Title: Different types of retinal inhibition have distinct neurotransmitter release properties

Running Title: Ca\textsuperscript{2+}-buffering differentially affects amacrine cell release

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Keywords: amacrine cell, bipolar cell, release
Abstract

Neurotransmitter release varies between neurons due to differences in presynaptic mechanisms such as Ca\textsuperscript{2+}-sensitivity and timing. Retinal rod bipolar cells respond to brief dim illumination with prolonged glutamate release that is tuned by the differential release of GABA and glycine from amacrine cells in the inner retina. To test if differences among types of GABA and glycine release are due to inherent amacrine cell release properties, we directly activated amacrine cell neurotransmitter release by electrical stimulation. We found that the timing of electrically evoked inhibitory currents was inherently slow and that the timecourse of inhibition from slowest to fastest was GABA\textsubscript{C} receptors > glycine receptors > GABA\textsubscript{A} receptors. Deconvolution analysis showed that the distinct timing was due to differences in prolonged GABA and glycine release from amacrine cells. The timecourses of slow glycine release and GABA release onto GABA\textsubscript{C} receptors were reduced by Ca\textsuperscript{2+}-buffering with EGTA-AM and BAPTA-AM, but faster GABA release onto GABA\textsubscript{A} receptors was not, suggesting that release onto GABA\textsubscript{A} receptors is tightly coupled to Ca\textsuperscript{2+}. The differential timing of GABA release was detected from spiking amacrine cells and not non-spiking A17 amacrine cells that form a reciprocal synapse with rod bipolar cells. Our results indicate that release from amacrine cells is inherently asynchronous and that the source of non-reciprocal rod bipolar cell inhibition differs between GABA receptors. The slow, differential timecourse of inhibition may be a mechanism to match the prolonged rod bipolar cell glutamate release and provide a way to temporally tune information across retinal pathways.
Abbreviations

BA, BAPTA-AM

EG, EGTA-AM

eIPSC, electrically-evoked inhibitory postsynaptic current

L-IPSC, light-evoked inhibitory postsynaptic current

IPSC, inhibitory postsynaptic current

R, receptor

TPMPA, 1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid hydrate

TTX, tetrodotoxin
Introduction

Neural systems rely on a proper balance between the timing of excitation and inhibition to function optimally (Buzsaki et al. 2007; Mann and Mody 2010; Scholl and Wehr 2008). In the retina, the excitatory pathway consists of light detection by photoreceptors that signal to bipolar cells and ganglion cells using glutamate (Masland 2001). The output of the excitatory pathway is modulated by inhibitory input from amacrine cells onto bipolar cells and ganglion cells in the inner retina (Masland 2001). GABAergic and glycinergic amacrine cells shorten the timecourse of bipolar cell glutamate release, which is essential to retinal temporal tuning (Dong and Werblin 1998). Bipolar cells use graded membrane depolarization to evoke sustained glutamate release (Heidelberger and Matthews 1992), therefore the timing of amacrine cell-mediated inhibition must be prolonged to contribute to shaping bipolar cell output.

Amacrine cells provide inhibition to bipolar cell axon terminals by releasing GABA or glycine onto GABA_A, GABA_C or glycine receptors (R) (Eggers and Lukasiewicz 2006a; b; Euler and Masland 2000). The timing of light-evoked inhibitory postsynaptic currents (L-IPSCs) in bipolar cells is prolonged relative to an initial light stimulus (Eggers and Lukasiewicz 2006b). The timecourse of inhibition has been shown to vary due to the kinetics of each receptor type (Chang and Weiss 1999; Eggers and Lukasiewicz 2006b; Frech and Backus 2004), and due to prolonged amacrine cell neurotransmitter release (Eggers and Lukasiewicz 2006b; Gleason et al. 1993). However, it is unclear whether prolonged release from amacrine cells is a result of the time it takes to process the light signal upstream of amacrine cells at the photoreceptor-bipolar cell or bipolar cell-amacrine cell synapses or if it is due to an inherent mechanism of amacrine cell function.

Unlike the graded membrane depolarization used by bipolar cells, many amacrine cell
types rely on action potentials to mediate transmitter release (Bloomfield 1992; Eggers et al. 2013; Shields and Lukasiewicz 2003; Taylor 1999). Spike-dependent neurotransmitter release is a fast, synchronous event that generally involves the rapid increase of intracellular Ca\(^{2+}\) near release sites and subsequent neurotransmitter release to occur within 10-20 ms (Kaeser and Regehr 2013). However, previous studies have established that release from amacrine cells can occur asynchronously over several hundred milliseconds after a single stimulus (Borges et al. 1995; Eggers et al. 2013; Eggers and Lukasiewicz 2006b; Gleason et al. 1994). In contrast to the fast, local rises in intracellular Ca\(^{2+}\) that mediate synchronous release, slow asynchronous release relies on global increases in intracellular Ca\(^{2+}\) far from the release site (Chung and Raingo 2013; Goda and Stevens 1994; Kaeser and Regehr 2013; Sakaba and Neher 2001; Scheuss et al. 2007). Amacrine cells may need to utilize asynchronous release in response to an action potential as a mechanism to match the timing of sustained bipolar cell glutamate release.

We have previously shown that asynchronous release after a single stimulus is a primary mechanism used by a population of spiking amacrine cells to release GABA onto rod bipolar cell GABA\(_C\)Rs (Eggers et al. 2013). However, although rod bipolar cell L-IPSCs, mediated by GABA\(_C\)Rs, GABA\(_A\)Rs and glycineRs are all prolonged relative to a brief light stimulus, the release kinetics of GABA and glycine underlying these light responses vary (Eggers and Lukasiewicz 2006b) and it is not known whether all amacrine cells use asynchronous release. Additionally, rod bipolar cells receive two types of inhibition: lateral inhibition from GABAergic and glycinergic amacrine cells that often have action potentials and reciprocal inhibition where rod bipolar cells activate a GABAergic amacrine cell called the A17 (Chavez et al. 2010; Chavez et al. 2006; Eggers and Lukasiewicz 2010; Grimes et al. 2009) that is non-spiking and uses Ca\(^{2+}\) entry through Ca\(^{2+}\)-permeable AMPA receptors to activate GABA release back onto the rod
bipolar cell (Chavez et al. 2006). Here, we ask if asynchronous release is the release mechanism inherent to all amacrine cells giving input to rod bipolar cell axon terminals by using an electrical stimulus to stimulate isolated input from many amacrine cell types and by electrically activating reciprocal feedback inhibition. In the present study, we show that the distinct timing of rod bipolar cell GABA_cR, glycineR and GABA_aR inhibitory currents is mediated by the distinct timing of release from amacrine cells independent of photoreceptor or bipolar cell signaling. We present evidence that asynchronous release due to prolonged calcium signaling is a primary mechanism of release from amacrine cells that synapse onto GABA_cRs and glycineRs on rod bipolar cells.

Materials and Methods

Ethical approval

Animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Retinal slice preparation

As previously described (Eggers and Lukasiewicz, 2006), C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME, USA) 35-60 days of age were euthanized using carbon dioxide. The eyes were enucleated, the cornea and lens removed, and the eyecup was incubated in cold extracellular solution (see Solutions and drugs) with 800 units/mL of hyaluronidase for 20 minutes. The eyecup was washed with cold extracellular solution and the retina was removed. The retina was trimmed into an approximate rectangle and mounted onto 0.45 µm nitrocellulose
filter paper (Millipore Billerica, MA, USA). The filter paper containing the retina was transferred to a hand chopper. The filter paper was sliced into 250 µm thick slices, rotated 90º, and mounted onto glass cover slips using vacuum grease.

Solutions and drugs

Extracellular solution used as a control bath for dissection and whole cell recordings was bubbled with a mixture of 95%/5% O₂/CO₂ and contained (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 20 Glucose, 26 NaHCO₃, 2 CaCl₂. The intracellular solution in the recording pipette used for monitoring electrically stimulated inhibitory rod bipolar cell currents contained (in mM): 120 CsOH, 120 Gluconic Acid, 1 MgCl₂, 10 HEPES, 10 EGTA, 10 TEA-Cl, 10 phosphocreatine-Na₂, 4 Mg-ATP, 0.5 Na-GTP, 50 µM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. The intracellular solution used for monitoring electrically stimulated amacrine cell depolarization contained (in mM): 120 KOH, 120 Gluconic Acid, 1 MgCl₂, 10 HEPES, 10 EGTA, 10 TEA-Cl, 10 phosphocreatine-Na₂, 4 Mg-ATP, 0.5 Na-GTP, 50 µM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. The intracellular solution used for monitoring reciprocal feedback inhibitory currents contained (in mM): 120 CsCl, 1 MgCl₂, 10 HEPES, 0.1 EGTA, TEA-Cl, 10 phosphocreatine-Na₂, 4 Mg-ATP, 0.5 Na-GTP, 50 µM Alexa Fluor 488 and was adjusted to pH 7.2 with CsOH. With these concentrations the driving force for Cl⁻ was calculated as 60 mV in all solutions.

Antagonists were used to isolate receptor input. 500 nM-1 µM strychnine was used to block glycineRs, 20 µM SR95531 was used to block GABAₐRs and 50 µM TPMPA ((1,2,5,6-Tetrahydropyrindin-4-yl)methylphosphinic acid hydrate) was used to block GABAₐRs.
Tetrodotoxin (TTX, 500 nM, Alomone Labs, Jerusalem, Israel) was used to block voltage-gated Na\(^+\) channels. EGTA-AM (50 \(\mu\)M, Invitrogen, Carlsbad, California, USA) and BAPTA-AM (50 \(\mu\)M, Invitrogen, Carlsbad, California, USA) were used to increase intracellular Ca\(^{2+}\) buffering. EGTA-AM and BAPTA-AM were applied to the bath for 5 min prior to the experiment and was present during the recordings for a total of 10 min. All antagonists were applied to the slice by a gravity-driven superfusion system (Cell Microcontrols, Norfolk, VA) at a rate of \(~1\)mL/minute. Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), unless otherwise indicated.

**Whole-cell recordings**

All recordings were performed in light adapted conditions. Retinal slices on glass cover slips were placed in a custom chamber and heated to 32° by temperature controlled thin stage and inline heaters (Cell Microcontrols, Norfolk, VA). Whole-cell voltage clamp recordings from rod bipolar cells in retinal slices were made as previously described (Eggers and Lukasiewicz 2006b). Electrically evoked inhibitory postsynaptic currents (eIPSCs) were recorded from rod bipolar cells clamped at 0 mV, the reversal potential for currents mediated by non-selective cation channels. Electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, Florida, USA) using a P97 Flaming/Brown puller (Sutter Instruments, Novato, California, USA) and had resistances of 5-7 M\(\Omega\). Liquid junction potentials of 20 mV were corrected for prior to recording.

For electrically evoked rod bipolar cell current responses, rod bipolar cells with intact axon terminals were identified morphologically by exciting the dye in the intracellular solution using a fluorescence microscope. A 1 ms, 4-20 \(\mu\)A stimulus was delivered through a stimulating pipette placed near rod bipolar cell axon terminals by an S48 stimulator (Grass, Warwick Rhode Island, USA).
Island, USA) with attached PSIU6 photoelectric isolation unit (Grass, Warwick Rhode Island, USA). The stimulus was delivered every 60 seconds. Electrically evoked amacrine cell voltage responses were recorded in current clamp mode in response to the same electrical stimulus from a stimulating electrode located near a fluorescently identified amacrine cell process in the distal IPL, where rod bipolar cell terminals are located. Electrically evoked responses were filtered at 5 kHz using a 4-pole low-pass Bessel filter on an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California, USA). The response was digitized at 10 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, California, USA) and Clampex software (Molecular Devices, Sunnyvale, California, USA). Reciprocal IPSCs were elicited every 45 to 60 sec by a 10 ms step depolarization from a holding potential of -60 mV to -20 mV. Confirmation of rod bipolar cell (Ghosh et al. 2004) or amacrine cell morphology was done at the end of each recording using an Intensilight fluorescence lamp and Digitalsight camera controlled by Elements software (Nikon Instruments, Tokyo, Japan).

Data analysis and Statistics

Traces of evoked and reciprocal IPSCs and evoked EPSPs for each condition were averaged using Clampfit (Molecular Devices, Sunnyvale, California, USA). The peak amplitude, charge transfer (Q), time to peak and decay to 37% of the peak (D37) were determined. The timecourse and amount of transmitter release mediating evoked and reciprocal IPSCs were calculated with custom written Matlab software. Release functions were calculated by convolution analysis (Diamond and Jahr 1995) using the relationship:

\[ eIPSC(t) = release(t) \otimes sIPSC(t) \]

such that
\( \text{release}(t) = F^{-1} \left[ \frac{F[eIPSC(t)]}{F[sIPSC(t)]} \right] \)

where \( sIPSC(t) \) is the average spontaneous GABA\(_C\)R, GABA\(_A\)R or glycineR-mediated IPSC and \( F \) and \( F^{-1} \) represent the Fourier transform and inverse Fourier transform of the function, respectively. Briefly, software data processing occurred as follows: data were down sampled from 10 KHz to 1 KHz and smoothed using a moving average filter (20 points). The software calculated vesicle release from current-time curves by performing deconvolution of the average rod bipolar cell GABA\(_A\)R, GABA\(_C\)R, or glycineR-mediated current evoked by a single vesicle release (sIPSC(t)), (Eggers and Lukasiewicz 2006b). The deconvolution was performed by dividing the fast Fourier transform (FFT) of the single vesicle release into the FFT of the electrically evoked response (eIPSC(t)) and again smoothed similar to the initial current trace. The resulting release traces were further analyzed in Clampfit to determine the number of vesicles released and the D37. The area under the curve was used to determine the amount of vesicle release. Release occurred in early and late phases. The early phase of release was determined to be the first 100 ms after the initial stimulus because it included the peak for release onto all receptor inputs. The late phase was determined to be the remaining release that occurred from 100 ms until the response returned to baseline.

Paired Student’s t-tests were used to compare values between conditions for the same cell. For each cell, a normalized data value of the % of total GABA\(_A\)R, GABA\(_C\)R or glycineR-mediated eIPSC that remained after the drug treatment was calculated. Non-paired Student’s t-tests were used to compare these values across 2 groups of cells. For comparisons of 3 or more groups of cells, these values were compared across drug conditions with ANOVA tests, using the SNK method for pair-wise comparisons. Differences were considered significant when \( p \leq 0.05 \). All data are reported as mean ± standard error of the mean (SEM).
Results

Release from GABAergic and glycinergic amacrine cells occurs with prolonged but distinct timing

Light-evoked GABA and glycine release have been shown to occur with prolonged, but distinct, timing and to mediate the sustained timecourse of GABAergic and glycinergic L-IPSCs (Eggers and Lukasiewicz 2006b). Previous work has shown that some amacrine cells use asynchronous release as a primary mechanism to release GABA onto GABA$_C$Rs (Eggers et al. 2013). It is not known whether GABAergic amacrine cells also use asynchronous release to release GABA onto GABA$_A$Rs or if glycinergic amacrine cells use asynchronous release. To determine whether prolonged GABA and glycine release is an inherent characteristic of amacrine cells, we isolated the amacrine cell inputs to rod bipolar cells using an electrical stimulus at the amacrine cell-rod bipolar cell synapse in the inner plexiform layer in the presence of antagonists to isolate GABA$_A$R, GABA$_C$R or glycineR mediated inputs (see Methods). We previously determined that this electrical stimulus activates isolated amacrine cell input to rod bipolar cells because the response was not reduced by blocking glutamate receptors with CNQX (Eggers et al. 2013). This electrical stimulus evoked a brief depolarization ($D37 = 1 \pm 0.1$ ms, $n = 6$) in recorded amacrine cells (see methods), suggesting that any prolonged signals are due to inherent release properties of the amacrine cell and not prolonged depolarization.

Electrically evoked IPSCs (eIPSCs) mediated by GABA$_A$Rs and glycineRs had a prolonged response that lasted much longer than the 1 ms stimulus (Figure 1A), similar to eIPSCs of GABA$_C$Rs (Eggers et al. 2013). However, there were significant timing differences between the three inputs. GABA$_C$R eIPSCs had the slowest decay time ($D37$) followed by
glycineR eIPSCs and GABA_A eIPSCs (p < 0.001 ANOVA, SNK post-hoc p < 0.05; Table 1, Figure 1B). The D37 times reported here follow the same hierarchy from the slowest time to fastest time as L-IPSCs measured in a previous study: GABA_CRs > glycineRs > GABA_ARs (Eggers and Lukasiewicz 2006b). To determine if differences in the eIPSCs were due to variability in stimulus electrode placement, we measured eIPSCs of GABAR and glycineR inputs from the same rod bipolar cell while sequentially applying GABA_C-R, GABA_A-R and glycineR antagonists. We found that the D37 times followed the same hierarchy as isolated receptor inputs compiled from multiple rod bipolar cells with GABA_C-R eIPSCs having the slowest D37 and GABA_A-R eIPSCs having the fastest D37 (GABA_C-R: 2365 ± 208 ms > glycineRs: 1307 ± 235 ms > GABA_A-Rs: 258 ± 97 ms; n = 3, p < 0.001 ANOVA, SNK post-hoc p < 0.01). This suggests that although the electrical stimulus evokes long-lasting IPSCs, it does not affect the relative timing of GABA_C-R, GABA_A-R and glycineR inputs.

The decays of spontaneous IPSCs mediated by GABA_A-Rs (2.0 ± 0.5 ms), GABA_C-Rs (34.1 ± 2.1 ms) and glycineRs (3.6 ± 0.5 ms) (Eggers and Lukasiewicz 2006b) that measure individual receptor kinetics are also different and share a similar relationship to the eIPSC and L-IPSC kinetics. However, the sIPSC decays are much briefer than the eIPSC D37s, in contrast to other synapses where the decay times of spontaneous and electrically evoked currents are similar (Lu and Trussell 2000). This suggests that, similar to GABA release onto GABA_C-Rs, prolonged GABA and glycine release likely contributes to the prolonged timecourse of GABA_A-R and glycineR eIPSCs.

To test this, we used deconvolution analysis (Diamond and Jahr 1995; Eggers et al. 2013; Eggers and Lukasiewicz 2006b) to estimate the timecourse of GABA and glycine release that underlies the eIPSCs. This analysis assumes that vesicles released are linearly summed by
postsynaptic receptors, which may not be the case. This analysis then represents the minimum
release required to create the eIPSCs measured. We found that the timecourse of GABA and
glycine release was prolonged relative to the stimulus (Figure 1C). We expected the timing of
amacrine cell transmitter release to follow the same hierarchy as that observed for the eIPSC
D37. Surprisingly, the D37 of GABA release onto GABA<sub>C</sub>Rs and glycine release were similar (p
= 0.9; Table 1; Figure 1D), despite the difference in GABA<sub>C</sub>R vs. glycine<sub>R</sub> eIPSCs. This is
likely because the slow kinetics of the GABA<sub>C</sub>Rs prolong the timecourse of the eIPSC in
response to the same release timecourse (Eggers et al. 2013; Eggers and Lukasiewicz 2006a).
The D37 of GABA release onto GABA<sub>A</sub>Rs was significantly faster than that of GABA<sub>C</sub>Rs and
glycine<sub>Rs</sub> (p < 0.05 ANOVA, SNK post hoc p < 0.05 Table 1; Figure 1D) so the hierarchy of the
timecourse of transmitter release was GABA<sub>C</sub>Rs = glycine<sub>Rs</sub> > GABA<sub>A</sub>Rs. This suggests that
GABA release onto GABA<sub>A</sub>Rs may originate from a different population of amacrine cells than
GABA release onto GABA<sub>C</sub>Rs. This also suggests that GABAergic amacrine cells that release
onto GABA<sub>C</sub>Rs and glycinergic amacrine cells may share similar mechanisms that determine the
timecourse of release. Although the timecourse of release onto GABA<sub>A</sub>Rs was faster than that
onto GABA<sub>C</sub>Rs or glycine<sub>Rs</sub>, all were consistent with the timing of asynchronous release that
occurs over several hundred milliseconds (Chung and Raingo 2013; Goda and Stevens 1994;
Kaeser and Regehr 2013; Sakaba and Neher 2001; Scheuss et al. 2007).

*Slow Ca<sup>2+</sup> buffering does not decrease GABA release onto GABA<sub>A</sub>Rs*

These data suggest that transmitter release from amacrine cells that mediates light-evoked
(Eggers and Lukasiewicz 2006a) and electrically evoked IPSCs (Figure 1) may be asynchronous
release. Similar to synchronous release, asynchronous release is regulated by changes in
intracellular Ca$^{2+}$ concentrations. However, asynchronous release often occurs in response to
tetanic stimulation that leads to a global accumulation of intracellular Ca$^{2+}$ (Chung and Raingo
2013; Kaeser and Regehr 2013; Sakaba and Neher 2001; Scheuss et al. 2007). Asynchronous
release is susceptible to Ca$^{2+}$ buffering by the slow Ca$^{2+}$ chelator EGTA because of its
dependence on global changes in intracellular Ca$^{2+}$. EGTA primarily affects asynchronous
release while sparing fast, synchronous release that occurs rapidly and in response to an increase
in Ca$^{2+}$ near Ca$^{2+}$ channels and release sites (Augustine et al. 2003; Chung and Raingo 2013;
Hefft and Jonas 2005; Kaeser and Regehr 2013; Neher 1998; Rozov et al. 2001; Sakaba and
Neher 2001).

The timecourse of amacrine cell GABA release onto GABA$\text{A}$Rs (Figure 1) is slow,
lasting for hundreds of milliseconds which is characteristic of asynchronous release. To test if
amacrine cell release onto GABA$\text{A}$Rs occurs asynchronously we used EGTA-AM, the membrane
permeant analogue of the chelator, to limit asynchronous release. We added low concentrations
(50 µM) of EGTA-AM to the bath and 10 mM EGTA was in the recording pipette so that EGTA
affected only the presynaptic amacrine cell terminal. If prolonged GABA release onto
GABA$\text{A}$Rs is occurring asynchronously, we would expect EGTA-AM to reduce the GABA$\text{A}$R-
mediated input by reducing GABA release and shortening the decay time.

As shown in Figure 2A, EGTA-AM did not reduce GABA$\text{A}$R-mediated eIPSCs. There
was no significant change in the Q, D37 or peak (Table 2, Figure 2C-E), although on average the
Q and D37 of GABA$\text{A}$R eIPSCs were increased compared to control. Although the underlying
GABA release onto GABA$\text{A}$Rs occurred with prolonged timing (125 ± 33 ms) that is
characteristic of asynchronous release (Figure 2F), EGTA-AM did not reduce the timing, peak or
the amount (Table 3, Figure 2H-J) of release. In comparison, EGTA-AM reduced the Q, peak
amplitude and D37 (Table 2, Figure 2C-E) of GABA<sub>C</sub>-R eIPSCs (Figure 2B) by shortening the
timecourse and limiting the amount of GABA release (Table 3, Figure 2G-J) (Eggers et al. 2013).

If GABA is released from the same population of amacrine cells, then GABA release
from GABAergic amacrine cells should occur by a similar mechanism. However, our data
indicate that GABA release onto rod bipolar cell GABA<sub>A</sub>Rs may occur by a mechanism other
than the asynchronous release, dependent on prolonged Ca<sup>2+</sup> signals, that is observed for
GABA<sub>C</sub>Rs (Eggers et al. 2013). Due to the faster timecourse of the GABA release that underlies
the GABA<sub>A</sub>R-mediated eIPSCs, it is possible that synchronous release of GABA onto
GABA<sub>A</sub>Rs accounts for majority of the release. Because EGTA-AM is a calcium chelator with
slow kinetics (Augustine et al. 1991; Tymianski et al. 1994) it cannot act quickly enough to limit
synchronous release. Therefore, we tested if GABA release onto GABA<sub>A</sub>Rs could be reduced by
BAPTA-AM a membrane permeant analogue calcium chelator with fast kinetics (Tymianski et
al. 1994). BAPTA-AM did not reduce the Q, D37 or peak (Table 2, Figure 2C-E) of GABA<sub>A</sub>R-mediated
eIPSCs, although on average the value was increased but not significantly different.
The amount, timing and peak (Table 3, Figure 2H-J) of GABA release onto GABA<sub>A</sub>Rs were also
not reduced in the presence of BAPTA-AM, in contrast with the effects on release onto
GABA<sub>C</sub>Rs. There was no significant difference between the effect of EGTA-AM or BAPTA-
AM on release onto GABA<sub>A</sub>Rs (Current Q p = 0.5, D37 p = 1, pk p = 1; Release Q p = 0.7, D37
p = 0.8, pk p = 0.4). GABA release onto GABA<sub>A</sub>Rs is prolonged and the timing is characteristic
of asynchronous release, but it is not susceptible to Ca<sup>2+</sup> buffering by the fast Ca<sup>2+</sup> chelator
BAPTA-AM or the slow Ca<sup>2+</sup> chelator EGTA-AM suggesting that release is tightly coupled to
Ca<sup>2+</sup> (Augustine et al. 2003). As we have isolated the amacrine cell-rod bipolar cell synapse
using a direct electrical stimulus, the differences in the effects of Ca\(^{2+}\) buffering on GABA release onto GABA\(_A\)Rs and GABA\(_C\)Rs is not a result of upstream signaling from photoreceptors or bipolar cells. Thus, amacrine cell GABA release onto GABA\(_A\)Rs and GABA\(_C\)Rs occurs with distinct timing that may reflect GABA release from two different populations of amacrine cells.

**Slow Ca\(^{2+}\) buffering reduces glycine release from amacrine cells**

Our results indicate that the timecourse of evoked GABA release onto GABA\(_C\)Rs and glycine release is similar (Figure 1D). Release of glycine from amacrine cells in response to glutamate puffs in the inner plexiform layer was shown to be enhanced by Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (Chavez and Diamond 2008) that mediates asynchronous GABA release (Eggers *et al.*, 2013), suggesting that glycine release may be mediated by slow Ca\(^{2+}\) signaling as well. We tested whether glycine release from amacrine cells could be reduced by slow Ca\(^{2+}\) buffering by recording glycine-IPSCs in the presence of EGTA-AM. We found that EGTA-AM reduced the eIPSC Q, D37 and peak amplitude (Table 2, Figure 3A-D). Deconvolution analysis showed that EGTA-AM also reduced the timing and amount of vesicle release (Table 3, Figure 3E-H).

These results show that glycine release from amacrine cells onto rod bipolar cell terminals occurs asynchronously.

We tested whether any additional glycine release from amacrine cells could be reduced by fast Ca\(^{2+}\) buffering with BAPTA-AM. BAPTA-AM reduced the glycine-IPSC Q, D37 and peak by similar amounts as EGTA-AM with no further decrease (EG vs. BA Q p = 0.3; D37 p = 0.7; pk = 0.4, Figure 3B-D). Consistent with these results, deconvolution analysis showed that BAPTA-AM also reduced the total, peak and timing of vesicle release (Figure 3F-H). Interestingly, BAPTA-AM, but not EGTA-AM reduced the peak value (Figure 3H) of
transmitter release. These results indicate that much of amacrine cell glycine release onto rod bipolar cell terminals is asynchronous with some synchronous release that can be reduced by fast Ca\(^{2+}\) buffering. This is similar to the results of GABA release onto GABA\(_C\)Rs. It is likely that amacrine cell glycine release and GABA release onto GABA\(_C\)Rs share similar mechanisms, and although GABA release onto GABA\(_A\)Rs still occurs more slowly than you would expect for purely synchronous release, it may require higher intracellular Ca\(^{2+}\) or those neurons have higher Ca\(^{2+}\) buffering capacities that our addition of EGTA-AM or BAPTA-AM did not disturb.

GABA release onto GABA\(_A\)Rs occurs in two phases

Deconvolution analysis suggested that GABA release onto GABA\(_A\)Rs occurred in two phases— an early fast phase represented by a transient spike of release occurring immediately after the stimulus followed by a later slow, sustained phase of vesicle release (Figure 2F). A fast and slow phase of GABA release onto GABA\(_A\)Rs was found in previous studies of depolarization-induced transmission between cultured chick retinal amacrine cells (Borges et al. 1995). We determined the number of vesicles that were released in each phase relative to total vesicle release. We defined the fast phase as release that occurred 100 ms after the stimulus because this timing included the peak of release onto each receptor input. The slow phase was defined as the amount of vesicle release remaining between 100 ms and the return of the response to baseline (see Methods). We found that on average 55 ± 12 vesicles (~33% of all vesicles) were released onto GABA\(_A\)Rs during the fast phase (Figure 4A). We compared the number of vesicles released onto GABA\(_A\)Rs during both phases to that released onto GABA\(_C\)Rs and glycineRs during the early (first 100 ms) and late (100 ms – baseline return) time periods. During the early time period of release, amacrine cells released significantly fewer vesicles onto
GABA\textsubscript{C}Rs (7 ± 1\%, ANOVA p<0.001, SNK post-hoc p < 0.001) and glycineRs (9 ± 2\%, p < 0.001) when compared to release onto GABA\textsubscript{A}Rs (Figure 4A). Most of the vesicle release onto GABA\textsubscript{C}Rs and glycineRs occurred during the later time period (Figure 4B). Although most of the GABA release onto GABA\textsubscript{A}Rs occurred during the fast phase, a large proportion of the release also occurred during the slow phase. At conventional synapses, much of synchronous release occurs within 10 to 20 ms. In our experiments, only 0.19\% of total vesicle release onto GABA\textsubscript{A}Rs, 0.07\% of glycine release and 3.3\% of release onto GABA\textsubscript{C}Rs (Eggers et al. 2013) occurred within 10 ms of the stimulus. These results support the hypothesis that amacrine cells predominantly use asynchronous release to release onto glycineRs and GABA\textsubscript{C}Rs, and may rely on a distinct mode of release onto GABA\textsubscript{A}Rs.

Electrical stimuli preferentially activate spiking GABAergic amacrine cells

Bipolar cells receive inhibitory input from slowly depolarizing (non-spiking) and spiking amacrine cells (Bloomfield 1992; Hartveit 1999; Ivanova et al. 2006; Taylor 1999). Some of the inhibitory input arises from a reciprocal rod bipolar cell-A17 amacrine cell circuit, where non-spiking A17 amacrine cells require direct activation by rod bipolar cell glutamate release (Chavez et al. 2006; Grimes et al. 2009; Hartveit 1999; Menger and Wässle 2000; Nelson and Kolb 1985; Singer and Diamond 2003). The remaining rod bipolar cell inhibition is mediated by non-reciprocal inhibition mediated by amacrine cells activated by cone bipolar cells (Chavez and Diamond 2008; Cui et al. 2003; Ivanova et al. 2006). It is likely that an electrical stimulus will activate multiple amacrine cell inputs. Previous studies have shown that non-reciprocal inhibition is due in part to spiking amacrine cells (Chavez and Diamond 2008; Chavez et al. 2010; Cui et al. 2003; Lukasiewicz and Shields 1998) and that using an electrical stimulus in the OPL to
activate bipolar cell inhibition predominantly recruits spiking amacrine cells (Shields and Lukasiewicz 2003). It is possible that asynchronous release may be used primarily by spiking amacrine cells, since it could be a way to match timing with the graded activation of bipolar cells.

We previously showed that the asynchronous release onto GABA$\text{C}_\text{R}$s that underlies GABA$\text{C}_\text{R}$ eIPSCs was due to the activation of spiking amacrine cells, as GABA$\text{C}_\text{R}$ eIPSCs were blocked by 500 nM TTX (Eggers et al. 2013). Here, we have extended these studies to test if eIPSCs mediated by GABA$\text{A}_\text{R}$s and glycineRs were also primarily due to input from spiking amacrine cells. We found that blocking action potentials with 500 nM TTX eliminated GABA$\text{A}_\text{R}$ eIPSCs similar to the significant decrease seen with TTX on GABA$\text{C}_\text{R}$ eIPSCs (Figure 5A,B) (Eggers et al. 2013). These results suggest that our electrical stimulus is primarily activating non-reciprocal connections between populations of spiking GABAergic amacrine cells and rod bipolar cells. However, blocking action potentials did not decrease glycineR eIPSCs (Figure 5C). On average TTX actually increased the glycineR eIPSC Q, potentially due to actions on inputs to glycinergic amacrine cells (Chavez and Diamond 2008), although the difference was not significant (p = 0.2). There was also no significant decrease in the peak (120 ± 40% of control, p = 0.5) or D37 (150 ± 40% of control, p = 0.2). This suggests that non-spiking glycinergic amacrine cells are activated by our electrical stimulus, and that both spiking and non-spiking amacrine cells demonstrate asynchronous release.

Timing of GABA release onto GABA$\text{A}_\text{R}$s and GABA$\text{C}_\text{R}$s at the rod bipolar cell-A17 reciprocal synapse

Our data suggest that the distinct timecourse of GABA release onto GABA$\text{A}_\text{R}$s and
GABACRs comes from different amacrine cells. To test this idea, we examined release at the reciprocal rod bipolar cell circuit, where both GABAR types are activated by one type of presynaptic amacrine cell called the A17 (Chavez et al. 2010; Chavez et al. 2006; Eggers and Lukasiewicz 2010; Grimes et al. 2009) that relies on Ca\(^{2+}\) entry through Ca\(^{2+}\)-permeable AMPA receptors to activate GABA release (Chavez et al. 2006). This reciprocal feedback inhibition will not be activated by our electrical stimulus as we show in Figure 5 that TTX blocks electrically stimulated GABAergic inhibition to rod bipolar cells and previous reports showed that the A17 amacrine cell is non-spiking (Chavez et al. 2006; Grimes et al. 2009; Hartveit 1999; Menger and Wässle 2000; Nelson and Kolb 1985; Singer and Diamond 2003) and that the A17 feedback inhibition is not affected by TTX (Chavez et al. 2006). Therefore, we asked if the distinct timing of GABA release also occurs at this reciprocal synapse where both receptor types are activated by the same presynaptic amacrine cell.

We used an electrode containing a high Cl\(^-\) intracellular solution (see Methods) to deliver a brief (10 ms) depolarizing step from -60 mV to -20 mV to a rod bipolar cell to activate feedback inhibitory currents (fIPSCs). This caused an initial transient inward Ca\(^{2+}\) current (Hartveit 1999) followed by a more sustained inward inhibitory current that lasted several hundred milliseconds after the initial 10 ms step (Figure 6A). The timing of fIPSCs was prolonged and had an average D37 of 132 ± 28 ms (n = 5) which is considerably faster than that detected in response to an electrical (Figure 1, (Eggers et al. 2013)) or light stimulus (Eggers and Lukasiewicz 2006b). When TPMPA was added to the bath to block GABACR input, the remaining GABAAR fIPSC (Figure 6A, light grey trace) was more transient and the D37 on average was 32 ± 13 ms (n = 5, Figure 6C). GABAAR input (Figure 6A, black trace) was determined by subtracting the GABAAR fIPSC from the total response. The D37 of the
GABACR fIPSC was 191 ± 58 ms (n = 5) and significantly longer than that of the GABAAR fIPSC (p < 0.05, Figure 6C). Deconvolution analysis (Figure 6B) indicated that GABA release underlying the fIPSCs occurred much faster than that induced by a light stimulus (Eggers and Lukasiewicz 2006b) or the summed inputs activated by an electrical stimulus (Figure 1). This is likely due to the rapid activation of Ca\(^{2+}\)-permeable AMPA receptors on the A17 amacrine cells by depolarization of the rod bipolar cell (Chavez et al. 2006). Although the timing of the GABAR currents was different, the timing of the underlying GABA release was not significantly different. On average release onto GABACRs was slightly faster than release onto GABAARs (p = 0.07, Figure 6D), in contrast with our results with an electrical stimulus (Figure 2) where release onto GABACRs is considerably slower. This shows that the slower timecourse of GABACR fIPSCs was due to slow GABACR kinetics, and not slow GABA release. These results suggest that at the rod bipolar cell-A17 synapse GABAR kinetics predominantly shape GABAergic input to rod bipolar cells and that GABA release onto GABAARs and GABACRs occurs by similar mechanism in contrast to GABA release from amacrine cells that provide non-reciprocal input to rod bipolar cells.

Discussion

Our results show that the slow release of glycine and GABA onto GABAARs is an inherent characteristic of amacrine cells, similar to GABA release onto GABACRs (Eggers et al. 2013). Glycine and GABA release onto GABACRs occurs asynchronously and can be reduced by both EGTA-AM and BAPTA-AM. Although the timing of GABA release onto GABAARs was also inherently slow, it occurred more rapidly than glycine release and GABA release onto GABACRs and was not reduced by slow or fast Ca\(^{2+}\) buffering. The differential timing of GABA
release was not observed at the reciprocal rod bipolar cell-A17 amacrine cell synapse indicating that different release mechanisms are employed in reciprocal and non-reciprocal rod bipolar cell-amacrine cell synapses, and that GABA release at non-reciprocal synapses likely comes from different GABAergic amacrine cells. The differences in the timecourse of release onto GABAR and glycineR inputs may provide another level of control on retinal inhibition and a way to tune information across retinal pathways.

Comparisons with release at other synapses

At some conventional synapses, fast synchronous release that occurs within tens of milliseconds is followed by prolonged asynchronous release that can last for several hundred milliseconds (Atluri and Regehr 1998; Cummings et al. 1996; Goda and Stevens 1994; Jiang et al. 2012; Lu and Trussell 2000; Otsu et al. 2004; Sakaba 2006), often in response to repetitive stimuli (Best and Regehr 2009; Cummings et al. 1996; Hefft and Jonas 2005; Jiang et al. 2012; Lu and Trussell 2000; Otsu et al. 2004; Sakaba 2006). Hippocampal neurons utilize asynchronous release at excitatory (Cummings et al. 1996; Otsu et al. 2004) and inhibitory synapses (Hefft and Jonas 2005; Jensen et al. 2000) to maintain synaptic transmission during high frequency stimulation after the depression of synchronous release (Otsu et al. 2004). We have shown here and in previous studies (Eggers et al. 2013; Eggers and Lukasiewicz 2006b) that a single brief stimulus can evoke prolonged GABAR and glycineR IPSCs. These prolonged responses are mediated by prolonged GABA and glycine release, and are not artifacts of our electrical stimulus because stimulated amacrine cells show a brief depolarization and the D37 of the eIPSCs followed the same hierarchy (GABA\(\text{C}\)-Rs > glycineRs > GABA\(A\)-Rs, Figure 1) as the D37 of L-IPSCs (Eggers and Lukasiewicz 2006b) even when each receptor input was measured.
from the same rod bipolar cell. Asynchronous release has also been observed to occur after step-
depolarization at the calyx of Held (Sakaba 2006) and after a high intensity stimulus in cerebellar
granule cells (Atluri and Regehr 1998), but neither rely on asynchronous release as a primary
mode of transmitter release. Amacrine cells belong to a unique subset of neurons in that they
inherently release transmitter with prolonged timing after a single stimulus. The use of
asynchronous release as a primary mode of transmitter release has so far been observed in only
one other neuronal system where asynchronous release accounts for >90% of all release from
neurons that project from the deep cerebellar nuclei to the inferior olive in response to low
frequency stimulation (Best and Regehr 2009).

Mechanisms of asynchronous release from glycinergic amacrine cells

Our results suggest that glycine release from amacrine cells is similar to GABA release
onto GABA\(\text{C}\)-Rs (Eggers et al. 2013). Amacrine cell glycine release occurs asynchronously
because it is attenuated by the slow Ca\(^{2+}\) buffer EGTA-AM (Augustine et al. 2003).

Asynchronous release of GABA has been observed at other synapses (Best and Regehr 2009;
Hefft and Jonas 2005; Jiang et al. 2012; Lu and Trussell 2000), but asynchronous release of
glycine has not been widely reported. A recent study in neurons of the avian auditory brainstem
that release both GABA and glycine showed that asynchronous glycine release could only be
evoked by high frequency stimulation after GABA was depleted (Fischl et al. 2014). The authors
suggest that this is due to the recruitment of glycine to vesicles in the absence of adequate
amounts of GABA. In the retina, colocalization and corelease of GABA and glycine has not been
observed and both transmitters are released from different populations of amacrine cells
(Masland 2012), therefore it is unlikely that GABA release influences glycine release from the
same amacrine cell.

In contrast to GABA release, electrically evoked glycine release from amacrine cells was not blocked by TTX suggesting that the glycinergic amacrine cells activated by our electrical stimulus do not rely on the spikes to trigger release. This is likely because the span of glycinergic amacrine cell processes is compact and they do not extend across the retina as widely as GABAergic amacrine cells (Masland 2001). Thus, they would not require spikes to support transmission of a signal across long distances as may be the case for some GABAergic amacrine cells (Eggers et al. 2013; Shields and Lukasiewicz 2003). Glycine release from amacrine cells is Ca$^{2+}$-dependent (Bieda and Maclver 2004; Chavez and Diamond 2008; Habermann et al. 2003) and relies in part on activation of slowly inactivating L-type Ca$^{2+}$ channels (Bieda and Maclver 2004; Chavez and Diamond 2008; Habermann et al. 2003) and Ca$^{2+}$-induced Ca$^{2+}$ release (Chavez and Diamond 2008) that would cause prolonged Ca$^{2+}$ signals. We have previously shown that the activation of L-type Ca$^{2+}$ channels and Ca$^{2+}$-induced Ca$^{2+}$ release underlies asynchronous release from GABAergic amacrine cells onto rod bipolar cell GABA$_C$Rs (Eggers et al. 2013), suggesting the mechanisms for slow release may be similar between glycinergic and GABAergic amacrine cells.

Differential GABA release from spiking GABAergic amacrine cells

An unexpected result from this present study shows that GABA release from amacrine cells onto GABA$_A$Rs occurs differently than release onto GABA$_C$Rs even though they both come from GABAergic amacrine cells. Differential release of the same transmitter from different types of neurons onto the same postsynaptic neuron has been reported in other neuronal systems (Best and Regehr 2009; Hefft and Jonas 2005). Our data showing different Ca$^{2+}$ buffering
sensitivity of release onto GABA\textsubscript{A}Rs and GABA\textsubscript{C}Rs suggest that the differences in the timing of GABA release from spiking amacrine cells may reflect differences in inputs from multiple types of GABAergic amacrine cells onto the same rod bipolar cell terminals. There are ~30 types of amacrine cells found in the retina that receive excitatory input from rod and cone pathways, and some subset of these could provide inhibitory input to rod bipolar cells (Masland 2001). Also, GABA\textsubscript{A}Rs and GABA\textsubscript{C}Rs cluster at different synapses on rod bipolar cell terminals (Koulen et al. 1998), so their inputs could be independent. It is possible that the electrically evoked GABAergic input on to each receptor arises from non-reciprocal amacrine cells that have distinct release mechanisms. A unique feature of rod bipolar cells is that in addition to receiving non-reciprocal amacrine cell inhibition, they also receive reciprocal inhibition from a non-spiking GABAergic amacrine cell, the A17 amacrine cell which accounts for ~50% of GABAergic inhibitory input (Chavez et al. 2010; Chavez et al. 2006; Hartveit 1999; Nelson and Kolb 1985). Although our focal electrical stimulus strongly activated non-reciprocal amacrine cell inputs to rod bipolar cells (Eggers et al. 2013), it likely did not induce GABA release from A17 amacrine cells that do not generate spikes (Chavez et al. 2006; Menger and Wässle 2000). A17 amacrine cells rely predominantly on the opening of Ca\textsuperscript{2+}-permeable AMPA receptors that are directly activated specifically by rod bipolar cell glutamate release to trigger GABA release (Chavez et al. 2006). We found that the timing of depolarization-induced GABA release from A17 amacrine cells onto GABA\textsubscript{A}Rs and GABA\textsubscript{C}Rs was not different (Figure 6). Therefore, our results indicate that rod bipolar cells receive non-reciprocal inhibition from different amacrine cells with distinct release mechanisms that differs from the release that mediates reciprocal inhibition.

Differences in spiking GABAergic amacrine cells release could be due to differences in Ca\textsuperscript{2+} signaling such as the expression of voltage-dependent Ca\textsuperscript{2+} channels, the extent of Ca\textsuperscript{2+} -
induced Ca$^{2+}$ release, vesicle availability, the Ca$^{2+}$ sensors found on the vesicles and the amount of Ca$^{2+}$ buffers that together influence how Ca$^{2+}$ triggers release (Kaeser and Regehr 2013). In addition to slow L-type Ca$^{2+}$ channels, amacrine cells also express N-type and P/Q-type Ca$^{2+}$ channels (Bieda and Maclver 2004; Chavez and Diamond 2008; Chavez et al. 2010; Vigh and Lasater 2004) which would not contribute to a prolonged Ca$^{2+}$ signal (Eggers et al. 2013; Randall and Tsien 1995). Our results suggest that GABA release occurs more rapidly from spiking amacrine cells that release GABA onto GABA$\_A$Rs because neither the slow Ca$^{2+}$ buffer EGTA-AM nor the fast Ca$^{2+}$ buffer BAPTA-AM affected GABA release onto GABA$\_A$Rs. Another potential explanation for the differential effects of the Ca$^{2+}$ buffers on amacrine cell GABA release is that it is due to differing sensitivities of amacrine cells to the AM ester analogues of EGTA and BAPTA that may cause varied accumulation of the buffers in amacrine cells. However, we think that this possibility is unlikely because a higher concentration of EGTA-AM (100 μM) did not affect GABA release onto GABA$\_A$Rs (unpublished results). We think it is more likely that in spiking GABAergic amacrine cells release onto GABA$\_A$Rs is more tightly coupled to Ca$^{2+}$ (Hefft and Jonas 2005) possibly due to the close proximity of vesicles to sites of Ca$^{2+}$ entry where Ca$^{2+}$ is present in concentrations as high as 100 μM (Augustine et al. 2003; Kaeser and Regehr 2013) that could saturate BAPTA-AM thereby reducing its buffering capacity (Cummings et al. 1996; Tymianski et al. 1994). Neurotransmitter release at the parvalbumin interneuron-granule cell synapse has also been shown to be only weakly sensitive to Ca$^{2+}$ buffering with BAPTA-AM (Hefft and Jonas 2005). Alternatively, GABAergic amacrine cells that release onto GABA$\_A$Rs could have a higher Ca$^{2+}$ buffering capacity that prevents a global buildup of Ca$^{2+}$, as has been seen in other retinal neurons (Mehta et al. 2014). In addition to various sources of Ca$^{2+}$ some studies have suggested that different synaptic proteins are
involved in mediating asynchronous release (Kaeser and Regehr 2013) including synaptotagmin 7 (Schonn et al. 2008; Wen et al. 2010), Doc2 (Yao et al. 2011) and the SNARE protein VAMP4 (Raingo et al.). These proteins could be expressed in distinct amacrine cells at different levels and may contribute to differential release. Additionally, although we saw no differences in the timing of amacrine cell depolarization, the possibility remains that different amacrine cells have distinct voltage responses to the electrical stimulus which would affect the timing of release. However, this hypothesis is difficult to test because the identities of all the amacrine cells that inhibit rod bipolar cell output are unknown.

Functional implications

Light stimuli are processed by the rod pathway when rod photoreceptors are active in dim light, and the cone pathways when cone photoreceptors are active at bright light intensities (Wang and Kefalov 2009). Amacrine cell-mediated inhibition limits the extent of bipolar cell output in the cone and rod pathways by releasing GABA or glycine onto different proportions of GABARs and glycineRs with distinct kinetics (Eggers and Lukasiewicz 2006b), and our results reported here and previously (Eggers et al. 2013; Eggers and Lukasiewicz 2006b) suggest that differential asynchronous release also contributes. Previous studies have shown that light-evoked rod bipolar cell glutamate release is prolonged with increasing light intensities (Berntson and Taylor 2000; Euler and Masland 2000). Given that light-evoked GABA release onto rod bipolar cell terminals is also increased and occurs on a slower timecourse at increasing light intensities (Eggers and Lukasiewicz 2006b), it is possible that differences in prolonged GABA and glycine release is a way to maintain long lasting inhibition at higher light intensities to match prolonged
rod bipolar cell glutamate release. At other inhibitory synapses, asynchronous release has been shown to increase after the depression of synchronous release to maintain tonic inhibitory input (Fischl et al. 2014; Hefft and Jonas 2005; Jensen et al. 2000; Otsu et al. 2004). Asynchronous release may also be more adaptable to changes in stimuli (light intensity) than receptor expression and kinetics because release can be adjusted rapidly by modulating vesicle availability and Ca\(^{2+}\) signaling (Kaeser and Regehr 2013).

Release onto GABA\(_A\)Rs and glycineRs provides the initial inhibitory input that shapes the peak of rod bipolar cell IPSCs (Eggers and Lukasiewicz 2006b) while prolonged release onto glycineRs and GABA\(_C\)Rs controls the slow timecourse of rod bipolar cell IPSCs, and maintains inhibition to match sustained rod bipolar cell glutamate release (Eggers and Lukasiewicz 2006b). Because the kinetics of light-evoked-IPSCs have been shown to affect the kinetics of rod bipolar cell output (Eggers and Lukasiewicz 2006a), increased inhibition would limit the output of the rod pathway as the light intensity increases to cone levels. Our results suggest that the differential timing of slow transmitter release is utilized in amacrine cells presumably to match the sustained rod bipolar cell excitatory output. Assuming that differential amacrine cell release occurs in the ON and OFF pathways, the difference in timing of release combined with known differences in receptor kinetics (Eggers et al. 2007) would make the kinetics of inhibition different between each pathway allowing for rapid adaptation to changes in light intensities.


Diamond JS, and Jahr CE. Asynchronous release of synaptic vesicles determines the time course of the AMPA receptor-mediated EPSC. *Neuron* 15: 1097-1107, 1995.


Competing interests:
The authors declare no competing interests.


Funding: This work was supported by the National Institutes of Health (EY-018131 to E.D.E; Graduate Training in Systems and Integrative Physiology grant 5T32GM008400 appointment to R.E.M; Cardiovascular Training Grant 2T32HL7249-36A1 appointment to J.M.M) and the Science Foundation Arizona (R.E.M.).

Tables

Table 1. Timecourse of eIPSCs and estimated transmitter release.

<table>
<thead>
<tr>
<th>eIPSC type</th>
<th>eIPSC $D_{37}$ (ms)</th>
<th>Release $D_{37}$ (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA$_c$R</td>
<td>1410 ±116***</td>
<td>907±108*</td>
<td>50</td>
</tr>
<tr>
<td>GlycineR</td>
<td>717±164*†</td>
<td>878±233*</td>
<td>13</td>
</tr>
<tr>
<td>GABA$_A$R</td>
<td>73.4±33.5†</td>
<td>288±154†</td>
<td>15</td>
</tr>
</tbody>
</table>

Data are average values of decay to 37% of peak ($D_{37}$) of eIPSCs and estimated vesicle release.

*p < 0.05, *** p < 0.001 for statistical comparisons with GABA$_A$R and †p < 0.05 for comparisons with GABA$_c$R.
Table 2. Average values for eIPSCs recorded in the presence of EGTA-AM or BAPTA-AM (normalized to percent of respective eIPSC control values).

<table>
<thead>
<tr>
<th>eIPSC type</th>
<th>$Q$ (norm.)</th>
<th>$D37$ (norm.)</th>
<th>Peak (norm.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA(_c)R</td>
<td>50 EGTA-AM</td>
<td>33.6±6.7***</td>
<td>57.3±5.8***</td>
<td>58.2±8.1**</td>
</tr>
<tr>
<td>GABA(_c)R</td>
<td>BAPTA-AM</td>
<td>31.6±5.2***</td>
<td>50.5±4.0***</td>
<td>53.5±8.3***</td>
</tr>
<tr>
<td>GlycineR</td>
<td>50 EGTA-AM</td>
<td>33.3±4.8***</td>
<td>47.4±9.1**</td>
<td>68.2±5.7**</td>
</tr>
<tr>
<td>GlycineR</td>
<td>BAPTA-AM</td>
<td>47.1±12.6**</td>
<td>42.2±10.7**</td>
<td>80±11.8(p=0.2)</td>
</tr>
<tr>
<td>GABA(_a)R</td>
<td>50 EGTA-AM</td>
<td>235±66.4 (p=0.09)</td>
<td>211.2±62.1 (p=0.13)</td>
<td>85.1±6.2 (p=0.06)</td>
</tr>
<tr>
<td>GABA(_a)R</td>
<td>BAPTA-AM</td>
<td>173±68.6 (p=0.34)</td>
<td>209.1±92.3 (p=0.3)</td>
<td>84.8±14.5 (p=0.4)</td>
</tr>
</tbody>
</table>

Data are average values of charge transfer ($Q$), $D37$ and peak amplitude of eIPSCs in the presence of the drug shown in the left column, normalized to the control value for each cell. GABA\(_c\)R data taken from Eggers et. al., 2013 are shown for comparison. ** p < 0.01, *** p < 0.001 compared to respective controls.

Table 3. Average values for release from deconvolution of eIPSCs recorded in the presence of EGTA-AM or BAPTA-AM (normalized to percent of respective control values).

<table>
<thead>
<tr>
<th>eIPSC type</th>
<th>$Q$ (number of vesicles, norm.)</th>
<th>$D37$ (norm.)</th>
<th>Peak (norm.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA(_c)R</td>
<td>50 EGTA-AM</td>
<td>56.6±14.7*</td>
<td>50.4±11.5**</td>
<td>65.4±15.0</td>
</tr>
<tr>
<td>GABA(_c)R</td>
<td>BAPTA-AM</td>
<td>37.0±5.0***</td>
<td>42.8±9.7***</td>
<td>62.1±12.5*</td>
</tr>
<tr>
<td>GlycineR</td>
<td>50 EGTA-AM</td>
<td>47.1±5.6***</td>
<td>58±11.6*</td>
<td>77.2±15.7(p=0.2)</td>
</tr>
<tr>
<td>GlycineR</td>
<td>BAPTA-AM</td>
<td>33.8±14.2**</td>
<td>37.5±14.1**</td>
<td>54.7±16.4*</td>
</tr>
<tr>
<td>GABA(_a)R</td>
<td>50 EGTA-AM</td>
<td>121±24 (p=0.4)</td>
<td>144±30(p=0.2)</td>
<td>140±34 (p=0.3)</td>
</tr>
<tr>
<td>GABA(_a)R</td>
<td></td>
<td>162±82 (p=0.6)</td>
<td>158.2±37.4(p=0.2)</td>
<td>224±75 (p=0.2)</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are average values of number of vesicles (Q), D37 and peak amplitude of release in the presence of the drug shown in the left column, normalized to the control value for each cell. GABA<sub>C</sub>R data taken from Eggers et. al., 2013 are shown for comparison. * P ≤ 0.05, ** p ≤ 0.01, *** p < 0.001 compared to respective controls.
Figure Legends.

Figure 1. Electrically evoked (e)IPSCs of rod bipolar cells are prolonged by slow GABA and glycine release. (A) eIPSCs mediated by GABA<sub>C</sub>Rs, glycineRs and GABA<sub>A</sub>Rs measured from rod bipolar cells were prolonged relative to the stimulus (1 ms, grey bar, stimulus artifact removed) similar to light-evoked IPSCs. eIPSCs shown were normalized to the peak of the response to emphasize the timecourse differences. (B) GABA<sub>C</sub>R eIPSCs had an average D37 significantly longer than the average D37 for glycineRs (1-way ANOVA p < 0.05, SNK post-hoc p < 0.05) and GABA<sub>A</sub>Rs (p < 0.01). (C) GABA and glycine release were estimated from eIPSCs with the deconvolution of an average GABA<sub>C</sub>R, glycineR or GABA<sub>A</sub>R spontaneous IPSC (sIPSC) from rod bipolar cells, respectively. Traces are shown normalized to the peak. (D) The timecourse of release onto GABA<sub>C</sub>Rs and glycineRs was similar and significantly longer than release onto GABA<sub>A</sub>Rs (SNK post-hoc p < 0.05). *p < 0.05, **p < 0.01

Figure 2. GABA release onto GABA<sub>A</sub>Rs is not reduced by Ca<sup>2+</sup> buffers. Representative traces of GABA<sub>A</sub>R (A) and GABA<sub>C</sub>R (B) eIPSCs before and after treatment with 50 µM EGTA-AM. EGTA-AM (EG) and BAPTA-AM (BA) reduced the charge transfer (Q; C), shortened the D37 (D) and reduced the peak (E) of GABA<sub>C</sub>R, but not GABA<sub>A</sub>R eIPSCs. Representative traces of the timecourse of GABA release estimated through deconvolution onto GABA<sub>A</sub>R (F) and GABA<sub>C</sub>Rs are shown (G). The amount (H), timing (I) and peak (J) of GABA release onto GABA<sub>A</sub>Rs after treatment with EGTA-AM or BAPTA-AM was similar to control values. In contrast, both Ca<sup>2+</sup> buffers significantly reduced the timing and amount of GABA release onto GABA<sub>C</sub>Rs and BAPTA-AM reduced the peak. All values are normalized to the control.
GABA<sub>A</sub>R or GABA<sub>C</sub>R eIPSC (C-E) or GABA release (H-J) for each cell. Grey bar = 1 ms electrical stimulus *p < 0.05, **p < 0.01

**Figure 3. Glycine release from amacrine cells is reduced by Ca<sup>2+</sup> buffering.** Representative traces of glycineR eIPSCs (A) and timecourse of glycine release (E) recorded before and after treatment with EGTA-AM are shown. Increased Ca<sup>2+</sup> buffering with EGTA-AM or BAPTA-AM reduced Q (B), shortened D37 (C) of glycineR mediated eIPSCs and EGTA-AM reduced the peak (D). EGTA-AM and BAPTA-AM also reduced the amount (F) and D37 (G) of vesicle release onto glycineRs and BAPTA-AM reduced the peak (H). All values are normalized to the glycineR eIPSCs (B-D) or the release onto glycineRs (F-H) for each cell. Grey bar = 1ms electrical stimulus *p < 0.05, **p < 0.01

**Figure 4. GABA release onto GABA<sub>A</sub>Rs occurs in the fast and slow phases of release.** The amount of vesicle release that occurred during early (A) and late (B) time periods normalized to the total amount of vesicle release is shown for release onto GABA<sub>C</sub>Rs, glycineRs and GABA<sub>A</sub>Rs. An example trace of vesicle release onto GABA<sub>A</sub>Rs with the fast and slow phases separated (grey dotted line) is shown in the inset. Vesicle release onto GABA<sub>C</sub>Rs and glycineRs occurred primarily during the late time period (B, GABA<sub>C</sub> = 93 ± 1%, n = 18; glycine = 91 ± 2%, n = 11) compared to release onto GABA<sub>A</sub>Rs (66 ± 8%, n = 11, 1-way ANOVA p < 0.01, SNK post-hoc p < 0.01). 33 ± 8% of vesicle release onto GABA<sub>A</sub>Rs occurred during the early time period (A). **p < 0.01
Figure 5. TTX sensitive GABAergic amacrine cells are activated in response to an electrical stimulus. Representative traces of rod bipolar cell eIPSCs recorded in the presence of TTX are shown for GABA_A (A), GABA_C (B) and glycineRs (C). (D) The Q of GABA_A (Student’s t-test p < 0.01 n = 4) and GABA_C (p < 0.01, n = 5) mediated eIPSCs are attenuated by TTX. TTX did not significantly change glycineRs (p = 0.2, n = 5) eIPSCs. All values are normalized to the control GABA_A (A), GABA_C (B), or glycineR (C) current for each cell. GABA_C eIPSCs modified from Eggers et. al., 2013. Grey bar = 1ms electrical stimulus **p < 0.01

Figure 6. The distinct timing of GABA release onto GABA_ARs and GABA_CRs can be measured at the rod bipolar cell-A17 amacrine cell synapse. (A) Representative traces of reciprocal feedback (f)IPSCs from A17 amacrine cells onto rod bipolar cells are shown. GABAergic fIPSCs of rod bipolar cells were evoked by a 10 ms step from -60 to -20 mV (top), and recorded in the absence (dark grey) and presence of TPMPA to isolate GABA_A -mediated IPSCs (light grey trace). The GABA_C (black trace) mediated input was determined after subtracting the GABA_A IPSC from the total response. (B) Estimated release onto GABA_ARs and GABA_CRs from the example in A. (C) GABA_A (n = 5) fIPSCs D37 was briefer than the total fIPSC D37 (n = 5) and isolated GABA_C fIPSCs (n = 5, p < 0.05 for both). The GABA_C fIPSC was not different from control (p = 0.2). (D) The timing of GABA release onto GABA_ARs and GABA_CRs was not significantly different. *p < 0.05
Figure 1

A. 

GABA release (GABAAR) 
GABA release (GABACR) 
Glycine release

B. 

GABAAR eIPSC
GABACR eIPSC 
GlycineR eIPSC

C. 

GABA release (GABA_C R) 
Glycine release 
GABA release (GABA_A R)

D. 

GABA release (GABA_C R) 
Glycine release 
GABA release (GABA_A R)

Figure 1
Figure 2
**Figure 3**

A. 
GlycineR eIPSC
GlycineR eIPSC + EGTA-AM

B. 
Q (norm.)

C. 
D37 (norm.)

D. 
Peak (norm.)

E. 
Glycine release
Glycine release + EGTA-AM

F. 
# Vesicles (norm.)

G. 
Release D37 (norm.)

H. 
Release peak (norm.)

Legend:
- **EG**
- **BA**
- **** indicates statistical significance
- * indicates statistical significance

5 pA
500 ms

0.02 quanta/ms
500 ms
Figure 4

A. Early

GABA release (GABA_{A}R)

B. Late

** **

GABA release
Figure 5

A. GABA_A and TTX

B. GABA_C and TTX

C. Glycine and TTX

D. TTX Q (norm.)

** Figure 5 **
Figure 6

A. Control fIPSC

B. GABA release (GABACR)

C. Current

D. Release

* indicates statistical significance. N.S. indicates not statistically significant.