Amacrine-to-Amacrine Cell Inhibition in the Rabbit Retina

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Hsueh H-A, Molnar A, Werblin FS. Amacrine-to-amacrine cell inhibition in the rabbit retina. J Neurophysiol 100: 2077–2088, 2008. First published July 30, 2008; doi:10.1152/jn.90417.2008. We studied the interactions between excitation and inhibition in morphologically identified amacrine cells in the light-adapted rabbit retinal slice under patch clamp. The majority of ON amacrine cells received glycineric OFF inhibition. About half of the OFF amacrine cells received glycineric ON inhibition. Neither class received any GABAergic inhibition. A minority of ON, OFF, and ON–OFF amacrine cells received both glycineric ON and GABAergic OFF inhibition. These interactions were found in cells with diverse morphologies having both wide and narrow processes that stratify in single or multiple layers of the inner plexiform layer (IPL). Most ON–OFF amacrine cells received no inhibition and have monostatified processes confined to the middle of the IPL. The most common interaction between amacrine cells that we measured was “crossover inhibition,” where OFF inhibits ON and ON inhibits OFF. Although the morphology of amacrine cells is diverse, the interactions between excitation and inhibition appear to be relatively limited and specific.

INTRODUCTION

Recent studies have elucidated the interactions between bipolar and amacrine cells in the mammalian retina: bipolar cells excite amacrine cells and amacrine cells provide GABAergic and glycineric feedback inhibition to bipolar cells (Eggers and Lukasiewicz 2006; Molnar and Werblin 2007). In other examples, amacrine cells provide feedforward inhibition to ganglion cells (Lukasiewicz and Werblin 1990; Roska and Werblin 2001, 2003), generating a set of about 12 different ganglion cell response forms (Roska et al. 2006). To what extent does the diversity of responses in ganglion cell responses reflect a diversity of amacrine cell activity? Indeed, there are >27 different morphological types of amacrine cells in the mammalian retina (Badea and Nathans 2004; MacNeil et al. 1999), and, in addition to inhibiting bipolar and ganglion cells, amacrine cells interact with one another. Starburst amacrine cells mutually inhibit each other via γ-aminobutyric acid (GABA) (Fried et al. 2005; Lee and Zhou 2006; Taylor and Wässle 1995) and feed forward to ganglion cells. All amacrine cells are electrically coupled to each other and to ON bipolar cells and they inhibit OFF cone bipolar cells via glycine (Strettoi et al. 1990, 1992). Polyaxonal amacrine cells are coupled via gap junctions to other amacrine cells (Völgyi et al. 2001). These amacrine cell studies in mammalian retina, along with a study in salamander retina (Pang et al. 2002), begin to elucidate the interactions between amacrine cells, although much has yet to be learned about amacrine-to-amacrine interactions.

METHODS

Preparation of slices

New Zealand white rabbits (2.5 kg) were anesthetized and killed in accordance with protocols approved by the Office of Laboratory Animal Care at University of California, Berkeley. The eyes were quickly enucleated and placed in physiological saline solution [1.9 g/L sodium bicarbonate (EMD Chemical), 0.05 g/L kanamycin sulfate (Invitrogen), 8.8 g/L Ames powder (Sigma)], and bubbled with 95% O₂-5% CO₂ (Bioblend PraxAir). Each eye was dissected in dim red light by first removing the vitreous then cutting away the periphery to preserve the visual streak, a region about 3 × 4 mm. The visual streak was cut into quarters then stored in the dark in Ames solution, constantly bubbled with Bioblend. Retinas stored in this manner remained light responsive for 6 h. To prepare the retina for patch recording, a chamber with an inlet and outlet was mounted on a slide, then filled with 2 mL Ames solution. One quarter of the visual streak was placed in the chamber, the retina peeled away and mounted, ganglion cell side down, on filter paper (Millipore). The retina was then sectioned into 250-μm-wide slices, turned on its side, and held stationary with silicone grease. A constant perfusion of Ames solution, bubbled with 95% O₂-5% CO₂, was provided to the chamber at a rate of 6 mL/min.

Patch recording

We used the whole cell patch-clamp method to examine the excitatory and inhibitory currents in amacrine cells. Patch pipettes were pulled from thin-walled glass tubes with a filament (1.5-mm diameter, 4 in long; World Precision Instruments) using a pipette puller (Sutter Instruments, Novato, CA). The intracellular solution contained the following (in mM): 112.5 cesium methanesulfonate (Sigma), 0.0078 calcium chloride, 10 HEPES (Fisher), 1.0 magnesium sulfate (Sigma), 0.5 BAPTA (Sigma), 4 ATP (Sigma–Aldrich), 0.5 GTP (Sigma), 5 potassium chloride, and 7.75 Neurobiotin Tracer
of the stimulus. A light bar denotes a light flash; a black bar (in later figures) denotes a dark flash.

The retina was light adapted (the background light level was 2.2 mW/cm²) for 10 min prior to the beginning of each experiment. Amacrine cell somas were identified by their proximity to the distal edge of the IPL. The patch pipette (6- to 8-MΩ resistance) was brought to the soma by visual guidance; negative pressure was applied to achieve a 1.5- to 2.5-GPa seal prior to breaking in with increasing voltage steps (increasing in 50-mV increments from 50 to 250 mV). The correction for junction potential was 5.6 mV. The EPC-7 (HEKA Electronik) patch amplifier was used to voltage- and current-clamp the cell. Excitatory currents were measured by holding the cell at -60 mV; inhibitory currents were measured at a holding potential of 0 mV. The retina was stimulated with a 200-μm stripe stimuli and the full-field stimuli, and/or a full-field light or dark flash for 2.5 s (100% contrast from the background light level of 2.2 mW/cm²). We saw no difference in response between the 200-μm stripe stimuli and the full-field stimuli, so we pooled these responses for our analyses. Current and voltage recordings were digitized and sampled at 10 kHz, as described previously (Molnar and Werblin 2007). All signals were postanalyzed in MATLAB (The MathWorks). Signals were filtered and down-sampled to a 60-Hz sample rate (by averaging over 16.7-ms bins), the same as the update rate of the stimulus. Once cells were dialyzed, no meaningful signals (light responsive or otherwise) were observed above this frequency.

**Defining on and off activity**

In the rest of this report, we will describe the polarity of excitation and inhibition as "ON" or "OFF". In general, ON activity refers to a response at the onset of a light flash (or the termination of a dark flash). OFF activity refers to the response at the offset of a light flash (or the initiation of a dark flash). Our experiments with 2-amino-4-phosphonobutyric acid (APB) show that ON activity is derived from ON bipolar cells and OFF activity is derived from OFF bipolar cells. We label an amacrine cell as ON, OFF, or ON-OFF by the polarity of the peak excitatory response. For instance, an ON amacrine cell is one in which the peak excitatory current occurs at light onset. An ON-OFF amacrine cell has peak excitatory currents at both light onset and offset.

To ensure consistency of our assignment of polarity based on current responses to full-field light and dark flashes, we assigned a polarity metric X, previously described (Molnar and Werblin 2007). Briefly, for each transition from background to light or dark flash, we averaged the current 600 ms prior to the flash and the 600 ms after the flash, and took the difference of the two averages. For each cell, four numbers are computed in this manner, each representing the change in current: from background to dark (a), dark to background (b), background to light (c), and light to background (d). The polarity metric was defined as follows: X = -(a + b + c - d)/(a + b + c + d). Herein, a response whose polarity metric X = -1 is an OFF response, X = +1 is an ON response, and -1 < X < +1 is an ON-OFF response (see Figs. 1B and 4B). Histograms shown in Figs. 1B and 4B show X for every single amacrine cell we recorded from. Data reported in pie charts (Figs. 1A and 4A) are cells whose responses remained robust for the entire duration of the experiment and thus are a subset of the cells represented in the histograms.

**Pharmacological studies**

After recordings under control conditions, we applied 10 μM strychnine to block glycine receptors (Molnar and Werblin 2007; Rotolo and Dacheux 2003), 100 μM picrotoxin to block GABA<sub>A</sub> and GABA<sub>C</sub> receptors (Molnar and Werblin 2007; Roska and Werblin 2001; Rotolo and Dacheux 2003), and 5 μM 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl) pyridazinium bromide (SR95531, gabazine) to block GABA<sub>A</sub> receptors (Roska and Werblin 2001). We also used 20 μM APB to block metabotropic glutamate receptor 6 (mGluR6)-mediated responses from ON bipolar cells (Slaughter and Miller 1983). Each drug was washed in for 3 min before recording light responses and washed out for 5 min (with the exception of strychnine, which did not wash out). The flow rate of Ames solution was 6 mL/min. Pharmacological studies were done on morphologically diverse amacrine cell types.

Studies have shown that at commonly used concentrations, some GABA-receptor antagonists can also block glycine receptors (Wang and Slaughter 2005). Our experiments show that a response blocked by strychnine is not blocked by either 5 μM SR95531 or 100 μM picrotoxin, which means that the GABA-receptor antagonists are not acting significantly on glycine receptors. In one study, 10 μM strychnine was applied to dialyze the retina; no change was observed in the ON-OFF amacrine cell responses to light and dark flashes.

**FIG. 1.** The majority of ON amacrine cells receive OFF inhibition. A: distribution of inhibition recorded in 153 ON amacrine cells derived from the histogram in B. Some of these ON cells were AII amacrine cells (34/153) that have been extensively described elsewhere and which we exclude from the analysis here. The majority of ON amacrine cells received OFF inhibition (90/119). Of the remaining ON cells, some received ON-OFF inhibition (16/29), others ON inhibition (4/29), and a few did not receive any inhibition (9/29). B: histogram of the polarity coefficient X for the inhibitory current responses to flash stimuli (see METHODS). X = -1 represents a pure OFF response and X = +1 represents a pure ON response. Almost all ON amacrine cells received OFF inhibition, with few other inhibitory interactions. C, left column: an example trace of excitatory current, measured by clamping the cell at -60 mV, is inward at light ON. Center column: inhibitory current for the same cell, measured by clamping at 0 mV, is outward at light OFF. Right column: voltage, elicited by the ON inward current and the OFF outward current, is depolarizing at ON and hyperpolarizing at OFF. In this and subsequent figures, the bar at the top of each trace represents the time course (2.5-s flash) of the stimulus. A light bar denotes a light flash; a black bar (in later figures) denotes a dark flash.
nine was shown to block GABA<sub>R</sub> receptors in neonatal rat brain stem hypoglossal motoneurons (O’Brien and Berger 1999), although in other studies, 10 μM strychnine did not affect GABA currents measured in OFF-alpha ganglion cells in the rabbit (Rotolo and DaCheux 2003). Our experiments using both drugs on single cells show that a response blocked by 5 μM SR95531 is not blocked by 10 μM strychnine and that we are able to selectively block either GABergic or glycineric inhibition at these concentrations.

To determine whether a pharmacological agent significantly influenced the physiological response, we calculated the response magnitude to each transition of light and dark flashes, as described earlier. We then compared the magnitude of the light response in control conditions with the magnitude of the response in drug conditions. For each cell tested, we computed the percentage change in the magnitude of the response that accompanies the application of the drug, normalized relative to the magnitude of the response in control conditions. A complete block yields a percentage change of 100%, and in some cases where the response inverts its polarity, changes >100% were measured. Then we computed the mean change (m<sub>d</sub>) and SE changes (s<sub>m</sub>) to obtain the test variable t = m<sub>d</sub>/s<sub>m</sub>. Using the paired t-test (Glantz 2005), we determined the drug to have a significant effect if the value of t exceeded the critical value for v = n − 1 degrees of freedom (where n = number of cells tested) for P = 0.05. If t was less than or equal to the critical value for P = 0.05 for v degrees of freedom, we accepted the null hypothesis of no effect, concluding that the drug had no significant effect on the measured light response. Results of statistical analyses are reported in the following format: sample size, mean percentage change in the light response due to the pharmacological blocker ± SE, P value.

Imaging the recorded cells

At the completion of electrophysiological measurements, we imaged cell morphology using Alexa Fluor 488 (excitation: 488 nm; emission: 519 nm), which we had included in the patch pipette, taking confocal z-stacks (Improvision Grid Confocal; images acquired and processed using Volocity software). For clearer presentation, faint dendrites were traced and contrast was adjusted using Adobe Photoshop (see Fig. 10). This allowed us to determine the stratification and other morphological characteristics of the recorded cell. We were unable to acquire quality images of all the cells from which we recorded due to poor dye diffusion and/or glare from the patch electrode. Two morphological parameters were quantified based on raw images (without contrast enhancement): the spread of the processes and their stratification. Narrow cells are those whose processes extend <100 μm. Medium-width cells have processes that extend between 100 and 200 μm. Wide cells are those whose processes extend >200 μm. These definitions are smaller than the definitions laid out by other morphological studies (Badea and Nathans 2004; MacNeil et al. 1999) because the retina slices were 250 μm thick; any morphology beyond 200 μm would be truncated and hard to visualize. Furthermore, we were limited to imaging processes that are within 20 μm of the slice surface. The IPL is divided into five sublamina and, for each cell, the sublamina in which their processes extended was measured. The distal layers 1 and 2 constitute the functional OFF sublamina (Bloomfield and Miller 1986; Kolb and Famiglietti 1974; Strettoi et al. 1990) and the proximal layers 3, 4, and 5 constitute the ON sublamina. The spread of the processes and their stratification allowed us to distinguish between different morphological types of amacrine cells.

RESULTS

We recorded from 292 amacrine cells in the retinal slice in response to 2.5-s full-field light or dark flashes. The main forms of interaction between excitation and inhibition for the majority of these cells are described in the following text.

on amacrine cells receive off inhibition

We recorded from 153 amacrine cells that responded with an inward excitatory current at light ON but not at light OFF. Of these ON cells, 34 of them were morphologically identified as AII amacrine cells (Wässle et al. 1993), which we excluded from this analysis. As shown in Fig. 1A and B, the majority (90/119 excluding AII amacrine cells) of ON amacrine cells received OFF inhibition. For these cells, both the excitatory and inhibitory currents, as well as voltage response, are shown in Fig. 1C. Typically, excitatory (inward) currents were greater at light ON than at light OFF, whereas the inhibitory (outward) currents were greater at light OFF than at light ON. The summation of these currents across the cell membrane yielded a voltage response that was more symmetrical than either of the currents: this voltage showed about equal but opposite magnitude response at light ON and OFF, as shown in Fig. 1C.

We confirmed that ON amacrine cells received excitation from ON bipolar cells by blocking OFF activity with APB, a highly specific glutamate agonist at the mGluR6 receptors of ON bipolar cells (Slaughter and Miller 1981). Figure 2B shows that in the presence of 20 μM APB, the ON excitatory current was blocked (n = 6, 101.05 ± 9.26%, mean percentage change in current amplitude ± SE, P < 0.001, paired t-test) but that the OFF inhibitory current was not significantly affected (n = 6, 38.16 ± 33.81%, P > 0.2). This demonstrates that this inhibition likely originates from the OFF pathway and not the ON pathway. We call this OFF inhibition to ON amacrine cells “crossover” inhibition.

FIG. 2. 2-Amino-4-phosphonobutyric acid (APB) blocks ON excitation, but not OFF inhibition. Current traces measured from an ON amacrine cell that received OFF inhibition. A: ON excitatory current in control conditions. B: 20 μM APB blocks ON excitatory current. C: APB wash shows recovery of ON excitatory current. D: OFF inhibitory current in control conditions. E: OFF inhibitory current persists in APB, demonstrating that inhibition originates from the OFF pathway and excitation originates from the ON pathway. F: APB wash step.
Since these cells were encountered so rarely, we were able to unaffected by either 5 \( \mu M \) strychnine. Current traces measured from another ON amacrine cell that received OFF inhibition. C: OFF inhibitory current in control conditions. D: OFF inhibitory current is not significantly blocked by SR95531.

Figure 3B shows that OFF inhibition was suppressed by 10 \( \mu M \) strychnine, an antagonist for glycine receptors \((n = 18, 75.02 \pm 10.89\%\), \( P < 0.001\), paired \( t \)-test) but was relatively unaffected by either 5 \( \mu M \) SR95531, an antagonist for GABA\(_A\) receptors, or 100 \( \mu M \) picrotoxin, an antagonist for both GABA\(_A\) and GABA\(_C\) receptors \((n = 12, 42.58 \pm 22.67\%\), \( P < 0.1\), Fig. 3D). This suggests that OFF inhibition to ON cells is mediated primarily by glycine and not by GABA.

As shown in Fig. 1A, 4/119 ON cells received ON inhibition. Since these cells were encountered so rarely, we were able to do only two experiments that showed that the ON inhibition was suppressed with picrotoxin \((n = 2, 57.23\) and 83.31\%).

**OFF amacrine cells receive ON inhibition**

We recorded from 86 amacrine cells that responded with an inward excitatory current at light OFF but not at light ON. As shown in Fig. 4, A and B, almost half of OFF cells received purely ON inhibition. Figure 4C shows an example of the response currents of these OFF cells. For these cells, the excitatory current was greater at light OFF than that at light ON, but the inhibitory current was greater at light ON than that at light OFF. When these two currents summed across the membrane, the voltage response was more symmetrical than either current, with relatively equal depolarizing and hyperpolarizing phases.

We used APB to show that this ON inhibition is derived from the ON pathway, initiated by ON bipolar cells. In the presence of APB, the ON inhibition was strongly blocked \((n = 6, 102.05 \pm 14.23\%\), \( P < 0.001\), Fig. 5E), whereas the OFF excitation was only reduced \((n = 5, 59.50 \pm 7.63\%\), \( P < 0.01\), Fig. 5B). In the wash step, the ON inhibition was restored \((-47.73 \pm 15.60\%\), Fig. 5F), but the OFF excitation was not \((0.013 \pm 17.42\%\), Fig. 5C). Throughout the course of the experiment, OFF excitation showed a reduction in response, but since this effect did not recover under wash, it is likely due to the light response running down over time, and not an effect of APB on the OFF pathway.

The ON inhibition was suppressed in the presence of strychnine \((n = 11, 67.00 \pm 8.55\%\), \( P < 0.002\)) but was relatively unaffected by either SR95531 or picrotoxin \((n = 11, 13.66 \pm 39.00\%\), \( P > 0.5\)) as shown in Fig. 6, B and D, respectively. This suggests that the crossover inhibition to OFF cells, like the crossover inhibition to ON cells, is mediated primarily by glycine and not by GABA.

As shown in Fig. 4A, a subset of OFF cells (15/86) received OFF inhibition. We were able to test six of these cells with pharmacological blockers. In five cases, the OFF inhibition to OFF cells was blocked by SR95531 \((121.05 \pm 6.40\%\), \( P < 0.001\)). In two cases, strychnine did not fully suppress the OFF inhibition \((45.41\) and 72.78\%). This includes a case where 10 \( \mu M \) strychnine did not block the OFF inhibition, but 5 \( \mu M \) SR95531 did. Taken together with the limited pharmacology of ON cells with ON inhibition, these results suggest that inhibition within a functional sublamina (e.g., ON cells that receive ON inhibition, OFF cells that receive OFF inhibition) is mediated by GABA. A recent study in bipolar cell inhibition (Molnar and Werblin 2007) found that GABA mediates inhibition within a sublamina. Our pharmacological experiments suggest that both ON and OFF amacrine cells adhere to this convention.

**FIG. 4. Many OFF amacrine cells receive ON inhibition.** A: distribution of inhibition recorded in 86 OFF cells derived from the histogram shown in B. About half (38/86) of the OFF amacrine cells received a crossover ON inhibition. Of the remaining 48 OFF cells, some received ON–OFF inhibition (24/48), OFF inhibition (15/48), or no inhibition at all (9/48). B: histogram of polarity coefficient \( X \) for the inhibitory current to OFF amacrine cells (see METHODS). \( X = -1 \) represents a pure OFF response and \( X = +1 \) represents a pure ON response. Most OFF amacrine cells received ON inhibition, although there is a greater diversity of inhibitory interactions than for ON cells. C: sample traces measured from a single amacrine cell. Left column: excitatory current is inward at light OFF. Center column: inhibitory current is outward at light ON. Right column: voltage is depolarizing at light OFF and hyperpolarizing at light ON. A dark bar indicates a dark flash in this figure and in Figs. 5–7.
Majority of ON–OFF amacrine cells receive little or no inhibition

We recorded from 53 ON–OFF amacrine cells, which received excitatory input at both light ON and OFF as shown in Fig. 7. For the majority of ON–OFF cells (30/53 shown in Fig. 7A), we observed no evidence of any outward inhibitory current when we held the cell membrane at 0 mV, as shown in the right traces of Fig. 7, B and C. Staining with Alexa Fluor 488 showed that this type of ON–OFF amacrine cell was monostratified, with some cells showing widely ramifying processes in the IPL. It seems likely that these monostratified amacrine cells are wide (MacNeil et al. 1999) and it is typically difficult to voltage-clamp cells with wide processes. In some cases, we were able to effectively voltage-clamp these ON–OFF cells (shown in Fig. 7B, right trace), showing clearly that there is little or no inhibitory input. In other cases, we could not adequately voltage-clamp the cell and we measured ON–OFF inward currents, even though we were holding the cell at 0 mV, as shown in the right trace of Fig. 7C. The inward currents measured at 0 mV suggested that this cell may not be adequately space-clamped and there may be a subtle inhibitory input masked by the residual inward current.

If there were a coincident outward inhibitory component superimposed on the inward currents we would expect to see an increase in inward current in the presence of inhibitory blockers. To test for a masked inhibition we measured whether inhibitory blockers could increase the magnitude of the re-
FIG. 8. ON–OFF amacrine cells receive no inhibition. Current traces measured from an ON–OFF cell: A: ON–OFF inward currents measured when holding the cell at 0 mV. B: ON–OFF inward currents are not increased in the presence of 100 μM picrotoxin. Current traces measured from another ON–OFF cell. C: ON–OFF inward currents measured when holding the cell at 0 mV. D: ON–OFF inward currents are not increased in the presence of strychnine. These results indicate that this cell type receives no significant inhibitory input and the inward currents are likely the result of inadequate space clamp.

A subset of ON–OFF amacrine cells received OFF inhibition (5/53), that was blocked by 100 μM picrotoxin (n = 3, 138.29 ± 43.65%). The morphology of these cells was very similar and they are likely of the same type: narrowly monosтратified in the OFF sublamina (and thus likely wide-field; MacNeil et al. 1999), in layer S2, as shown in Fig. 11G. These cells depolarized at the onset and offset of a light flash and the response was very transient. The physiological and morphological characteristics suggest that these may be analogs to the A19 amacrine cells measured in cat retina (Freed et al. 1996).

Amacrine cells that receive ON–OFF inhibition

We recorded from 49 amacrine cells that received ON–OFF inhibition. Example inhibitory traces are shown in Fig. 9, A and C. Of these amacrine cells, 16 received ON excitation, 24 received OFF excitation, and 9 received ON–OFF excitation. We were able to characterize the pharmacology of 22 of these cells. In a few cases (3/22), the ON–OFF inhibition was blocked by strychnine (n = 3, ON phase: 85.27 ± 84.45%; OFF phase: 92.80 ± 54.54%). Surprisingly, for most of these cells (19/22), we found that the ON and OFF phases of inhibition were mediated by different transmitters, regardless of the polarity of their excitation. When we applied strychnine, the ON phase of the inhibition was blocked but the OFF phase persisted, shown in Fig. 9B (n = 12; ON phase: 90.77 ± 20.30%, P < 0.001; OFF phase: −9.93 ± 56.20%, P > 0.5). When we applied SR95531, the OFF phase of the inhibition was mostly blocked, but the ON phase persisted, shown in Fig. 9D (n = 10; OFF phase: 81.15 ± 36.24%, P < 0.05; ON phase: 9.56 ± 87.62%, P > 0.5).

The ON–OFF inhibition in these amacrine cells does not derive from a single class of amacrine cell, but rather from two distinct classes: the ON inhibition is derived from a glycinergic amacrine cell class and the OFF inhibition from a GABAergic amacrine cell class. It was fortuitous that the two amacrine cell inputs were mediated by different neurotransmitters: if both inputs used the same transmitter, we would not have been able to discern the separate sources of inhibition at ON and OFF, as in the case of the cells with glycinergic ON–OFF inhibition.

FIG. 9. Some amacrine cells receive glycinergic ON and GABAergic OFF inhibition. Current traces measured from an amacrine cell that received ON–OFF inhibition: A: ON–OFF inhibition in control conditions. B: the ON phase of inhibition was blocked by strychnine, whereas the OFF phase persisted. Current traces measured from another amacrine cell that received ON–OFF inhibition. C: ON–OFF inhibition in control conditions. D: OFF phase of inhibition is blocked by SR95531, whereas the ON phase persists.
Taken together, these results suggest that the majority of amacrine cells were not inhibited by the ON–OFF amacrine cells we measured in Fig. 7.

**All amacrine cells**

We were able to identify all amacrine cells by their morphology and physiology (n = 34). They responded by depolarizing at light onset and hyperpolarizing at light offset, as previously reported (Kolb 1997; Xin and Bloomfield 1999). All amacrine cells have been extensively studied elsewhere (Bloomfield and Xin 2000; Bloomfield et al. 1997; Boos et al. 1993; Famiglietti and Kolb 1975; Gill et al. 2006; Manookin et al. 2008; Mørkve et al. 2002; Wässle et al. 1995) and we excluded them from the analyses in our study.

**Morphology of recorded amacrine cells is diverse**

Examples of images obtained from 158 physiologically studied amacrine cells are shown in Fig. 10. ON amacrine cells that received crossover OFF inhibition included cells with dendritic spreads that varied from narrow to wide, shown in Fig. 11A. These amacrine cells appeared in monostatified, bistratified, and diffusely stratified forms, extending to every sublamina of the retina, as shown in the left column of Fig. 10A. OFF cells that received ON inhibition had both narrow and wide processes, shown in Fig. 11B, that stratified primarily in the distal OFF sublamina (layers S1 and S2 and the distal part of S3), seen in Fig. 10B. These studies suggest that glycineergic crossover inhibition appears to span a diversity of amacrine cell morphologies. This is also consistent with earlier studies that found glycine receptors on both narrow and wide mammalian amacrine cells processes (Heinze et al. 2007; Veruk et al. 2007).

ON–OFF amacrine cells that did not receive inhibition were monostatified, with their processes largely confined to layers S2 and S3 of the IPL, as shown in Fig. 10C. The dendritic spread reported in Fig. 11C is likely a lower bound estimate on the actual spread of these cells, since slicing the retina often destroys the processes closest to the cut. There were cases in which we could see the processes extend for hundreds of microns across the slice, but were unable to obtain brightly stained images of them. Nevertheless, abundant morphological studies have shown that wide amacrine cells are usually monostatified (Badea and Nathans 2004; MacNeil et al. 1999), which leads us to believe that these ON–OFF amacrine cells are likely to be wide-field as well. In contrast with the amacrine cells that receive inhibition, it appears that these ON–OFF cells represent a limited morphological subset of amacrine cells.

Included in this broad class of ON–OFF cells were a set of cells (n = 8) whose somas resided interstitially in the IPL in S3. Where the processes were imaged (n = 4), these cells were consistently wide-field, monostatified in S3, and showed unusually thick processes. It is also noteworthy that these cells showed clear dye coupling (n = 3) of both Alexa Fluor 488 and Neurobiotin to additional interstitial cell bodies, typically about 200 μm away, suggesting that this subtype formed a distinct electrically coupled network. This unusual morphology has been reported elsewhere (Famiglietti 1992; Völgyi et al. 2001; Wright and Vaney 2004) as a subset of the type I polynodal amacrine cell.

Amacrine cells that received ON–OFF inhibition had processes that were mostly confined to the OFF layer, or that spanned both the ON and OFF layers, as shown in Figs. 10D and 11D. The unshaded cells in Fig. 11D received ON excitation and the shaded cells received OFF excitation. The ON cells in Fig. 11D stratified throughout the IPL, but the OFF cells had most of its processes in the OFF sublamina. Although these cells varied in their dendritic width, they were mostly narrow-field. This correlates with morphological studies showing that amacrine cells with diffusely stratified processes usually have smaller dendritic fields (Badea and Nathans 2004; MacNeil et al. 1999). From the variability in stratification and dendritic spread, it appears that ON–OFF inhibition impinges on multiple morphological types of amacrine cells.

OFF amacrine cells that received OFF inhibition had most of their processes in the OFF sublamina, as shown in Fig. 11E. The majority of these cells were monostratified or bistratified. It would appear that these OFF cells with OFF inhibition were not of a single morphological class.

**DISCUSSION**

We describe three predominant forms of interaction between excitation and inhibition measured in amacrine cells in the light-adapted retina, as summarized in Fig. 12: ON amacrine cells receive OFF inhibition, shown in Fig. 12A, whereas OFF amacrine cells receive ON inhibition, shown in Fig. 12B. These crossover inhibitory signals are carried by glycine. The processes of most OFF amacrine cells are confined to the OFF sublamina, so most of the crossover interactions between the ON and OFF amacrine cells must occur within the OFF sublamina where the processes of ON and OFF cells are in close proximity. This is consistent with earlier findings that glycine receptors with different subunits exist in bands throughout the entire IPL and that the distal layers contain the highest density of glycine receptors of all types (Heinze et al. 2007). Other studies that examined the uptake of glycine in retina showed that glycine accumulates in layers 2 and 3 of the IPL (Pourcho 1980).

Most amacrine cells with monostatified processes received ON–OFF excitatory input, but no measurable inhibitory input, shown in Fig. 12C. ON–OFF inhibition to amacrine cells was generally not mediated by a single ON–OFF amacrine cell type, but by an ON glycineergic amacrine cell and an OFF GABAergic cell, as shown in Fig. 12D. ON–OFF inhibition was found in a variety of amacrine cell types. Overall, these forms of excitation/inhibition interaction account for >70% of the interactions recorded in amacrine cells, excluding all amacrine cell interactions.

**Crossover inhibition facilitates excitation**

We measured crossover inhibition in about 50% of the 292 amacrine cells studied in the light-adapted retina. By convention, we designated the measured currents as “inhibitory” because they were carried by glycine, an inhibitory transmitter, and glycine increases chloride conductance, also characteristic of inhibition. However, the functional role of this signal is not antagonistic, but instead serves to enhance excitation: These crossover inhibitory currents drive membrane voltage with the same polarity as excitation: the “inhibitory” currents become less positive when excitation becomes more negative and they become more positive when excitation becomes less negative. Although these currents are pharmacologically and ionically...
inhibitory, in these cases “inhibition” serves to enhance, rather than suppress or oppose, excitation.

**ON–OFF inhibition in amacrine cells is distinct from ON–OFF inhibition in ganglion cells**

ON–OFF inhibition to ganglion cells has been shown to be GABAergic and wide-field (Roska and Werblin 2003). In this study, we have measured from ON–OFF amacrine cells that are monostratified—and we suspect that they may be the amacrine cell class that provides feedforward inhibition to ganglion cells. Unfortunately, in retina slice we cannot verify that these ON–OFF cells are indeed wide-field, but if they were, they could potentially be the cells that provide GABAergic ON–OFF inhibition to ganglion cells during rapid scene shifts (Roska and

**FIG. 10.** Multiple morphological types receive these general forms of inhibition. These are sample tracings of compressed grid confocal z-stacks of the cells we measured from. The dotted guidelines delineate the borders of the inner plexiform layer (IPL). For each interaction, there are multiple types of amacrine cells. The histograms represent the number of cells whose processes terminate in each of the five layers in the IPL. A: ON cells that receive OFF inhibition have processes that terminate throughout the entire depth of the IPL, with most of the processes terminating in the ON sublamina (layers 4, 5, and proximal region of layer 3). B: OFF cells that receive ON inhibition have processes that terminate in the OFF sublamina (layers 1 and 2 and the distal region of layer 3). C: ON–OFF cells that do not receive any inhibition have processes that terminate in the middle of the IPL (layer 3), along the border between the functional OFF and ON sublamina. D: cells that receive ON–OFF inhibition have processes that terminate throughout the entire IPL, indicating that many different types of amacrine cells receive glycinergic ON and GABAergic OFF inhibition.
Werblin 2003). ON–OFF inhibition to amacrine cells, however, does not arise in the same way as the ON–OFF inhibition to ganglion cells. We have shown that the majority of ON–OFF inhibition to amacrine cells is mediated by an ON glycinergic amacrine cell and an OFF GABAergic amacrine cell.

It has been suggested that in the mammalian retina, glycinergic amacrine cells tend to be narrow-field and are involved in local interactions between ON and OFF sublamina (Menger et al. 1998; Weiss et al. 2008). We have found this to be true in our study: OFF inhibition to ON cells and ON inhibition to OFF cells are both glycinergic. There is also evidence that in mammalian retina, GABAergic amacrine cells are often wide-field and are involved in lateral interactions within a given sublamina (Majumdar et al. 2008). Based on these precedents, ON–OFF inhibition to amacrine cells could merely be a combination of these two forms of interactions. For the off amacrine cells that receive ON–OFF inhibition, these rules are consistent: glycine mediates a cross-lamina ON inhibition and GABA mediates a lateral OFF inhibition. However, this schema is inconsistent with the pharmacology of ON and ON–OFF amacrine cells that receive ON–OFF inhibition. For an ON cell that receives ON–OFF inhibition, we would expect GABA to mediate the lateral ON inhibition and glycine to mediate the cross-lamina OFF inhibition. Our experiments show that this is not the case. Regardless of polarity of the excitation of an amacrine cell that receives ON–OFF inhibition, the ON phase is always mediated by glycine and the OFF phase is always mediated by GABA. It seems these amacrine cells represent a special subclass that may be in-

**FIG. 11.** A diversity of amacrine cells receive these general forms of inhibition. Each column represents the morphology of a recorded amacrine cell: the location of the bar(s) along the vertical axis indicates the level of stratification and the bar length represents the width of the cell’s processes. A: ON cells that received OFF inhibition were of diverse types and their processes extended throughout the entire IPL. The width of the cells varied from <100 to >200 μm. B: OFF cells that received ON inhibition also show a diversity in stratification, although the stratification was largely limited to the OFF sublamina. The width of the cells varied from narrow to wide. C: ON–OFF cells that do not receive inhibition are typically monostratified in layer 3. The images show varying lateral spread, although this is likely an underestimate of the actual dendritic spread. D: cells that receive ON–OFF inhibition frequently span multiple sublamina and also vary in the width of their processes. The unshaded cells received ON excitation and the shaded cells received OFF excitation. E: cells that receive OFF excitation and OFF inhibition have processes that stratify in the OFF sublamina. They may represent a few subclasses of amacrine cells. F: ON–OFF cells that received ON inhibition primarily stratify in layers S2 and S3 and have narrow to medium dendritic spread. G: ON–OFF cells that receive OFF inhibition have processes that stratify primarily in S2. This physiology and morphology suggest that these cells may be analogs to the A19 amacrine cells found in cat retina (Kolb 1997).
involved in specific processing, separate and pharmacologically distinct from the rules that seem to apply to other amacrine cells.

The distinct pharmacology of ON–OFF inhibition in amacrine cells also raises the question of spatial processing. Glycinergic interactions are likely involved in local processing, whereas GABAergic interactions are postulated to mediate interactions across larger regions of the retina. We would expect that each phase of ON–OFF inhibition in these amacrine cells would take on different spatial properties as well, with ON inhibition generated by a narrow amacrine cell, and OFF inhibition is generated by a wide, likely monostratified, amacrine cell. Further measurements of amacrine cells in whole mount would be required to examine this hypothesis.

Asymmetries between the ON and OFF channels in the inner retina

Almost all ON amacrine cells receive purely OFF inhibition, whereas only about half of OFF amacrine cells receive pure ON inhibition. Furthermore, almost one half of OFF amacrine cells receive OFF inhibition (either alone or in combination with ON inhibition). In contrast, <20% of (non-AII) ON amacrine cells receive ON inhibition. This points to an asymmetry where OFF inhibition is more common than ON inhibition. This asymmetry is exactly contrary to the interaction between the ON and OFF pathways in bipolar and ganglion cells. In bipolar cells, only about half of ON bipolar cells receive OFF inhibition, whereas almost all OFF bipolar cells and half of ON bipolar cells receive ON inhibition (Molnar and Werblin 2007). Similarly in ganglion cells, few ON cells receive OFF inhibition, whereas most OFF cells receive ON inhibition (Roska et al. 2006). For bipolar and ganglion cells, ON inhibition is more common than OFF inhibition.

This reversed asymmetry may have a functional rationale: OFF ganglion cells receive the majority of crossover compensation from the ON amacrine cells. This requires that the ON amacrine cells themselves be compensated by OFF inhibition. The majority of ON amacrine cells receive OFF crossover inhibition so that they can compensate the OFF bipolar and ganglion cells with ON inhibition. The pathways that mediate this predominant interaction are outlined in Fig. 13.

Other studies identify additional asymmetries between ON and OFF pathways in the retina. In salamander retina, it has been shown that there is a bias in synaptic circuitry toward ON channels (Pang et al. 2002). In guinea pig retina, ON brisk transient (Y) cells exhibit contrast sensitivity differently from OFF brisk transient (Y) cells (Zaghloul et al. 2003). It has been shown in rabbit retina that there is a clear difference in the gap-junction coupling in ON and OFF alpha ganglion cells (Völgyi et al. 2005). Very recent work on a class of wide-field ON–OFF amacrine cells reveals that they have different spatial properties: the ON phase of the response has a larger receptive field than that of the OFF phase (Bloomfield and Völgyi 2007). The role of these striking asymmetries in processing the visual signal is not yet fully understood.

Slicing the retina may truncate extended spatial processing

Measuring from amacrine cells in slice allows us to carefully examine local inhibitory circuits and interactions, but we exclude the circuitry formed by neuronal processes that are >250 μm away from the cell of interest. This limits our ability to measure broader spatial interactions between amacrine cells, especially surround inhibition. The process width of ganglion cell dendrites varies from narrow for the local edge detectors (150 μm) to wide for alpha cells (~1 mm) (Peichl et al. 1987; Rockhill et al. 2002). These dendrites receive input from a variety of amacrine cells, integrated in space, giving rise to nuanced light responses. Temporal diversity also exists due to the variable time course of different types of neurotransmitter receptors. As an example, consider the different subunits of glycine receptors. Glycine receptors consisting of the GlyRα1 subunit display fast kinetics, whereas receptors with GlyRα2 are slow by comparison (Weiss et al. 2008). Even among the different subunits that constitute the receptor for a given neurotransmitter, there is temporal variety. The light stimulus used in this study was a simple probe to characterize the basic interactions between amacrine cells in the light-adapted retina to a first approximation and may overlook interactions that can...
be revealed only with a more tailored stimulus. Even though this study does not address these sources of spatial and temporal diversity, we have shown the fundamental wiring diagram on which these more nuanced inhibitory responses are formed.

**ON–OFF amacrine cells that receive OFF inhibition may be A19 amacrine cells**

An amacrine cell type that depolarizes at ON and OFF and has a similar morphology has been reported previously and identified as a wide-field A19 amacrine cell (Freed et al. 1996; Kolb 1997). The A19 synaptic structure suggests that it receives excitatory input from OFF-center cb2 and ON-center cb5 bipolar cells, yet it has a significant inhibitory input from possibly OFF-center A2 amacrine cells (not to be confused with ALL amacrine cells, which are ON-center cells). Furthermore, A2 cells are believed to contain GABA (Pourcho and Goebel 1983). Our data confirm that this amacrine cell type does receive ON–OFF excitation from bipolar cells and that it does receive GABAergic OFF inhibition. The question is then directed at the function of the OFF inhibition, since the cell responds by depolarizing at ON and OFF.

**Comparing rabbit and salamander amacrine cells**

An earlier study (Pang et al. 2002) characterized the inhibitory inputs to salamander amacrine cells. Some similarities exist: like rabbit, salamander OFF amacrine cells receive ON inhibition and ON amacrine cells receive OFF inhibition. ON–OFF inhibition exists in multiple strata in the IPL. However, there are many differences: in salamander, ON–OFF amacrine cells are diffusely stratified, whereas in rabbit, ON–OFF cells are monosynaptically near the middle of the IPL. Almost all OFF amacrine cells in salamander receive ON inhibition and almost NO ON amacrine cells receive OFF inhibition. In the rabbit, this relationship is reversed: just half of the OFF cells get ON inhibition and almost all ON cells get OFF inhibition. There also appears to be a prevalence of ON–OFF inhibition in salamander (about half the strata in the IPL carry ON–OFF inhibition), whereas in rabbit, ON–OFF inhibition is less common. A given salamander amacrine cell integrates inhibition from every layer in the IPL that its processes pass through, but rabbit amacrine cells do not appear to integrate inhibition from every layer. For example, a vertically oriented amacrine cell in salamander whose processes lie in both ON and OFF layers will receive ON–OFF inhibition. Most vertically oriented amacrine cells in rabbit receive only OFF or ON inhibition. It appears that rabbit amacrine cell processes span many layers diffusely, but their inputs are specific and selective, whereas salamander amacrine cell processes lie in selected layers, but receive diffuse inputs. The different pharmacological basis for the ON and OFF components of inhibition was not studied in salamander.

**Crossover inhibition exists at higher visual areas**

Crossover inhibition is the most common inhibitory interaction that we measured between amacrine cells. Additionally, crossover inhibition has been also observed in ganglion cells (Ikeda and Sheardown 1983; Roska et al. 2006; Sterling 1983) and bipolar cells (Molnar and Werblin 2007). This interaction appears to be present at all levels of processing in the inner retina. Push–pull activity has also been measured in cortex, suggesting a similar form of crossover inhibition (Anderson et al. 2000; Hirsch 2003; Lauritzen and Miller 2003). Crossover inhibition appears to be a common and repeated circuit motif, appearing at each level of visual processing.

**References**


