized to various brain regions including the midbrain, thalamus, hippocampus and cerebellum [18]. BC GABA<sub>C</sub>-Rs are believed to be p1-p2 heteromers, although p subunits can also co-assemble with GABA<sub>C</sub>-R γ subunits [19,20]. Heterologous expression of p1 and/or p2 subunits reveals differences in receptor properties, for example p1 homomers exhibit higher GABA sensitivity, lower conductance and slower deactivation than p2 homomers, with heteromeric p1-p2 receptors generally showing intermediate...
properties [21–24]. However, it is unknown whether receptor subunit diversity contributes to the different forms of GABA<sub>C</sub>-R-mediated inhibition in BCTs.

To further investigate the activation and receptor properties of GABA<sub>C</sub>-Rs mediating the tonic current in BCTs, we have examined the effect of anion channel inhibitors and subunit-selective antagonists on spontaneous and evoked GABA<sub>C</sub>-R currents recorded directly from BCTs in goldfish retinal slices. We find evidence for a role of DIDS-sensitive anion channels/exchangers in tonic GABA release, and for a contribution of homomeric ρ1 receptors to the tonic GABA<sub>C</sub>-R current.

**Methods**

Goldfish (*Carassius auratus*) were maintained in a 12 hour dark/light cycle at 16°C. Prior to use, light-adapted goldfish were dark-adapted for 1 hour to facilitate removal of the pigment epithelium. Goldfish were killed by decapitation followed immediately by destruction of the brain and spinal cord under Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The experiments were approved by Keele University’s Central Animal Facility Management Committee. The eyeballs were removed and retinae dissected out and treated for 25 minutes with hyaluronidase to remove vitreous humor. Each retina was quartered, placed ganglion cell layer down on filter paper and kept until needed at room temperature (18–22°C), in daylight conditions.

Methods

Drugs were bath-applied via the extracellular solution and locally-applied via pressure application from a low resistance glass micropipette (<5 μm tip diameter) positioned 25-50 μm from the recording chamber and perfused (1 ml.min<sup>-1</sup>) with medium comprising (mM): NaCl (127), KCl (2.5), MgCl<sub>2</sub> (1.0), CaCl<sub>2</sub> (0.5), Hepes (5), glucose (12), adjusted to pH 7.45 with NaOH. Slices were cut at 250 μm intervals, transferred to the recording chamber and perfused (1 ml.min<sup>-1</sup>) with medium comprising (mM): NaCl (108), KCl (2.5), MgCl<sub>2</sub> (1.0), CaCl<sub>2</sub> (2.5), NaHCO<sub>3</sub> (24), glucose (12), gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4. For Ca<sup>2+</sup>-free extracellular solution, CaCl<sub>2</sub> was omitted and MgCl<sub>2</sub> was increased to 3.5 mM, to maintain the divalent cation concentration. The osmolalities of the 2.5 mM Ca<sup>2+</sup> and Ca<sup>2+</sup>-free extracellular solutions were 267 mOsm and 269 mOsm respectively. Slice preparation and recordings were performed at room temperature (18–22°C), in daylight conditions.

Results

The role of anion channels: Effects of NPPB and flufenamic acid

In order to investigate the role of Best1 and other anion channels in non-vesicular GABA release in the retina, we tested the effect of anion channel inhibitors on GABA<sub>C</sub>-R-mediated currents in BCTs. Recordings were made with CsCl-based intracellular solution at a holding potential of −60 mV, in the presence of bicuculline (50 μM) to block GABA<sub>B</sub>-R-mediated spontaneous IPSCs (sIPSCs). The anion channel inhibitors were tested under both normal (2.5 mM) Ca<sup>2+</sup> and Ca<sup>2+</sup>-free extracellular conditions; when no differences were observed between these conditions, the data has been pooled.

Application of the anion channel inhibitor NPPB (50–100 μM) to axon-severed BCTs initially evoked a small decrease, followed by an increase, in the holding current over the course of about 20 minutes (2.5 mM Ca<sup>2+</sup> n = 2, Ca<sup>2+</sup>-free n = 2; fig. 1A). Application of flufenamic acid (FFA; 100–200 μM), either alone (2.5 mM Ca<sup>2+</sup> n = 2) or in combination with NPPB (Ca<sup>2+</sup>-free n = 2), evoked the same biphasic effect (fig. 1A). The potentiated current in NPPB and/or FFA was subsequently inhibited by the addition of the GABAR antagonist picrotoxin (200 μM; 2.5 mM Ca<sup>2+</sup> NPPB n = 1, FFA n = 1; Ca<sup>2+</sup>-free NPPB n = 1, NPPB+FFA n = 2, fig. 1A), confirming that it was mediated by GABA<sub>C</sub>-Rs. Responses to locally-applied GABA (100 μM, 50–100 ms application) were monitored in the same experiments to check for direct inhibition of GABA<sub>C</sub>-Rs by the anion channel blockers. The charge of GABA-evoked responses was not reduced by NPPB (n = 3), FFA (n = 1) or combined application (n = 2). Instead, a significant potentiation of GABA-evoked responses was observed, which occurred in parallel with the tonic current increase (fig. 1B). The GABA-evoked responses were subsequently fully blocked by picrotoxin (n = 4; fig. 1B).

The potentiating effects of NPPB and FFA on both the tonic current and exogenous GABA responses may result from inhibition of GABA uptake, as inhibition of GAT-1 by NO-711 (3 μM) exerts a similar, though more pronounced, potentiating effect on the tonic current [4] and on the charge of GABA-evoked responses (n = 6; fig. 1C). FFA and the related compound nimloc acid have previously been found to inhibit certain GAT isoforms to variable extents [27]. The small initial decrease in the holding current may indicate a minor contribution of NPPB/FFA-sensitive anion channels to non-vesicular GABA release, or may result from a non-specific effect of these drugs on other ion channels (see below).

A markedly different effect of NPPB and FFA was observed in recordings made from the terminals of intact BCs. Application of NPPB (50 μM; n = 3), FFA (100–200 μM; n = 2) or both in combination (n = 2) resulted in a significant reduction in the holding current (2.5 mM Ca<sup>2+</sup> n = 3, Ca<sup>2+</sup>-free n = 4), which was subsequently further reduced by application of picrotoxin (200 μM; fig. 1D). Conversely, responses evoked by local application of GABA...
Figure 1. The effect of NPPB and FFA on GABA$_R$ currents. A, Example experiments and mean data (2.5 mM Ca$^{2+}$ n = 4, Ca$^{2+}$-free n = 4) showing the effects of NPPB (50–100 μM) and FFA (100–200 μM) on the holding current in recordings from axon-severed BCTs, with subsequent addition of picrotoxin (PTX; 200 μM; NPPB/FFA(2) was measured 5–10 mins after drug application; NPPB/FFA(2)) was measured 10–20 mins after application. B, Example GABA$_R$ responses evoked by local application of GABA (100 μM, 100 ms) and the charge of GABA$_R$-evoked responses against time for the recording in Ca$^{2+}$-free conditions in A, with mean data (2.5 mM Ca$^{2+}$ n = 2, Ca$^{2+}$-free n = 4) showing the effect of NPPB and/or FFA on the charge of GABA-evoked responses. C, Example responses and mean data (n = 6) showing the effect of the GAT-1 inhibitor NO-711 (3 μM) on GABA-evoked responses (100 μM, 50–100 ms), and the associated increase in the tonic GABA$_R$ current. D, An example experiment in Ca$^{2+}$-free extracellular solution and mean data (2.5 mM Ca$^{2+}$ n = 3, Ca$^{2+}$-free n = 4) showing the effect of NPPB (50 μM) and/or FFA (100–200 μM) on the holding current in recordings from the terminals of intact BCTs, with subsequent addition of picrotoxin (200 μM). E, Mean data showing the effect of NPPB (50 μM) and/or FFA (100–200 μM) on the charge of GABA$_R$-evoked responses (2.5 mM Ca$^{2+}$ n = 2, Ca$^{2+}$-free n = 3) in recordings from the terminals of intact BCTs. All experiments in this and subsequent figures were performed with CsCl-based intracellular solution at a holding potential of ~60 mV in the presence of bicuculline (50 μM), unless stated otherwise. Example evoked currents show the average of 2–5 responses in each condition. Error bars represent SEM; * denotes P<0.05.
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**Figure A**
- 2.5 mM Ca<sup>2+</sup>
- DIDS
- PTX

**Figure B**
- Ca<sup>2+</sup>-free
- DIDS
- PTX

**Figure C**
- DIDS
- PTX

**Figure D**
- Baseline
- DIDS

*Note: The figures illustrate the current (I<sub>M</sub>) and GABA charge (I<sub>GABA</sub>) changes over time and across different conditions. The asterisks (*) indicate significant differences.*
GABA uptake rather than via any direct action on GABACRs, as GABACR-mediated mIPSCs [5] are not affected by application of NO-711 (fig. 4C). Following the establishment of a stable baseline tonic current in NO-711, picrotoxin was applied at a concentration of 0.5, 2, 10, 50, 100 or 200 \( \mu M \), followed by a maximal concentration of 250 \( \mu M \) (n = 3-6 for each concentration; fig. 4B). The amplitude of the GABACR-mediated tonic current was normalized to the baseline current and plotted versus picrotoxin concentration (fig. 4D). A fit of the dose-response plot with a Hill equation gave an IC\(_{50}\) value of 8.5 \( \mu M \). The amount of inhibition of the tonic GABACR current was statistically different from that of glu-evoked GABA CR currents at picrotoxin concentrations between 0.5 \( \mu M \) and 50 \( \mu M \) (\( P < 0.05 \)). The approximately 6-fold difference in picrotoxin sensitivity suggests that homomeric \( \rho 1 \) receptors may contribute more to the tonic GABACR current than to synaptic GABACR currents.

GABACR subunit composition: Cyclothiazide-sensitivity

Cyclothiazide has recently been shown to be a selective inhibitor of \( \rho 2 \) receptors, acting as a non-competitive antagonist with an \( IC_{50} \) of \( \approx 12 \mu M \). At a concentration of 300 \( \mu M \), cyclothiazide abolishes GABA responses mediated by \( \rho 2 \) homomers but has no significant effect on the responses of \( \rho 1 \) homomers [42]. We therefore examined the effect of cyclothiazide on GABACR-mediated currents in BCTs. Bath-application of cyclothiazide (300 \( \mu M \)), in the presence of bicuculline (50 \( \mu M \)), significantly reduced the amplitude of the holding current (n = 10), and also reduced the spontaneous fluctuations of this current (fig. 5A). Synaptic feedback currents evoked by brief BCT depolarization (to \(-10 mV\) for 5 ms) were initially potentiated during cyclothiazide wash-on, as observed previously [43], due to the activity of cyclothiazide as an inhibitor of AMPA receptor desensitization. However, the feedback currents were subsequently virtually eliminated (n = 8; fig. 5B), although it is likely that run-down of BCT exocytosis contributed to the feedback current reduction [26]. GABACR currents evoked by local application of GABA (100 \( \mu M \), 50–100 ms) were also significantly reduced by cyclothiazide, but not completely eliminated (n = 7; fig. 5C). GABA-evoked responses had a slower rate of decay in the presence of cyclothiazide than in control conditions (n = 7; fig. 5C).

Figure 2. The effect of DIDS on GABA\(_{\rho}\)R currents. A, Example experiment and mean data (n = 9) showing the biphasic effect of DIDS (500 \( \mu M \)) on the holding current in normal Ca\(^{2+}\) extracellular solution, with subsequent application of picrotoxin (200 \( \mu M \)). DIDS(1) was measured at the peak of the tonic current potentiation, DIDS(2) was measured following 15-30 mins of DIDS application, just prior to addition of picrotoxin. B, Example experiment and mean data (n = 11) showing a similar effect of DIDS (500 \( \mu M \)) in Ca\(^{2+}\)-free extracellular solution. C, The charge of GABA-evoked responses (100 \( \mu M \), 100 ms) against time and example responses for the experiment in A, with mean data (2.5 mM Ca\(^{2+}\); n = 7, Ca\(^{2+}\)-free n = 7) showing the effect of DIDS on the charge and the decay time-constant of GABA-evoked responses. D, Example responses and mean data showing the effects of DIDS (500 \( \mu M \); n = 5) and NO-711 (3 \( \mu M \); n = 6) on the charge of GABA\(_{\rho}\)R-mediated synaptic feedback responses evoked by brief BCT depolarization (to \(-10 mV\) for 5 ms).

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Figure 3. The effect of DIDS on GABA\(_{\rho}\)R currents. A, An example recording and mean data (n = 4) showing that application of DIDS (500 \( \mu M \)) in the presence of TPMPA (200 \( \mu M \)) but not bicuculline has no effect on the holding current but inhibits spontaneous GABA\(_{\rho}\)R-mediated IPSCs (sIPSCs). B, Example current traces from recordings with and without DIDS (500 \( \mu M \)) included in the intracellular solution, with average sIPSCs from a different recording with intracellular DIDS (500 \( \mu M \)), and mean sIPSC amplitude and frequency data in control recordings (n = 5) and recordings with intracellular DIDS (0.5–1 mM; n = 5). Control(1) and DIDS(1) were measured during the 2nd minute after gaining whole-cell access, DIDS(2) was measured during the 6th minute.

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To further investigate the remaining cyclothiazide-resistant tonic and GABA-evoked currents, NO-711 (3 μM) was applied in the continuing presence of cyclothiazide. NO-711 increased the holding current (n = 10), which was subsequently inhibited by application of picrotoxin (200–250 μM; fig. 5A). In addition, NO-711 significantly increased the charge and slowed the decay of responses evoked by exogenous GABA (n = 7; fig. 5C). These results support the view that the majority of BCT GABA<sub>C</sub>Rs are α<sub>1</sub>-β<sub>2</sub> heteromers, but provide evidence that a population of homomeric α<sub>1</sub> receptors contributes to the tonic current.

**Discussion**

The aim of the current experiments was to further our understanding of two unknown properties of the tonic GABA<sub>C</sub>R current in BCTs: the non-vesicular source of GABA for activating the current and the identity of the receptors mediating the current. Following recent reports of non-vesicular GABA release via Best1 anion channels [12], we tested the effects of anion channel inhibitors on the tonic GABA<sub>C</sub>R current. The results indicate that the GABA release mechanism is insensitive to NPPB and FFA but sensitive to DIDS. All three drugs inhibited to some extent the activity of GABA transporters, as evidenced by the potentiation of tonic, GABA-evoked and synaptic feedback currents mediated by GABA<sub>C</sub>Rs. In addition, NPPB and FFA exerted effects on intact BCs via inhibition of hemichannels, and DIDS was found to inhibit GABA<sub>A</sub>R-mediated sIPSCs. However, there appeared to be no direct inhibitory effect of NPPB, FFA or DIDS on GABA<sub>C</sub>Rs.

There is increasing evidence for the release of neurotransmitters, in particular glutamate and ATP, from astrocytes [44,45]. GABA is also known to be released from astrocytes in the hippocampus, cerebellum, thalamus and olfactory bulb, with consequent activation of neuronal GABA<sub>A</sub>Rs [12,46-48]. Astrocytes release ‘gliotransmitters’ via several mechanisms including Ca<sup>2+</sup>-dependent vesicular exocytosis, reversal of transporters, and release via hemichannels, ionotropic purinergic receptors and anion channels [49]. Various different types of anion channel have been implicated in gliotransmitter release including volume-regulated anion channels (VRACs) [15] and more recently Ca<sup>2+</sup>-activated anion channels such as Best1, which are present in hippocampal and cerebellar astrocytes, and which can mediate tonic GABA release [12,13].

Distinguishing pharmacologically between mechanisms of non-vesicular release and between different types of Cl<sup>-</sup> channel is challenging due to the cross-reactivity of commonly-used anion channel inhibitors with other release mechanisms, for example the block of hemichannels by NPPB [50], and due to the lack of selectivity of inhibitors between Cl<sup>-</sup> channel classes [51]. However, the insensitivity of the tonic GABA<sub>C</sub>R current to carbenoxolone, PPADS and Brilliant Blue G [11], and to NPPB and FFA indicates that hemichannels, P2X<sub>7</sub> receptors, VRACs and Best1 anion channels are not major contributors to the non-vesicular GABA release that activates this current. Reversal of GABA transporters also does not seem to be involved [11]. The non-vesicular release of GABA in the cerebellum that activates a tonic GABA<sub>C</sub>R current in granule cells was similarly found to be independent of GABA transporter reversal and VRACs, and to be potentiated rather than inhibited by NPPB [9].

**Figure 4. Picrotoxin-sensitivity of GABA<sub>C</sub>R currents.** A, Example GABA<sub>C</sub>R responses evoked by local application of L-glutamate (glu; 100 μM, 10 ms) to activate reciprocal amacrine cell synapses, and their inhibition by picrotoxin. Three individual responses (grey) and the mean response (black) are shown for each condition. B, An example experiment showing inhibition of the tonic GABA<sub>C</sub>R current by the same concentrations of picrotoxin, following potentiation of the current by NO-711 (3 μM). C, Example average GABA<sub>C</sub>R-mediated mIPSCs during baseline and following application of NO-711 (3 μM), with mean data for average mIPSC charge under these conditions (n = 4). GABA<sub>C</sub>R mIPSCs were recorded in Ca<sup>2+</sup>-free extracellular solution to facilitate their detection, in the presence of bicuculline. D, Dose-response curves for picrotoxin inhibition of glu-evoked (n = 4–6) and tonic (n = 3–6) GABA<sub>C</sub>R currents, fit with Hill equations to give IC<sub>50</sub> values. doi:10.1371/journal.pone.0024892.g004
The tonic GABACR current in BCTs was significantly inhibited by DIDS, but the identity of the DIDS-sensitive anion channel or exchanger that mediates tonic GABA release in the retina is not known. Interestingly, a similar NPPB-resistant but DIDS-sensitive mechanism underlies the tonic release of glutamate in the hippocampus [33]. One potential candidate is a type a large-conductance Cl⁻ channel (maxi-Cl⁻) that was identified in drosophila and has three mammalian homologs that are activated by either Ca²⁺ or cell swelling, which is sensitive to DIDS but resistant to niflumic acid [52]. In the current experiments, DIDS failed to completely block the tonic GABACR current in BCTs, even in Ca²⁺-free extracellular solution, suggesting that either DIDS at this concentration does not completely block the non-vesicular release mechanism, or it blocks only one of two or more contributing mechanisms. Alternatively, in the presence of DIDS the release of GABA may be blocked but, due to the additional action of DIDS as an inhibitor of GABA uptake, the ambient extracellular GABA concentration remains sufficient to evoke some tonic GABACR current.

The cellular source of GABA for activating the tonic GABACR current in BCTs is also unknown, but the most likely sources are amacrine cells and Müller cells. BCTs are surrounded by amacrine cells.
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recently been shown to be a selective inhibitor of responses, consistent with most BCT GABA CRs being Cyclothiazide reduced the amplitude of the tonic current, inhibited with a greater contribution of different (though probably overlapping) populations of GABACRs, currents in BCTs suggests that these currents may be mediated by BCTs by comparing the picrotoxin-sensitivity of glu-evoked and m

modulating synaptic and tonic forms of GABA CR-mediated release activating heteromeric GABA<sub>R</sub>-Rs contributes to the tonic current in BCTs [5]. Spontaneous GABA release occurs at a high rate at amacrine cell to BCT synapses in retinal slices, as evidenced by the high frequency of GABA<sub>R</sub>-mediated IPSCs observed in the absence of bicuculline [11] (fig. 3). In the presence of bicuculline, synaptic GABA release and the tonic GABA<sub>R</sub>-R current tend to be potentiated due to amacrine cell disinhibition [6].

However, a small constant tonic current remained in the presence of 300 μM cyclothiazide that was potentiated by inhibition of GABA uptake and is likely to be mediated by homomeric p1 receptors [42]. Small GABA-evoked currents were also observed in the presence of cyclothiazide that were potentiated by NO-711 and inhibited by picrotoxin. The slower decay rate of GABA-evoked currents in cyclothiazide compared with control conditions is consistent with reports of subunit-specific kinetics. For example, the deactivation rate of homomeric p1 receptors is slower than for p1-2 heteromers, with respective time-constants of 14 s and 9 s for human subunits, and 234 s and 75 s for perch subunits (B form) [22,23]. The change in decay kinetics also provides evidence against an incomplete block of heteromeric GABA<sub>R</sub>-Rs by cyclothiazide. The lack of desensitization of BCT GABA<sub>R</sub>-Rs [4] and the slow deactivation of p1 subunits are both likely to contribute to the very slow decay rate of GABA-evoked responses in the absence of GABA uptake (fig. 5). These properties, combined with a high affinity for GABA [21,22], make homomeric p1 receptors particularly suitable for mediating a tonic current in BCTs.

Given the lack of dependence on vesicular release of the tonic GABA<sub>R</sub>-R current [11], it is likely that the population of homomeric p1 receptors that contributes to this current is located extrasynaptically. An analogous situation is found in central neurons, where tonic GABA<sub>R</sub>-R currents are mediated by extrasynaptic receptors [16]. Fluorescence imaging of immunolabeled p subunits in BCTs has shown 'punctate' labeling in several species including goldfish, with labeling within the synaptic cleft at the electron microscope level [65-68], reflecting the synaptic localization of heteromeric receptors that mediate GABA<sub>R</sub>-R feedback currents and spontaneous IPSCs. However, it has been noted that rat BCTs also exhibit diffuse extrasynaptic p subunit labeling [68], which may correspond with a population of homomeric p1 receptors that contributes to the tonic current. Identification of the subcellular localization of GABA<sub>R</sub>-R subunits in BCTs, and mechanisms that target specific receptors to synaptic or extrasynaptic sites, requires further investigation. In addition, it will be interesting to determine whether synaptic and extrasynaptic GABA<sub>R</sub>-Rs are differentially regulated, and the relative importance of factors such as changes in receptor number or properties, or in the rates of GABA release and uptake, in modulating synaptic and tonic forms of GABA<sub>R</sub>-mediated inhibition in BCTs.

In summary, these experiments indicate that tonic GABA<sub>R</sub>-R currents in BCTs are activated by GABA released, in part, via a DIDS-sensitive mechanism, and that homomeric p1 receptors contribute to this current. Tonic inhibition regulates the ability of BCTs to fire Ca<sup>2+</sup>-dependent action potentials [4], and is likely to modulate the transmission of light responses to ganglion cells. However, how this form of inhibition interacts with synaptic GABA<sub>R</sub>-R and GABA<sub>R</sub>-R-mediated inhibition, and with the multiple additional forms of synaptic feedback that exist in BCTs, in the processing of visual information in the retina remains to be determined.

Author Contributions
Conceived and designed the experiments: SMJ MJJP. Performed the experiments: SMJ MJJP. Analyzed the data: SMJ MJJP. Wrote the paper: MJJP.
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