Inhibitory Feedback Shapes Bipolar Cell Responses in the Rabbit Retina

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Submitted 26 July 2007; accepted in final form 5 October 2007

Molnar A, Werblin F. Inhibitory feedback shapes bipolar cell responses in the rabbit retina. J Neurophysiol 98: 3423–3435, 2007. First published October 10, 2007; doi:10.1152/jn.00838.2007. Retinal bipolar cells can be divided into on and off types based on the polarity of their response to light. Bipolar activity is further shaped by inhibitory inputs, characterized here by the events that occur immediately after the onset of a light step: 1) in most off bipolar cells, excitatory current decreased, whereas inhibitory current increased. These currents reinforced each other, enhancing the light response. 2) In about half of the on cone bipolar cells, the excitatory current increased, whereas inhibitory current decreased, also reinforcing the light response. Both of these reinforcing interactions were mediated by glycinergic inhibition. 3) In the remaining on cone bipolar cells, excitation and inhibition both increased, but inhibition was delayed so that these cells responded transiently. 4) Finally, in rod bipolar cells, excitation and inhibition both increased so that inhibition suppressed excitation, reducing the light response at all time scales. The suppressive inhibition seen in on cone and rod bipolar cells was mediated by GABA. Thus morphologically diverse bipolar cells receive only four main types of inhibitory input, and the majority of “inhibitory” inputs actually serve to enhance excitation.

INTRODUCTION

Mammalian retinal bipolar cells show considerable morphological diversity. MacNeil et al. (2004) have distinguished 12 morphological types of bipolar cell based on the stratification and morphology of their synaptic terminals. Bipolar cells also display a variety of excitatory receptors and synaptic morphologies that lead to diverse physiological responses. On and off bipolar cells can be distinguished by their excitatory receptors: ionotropic, sign-preserving AMPA and kainate receptors are found in off bipolar cells, whereas sign-inverting metabotropic glutamate receptors are found in on bipolar cells (Nawy and Jahr 1990, 1991). At a finer level, DeVries (2000) has shown that different ionotropic glutamate receptors in off bipolar cells generate temporally distinct excitatory currents. Bipolar cells also receive inhibitory inputs from, and deliver excitation to a variety of amacrine cell types (Dong and Werblin 1998; Euler and Masland 2000; Volgyi et al. 2002), creating an additional level of physiological diversity (Łukasiewicz and Werblin 1994; Łukasiewicz et al. 1994). Because bipolar cells are the first cells in the visual pathway that display significant morphological and physiological diversity, understanding bipolar cell physiology could provide insight into the origin of the broad range of signals that the retina sends to higher visual centers (Roska et al. 2006). The inhibitory inputs bipolar cells and their interaction with the cells’ excitatory inputs are the subject of this study.

We characterized the light-evoked excitatory and inhibitory inputs that shape bipolar cell responses in the rabbit retina. Our measurements show that the responses of all 12 morphological types of bipolar cell are shaped by only four dominant forms of excitatory-inhibitory interaction and that these interactions are distributed very differently in the on and off pathways. Application of a variety of specific inhibitory blockers revealed that these interactions are pharmacologically distinct. The inhibition that acts between the on and off sublaminae is glycinegic and, surprisingly, enhances rather than suppresses the bipolar light response. The inhibition that acts within the on sublamina is GABAergic and suppresses the overall light response. We found very little inhibition that acted within the off sublamina, revealing a striking asymmetry between the on and off pathways of the retina.

METHODS

Rabbits were killed, and their eyes were removed and hemisected as described previously (Roska and Werblin 2001, 2003; Roska et al. 2006). Segments of the visual streak along with their associated sclera were stored in oxygenated Ames’ medium in the dark. Individual segments were then removed from the sclera, mounted on Millipore paper, and sliced with a razor into 250-μm-thick slices. The slices were mounted on their sides so that a cross-section was visible through the microscope. The slices were perfused with Ames’ solution at 35°C. The solution was saturated with a mixture of O2 (95%) and CO2 (5%) and pH buffered with NaCO3 to a pH of 7.4.

Patch clamp

Bipolar cells, identified as having cell bodies near the outer edge of the inner nuclear layer (INL), were whole cell patch-clamped with glass electrodes of resistance between 5 and 10 MΩ. For the majority of our experiments, the electrodes were filled with an intracellular solution that was potassium-based [in mM: 113 KMeSO4 (Fluka), 1 Mg SO4 (Fisher Scientific), 7.8 10⁻³ CaCl2 (Fisher Scientific), 0.5 BAPTA (Fisher Scientific), 10 HEPES (Sigma), 4 ATP-Na2 (Sigma), 0.5 GTP-Na3 (Sigma), 5 KCl (Fisher Scientific), 7.5 Neurobiotin-Cl (Vector Labs), pH 7.2]. However, to confirm that our results were not simply a consequence of imperfect voltage clamping due to potassium leakage currents, we performed 59 of 163 experiments using a cesium-based solution [in mM: 113 CsMeSO4 (Sigma), 1 Mg SO4 (Fisher Scientific), 7.8 10⁻³ CaCl2 (Fisher Scientific), 0.5 BAPTA (Fisher Scientific), 10 HEPES (Sigma), 4 ATP-Na2 (Sigma), 0.5 GTP-Na3 (Sigma), 5 QX314 (Sigma), 7.5 Neurobiotin-Cl (Vector Labs), pH 7.2]. The same forms of excitatory and inhibitory currents were found while using either solution, so these two data sets have been pooled in all subsequent analyses. Alexa Fluor 488 (Invitrogen)

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was also included in the intracellular solution to allow imaging of the cells after physiological measurements were complete.

Excitatory currents were measured by voltage clamping the cell at the calculated reversal potential for chloride (−60 mV). Inhibitory currents were recorded by voltage clamping the cell at the cation reversal potential (0 mV). Finally, the voltage response of the cell was recorded at a current clamp of 0 pA. The chloride reversal potential was confirmed by inhibitory synaptic noise, which reversed polarity at −60 mV. The cation reversal potential was confirmed under pharmacological blockade of inhibition, described in RESULTS, where light-evoked currents reversed at 0 mV, indicating a cation reversal potential of 0 mV. These results were consistent using both cesium- and potassium-based solutions and with previous studies (Fried et al. 2005; Roska et al. 2006; Wu et al. 2004). In most cases, excitation was recorded first, followed by inhibition, followed by voltage (under current clamp). Because voltage was recorded last, it was sensitive to slow degradation of patch quality and cell health and in many cases was observed to slowly depolarize over time. To avoid artifacts due to cell degradation, voltage recordings were only considered to accurately reflect the interaction between excitation and inhibition if the observed resting potential of the cells was more hyperpolarized than −25 mV during these recordings, corresponding to the upper end of the range of resting potentials reported elsewhere (Euler and Masland 2000). Recordings were digitized and sampled at 10 kHz. All signals were postanalyzed in MATLAB (The Mathworks). Signals were filtered and down sampled to a 60-Hz sample rate, the same as the update rate of the stimulus. No meaningful signals (light responsive or otherwise) were observed above this frequency.

**Stimulus paradigms**

For each of the clamp states, a variety of stimuli were presented against a background brightness of 3 \times 10^7 photons per \textmu m^2/s. The most basic stimulus was a pair of 2-s flashes at \pm100% contrast, specifically: 6 \times 10^7 photons per \textmu m^2/s and 3 \times 10^7 photons per \textmu m^2/s. The stimulus took the form of a 200-\textmu m-wide stripe projected on the cross-section of the retina. Full-field flashes were also used to explore possible wide-field effects but did not yield responses that were significantly different from those observed for 200-\textmu m-wide stripes.

The size and polarity of a cell’s response to given transition in stimulus brightness was found by averaging current for the 600 ms immediately preceding and the 600 ms immediately after each transition and taking the difference between these averages. We denoted the resulting numbers for the four transitions (shown in Fig. 2A) with the variables a, b, c, and d. A metric for the overall polarity of a given response was then defined as the ratio: \( X = \frac{a + b + c - d}{(a + |b| + |c| + |d|)} \). A purely OFF response, where light-to-dark transitions cause an increase in inhibition and dark-to-light transitions cause a decrease in inhibition, yields a value of \( X = -1 \). A purely ON response, where light-to-dark transitions cause an decrease in inhibition and dark-to-light transitions cause a increase in inhibition, yields a value of \( X = +1 \). A response with the same polarity at both onset and offset of light (an ON-OFF response) yields a value of \( X \) between +1 and −1. For simplicity, these responses were categorized according to which part of the response (ON or OFF) dominated. Specifically values of \( X < 0 \) were categorized as representing OFF responses and values of \( X > 0 \) were categorized as representing ON responses.

Stripes (200 \textmu m wide) were also stimulated with a sinusoidally varying intensity to assess temporal behavior in more detail. Sinusoids with temporal frequencies ranging from 0.3 to 15 Hz (incremented by a factor of 2) and contrasts of 100 and 50% were used. Responses to these sinusoidal stimuli were analyzed by performing a Fourier transform of the response (current or voltage) during the final 3/4 of a given frequency stimulus. Sinusoidal stimuli were in the form of 4, 8, or 12 cycles with higher frequency stimuli having more cycles; hence, 3, 6, or 9 cycles were analyzed—the first quarter was eliminated to avoid settling effects. The magnitude and phase of the Fourier-transformed response at the stimulus frequency were extracted for additional analysis. The power (magnitude squared) of the response at this frequency was compared with the sum of powers at the harmonics of the stimulus frequency. In most cases, the power associated with these harmonics was much less than associated with the stimulus frequency, as described in RESULTS.

The magnitude of responses across the various stimulus frequencies and cells were categorized as responding best to low frequencies (low-pass), high frequencies (high-pass), or roughly equally to both high and low frequencies (wide-band). This categorization was quantified by first normalizing the response magnitude of each cell so that average magnitude across frequency equaled 1. Then the magnitude of the two lowest and two highest frequencies (<10 Hz) were averaged and the difference between the low-frequency average and the high-frequency average was calculated. For a given set of responses (i.e., excitation in OFF cells), the average response could be categorized according to the average difference between the magnitude of responses to high- and low-frequency stimuli. The significance of this difference was assessed using the t-test. Interactions between excitation and inhibition were analyzed by comparing the phase of excitatory and inhibitory currents across the range of frequencies tested. A 0° phase difference implies that when excitation (an inward current) is maximum, inhibition (an outward current) is minimum, and when excitation was minimum, inhibition was maximum. In this case, the synaptic currents interfere constructively and drive the membrane voltage with the same polarity. In cases where phase shifted with increasing frequency, an equivalent time delay was calculated by dividing the change in phase (relative to that measured at the lowest frequency) by frequency and by 360°.

**Morphological reconstruction**

Once physiological recordings were complete, intact neurons were imaged with Alexa Fluor 488. Digital images were captured under fluorescence and under bright field white light (transmitted illumination, focused through a condenser) and superimposed. The dimensions of the inner plexiform layer (IPL) could be extracted in each case from the boundaries of the cell bodies in the INL and ganglion cell layer (GCL), visible under white light. By comparing the stratification of axon terminals to the edges of the IPL, their depth of stratification could be measured, which along with their lateral width and vertical diffuseness (see Fig. 5), providing a quantitative morphological description comparable with previously published results. “Width” was defined as the distance, in micrometers, from one tip of the axonal tree to the other, in the dimension parallel to the edges of the IPL. “Spread” was defined as the vertical dimension from the most distal branch-point to the most proximal terminal in the axonal tree, measured in micrometers. “Depth” was defined as the distance from the distal edge of the IPL to the middle of the most proximal terminal in the axonal tree, measured in micrometers. “Width” was defined as the distance from the first branch and the most proximal terminal, normalized to the total thickness of the IPL. Images were also compared directly with published examples of different identified morphological types (MacNeil et al. 2004) to match our physiological observations to known morphological classes. Finally, example cells of each type were traced for easier comparison with existing images.

**General cell type identification**

ON and OFF cells were distinguished by the polarity metric, \( X \), of their excitatory input in response light and dark flashes, as described above. Rod bipolar cells were distinguished from ON cone bipolar cells primarily based on morphology. Cells whose axon terminals projected >83% into the IPL and were <18 \textmu m wide were identified as rod bipolar cells, based on previous work by MacNeil et al. (2004) Cells the axon terminals of which projected >70% into the IPL or were >25 \textmu m wide were identified as cone bipolar cells. For cells with...
ambiguous morphologies or where the cell died before imaging, we identified the cells based on measured membrane currents in response to 50-ms voltage steps (relative to −60 mV) from −80 to +20 mV in 10-mV steps. For morphologically identified rod- and cone-bipolar cells, we found clear differences between their average current responses to voltage steps, as shown in Fig. 1, A and D. Presumably these differences reflect differences in the relative concentrations of voltage-gated ion channels in the membranes of the rod and cone bipolar cells (Karschin and Wassle 1990; Ma et al. 2005). In particular we quantified the turn-on voltage for the inward rectified current and 70% rise time. As can be seen in Fig. 1, these numbers were distributed very differently between the morphologically identified rod- and cone-bipolar cells. Based on these results, we identified cells with ambiguous morphologies as rod bipolar cells if their 70% rise time exceeded 2 ms and as cone bipolar cells otherwise.

**Pharmacology**

Experiments were repeated in the presence of pharmacological blockers of excitation and inhibition. To block metabotropic glutamate receptors, and so selectively inactivate the ON system, 10 μM 2-amino-4-phosphonobutyric acid (APB, L-AP4; Tocris) was added to Ames’ medium and perfused across the preparation. Recordings were repeated a third time under normal Ames’ as a control. To block ionotropic GABA receptors (GABA_A and GABA_C), 100 μM picrotoxin (Sigma) was added to Ames’ medium and perfused across the preparation. Recordings were repeated a third time under normal Ames’ as a control. To block ionotropic glycine receptors, 10 μM strychnine (Sigma) was added to Ames’ medium and perfused across the preparation. A wash step was repeated here, but we found that strychnine was very slow to wash out and so was rarely reversible. To confirm our picrotoxin findings [because picrotoxin has recently been reported to block glycine receptors in some cases (Wang and Slaugh- ter 2005)] and to separate GABA receptor types, we performed experiments using 10 μM SR95531 (Sigma), a GABA_A receptor-specific blocker (Gynther and Curtis 1986) with little glycine receptor reactivity (Wang and Slaught 2005) and 10 μM TPMPA (Sigma), a GABA_C receptor specific blocker (Ragozino et al. 1996) each in Ames’ medium. We followed each drug with a wash step and found that effects were completely reversed for TPMPA and more slowly washed out for SR95531. In several cases, we combined strychnine with one or another of the GABA blockers using the same concentrations described in the preceding text.

Efficacy of a given pharmacological agent was assessed using the same measure of response to a stimulus transition as used in the preceding text when finding polarity. To assess the effect of a drug, each transition was assessed under control and drug conditions, yielding transition numbers $a_d$, $b_d$, $c_d$, and $a_o$, $b_o$, $c_o$, $d_o$. The percent change in response was taken to be $100\%[(a_0 - b_o - c_o + d_o) - (a_d - b_d - c_d + d_d)]/(a_0 - b_0 - c_0 + d_0)$.

Thus complete elimination of a response yields a change of −100%. In cases where the response actually inverts its polarity (as seen for some ON bipolar cells such as in Fig. 7, H and L), changes >100% were found. Changes associated with drug washout were described similarly, but were normalized relative to the original control condition: $100\%[(a_0 - b_0 - c_0 + d_0) - (a_d - b_d - c_d + d_d)]/(a_0 - b_0 - c_0 + d_0)$.

Thus a complete reversal of a drug effect under wash will yield a change of equal size and opposite sign to the original drug effect.

**RESULTS**

**Physiological and morphological identification of basic bipolar cell types**

Whole cell patch-clamp recordings were made from 163 bipolar cells, and each cell was classified as ON or OFF based on excitatory responses to high-contrast light and dark flashes (see METHODS). Of these, 58 were identified as OFF cells and 105 as ON cells. Among the ON cells, 56 were identified as rod bipolar cells, and the remaining 49 were identified as ON cone bipolar cells. Rod bipolar cells were identified based on of the location and shape of their axon terminals, which characteristically lie along the proximal border of the IPL (MacNeil et al. 2004). Morphological identification was confirmed by the time course of the basic membrane currents of ON cells in response to depolarizing voltage steps as described in METHODS. Rod bipolar cells showed an outward rectifying component that activated at less depolarized voltages than cone bipolar cells (Ma et al. 2005) and showed a slower activation time (Karschin and Wassle 1990) than that in ON cone bipolar cells. In ON cells where morphology was unavailable or ambiguous, membrane currents alone were used to identify rod bipolar cells.

**OFF cone bipolar cells receive reinforcing inhibition**

OFF bipolar cells showed a common response form: at the onset of a dark flash, the amount of excitation (an inward current) increased and the amount of inhibition (an outward current) decreased. At the onset of a bright flash, the amount of
excitation decreased and the amount of inhibition increased. These results imply that excitation in these cells was an off signal, while inhibition was an on signal. However, because excitatory and inhibitory synapses act on the cell with opposite polarities, both currents drove the cell membrane with the same polarity, becoming more inward at light off and more outward at light on as shown in Fig. 2E. Thus the inhibitory current acts to reinforce the effects of the excitatory current. This reinforcing interaction appeared in 48/58 off cells as shown in Fig. 2B. Of the remaining cells, two received no inhibition and eight received on-off inhibition, with on (reinforcing) inhibition dominating in four of eight cases.

**Rod bipolar cells receive canceling inhibition**

Rod bipolar cells showed a common interaction: the amount of both excitation and inhibition increased in response to bright flashes, and both decreased in response to dark flashes, as shown in Fig. 2F. Thus in rod bipolar cells, inhibitory current acted to cancel the excitatory current. This canceling interaction was seen in 51 of the 56 rod bipolar cells recorded as shown in Fig. 2C.

**On cone bipolar cells receive reinforcing or canceling inhibition**

On cone bipolar cells showed both on and off types of inhibition, as can be seen in Fig. 2D. In 28/49 on cells, off inhibition was dominant with a purely off response (X = −1) in 17 of these cases: bright flashes increased the amount of excitation and decreased the amount of inhibition, whereas dark flashes decreased the amount of excitation and increased the amount of inhibition as shown in Fig. 2G. In 8 of the 11 on cone bipolar cells that showed off-dominated on-off inhibition, the polarity metric X took values more negative than −0.5, indicating that inhibition in response to off transitions (Fig. 1A, a and d) was at least three times larger than to on transitions (Fig. 1A, b and c). In this general class of on bipolar cells, off-dominated inhibition acted to reinforce excitation similar to the interaction described for the off cells.

In the remaining on cone bipolar cells (20/49), on inhibition dominated. In 10 of these cases, inhibition was purely on (X = 1), such that both excitation and inhibition increased in response to bright flashes and decreased in response to dark flashes (see Fig. 2H). These on-dominated inhibitory currents to on cone bipolar cells cancelled excitatory currents in a manner similar to the interaction measured in rod bipolar cells.

Thus although virtually every off bipolar cell received reinforcing on inhibition, on bipolar cells received a wider array of inhibitory inputs: rod bipolar cells and half of the cone bipolar cells received canceling on inhibition, whereas the other half of on cone bipolar cells received reinforcing, off inhibition.

**Sinusoid responses reveal the same four distinct interactions**

Flash responses like those shown in Fig. 2 fail to resolve fine temporal detail. To better characterize the temporal aspects of bipolar cell responses, we stimulated 152 preparations with stripes the intensity of which varied sinusoidally in time as shown in Fig. 3A. We characterized each cell’s excitatory and inhibitory currents and voltage (where appropriate) by extract...
ing the magnitude of their fundamental frequency term (using the Fourier transform: see METHODS), as a function of temporal frequency, (see Fig. 3B). To analyze the interaction between excitation and inhibition at different temporal frequencies, we calculated the relative phase of the excitatory and inhibitory currents, with 0° corresponding to reinforcement and 180° corresponding to cancellation (see Fig. 3, C–E).

All measured responses showed a significant reduction in amplitude at frequencies > 10 Hz, as shown in Fig. 4, probably indicating a roll-off in the underlying signals generated in the outer retina (Kamermans et al. 2001). Subsequent analyses were only performed on frequencies < 10 Hz. We primarily analyzed the magnitude and phase of the response at the fundamental frequency of the stimulus. Higher harmonics were also investigated, but in most cases (90% of cells), the magnitude of harmonic terms was <10% of the magnitude of the fundamental term (see METHODS) and so contributed little to the measured response.

OFF cone bipolar cells showed clear excitatory and inhibitory activity at all frequencies < 10 Hz. The average excitatory response in OFF cells was mildly low-pass, showing an average response the amplitude of which peaked at 1 Hz and decreased at higher frequencies. At the highest frequency analyzed (8 Hz), this amplitude decreased (n = 53, P < 0.005, t-test) by a factor of 1.6 relative to the maximum response as shown in Fig. 4A. Inhibition, by comparison, showed a wide-band response, varying little across the range of frequencies investigated. OFF cells that received ON-dominated inhibition to light flashes (X > 0, n = 50) showed a phase difference between excitatory and inhibitory input currents of 25° ± 27° (mean ± SD). This phase difference is close to 0° and did not vary significantly across frequency (see Fig. 4A), indicating that inhibition acted to reinforce the excitatory response in OFF cells at all of these frequencies.

Rod bipolar cells (n = 53) showed a strong low-pass response peaking at 1 Hz and rolling off sharply at higher frequencies for both excitation and inhibition (see Fig. 4B). Excitation on average decreased (P < 10−7) by a factor of 2.6 from its peak response to the highest frequency analyzed (8 Hz). Inhibition tended to be even more strongly biased toward low frequencies than excitation, such that on average it peaked at a lower frequency of 0.6 Hz and decreased (P < 10−7) by a
factor of 3.4 at the highest frequency analyzed. In those rod bipolar cells that received on-dominated inhibition to light flashes \( n = 49 \), the phase relationship between excitatory and inhibitory currents maintained a constant phase shift close to 180° \( (156 \pm 23°) \) as shown in Fig. 4B. This shows that inhibition acts to cancel the excitatory response of rod bipolar cells at all of the frequencies where a response was elicited.

For those on cells identified as having off-dominated inhibition to flashed stripes (defined as having a polarity metric \( X < 0 \), \( n = 26 \)), the average excitatory response was wideband, and its magnitude did not vary significantly between the lowest and highest frequencies analyzed \( (P > 0.15, \text{ see METHODS}) \). Inhibition was on average mildly high-pass, increasing by a factor of 1.6 between the lowest and highest frequencies analyzed \( (P < 0.003) \). The phase relationship between excitatory and inhibitory currents was consistently close to 0° at all frequencies \( (9 \pm 23°) \), see Fig. 4C). Thus inhibition reinforced excitation at all of the time scales represented by these frequencies. Only three cells significantly deviated from this trend, and all three were cells with polarity metrics close to zero, implying that a strong on inhibitory signal was also present.

The sinusoidal responses were more complex in those on cone bipolar cells showing on-dominated inhibition (polarity metric \( > 0 \), \( n = 18 \)). Excitation and inhibition both responded in a wide-band fashion. No significant variation in the mean excitatory and inhibitory responses was seen between the lowest and highest frequencies analyzed \( (P > 0.5, \text{ t-test}) \), as shown in Fig. 4D. The phase relationship between excitatory and inhibitory currents, however, changed dramatically across frequencies. At low frequencies, excitation and inhibition were on average 182 ± 38° \( (\text{mean} \pm \text{SD}) \) out-of-phase as shown in Figs. 3C and 4D and so the currents cancelled. As a consequence of this cancellation, the membrane voltage, recorded under current clamp, showed little response to low-frequency sinusoidal stimuli. At high frequencies, the phase relationship shifted to 46 ± 42° as shown in Figs. 3E and 4D, so that excitatory and inhibitory currents reinforced one another, resulting in a significantly larger voltage response. Phase changed smoothly with frequency and can be well approximated by a simple time delay of 50 ms as shown in Fig. 3D. This change in phase was significant \( (n = 18, P < 10^{-8}, \text{ t-test}) \) as the high-pass nature of the voltage response \( (n = 10, P < 0.005) \), which increased on average by a factor of 2.6 between the lowest and highest frequencies analyzed. This voltage response was much more high-pass than either excitation or inhibition alone, as shown in Figs. 3B and 4D.

In summary, we measured four common types of excitatory-inhibitory interaction in bipolar cells: reinforcement in nearly all off cells, reinforcement in approximately half of on cone
bipolar cells, cancellation, which appears in all rod bipolar cells, and delayed cancellation, which appears exclusively in a subset of ON cone bipolar cells.

Bipolar cells of different morphologies show the same basic interactions

We imaged each bipolar cell after electrophysiological recordings were complete and recorded good images of 56 OFF cells, 52 rod bipolar cells, 27 reinforcing ON cells, and 15 delayed canceling ON cells. Although we could not find a one-to-one correspondence between every cell we imaged and morphological classes described elsewhere, we did find individual examples that corresponded closely to each class described by MacNeil et al. (2004). These examples appear in Fig. 5A, along with tentative classification according to the system of MacNeil et al. (2004). We also characterized each cell according to its axonal morphology, measuring three parameters: the depth of the axon terminal, its lateral width, and its vertical spread within the IPL, as shown in Fig. 5B. A scatter plot of cells, showing these morphological parameters and color coded by interaction types, is shown in Fig. 5C. The distribution of morphological parameters is similar to that reported previously by MacNeil et al. (2004) leading us to believe that our dataset encompasses most, if not all of the major morphological types. As reported previously (Euler and Masland 2000), the axon terminals of ON and OFF cells were confined to different sublamina of the IPL with OFF cells stratifying in the outer 40% of the IPL, and ON cells stratifying in the inner 60%.

The OFF cells (blue in Fig. 5C) displayed a wide variety of morphologies including: mono-stratified cells with axonal processes 20 to 45 μm wide, stratifying close to the INL; narrower, diffusely stratifying cells, spanning various parts of the OFF sublamina, and a set of more monostratified cells close to the ON-OFF boundary. The OFF cells that showed an ON-OFF inhibition (shown as cyan in Fig. 5C) did not fall into a single clear morphological class.

Rod bipolar cells showed a characteristic morphology: they had a few large, closely spaced axon terminals close to the GCL, corresponding to descriptions elsewhere (Euler and Wassle 1998; MacNeil et al. 2004). ON cone bipolar cells, like OFF cone bipolar cells, showed a great deal of variation in axonal width and diffuseness, but only average ON cells had slightly wider, more monostratified axon arbors than OFF cells. As shown in Fig. 5, A and C, delayed canceling cells appeared in two distinct morphological types: a slightly diffuse, narrow (width = 25 ± 5 μm) type stratifying close to the IPL midline (depth = 55 ± 5%) and a wider-field monostratified type (width = 35–50 μm), stratifying closer to the ganglion cell layer (depth = 73 ± 5%), corresponding, respectively, to types CBb3 and CBb4 (MacNeil et al. 2004). ON reinforcing cells appeared to include multiple morphologies with both monostratified and diffuse forms at depths from the
ON-OFF boundary to ~85% depth, likely corresponding to the remaining ON morphological types described by MacNeil et al. (2004). It is noteworthy that from Fig. 5C, there is no clear morphological division between reinforcing and delayed canceling cells among cells close to the ON-OFF boundary, and indeed, the majority (12/17) of ON cells stratifying more distally than 65% of the IPL show both ON and OFF inhibition (1 > X > −1, see METHODS), though with a variable ratio between the ON and OFF components. Thus it may be more accurate to describe both CBb3 and CBb3n as receiving a combination of reinforcing and delayed canceling inhibition. This is distinct from the more proximal cell types, where CBb4 seems to receive almost exclusively delayed canceling inhibition, and CBb3-4 and CBb5 receive almost exclusively reinforcing inhibition.

**APB confirms ON inhibitory pathways**

It seems likely that synaptic signals the strength of which increases at light onset originate in ON bipolar cells. We tested this notion by recording in the presence of 10 μM 2-amino-4-phosphonobutyric acid (APB), an agonist of metabotropic glutamate receptors (Slaughter and Miller 1981). As shown in Fig. 6, APB eliminated excitation in ON cone bipolar cells (n = 5, change = −99 ± 32%, mean ± SD, P < 0.01, t-test) and rod bipolar cells (n = 2, changes: −100, and −149%), but not OFF bipolar cells (n = 7, change = −7 ± 64%). This effect partially washed out in both ON cone bipolar cells (n = 4, change = 42 ± 19%, P < 0.03) and rod bipolar cells (n = 2, changes: 17 and 120%). APB eliminated the reinforcing inhibition to OFF cells (n = 10, change = −111 ± 12%, P < 4 × 10−8; wash: n = 7, change = 42 ± 35%, P < 0.02), indicating that this inhibitory signal originates in the ON bipolar cells and represents an interaction between the ON and OFF pathways (see Fig. 6A).

The canceling inhibition seen in ON rod bipolar cells was also eliminated by APB (n = 4, change = −109 ± 6%, P < 6 × 10−5; wash: n = 4, change = 97% ± 24%, P < 0.004), indicating that this signal originates in the ON bipolar cells as well (see Fig. 6B).

Reinforcement to ON cone bipolar cells was eliminated in 3/5 cells (changes = −124, −127, and −104%) but was largely preserved in 2/5 cells (changes = −44, 53%), and the effectiveness of APB was strongly correlated with the depth of the cells’ axonal stratification. As shown in Fig. 8, cells stratifying close to the GCL were strongly affected by APB, whereas those stratifying more distally showed APB-insensitive reinforcing inhibition. This seems to indicate that reinforcing signals to some cells originate in the OFF system, whereas others appear to originate in the ON system.

Delayed cancellation, seen in many ON cone bipolar cells, was always suppressed by APB (n = 7, change = −123 ± 5%, P < 0.001, wash: n = 2, changes = 56 and 140%), indicating that this signal originated in the ON system bipolar cells.

**Pharmacological identification of GABAergic and glycineergic inhibitory components**

The inhibitory interactions described in the preceding text are likely mediated by amacrine cell interneurons that release either glycine or GABA (Bloomfield and Xin 2000; Bolz et al. 1985; Boos et al. 1993; Cueva et al. 2002; Fletcher et al. 1998; Freed et al. 1983; Greferath et al. 1993, 1995; Grunert and Wasse 1993; Haverkamp et al. 2003; Jager and Wasse 1987; Koul et al. 1998; Lukasiewicz and Werblin 1990; Muller et al. 1988; Wasse et al. 1986, 1998; Yang et al. 1991; Zhou and Dacheux 2005). We explored which neurotransmitters mediated different interactions using specific antagonists for the receptors of each of these transmitters, as illustrated in Fig. 7.

**Inhibition to OFF cells is blocked by strychnine but not by GABA receptor antagonists**

In all 7/7 ON OFF bipolar cells tested, reinforcing inhibition was blocked by 10 μM strychnine (change = −83 ± 16%, P < 8 × 10−4), a glycine receptor antagonist (see Fig. 7A). This effect was seen in multiple morphological types, including cells with both wide and narrow ramifications stratifying at

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**FIG. 6.** Effect of 2-amino-4-phosphonobutyric acid (APB) on excitation and inhibition in response to flashed stimuli. Columns correspond to different cell types: OFF reinforcing, rod bipolar (RB) canceling, ON reinforcing, and ON delayed canceling. ---, control condition; - - -, with APB; and + + +, post-APB wash. APB eliminated excitation to all ON cells (B–D) but not OFF cells (A). APB eliminated light responsive inhibition to OFF cone bipolar cells (E), rod bipolar cells (F), and delayed canceling ON cone bipolar cells (H). Inhibition to ON reinforcing cells was not eliminated (G) in roughly 1/3 of cases. Note that for excitation to OFF cells (A) and inhibition to ON reinforcing cells (H), the response shown is for a light-to-dark stimulus to maximize the OFF response, whereas in all other cases, the stimulus was dark-to-light, maximizing the ON response. The y axis represents picoamps scaled as shown by scale bar, x axis represents time, in seconds.
various depths. Picrotoxin (100 μM), a GABA receptor antagonist, acted to reduce the high-frequency component of reinforcing inhibition to some OFF cells but did not block the basic inhibitory flash response (Fig. 7E). This lack of effect was found in 11/13 trials (n = 13, change = −27 ± 61%, P > 0.1); in the 2 remaining cases, inhibition was partially suppressed, and in 1 of those 2 cases, when picrotoxin was washed out and replaced by strychnine, inhibition was completely eliminated. Thus any picrotoxin effect in these two cases is likely presynaptic. Neither SR95531 (10 μM), a GABAA receptor antagonist (n = 5, change = −32 ± 26%, P > 0.05) nor TPMPA (10 μM), a GABA-C receptor antagonist (n = 3 change = −11 ± 36%, P > 0.33) significantly suppressed inhibition to OFF bipolar cells. These results strongly suggest that the reinforcing inhibition to OFF cells is carried by glycine and not by GABA.

Inhibition to rod bipolar cells is blocked best by TPMPA

The canceling inhibition seen in rod bipolar cells was largely unaffected by strychnine (n = 13, change = −21 ± 74%, P > 0.3). Picrotoxin suppressed canceling inhibition in rod bipolar cells significantly but not completely (n = 22, change = −66 ± 35%, P < 3 × 10⁻⁸). This effect was reversed by washing in control Ames’ solution (n = 13, change = 56 ± 81%, P < 0.03). SR95531 did not significantly suppress ON inhibition to rod bipolar cells in (n = 6, change = 27 ± 54%, P > 0.25). The GABAC antagonist TPMPA, however, strongly suppressed canceling inhibition to rod bipolar cells (n = 9, change = −83 ± 30%, P < 3 × 10⁻⁹), and this effect also washed out under normal Ames’ solution (n = 7, change = 81 ± 38%, P < 0.003). Thus it seems that canceling inhibition to rod bipolar cells is mediated primarily by GABAC receptors and that picrotoxin provided an incomplete blockade of these receptors, consistent with previous reports (Wassle et al. 1998).

Reinforcing inhibition to ON cone bipolar cells is reduced by strychnine

In ON cells, reinforcing inhibition was significantly reduced by strychnine on average (n = 11, change = −50 ± 60% P < 0.02). This result was variable across individual cells, however: the response was reduced by >50% in 6/11 cases but was largely unaffected or even enhanced in the remaining 5 cases. In those cases where strychnine was effective, it acted to convert broad-band inhibitory responses to sinusoidal stimulation into a high-pass response. As shown in Fig. 7C, this effect corresponds to strychnine eliminating the sustained part of the inhibitory flash response leaving only a very transient inhibitory current. As with APB, the effectiveness of strychnine was correlated with axonal stratification (see Fig. 8). Among cells where morphology was available, all of the cells in which strychnine reduced inhibition by >50%, stratified in the middle third of the IPL (depth < 67%), whereas all of those where inhibition was reduced by <50% stratified in the inner third (depth > 67%). In two cases, both strychnine and APB were applied to the same ON cell. In one of these cases, strychnine blocked inhibition but APB did not. In the other case, APB blocked inhibition, but strychnine did not. Thus it appears that there are two pharmacologically distinct signals that appear as reinforcing inhibition to ON cone bipolar cells: a strychnine-sensitive APB-insensitive signal that acts on more distal ON
cells and a strychnine-insensitive APB-sensitive signal that acts on the more proximal ON cells.

Picrotoxin had little effect in ON reinforcing cells (n = 6, change = −11 ± 65%, P > 0.5). Neither SR95531 (n = 7, change = 0 ± 88% P > 0.5) nor TPMPA (n = 7, change = 19 ± 38%, P > 0.5) blocked reinforcing inhibition to ON cells. Thus it seems that glycine is involved in reinforcing inhibition to ON cells but GABA is not.

Delayed cancellation is suppressed selectively by GABA receptor antagonists

Delayed canceling inhibition in ON cells was weakly suppressed by strychnine (n = 4, change = −44 ± 41%, P > 0.1), maintaining a wide-band inhibition with the appropriate delay. Picrotoxin more strongly suppressed delayed canceling inhibition (n = 2, changes = −145 and −37%), and this effect washed out in the one case that survived (change = 203%). SR95531 strongly suppressed delayed cancellation in three cells (n = 3, change = −160 ± 104%), and this partially washed out (n = 3, change = 50 ± 63%). In a fourth cell, however, SR95531 actually enhanced delayed cancellation (change = 323%), and this effect also washed out (change = −302). TPMPA strongly suppressed delayed cancellation in two cells (−132 and −116%) but weakly enhanced two others (20 and 23%). Of the cells that were treated with SR, three also received TPMPA, and in each case, only one blocker was effective. The fact that one of the GABA blockers suppressed inhibition in all seven delayed canceling cells tested indicates that the delayed cancellation signal is always GABAergic. The variability as to which blocker is effective, however, indicates that this inhibition may act through either GABA_A or GABA_C receptors.

In summary, glycinergic inhibition is predominant in reinforcing interactions in bipolar cells, where ON inhibition reinforces OFF excitation and OFF inhibition reinforces ON excitation. GABAergic inhibition is predominant in interactions where ON inhibition cancels ON excitation.

DISCUSSION

There are more than 27 morphologically distinct classes of amacrine cell in the rabbit retina (MacNeil et al. 1999), yet we show here that the inhibition feed back to bipolar cells interacts with excitation in only four main ways. We infer the neural circuitry that underlies these interactions below.

Off bipolar cells receive reinforcing glycinergic ON inhibition

In OFF bipolar cells, inhibition both increases at the onset of light and is eliminated by APB (Fig. 6), suggesting that it is derived from the ON pathway. OFF bipolar cell axon terminals are confined to the OFF sublamina, so ON inhibition must be carried by amacrine cells the processes of which receive excitatory input in the ON sublamina and deliver inhibition to the OFF sublamina. Because these cells must span the ON and OFF sublaminae, they are likely to be the diffuse amacrine cells described by MacNeil and Masland (1998). The inhibition to OFF bipolar cells is eliminated by strychnine but not by GABA blockers, as shown in Fig. 7, suggesting that these diffuse amacrine cells are glycinergic as shown in Fig. 9A. These results are consistent with previous studies demonstrating that diffusely stratifying cells have narrow field ramifications (MacNeil and Masland 1998; MacNeil et al. 1999).
narrow field amacrine cells are glycinergic (Menger et al. 1998), and off cone bipolar cells show significant glycine sensitivity (Zhou and Dacheux 2005).

The majority of off bipolar cells receive inhibition over a wide range of temporal frequencies. The inhibitory signals to off bipolar cells most likely originate in on cone bipolar cells that themselves receive reinforcing inhibition because these cells also show a wide-band response. This wide-band response is unlikely to originate in the delayed canceling on bipolar cells because they carry a high-pass response or from rod bipolar cells because they carry a low-pass response.

The AII amacrine cell is known to inhibit off bipolar cells. All cells are glycinergic and under dark-adapted conditions receive on excitation from rod bipolar cells and inhibit off bipolar cells (Mills and Massey 1991, 1995). It is unlikely that the (wide-band) inhibition we have recorded in off cells originates in (low-pass) rod bipolar cells. However, all cells are also driven by electrical coupling from on cone bipolar cells and could supply wide-band on inhibition to the off bipolar cells via this pathway (Xin and Bloomfield 1999).

**Rod bipolar cells receive GABAergic on inhibition**

In rod bipolar cells, both excitation and inhibition increase in response to the onset of light, and both are eliminated by APB as shown in Fig. 6, indicating that both excitation and inhibition derive from the on system. This on inhibition may be feedback from other rod bipolar cells as shown in Fig. 9D, perhaps carried by A17 cells (Hartveit 1999; Nelson and Kolb 1985). This inhibition does not reflect the action of a purely reciprocal synapse to the recorded rod bipolar cell, such as described by Chavez et al. (2006) because that cell is voltage clamped, preventing any modulation of its own voltage-dependent glutamate release. However, the amacrine cells that supply inhibition to rod bipolar cells could (and probably do) make reciprocal synapses with multiple rod bipolar cells, resulting in the on inhibition we measure.

The generally low-pass nature of excitation to rod bipolar cells probably reflects the slow release dynamics in rods (Rabl et al. 2005, 2006; Schnapf and Copenhagen 1982). The extreme low-pass nature of inhibition to rod bipolar cells must be a consequence of slow inhibitory receptors and/or of a very slow inhibitory interneuron. Rod inhibition was suppressed or completely eliminated by picrotoxin and TPMPA as shown in Fig. 7 and is therefore likely carried by GABAergic amacrine cells acting on GABA<sub>C</sub> receptors. This is consistent with studies showing that rod bipolar cells are GABA sensitive (Karschin and Wassef 1990) and are dominated by GABA<sub>C</sub> receptors (Eggers and Lukasiewicz 2006a; Frech and Backus 2004) with slow dynamics (Eggers and Lukasiewicz 2006b). Other studies (Cui et al. 2003; Karschin and Wassef 1990) have also shown strychnine-sensitive glycine channels in rod bipolar cells; however, these channels do not appear to play a primary role in mediating the canceling inhibition we observe.

**Some on bipolar cells receive reinforcing glycinergic off inhibition**

In on reinforcing cone bipolar cells, the amount of outward current increases at light off and is resistant to APB about one half of the cells, indicating that it originates in the off pathway.

In many cases, strychnine suppressed this off inhibition, making it much more transient, as shown in Fig. 7C, but in no case was it blocked by GABA receptor antagonists. Thus the inhibitory signal in these on bipolar cells is most likely carried from the off sublamina by glycinergic amacrine cells as shown in Fig. 9B.

However, off glycineric inhibition to on bipolar cells can only partially explain these results because in approximately one half of cases reinforcement is eliminated by APB and strychnine does not eliminate this inhibition in some cases. Recent studies in mouse have shown little or no glycine sensitivity in on cone bipolar cells (Eggers et al. 2007; Ivanova et al. 2006); this would seem to indicate that none of these signals should be glycineric, casting doubt on our strychnine results. However, the only published study of inhibitory receptors across cone bipolar cells in rabbits (Zhou and Dacheux 2005) shows clear glycine sensitivity in all on cone bipolar cells except for the morphological class that stratifies closest to the GCL, known as “CBB5.” This observation corresponds with ours of reduced strychnine sensitivity in on cells with terminals close to the GCL as shown in Fig. 8. What, then, is the source of apparent reinforcing inhibition in these cells?

Electrical coupling via AII amacrine cells may provide an explanation: on cone bipolar cells make electrical synapses with all amacrine cells (Bloomfield and Xin 1997; Massey and Mills 1999; Mills and Massey 1995; Trexler et al. 2001), and it is unlikely that these AII amacrine cells are voltage clamped across the gap junctions. So the apparent off inhibition measured in proximally stratifying on cone bipolar cells may be transmitted via unclamped AII amacrine cells receiving input from other on bipolar cells. These signals would contain an APB-sensitive component but would not necessarily be sensitive to strychnine because they do not represent a true inhibitory signal. Because both APB and strychnine results are correlated with depth of stratification (Fig. 8), it seems likely that the reinforcement seen in on cells near the middle of the IPL can be best explained by conventional glycineric, off inhibition, but that apparent reinforcing inhibition to on cells near the GCL is more likely due to on signals coupled via gap junctions.

**Some on cone bipolar cells receive delayed GABAergic on inhibition**

The inhibition to on delayed canceling cone bipolar cells increases at the onset of light and is eliminated by APB as shown in Fig. 6D, indicating that this inhibition originates in the on system. As with rod bipolar cells, the presence of on inhibition to these on cone bipolar cells could be explained by the action of an amacrine cell forming reciprocal synapses with multiple on bipolar cells. However, the mismatch in frequency responses between the (wide-band) inhibitory input and (high-pass) voltage response, shown in Fig. 4D, make it unlikely that reciprocal synapses are the dominant pathway behind this inhibition. It seems more likely that the primary source of delayed canceling inhibition is from wide-band reinforcing on bipolar cells as shown in Fig. 9C. This inhibition is eliminated by picrotoxin and SR95531 or TPMPA and so is likely mediated by GABAergic amacrine cells, consistent with recent work indicating that on cone bipolar cells have significant GABA sensitivity (Zhou and Dacheux 2005).
GABAergic inhibition to cone bipolar cells acts to suppress low-frequency or sustained responses while enhancing the response to high-frequency or transient stimuli. This is distinct from the signals seen in rod bipolar cells that suppress response across the whole frequency band. These high-pass bipolar cells stratify at depths corresponding to the dendritic arbors of the ON beta, ON alpha, and ON parasol cells (Roska et al. 2006) and may provide the transient excitatory input to these ganglion cell classes.

**Asymmetries in the inhibitory circuits to bipolar cells**

Figure 9 summarizes our understanding of the amacrine cell pathways that underlie inhibition to bipolar cells. The cross-lamina inhibition carried between the ON and OFF cone bipolar cells is predominantly glycinegic (blue arrows). There is much less OFF-to-ON inhibition than ON-to-OFF inhibition, a significant asymmetry in signal flow between ON and OFF sublaminae. The inhibition carried within the ON sublamina is predominantly GABAergic (bold red arrows). Strikingly, although this ON-to-ON inhibition is common, there is little OFF-to-OFF inhibition, suggesting an additional asymmetry between the ON and OFF pathways. This asymmetry in within-layer inhibition has previously been inferred from recordings of ganglion cells under GABA blockers (Sagdullaev et al. 2006).

**Ganglion cells show similar asymmetries to bipolar cells**

The asymmetries found in inhibition to bipolar cells between ON and OFF systems are reflected in the inputs to ganglion cells as well: Zaghhloul et al. (2003) showed that in alpha ganglion cells, OFF cells received ON inhibition, whereas ON cells also received ON inhibition. Among other ganglion cell types, delayed canceling interactions have also been seen in a subpopulation of ON ganglion cells (Roska et al. 2006), where the delay serves to truncate the sustained components of excitatory inputs, leading to a more transient output. This interaction appears in ~50% of ON ganglion cell types but is rarely seen in OFF ganglion cells. Similarly, the prevalence of reinforcing inhibition in cone bipolar cells (all OFF cells and ~50% of ON cells) closely matches the presence of a similar interaction in ganglion cells, where ON inhibition appears in all OFF cells, but OFF inhibition is seen in <50% of ON cells (Roska et al. 2006). Remarkably, the inhibitory asymmetries apparent in bipolar cells are apparent in ganglion cells in similar proportions, revealing a general organizational property of the mammalian inner retina.

**Push-pull interactions are prevalent at every stage of the visual system**

Reinforcing interactions, often called push-pull interactions, were by far the most common class of interaction in cone bipolar cells, appearing in every type of OFF cell and in about half of the ON cells, either alone, or in combination with delayed cancellation. This inhibition maintains its complimentary phase relationship with excitation across a wide range of time-scales, indicating that it truly functions to enhance the basic excitatory input rather than shape or suppress it. Push-pull interactions have also been reported in ganglion cells (Arkin and Miller 1988a,b; Belgum et al. 1987; Muller et al. 1988) and in the thalamus and in early visual cortex (Hirsch 2003). Thus ON and OFF pathways appear to cross-inhibit each other at every stage of visual processing, starting in the bipolar cells where the ON and OFF signals are first established.

**GRANTS**

This work was supported by grants from the National Eye Institute, Office of Naval Research, and Defense Advanced Research Projects Agency.

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