Title

The Temporal and Spatial Characteristics of Amacrine-to-Amacrine Inhibition: Mapping Amacrine Cell Circuitry With Electrophysiology, Pharmacology, Morphology and Immunocytochemistry

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The Temporal and Spatial Characteristics of Amacrine-to-Amacrine Inhibition: Mapping Amacrine Cell Circuitry With Electrophysiology, Pharmacology, Morphology and Immunocytochemistry

by

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DISSERTATION

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[redacted]

A Note of Appreciation

This dissertation would have been a figment of my imagination without the help of the individuals I had the good fortune of working with at Berkeley. First, I'm grateful to my advisor Frank Werblin, for patiently explaining the basics of electrophysiology and neuroscience to a naïve engineer, and a female one at that! From him, I also learned how to give clear presentations, and to write concisely (unfortunately, this note isn't going to be an example of that). Tuesday morning lab meetings were a weekly source of stress, but they provided the backbone upon which ARVO presentations were built. I wish him many more years of fruitful research, and am looking forward to reading the latest findings out of the Werblin lab (especially the work on local edge detectors!). Next, I had the awe-inspiring experience of collaborating with Alyosha Molnar, who is now an assistant professor at Cornell University. He taught me everything I know about making retinal slices, and how to get those fickle inner retinal neurons to reveal just what other neurons they were listening to, one sinusoidal light stimulus at a time. A twenty minute coffee break to Yali's with Al usually yielded enough intellectual fodder for three or four months. Before Al, I worked with Shelley Fried, who introduced me to the challenge of patch clamping ganglion cells, and got me up to speed in retinal prosthetics. I also thank him for dragging me to each and every conference he attended, and abandoning me at our poster so that I could get the experience of explaining our work to other researchers. He was an infinite reservoir of encouragement and confidence, which was just what I needed as an insecure, novice researcher. I would not have lasted even a year in retinal research without Shelley and Al. Furthermore, the whole "locked-in-a-dark-room-with-a-patch-electrode" experience would have been far less interesting without the other members of

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On a more personal note, I absolutely must thank my husband Will, for putting up with my murderous mood after a long day of patching amacrine cells. The audience at my ARVO talks, and the readers of the work I have published have all witnessed his work: the refining and beautification of my MATLAB generated figures. Every single figure and/or slide had to pass his stringent standard of clarity and aesthetics before I could present it to the public. Graduate school was not only hard on me, but it was hard on him, which spurred him to remind me everytime I was having fun, *"Aren't you supposed to be writing your thesis?!"* Yes, I am, and now here it is. Without his persistent reminders, I may have ended up as an ABD student.

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Last, but not least, thank God for blessing me with all these people, and for everything I am today.

Abstract

We studied the interactions between excitation and inhibition in morphologically-identified amacrine cells in the light-adapted rabbit retinal slice and wholemount using the patch clamp technique with pharmacology.

We found that the majority of ON amacrine cells received glycinergic OFF inhibition. About half of the OFF amacrine cells receive glycinergic ON inhibition. By mapping the receptive field of inhibition, we found that this glycinergic inhibition was typically smaller than the receptive field of excitation. In retinal slice, a minority of ON, OFF, ON-OFF amacrine cells received both glycinergic ON and GABAergic OFF inhibition. In retinal wholemount, ON-OFF inhibition was found to be glyceringic. These interactions were found in cells with diverse morphologies (wide and narrow cells, with either monostratified or diffuse processes). Most ON-OFF amacrine cells received no inhibition and had monostratified processes confined to the middle of the inner plexiform layer. Glycinergic inhibition was the dominant type of inhibition we measured in amacrine cells. The most common interaction between amacrine cells that we measured is “crossover inhibition,” where OFF inhibits ON, and ON inhibits OFF.

After we recorded from amacrine cells, we identified their neurotransmitter content using immunocytochemical techniques, and correlated this with their morphological and physiological characteristics. We found that narrow cells, or cells that conveyed local information, usually contained glycine. Wide cells, or cells that conveyed global information, usually contained GABA. Some narrow field cells that received wide-field inhibition contained GABA. Some cells had receptive fields smaller than their dendritic fields, and appeared to be able to generate action potentials.

Data from electrophysiology, pharmacology, morphology and immunocytochemistry revealed that amacrine cells interact with one another using primarily glycine. GABAergic amacrine cells play a larger role in feedback and feedfoward inhibition to bipolar and ganglion cells than in amacrine cells. Narrow-field, glycinergic amacrine cells are primarily involved in crossover inhibition, which is also a common circuit topology in electronics. These interactions between amacrine cells forms the basis for the diverse inhibitory inputs to ganglion cells. With a deeper understanding of retinal circuits, we hope to design more effective retinal prostheses for the treatment of retinal disease.

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Chapter 1

Introduction

The morphology of amacrine cells

"The great change in the method dates mainly from when Cajal began to use it on the central nervous system of young animals and embryos. He showed the scientific world that the most beautiful and clear pictures could be obtained with this method, thereby offering a clearer and simpler account of the nervous system than had ever been imagined. A comprehension of such pictures would give us a schema, so to speak, by which we might be able to understand more complex relations."

From the Introduction by Richard Greeff to The Structure of the Retina by Ramon y Cajal, in reference to Cajal's use of the Golgi method to characterize neuronal structures. (pg.xviii)

The meticulous morphological studies by Santiago Ramon y Cajal revealed neural structures previously unknown. The mysteriously selective chromium-silver impregnation of neurons (Ramâon y Cajal 1972) provided beautifully clear pictures of the morphology of neurons, allowing researchers to identify the specific structures of a few types of neurons. With this method, the neurons of the retina were revealed, and it became evident that amacrine cells were the most diverse of all the cell types. Not only were their somas found in various places in the retina, from the inner nuclear layer, inner plexiform layer, and even displaced to the ganglion cell layer, their processes ranged greatly in stratification, spread, and branch qualities. With the Golgi method, Ramon y Cajal was able to surmise quite accurately that the inner plexiform layer was composed of at least four sublamina, and that bipolar and amacrine cell processes spanned some or all

of these layers. However, the diversity of amacrine cell morphologies proved to be somewhat confusing:

"At present the role of the amacrine cells has not been determined; it can be said that they must exert some influence on the clusters of the ganglion cells and perhaps have its origin in the higher nerve centers and communicate with the amacrine cells by means of the terminal ramifications of the centrifugal fibers." (Ramâon y Cajal 1972)

Since Ramon y Cajal used the Golgi method to study the morphological structures of the retina, new methods have been employed to further examine the structures of amacrine cells. A fluorescence-based "photofilling" method in which microirradiation of a single cell by a spot of light causes oxidation within that cell of dihydrorhodamine 123 to rhodamine 123, allowed for quantitative sampling of the entire population of amacrine cells (MacNeil and Masland 1998; MacNeil, Heussy et al. 1999). This method, in conjunction with the Golgi method, showed that there were at least 27 different morphologic types (MacNeil, Heussy et al. 1999). More recently, an alkaline phosphatase (AP) reporter was used to visualize detailed morphologies for retinal neurons in the adult mouse (Badea and Nathans 2004). The expression of AP was activated by Cre-mediated DNA recombination which occurred only in a small fraction of cells, allowing for the complete visualization of the morphology of a few cells. This study clustered amacrine cells by morphological parameters (spread of processes and stratification) into 23 clusters, and found that many morphological types were conserved between mouse and rabbit (Badea and Nathans 2004). These studies have comprehensively characterized the

morphology of all amacrine cells types, but ask the same question posed by Ramon y Cajal: what role(s) do amacrine cells play in the retina?

The question of functionality cannot be implied simply by looking at the morphology of amacrine cells. Unlike bipolar and ganglion cells which have clear structures for synaptic input (dendrites) and synaptic output (axons), most amacrine cell processes do not possess structural characteristics that identify input vs. output polarity (with a few exceptions, such as primate A1 amacrine cells (Davenport, Detwiler et al. 2007)). Furthermore, unlike bipolar cells and photorecepters that release only glutamate, amacrine cells release either GABA or glycine, and in fewer instances, dopamine and acetylcholine. These factors added a layer of complexity to the analysis of amacrine cell function, and required that multiple techniques be employed to study these cells.

The physiology of amacrine cells as revealed by electron micrographs

The first studies directed at determining the synaptic inputs of amacrine cells did so by revealing the synaptic structures using electron micrographs (Kolb 1997). By locating the synaptic ribbons of bipolar cells and the post-synaptic structures in amacrine cells, it could be determined which bipolar cells provided glutamertergic input to which amacrine cells. In this way, amacrine cells of the rod bipolar pathway could be distinguished from the amacrine cells of the cone bipolar pathway. For instance, examining the electron micrographs of the synaptic structures of AII and A17 amacrine cells, ribbon synapses in rod bipolar cells could be identified and observed to make synaptic contact with postsynaptic structures of both these amacrine cell types in a dyad (Kolb 1997). Furthermore, amacrine cell synaptic contacts could be distinguished from

the synaptic contacts of bipolar cells, allowing a first look at the connectivity between amacrine cells. Indeed, it could be clearly seen that amacrine cells frequently synapse onto other amacrine cells (Freed, Pflug et al. 1996), form gap junctions with one another (Kolb 1997), and form chemical synapses with bipolar and ganglion cells (Kolb and Nelson 1993). The stratification of bipolar and amacrine cell processes in the inner plexiform layer also constrains the connectivity of these difference cell types to a first approximation. The inner plexiform layer is functionally divided into two sublamina: the OFF layers and the ON layers (Werblin and Dowling 1969; Kaneko 1970; Nelson, Famiglietti et al. 1978). The terminals of OFF bipolar cells terminate in the distal portion of the inner plexiform layer, which is called the OFF sublamina. The proximal portion of the inner plexiform layer is called the ON sublamina because it contains the axon terminals of the ON bipolar cells (Nelson, Famiglietti et al. 1978). ON-center ganglion cell dendrites reach up into the ON sublamina to receive synaptic input from ON bipolar cells, and OFF-center ganglion cell dendrites reach up into the OFF sublamina to receive synaptic input from OFF bipolar cells. The physical location of cell processes in the IPL, i.e. their stratification, gives clues about synaptic connectivity of that cell. Cells can form chemical synapses only with other cells whose processes reside in the same layer, and can only receive synaptic inputs from other cells whose processes are in the same layer. Electrical synapses may not adhere to these conventions, but their participation in retinal circuitry can be determined with pharmacological blockers (Pan, Mills et al. 2007; Manookin, Beaudoin et al. 2008), connexin knock-out animal models (Volgyi, Abrams et al. 2005), or through electron micrographs.

The neurotransmitters contained in amacrine cells

The major neurotransmitters contained in amacrine cells are GABA and glycine (Ehinger and Lindberg-Bauer 1976; Miller, Dacheux et al. 1977). Other less common neurotransmitters released by amacrine cells are dopamine and acetylcholine (Haeggendal and Malmfors 1963; Nichols, Jacobowitz et al. 1967; Ehinger and Falck 1971; Ehinger 1983; Masland, Mills et al. 1984). Almost all amacrine cells contain only one type of neurotransmitter, except for the starburst amacrine cell, which has been shown to contain both GABA and acetylcholine (Masland and Mills 1979; Masland, Mills et al. 1984; Brecha, Johnson et al. 1988; O'Malley, Sandell et al. 1992). Dopamine acts as a neuromodulator in the retina, for instance, modulating amacrine responses to changes in ambient light levels (Voigt and Wassle 1987; Wellis and Werblin 1995). GABA and glycine receptors, which are found on bipolar, amacrine and ganglion cells, are ionotropic, directly gating a chloride channel (Miller, Frumkes et al. 1981; Huba and Hofmann 1991; Wu and Maple 1998). When amacrine cells release either of these two neurotransmitters to their post-synaptic partners, the chloride channels of the post-synaptic neurons open, allowing an influx of chloride. For this reason, GABA and glycine have been called inhibitory neurotransmitters, and amacrine cells are the inhibitory interneurons of the inner retina (Bonaventure, Wioland et al. 1974; Miller, Dacheux et al. 1977). To fully characterize the role an amacrine cell plays in retinal circuitry, it is important to consider the neurotransmitter content of that cell using either pharmacology to block the input of amacrine cells to the post-synaptic neuron, or to use immunocytochemistry to identify the neurotransmitter contained in an amacrine cell. However, prerequisite to using either of these techniques to characterize the

neurotransmitters involved in a given retinal circuit, one needs to first gain access to a single neuron. Without such access, it would be difficult to draw conclusions from a pharmacological experiment, nor would it be possible to examine the neurotransmitter content of a single type of amacrine cell separately from the general population of cells.

Electron microscopy allowed for the visualization of retinal microcircuits, revealing pre-synaptic and post-synaptic partners, but did not indicate which neurotransmitters were involved in that circuit, nor did it shed light on the characteristics of the light response (i.e. timing of excitation and inhibition, kinetics of the response, the relative input of neurons near the cell of interest vs. the neurons far away). How do all the bipolar and amacrine cell inputs to a post-synaptic amacrine cell shape its response, and what neurotransmitters are involved in this microcircuit? And, in light of the immense morphological diversity of amacrine cells, are the connections between these amacrine cells just as diverse? Are there amacrine cell types that just feed back to bipolar cells, or just feed forward to ganglion cells, or interact exclusively with other amacrine cells? Or is amacrine cell circuitry an incomprehensible plexus of connections?

Retinal circuitry revealed by intracellular recordings

Intracellular recording using microelectrodes provides the answer to many of these questions. As an example, the circuitry of AII amacrine cells was first studied using electron micrographs, where it was determined that rod bipolar cells provided excitatory input to AII amacrine cells (Kolb and Famiglietti 1974), and that AII amacrine cells were extensively coupled via gap junctions to cone bipolar cells (Famiglietti and Kolb 1975; Strettoi, Raviola et al. 1992). Combining both Golgi staining and electron microscopy

revealed that AII amacrine cells provided inhibitory input to OFF cone bipolar cells (Pourcho and Goebel 1985). Electrophysiological recordings using sharp electrodes not only confirmed these findings from anatomical studies, they revealed how AII amacrine cells responded to light stimuli of varying intensities (Dacheux and Raviola 1986). In addition to its role of conveying rod signals to cone pathways in scotopic conditions, intracellular recordings, along with pharmacology, in ganglion cells have implicated AII amacrine cells in other functions, such as disinhibition to OFF ganglion cells to extend their operating range, and perhaps to diminish simultaneous action potential generation in nearby ON and OFF ganglion cells (Manookin, Beaudoin et al. 2008; Murphy and Rieke 2008). With access to just one neuron, it is possible to parse the specific microcircuit in which that neuron participates in by voltage clamping it to reversal potentials for excitatory and inhibitory input. Furthermore, it can be specifically stained with a fluorescent dye to map its dendritic spread and stratification. These techniques have been applied to study the starburst amacrine cell circuitry driving directional selectivity in ganglion cells (Fried, Munch et al. 2002; Fried, Munch et al. 2005; Lee and Zhou 2006), the physiology and morphology of polyaxonal amacrine cells (Volgyi, Xin et al. 2001), and signal propagation in A1 amacrine cells (Davenport, Detwiler et al. 2007).

Understanding amacrine to amacrine interactions

There are at least 27 morphological types of amacrine cells (MacNeil, Heussy et al. 1999; Badea and Nathans 2004), and for the majority of these cells, the circuitry that drives their responses has yet to be determined. There are many studies that focus on the inhibition that arrives at bipolar cells (Eggers and Lukasiewicz 2006; Eggers, McCall et

al. 2007; Molnar and Werblin 2007) and at ganglion cells (Demb, Haarsma et al. 1999; Roska and Werblin 2001; Roska, Molnar et al. 2006), but few studies examine the inhibition in amacrine cells that shapes amacrine cell responses (Pang, Gao et al. 2002; Pang, Gao et al. 2007). The goal of this body of research is to characterize the inhibitory circuitry between amacrine cells that drives the feedback inhibition to bipolar cells, and feed forward inhibition to ganglion cells in the rabbit retina. The whole cell patch clamp technique is used to characterize the excitatory and inhibitory *inputs* to the cell. The excitatory input reveals the general class of bipolar cell that is driving the amacrine cell (i.e. ON or OFF bipolar cells), and the inhibitory input allows for the characterization of the amacrine cell(s) pre-synaptic to the cell of interest. Combining whole cell patch clamp with pharmacology is especially useful to determine the neurotransmitter that mediates the inhibitory *input*. To characterize the *output* of a given amacrine cell, the voltage response measured in current clamp mode gives the polarity (ON vs. OFF vs. ON-OFF) of the response, and how the cell sums the excitatory and inhibitory inputs to form this response. The morphology revealed by fluorescent staining also provides constraints as to which cells the amacrine cell of interest can synapse with. A diffusely stratified cell can form contacts with neurons of both the ON and OFF type, while a monostratified cell can form contacts only with neurons whose processes reside in that same stratum. The *output* of an amacrine cell can be further characterized by the neurotransmitter (GABA vs. glycine) released by the cell, which can be determined by immunocytochemical staining. Additionally, the contribution of local vs. wide circuitry can be studied by using different retinal preparations. The retinal slice preparation is ideally suited to study local interactions between amacrine cells, since circuitry greater

than 250μm away will likely be truncated. The retinal wholemount preparation is appropriate for studying spatial interactions that extend beyond 250μm. Each of these techniques characterizes either the input or output parameters of an amacrine cell, and can be applied generally to all amacrine cells to examine their circuitry. We can begin to define the different roles of GABA vs. glycine, how excitatory and inhibitory inputs interact in time, and how different amacrine cells inhibit other amacrine cells in space.

Ultimately, with a better understanding of how amacrine cells interact with each other to form the inhibition that shapes the responses of ganglion cells, we hope to be able to design more effective prosthetic devices and treatments for degenerative retinal diseases.

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Chapter 2

Local Amacrine-to-Amacrine Cell Inhibition

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 glycinergic feedback inhibition to bipolar cells (Eggers and Lukasiewicz 2006; Molnar and Werblin 2007). In other examples, amacrine cells provide feed forward inhibition to ganglion cells (Lukasiewicz and Werblin 1990; Roska and Werblin 2001; Roska and Werblin 2003), generating a set of about 12 different ganglion cell response forms (Roska, Molnar 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 at least 27 different morphological types of amacrine cells in the mammalian retina (MacNeil, Heussy et al. 1999; Badea and Nathans 2004), and in addition to inhibiting bipolar and ganglion cells, amacrine cells interact with one another. Starburst amacrine cells mutually inhibit each other via GABA (Taylor and Wassle 1995; Fried, Munch et al. 2005; Lee and Zhou 2006) and feed forward to ganglion cells. AII amacrine cells are electrically coupled to each other and to ON bipolar cells, and they inhibit OFF cone bipolar cells via glycine (Strettoi, Dacheux et al. 1990; Strettoi, Raviola et al. 1992). Polyaxonal amacrine cells are coupled via gap junctions to other amacrine cells (Volgyi, Xin et al. 2001). These amacrine cell studies in mammalian retina, along with a study in salamander retina (Pang, Gao et al. 2002), begin to elucidate the interactions between amacrine cells, however, much has yet to be learned about amacrine to amacrine interactions.

We found that amacrine cells inhibit each other in just a few distinct ways. The most common form of interaction that we have measured is “crossover inhibition”, where OFF amacrine cells receive ON inhibition, or ON amacrine cells receive OFF inhibition. Both of these interactions are mediated by glycine. Additionally, some ON and other OFF amacrine cell classes receive a combination of ON glycinergic and OFF GABAergic inhibition. These general forms of interaction are found across a variety of morphologies. Other classes of amacrine cells, with broadly ramifying processes confined to a single stratum in the IPL, receive ON-OFF excitation but receive no significant inhibition. Thus, although the morphologies of amacrine cells are diverse, the forms of amacrine to amacrine cell interaction and pharmacology seem relatively limited.

Methods

Preparation of slices. New Zealand white rabbits (2.5 kg) were anesthetized and euthanized 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), 0.05 g/L kanamyacin sulfate (Invitrogen), 8.8 g/L AMES powder (Sigma) and bubbled with 95% oxygen, 5% carbon dioxide (BIOBLEND by Praxair). Each eye was dissected in dim red light by first removing the vitreous, then cut away the periphery to preserve the visual streak, a region approximately 3 mm by 4 mm. The visual streak was cut into quarters, then stored in the dark in AMES solution, constantly bubbled with BIOBLEND at room temperature (approximately 25 degrees centigrade). Retinas stored in this manner remained light responsive for 6 hours. To prepare the retina for patch recording, a

chamber with an inlet and outlet was mounted on a slide, then filled with 2mL AMES solution. One quarter of the visual streak is placed in the chamber, retina peeled away and mounted, ganglion cell side down, on filter paper (Millipore). The retina was then sectioned into 250µm wide slices, turned on its side, and held stationary with silicone grease. A constant perfusion of AMES solution, bubbled with 95% oxygen and 5% carbon dioxide, was provided to the chamber at a rate of 6 mL/min at a temperature of between 34.5 and 35.0 degrees centigrade.

Patch recording. We use 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 Instrument) 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, 7.75 Neurobiotin Tracer (Vector Labs, Burlingame, CA), pH-balanced to 7.2 using cesium hydroxide. Alexa Fluor 488 was also added to the intracellular solution. The retina was light adapted (the background light level was 2.2mW/cm^2) for 10 minutes prior to the beginning of each experiment. Amacrine cell somas were identified by their proximity to the distal edge of the inner plexiform layer. The patch pipette (6-8 MΩ resistance) was brought to the soma by visual guidance, negative pressure was applied to achieve a 1.5-2.5 GΩ seal prior to breaking in with increasing voltage steps (increasing in 50mV increments from 50mV to 250mV). The correction for junction potential was 5.6mV. The

EPC-7 (HEKA Electronics, Inc) patch amplifier was used to voltage and current clamp the cell. Excitatory currents were measured by holding the cell at -60mV; inhibitory currents were measured at a holding potential of 0mV. The retina was stimulated with a 200µm light or dark stripe, and/or a full-field light or dark flash for 2.5 seconds (100% contrast from the background light level of 2.2mW/cm^2). We saw no difference in response between the 200µm stripe stimuli and the full-field stimuli, so we pooled these responses for our analyses. Current and voltage recordings were digitized and sampled at 10kHz, as described previously (Molnar and Werblin 2007). All signals were post-analyzed 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 paper, 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 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 600ms prior to the flash, and the 600ms 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*), light to background (*d*). The polarity metric was defined as follows: $X = -(a - b + c - d) / (|a| + |b| + |c| + |d|)$. In this paper, a response whose polarity metric X = -1 is an OFF response, and X = +1 is an ON response, and $-1 < X < +1$ is an ON-OFF response (see Fig. 2-1B and Fig. 2-4B). Histograms shown in Fig. 2-1B and 2-4B show X for every single amacrine cell we recorded from. Data reported in pie-charts (Fig. 2-1A and Fig. 2-4A) 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 10μM strychnine to block glycine receptors (Rotolo and Dacheux 2003; Molnar and Werblin 2007), 100μM picrotoxin to block $GABA_A$ and $GABA_C$ receptors (Roska and Werblin 2001; Rotolo and Dacheux 2003; Molnar and Werblin 2007), and 5μM SR95531 to block $GABA_A$ receptors (Roska and Werblin 2001). We also used 20μM APB to block mGluR6 mediated responses from ON bipolar cells (Slaughter and Miller 1983). Each drug was washed in for 3 minutes before recording light responses, and washed out for 5 minutes (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 5μM SR95531 and/or 100μM picrotoxin, which means that the GABA receptor antagonists are not acting significantly on glycine receptors. In one study, 10μM strychnine was shown to block $GABA_A$ receptors in neonatal rat brainstem hypoglossal motoneurons (O'Brien and Berger 1999), however in other studies, 10μM 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 5μM SR95531 is not blocked by 10μM strychnine, and that we are able to selectively block either GABAergic or glycinergic inhibition at these concentrations.

To determine if a pharmacological agent significantly impacted the physiological response, we calculated the response magnitude to each transition of light and dark flashes, as described above. 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 percent 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 change of 100%, and in some cases where the response inverts its polarity, changes greater than 100% were measured. Then we computed the mean change (m_d) and standard error of the mean changes (s_{md}) to obtain the test variable $t = m_d / s_{md}$. 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 ν = 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 ν degrees of freedom, we accepted the null hypothesis of no effect, and conclude that the drug had no significant effect on the measured light response. The result of statistical analyses is reported throughout this paper in the following format: sample size, mean percent change in the light response due to the pharmacological blocker ± SEM, P-value.

Imaging the recorded cells. At the completion of electrophysiological measurements, we imaged cell morphology using Alexa Fluor 488 (excitation: 488nm, emission: 519nm) which we had included in the patch pipette and 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. 2-10). 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 we recorded from 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 less than 100µm. Medium width cells have processes that extend between 100 and 200µm. Wide cells are those whose processes extend greater than 200µm. These definitions are smaller than the definitions laid out by other morphological studies (MacNeil, Heussy et al. 1999; Badea and Nathans 2004) because the retina slices were 250µm thick; any morphology beyond 200µm would

be truncated and hard to visualize. Furthermore, we were limited to imaging processes are within 20μm of the slice surface. The inner plexiform layer is divided into five sublamina, and for each cell, the sublamina in which their processes extended was measured. The distal layers 1, 2 comprise the functional OFF sublamina (Kolb and Famiglietti 1974; Bloomfield and Miller 1986; Strettoi, Dacheux et al. 1990) and the proximal layers 3, 4, 5 comprise 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 second full field light or dark flashes. The main forms of interaction between excitation and inhibition for the majority of these cells are described below.

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 (Wassle, Grunert et al. 1993), which we exclude from this analysis. As shown in Fig. 2-1A and 2-1B, the majority (90/119 excluding AII amacrine cells) of ON amacrine cells received OFF inhibition. For these cells, the excitatory and inhibitory currents, as well as voltage response, are shown in Fig. 2-1C. Typically, excitatory (inward) currents were greater at light ON than at light OFF,

while 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. 2-1C .

We confirmed that ON amacrine cells received excitation from ON bipolar cells by blocking ON activity with APB, a highly specific glutamate agonist at the mGluR6 receptors of ON bipolar cells (Slaughter and Miller 1981). Fig. 2-2B shows that in the presence of 20 μM APB, the ON excitatory current was blocked (n=6, 101.05% ± 9.26%, mean percent change in current amplitude ± SEM, $P < 0.001$, 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. 2-3B shows that OFF inhibition was suppressed by 10μM strychnine, an antagonist for glycine receptors (n=18, 75.02% ± 10.89%, $P < 0.001$, paired t-test) but was relatively unaffected by either 5μM SR95531, an antagonist for $GABA_A$ receptors, or 100μM picrotoxin, an antagonist for both $GABA_A$ and $GABA_C$ receptors (n=12, 42.58% ± 22.67%, $P < 0.1$, Fig. 2-3D). This suggests that OFF inhibition to ON cells is mediated primarily by glycine and not by GABA.

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

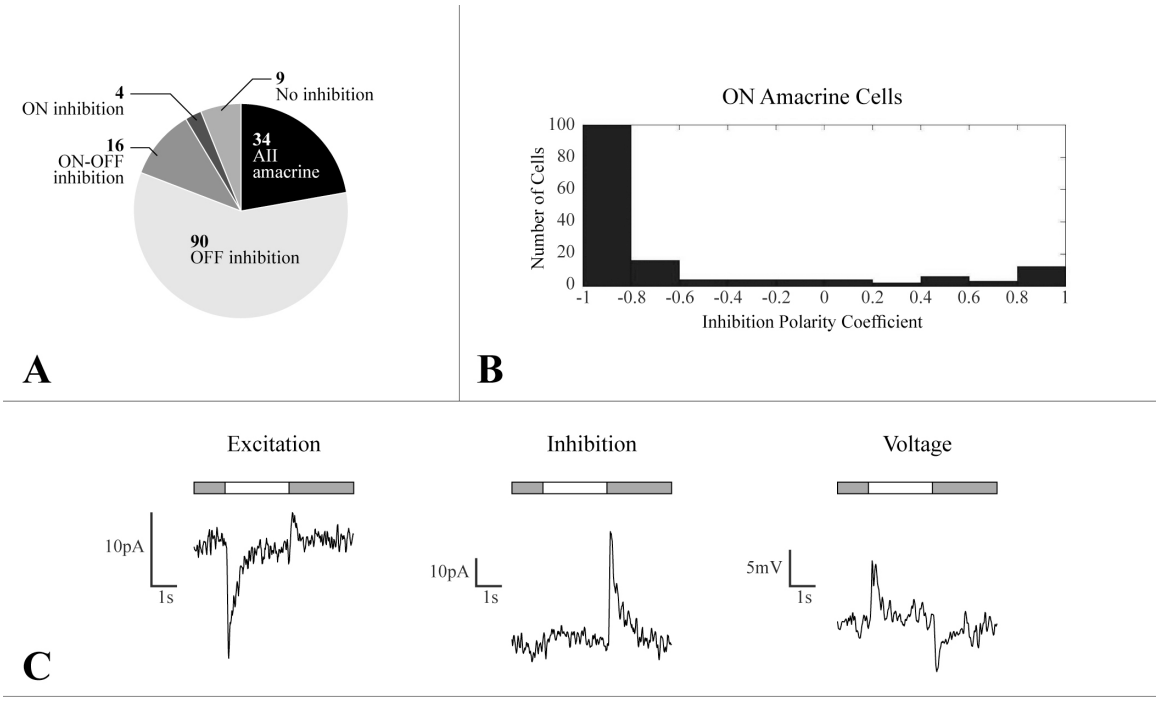

Figure 2-1. The majority of ON amacrine cells receive OFF inhibition. A) Distribution of inhibition recorded in 153 ON amacrine cells derived from the histogram in part B. Some of these ON cells were AII amacrine cells (34/153) which have been extensively described elsewhere, and which we exclude from 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 second flash) of the stimulus. A light bar denotes a light flash, a black bar (in later figures) denotes a dark flash.

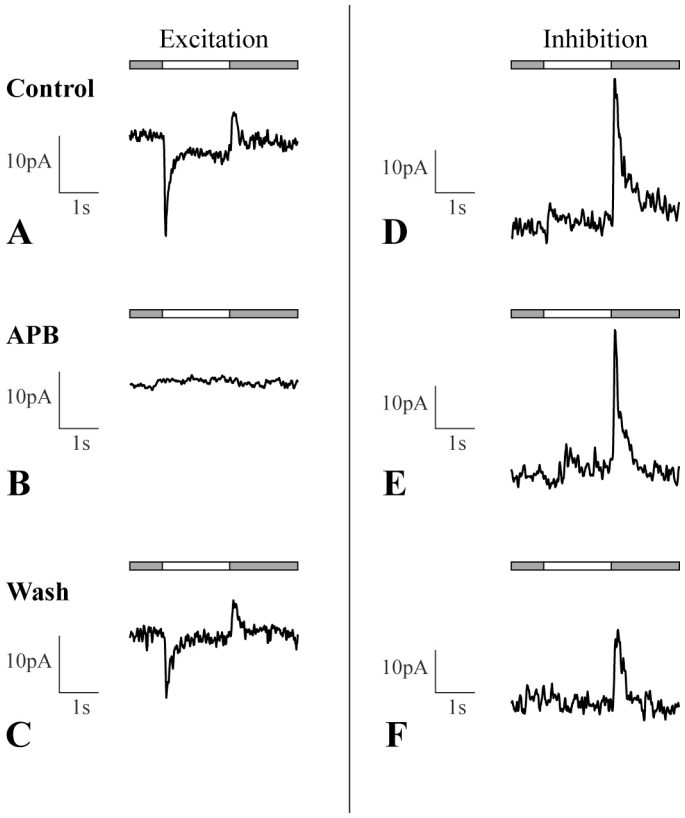

Figure 2-2. APB blocks ON excitation, but not OFF inhibition. Current traces measured from an ON amacrine cell that received OFF inhibition. A) ON excitatory current in control conditions. B) 20μM APB blocks ON excitatory current. C) APB wash shows recovery of ON excitatory current. D) OFF inhibitory 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.

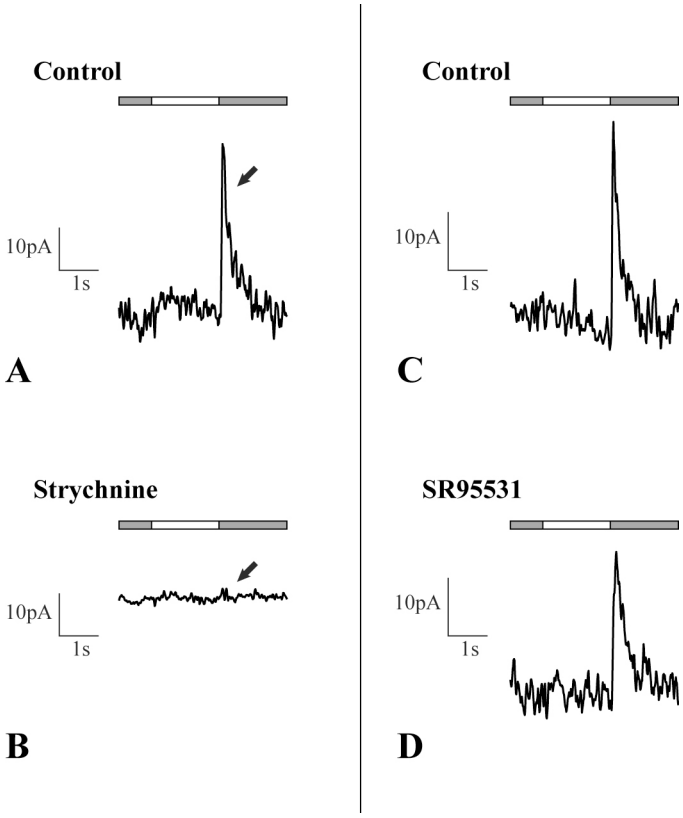

Figure 2-3. ON amacrine cell receives glycinergic OFF inhibition. Current traces measured from an ON amacrine cell that received OFF inhibition. A) OFF inhibitory current in control conditions. B) OFF inhibitory current is blocked by strychnine. Current traces measured from another ON amacrine cell that received OFF inhibition. C) OFF inhibitory current in control conditions. D) OFF inhibitory current is not significantly blocked by SR95531.

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. 2-4A and 2-4B, almost half of OFF cells received purely ON inhibition. Fig. 2-4C shows an example of the response currents of these OFF cells. For these cells, the excitatory current was greater at light OFF than at light ON, but the inhibitory current was greater at light ON than 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% ± 14.23%, $P < 0.001$, Fig. 2-5E), while the OFF excitation was only reduced (n=5, 59.50% ± 7.63%, $P < 0.01$, Fig. 2-5B). In the wash step, the ON inhibition was restored (-47.73% ± 15.60%, Fig. 2-5F), but the OFF excitation was not (0.013% ± 17.42%, Fig. 2-5C). 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% ± 8.55%, $P < 0.002$) but was relatively unaffected by either SR95531 or picrotoxin (n= 11, 13.66% ± 39.00%, $P > 0.5$) as shown in Fig. 2-6B, 2-6D, respectively. This suggests that the crossover inhibition to OFF cells, like the crossover inhibition to ON cells, is mediated primarily by glycine and not GABA.

As shown in Fig. 2-4A, 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% ± 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 10µM strychnine did not block the OFF inhibition, but 5µM SR95531 does. 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.

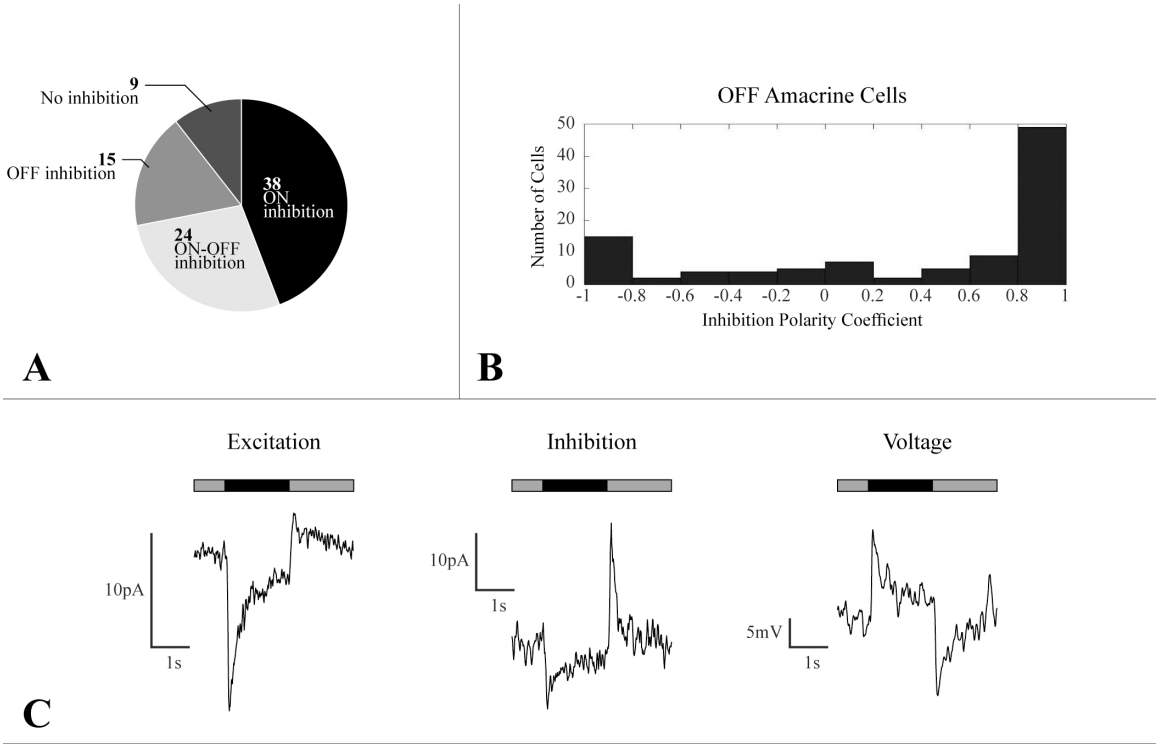

Figure 2-4. Many OFF amacrine cells receive ON inhibition. A) Distribution of inhibition recorded in 86 OFF cells derived from histogram shown in part B. About half (38/86) of the OFF amacrine cells received a crossover ON inhibition. Of the remaining 48 OFF cells, some received ON-OFF inhibition (24/48), 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, though 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 Figures 5, 6 and 7.

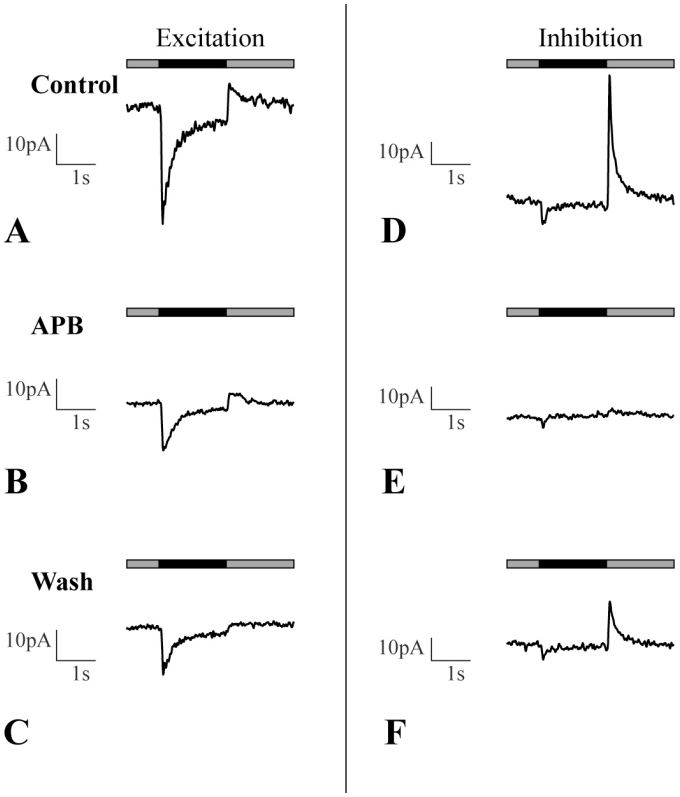

Figure 2-5. APB blocks ON inhibition, but not OFF excitation.

Traces measured from a single amacrine cell. A) OFF excitatory current in control conditions. B) OFF excitatory current persists in APB. C) OFF excitation is unaffected under a wash step. D) ON inhibitory current in control conditions. E) 20μM APB blocks ON inhibitory current. F) ON inhibition recovers in the wash step. These results show that excitation and inhibition are derived from two separate pathways: excitation originates from the OFF pathway and inhibition originates from the ON pathway.

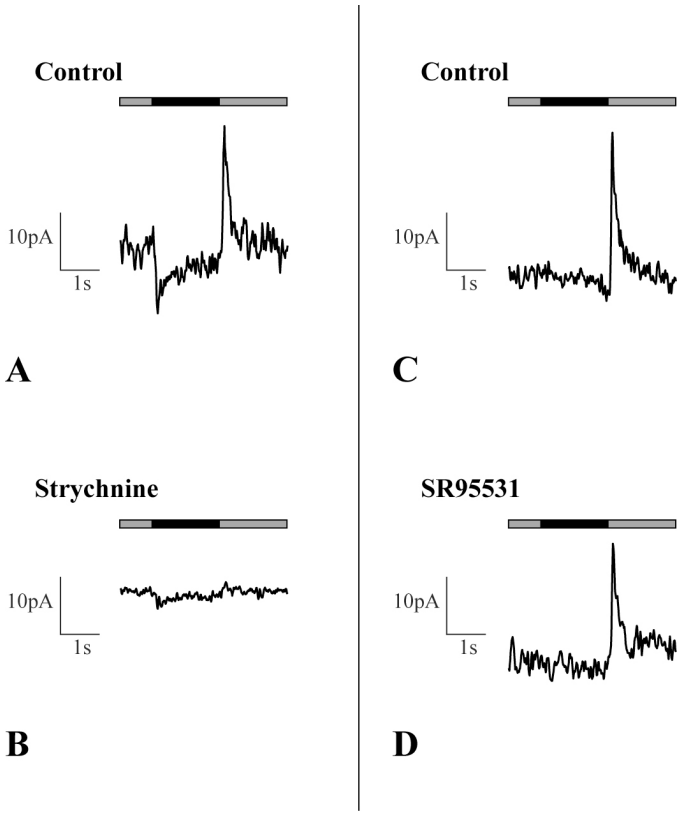

Figure 2-6. OFF amacrine cells receive glycinergic ON inhibition. Current traces measured from an OFF amacrine cell that received ON inhibition. A) ON inhibitory current in control conditions. B) ON inhibitory current is blocked by strychnine. Current traces measured from another OFF amacrine cell that received ON inhibition. C) ON inhibitory current in control conditions. D) ON inhibitory current is not blocked by SR95531.

The 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. 2-7. For the majority of ON-OFF cells (30/53 shown in Fig. 2-7A), we observed no evidence of any outward inhibitory current when we held the cell membrane at 0mV, as shown in the right traces of Fig. 2-7B and 2-7C. 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 inner

plexiform layer. It seems likely that these monostratified amacrine cells are wide (MacNeil, Heussy 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. 2-7B, 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 0mV, as shown in the right trace of Fig. 2-7C. The inward currents measured at 0mV 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 upon 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 response waveform. Instead of an *increase* in inward current, we saw a *decrease* in the magnitude of the inward current. This decrease in the inward current was observed in the presence of either 100μM picrotoxin or 5μM SR95531, shown in Fig. 2-8B (n=6, OFF phase: 30.27% ± 14.86%, $P < 0.1$; ON phase: 15.21% ± 49.71%, $P > 0.5$), or 10μM strychnine, shown in Fig. 2-8D (n=8, OFF phase: 51.30% ± 17.91%, $P < 0.05$; ON phase: 94.07% ± 102.70%, $P > 0.2$). This reduction in current (measured by clamping the cell at 0mV) was not due to inhibition or serial amacrine to amacrine synapses because we saw similar magnitude reductions in the excitatory current (measured by clamping at -60mV). The reduction in excitation was measured in the presence of picrotoxin or SR95531 (n=6, OFF phase: 20.36% ± 15.76%; ON phase: 26.47% ± 29.51%), or strychnine (n=8, OFF phase: 49.77% ± 9.98%; ON

phase: 139.49% ± 65.68%). Therefore, this current reduction is most likely due to slow degradation of the light response. These results suggest that if there were any direct inhibitory input impinging on this cell class, they are significantly weaker than the excitatory input and have little effect on the behavior of the cell.

Some ON-OFF amacrine cells received ON inhibition (9/53). We were unable to test the neurotransmitter mediating this inhibition. The majority of these cells (8/9) had similar morphology: they stratified in layers S2 and S3, as shown in Fig. 2-11F, and their processes spanned approximately 100µm. It would seem likely that these ON-OFF cells that received ON inhibition represent a single class of amacrine cell.

A subset of ON-OFF amacrine cells received OFF inhibition (5/53), that was blocked by 100µM picrotoxin (n=3, 138.29% ± 43.65%). The morphology of these cells was very similar, and they are likely of the same type: narrowly monostratified in the OFF sublamina (and thus likely wide-field (MacNeil, Heussy et al. 1999)), in layer S2, as shown in Fig. 2-11G. 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, Pflug et al. 1996).

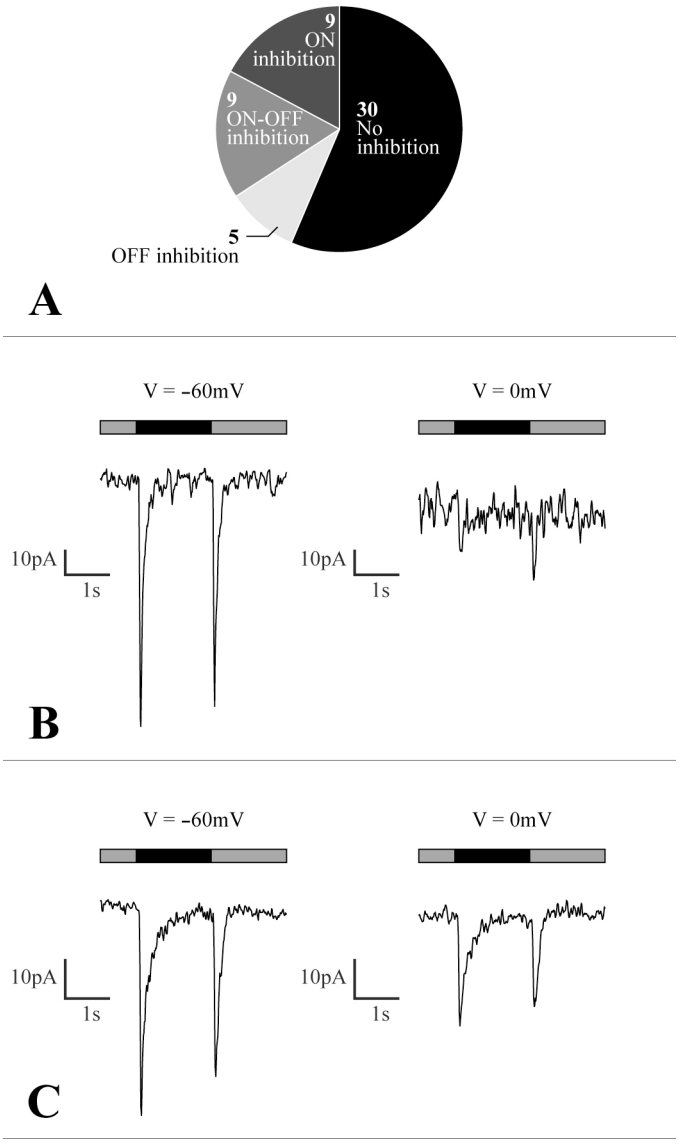

Figure 2-7: Light response of ON-OFF amacrine cells. A) Distribution of inhibition for ON-OFF amacrine cells. Most ON-OFF cells did not receive any inhibition (30/53). The remaining ON-OFF cells (n=23) did receive a measurable inhibition when we voltage clamped the cell at 0mV. Of these ON-OFF cells, (5/23) received OFF inhibition, while (9/23) received ON-OFF inhibition and (9/23) received ON inhibition. B) Example traces of light responses from one ON-OFF amacrine cell. Left: Inward excitatory currents at light onset and offset. Right: No evidence of outward currents, while clamped at 0mV, the reversal potential for non-specific cation channels.

C) Example traces of light responses from another ON-OFF amacrine cell. Left: Inward excitatory currents at light onset and offset. Right: Smaller inward currents at light onset and offset, no evidence of outward currents while clamped at the reversal potential for non-specific cation channels.

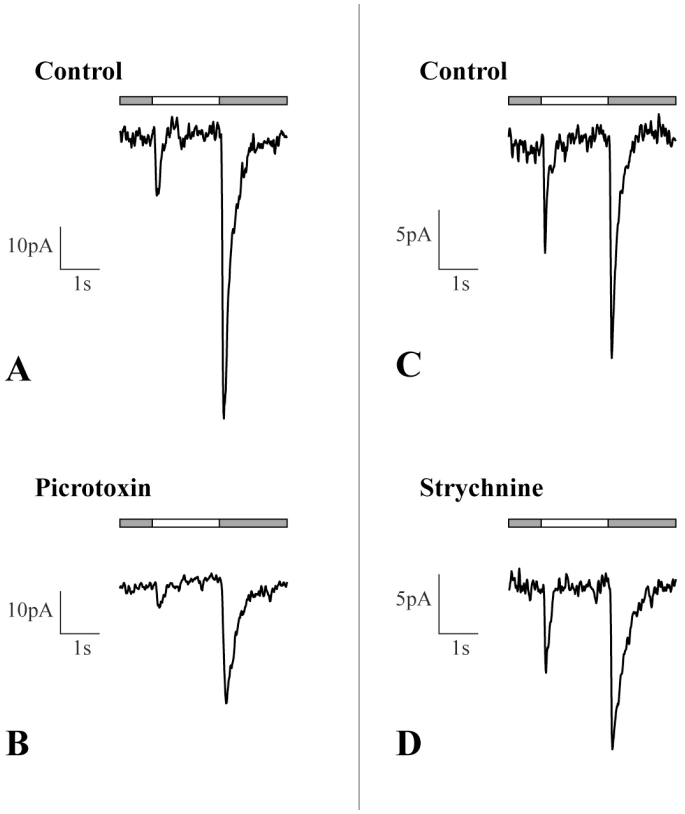

Figure 2-8: ON-OFF amacrine cells receive no inhibition. Current traces measured from an ON-OFF cell. A) ON-OFF inward currents measured when holding the cell at 0mV. B) ON-OFF inward currents are not increased in the presence of 100μM picrotoxin. Current traces measured from another ON-OFF cell. C) ON-OFF inward currents measured when holding the cell at 0mV. 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.

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. 2-9A and 9C. 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: 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. 2-9B (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. 2-9D (n=10; OFF phase: 81.15% ± 36.24%, $P < 0.05$; ON phase: 9.56% ± 87.62%, $P > 0.5$).

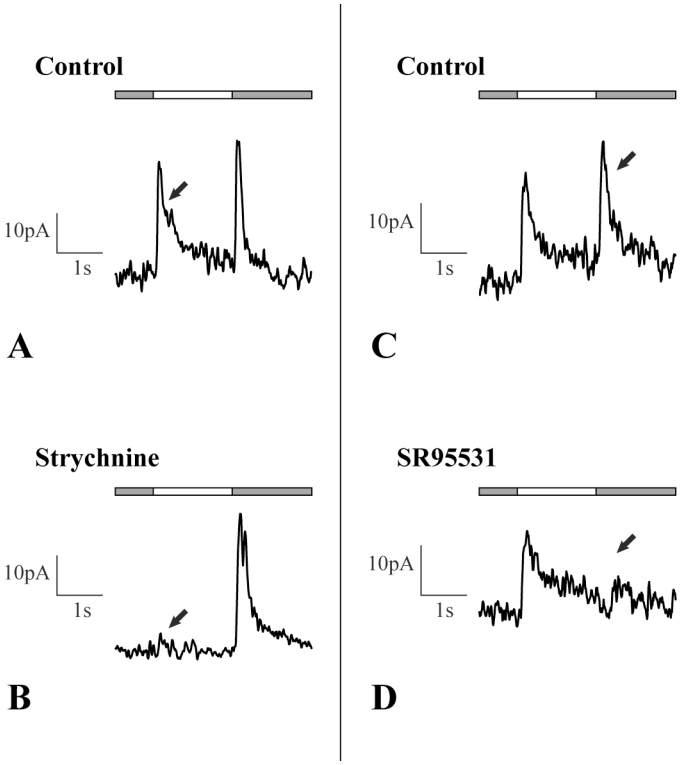

Figure 2-9: Some amacrine cells receive glycinergic ON and GABAergic OFF inhibition. Current traces measured from an amacrine cell that received ON-OFF inhibition. A) ON-OFF inhibition in control conditions. B) The ON phase of inhibition was blocked by strychnine, while the OFF phase persisted. Current 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, while the ON phase persists.

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.

Taken together, these results suggest that the majority amacrine cells were not inhibited by the ON-OFF amacrine cells we measured in Fig. 2-7.

AII Amacrine Cells

We were able to identify AII 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). AII amacrine cells have been extensively studied elsewhere (Famiglietti and Kolb 1975; Boos, Schneider et al. 1993; Wassle, Grunert et al. 1995; Bloomfield, Xin et al. 1997; Bloomfield and Xin

2000; Morkve, Veruki et al. 2002; Gill, Veruki et al. 2006; Manookin, Beaudoin et al. 2008), and we exclude 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. 2-10. ON amacrine cells that received crossover OFF inhibition included cells with dendritic spreads that varied from narrow to wide, shown in Fig. 2-11A. These amacrine cells appeared in monostratified, bistratified and diffusely stratified forms, extending to every sublamina of the retina, as shown in the left column of Fig. 2-10A. OFF cells that received ON inhibition had both narrow and wide processes, shown in Fig. 2-11B, that stratified primarily in the distal OFF sublamina (layers S1, S2, and the distal part of S3), seen in Fig.10B. These studies suggest that glycinergic 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, Harvey et al. 2007; Veruki, Gill et al. 2007).

ON-OFF amacrine cells that did not receive inhibition were monostratified, with their processes largely confined to layers S2 and S3 of the inner plexiform layer, as shown in Fig. 2-10C. The dendritic spread reported in Fig. 2-11C 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 monostratified (MacNeil, Heussy et al. 1999; Badea and

Nathans 2004), which leads us to believe that these ON-OFF 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 inner plexiform layer in S3. Where the processes were imaged (n=4), these cells were consistently wide-field, monostratified 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 ~200µm away, suggesting that this subtype formed a distinct electrically coupled network. This unusual morphology has been reported elsewhere (Famiglietti 1992; Volgyi, Xin et al. 2001; Wright and Vaney 2004) as a subset of the Type I polyaxonal 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 Fig. 2-10D and Fig. 2-11D. The unshaded cells in Fig. 2-11D received ON excitation, and the shaded cells received OFF excitation. The ON cells in Fig. 2-11D stratified throughout the inner plexiform layer, 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 (MacNeil, Heussy et al. 1999; Badea and Nathans 2004). 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. 2-11E. 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.

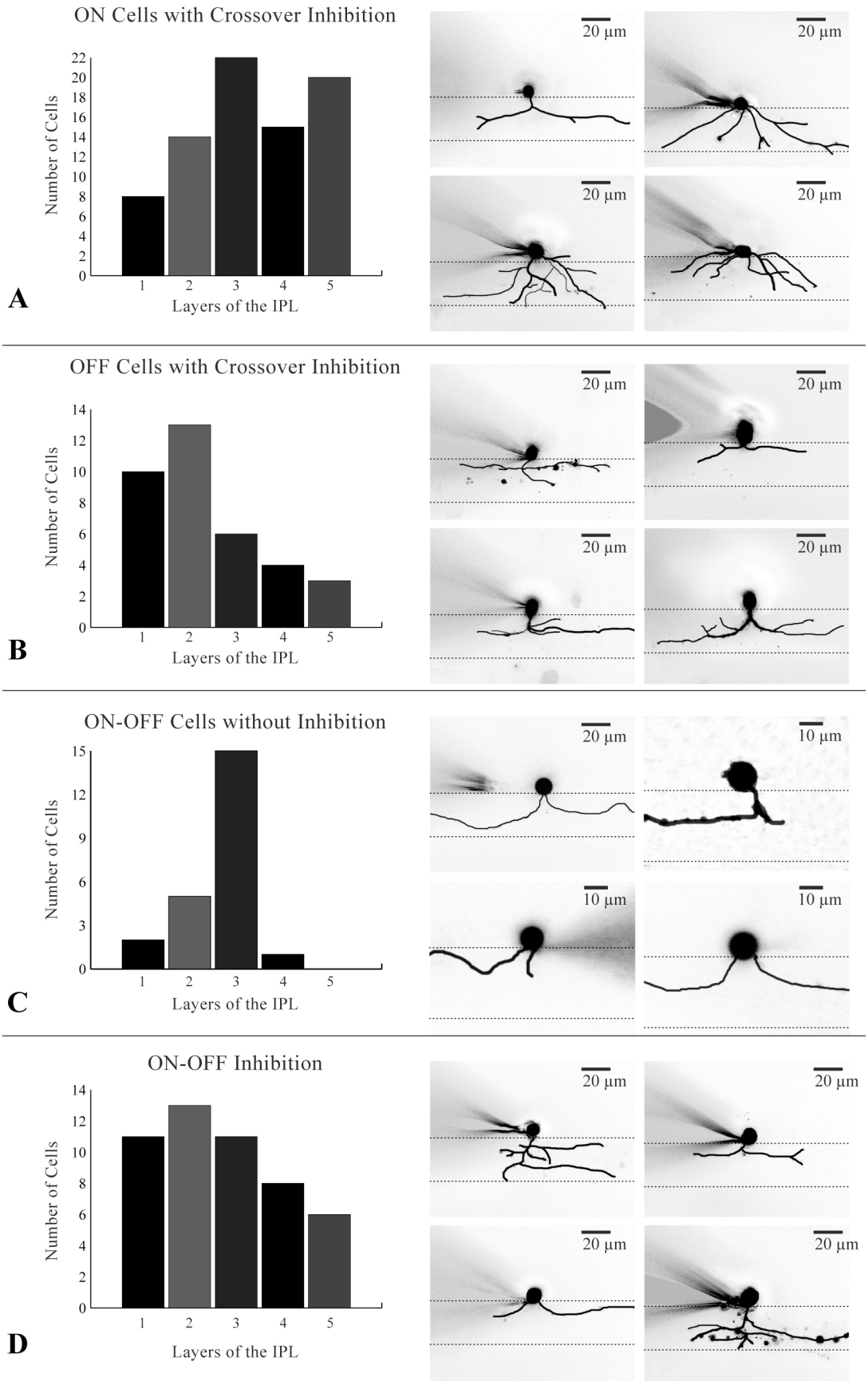
ON Cells with Crossover Inhibition
Number of Cells
Layers of the IPL
A
20 µm
OFF Cells with Crossover Inhibition
Number of Cells
Layers of the IPL
B
20 µm
ON-OFF Cells without Inhibition
Number of Cells
Layers of the IPL
C
20 µm
10 µm
ON-OFF Inhibition
Number of Cells
Layers of the IPL
D
20 µm

Figure 2-10. 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. 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 inner plexiform layer. A diversity of amacrine cells receive the general forms of inhibition we have described. A) ON cells that receive OFF inhibition have processes that terminate throughout the entire depth of the inner plexiform layer, 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 inner plexiform layer (layer 3), along the border between the functional OFF and ON sublamina. D) Cells that receive ON-OFF inhibition have processes that terminate in throughout the entire inner plexiform layer, indicating that many different types of amacrine cells receive glycinergic ON and GABAergic OFF inhibition.

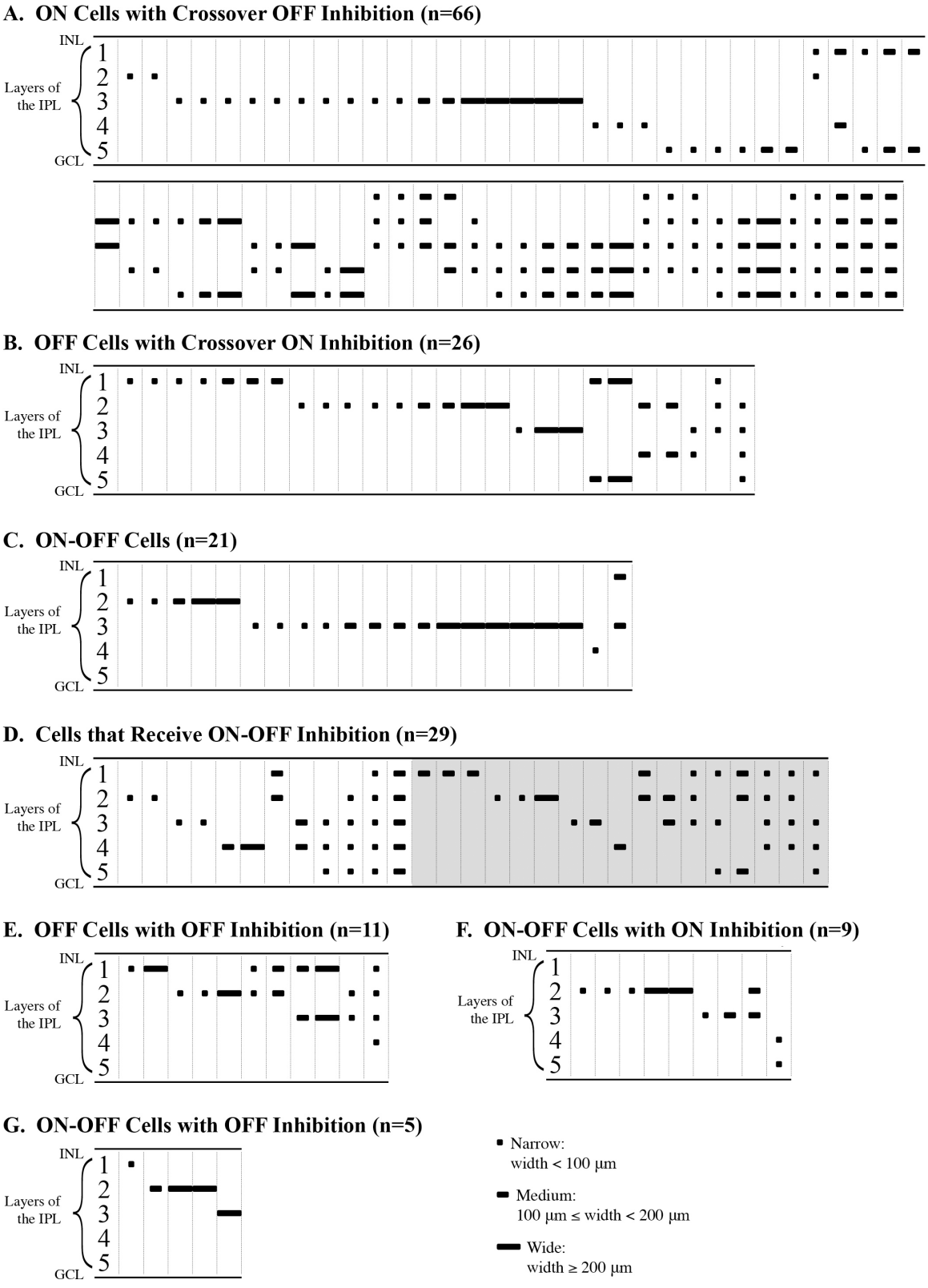

Figure 2-11. A diversity of amacrine cells receive these general forms of inhibition

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 inner plexiform layer. The width of the cells varied from less than 100μm to over 200μm. B) OFF cells that received ON inhibition also show a diversity in stratification, though 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, however, 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).

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. 2-12: ON amacrine cells receive OFF inhibition, shown in Fig. 2-12A, whereas OFF amacrine cells receive ON inhibition, shown in Fig. 2-12B. 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 inner plexiform layer and that the distal layers contain the highest density of glycine receptors of all types (Heinze, Harvey 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 monostratified processes received ON-OFF excitatory input, but no measurable inhibitory input, shown in Fig. 2-12C. ON-OFF inhibition to amacrine cells was generally not mediated by a single ON-OFF amacrine cell type, but by an ON glycinergic amacrine cell and an OFF GABAergic cell as shown in Fig. 2-12D. ON-OFF inhibition was found in a variety of amacrine cell types. Overall, these forms of excitation/inhibition interaction account for over 70% of the interactions recorded in amacrine cells, excluding AII amacrine cell interactions.

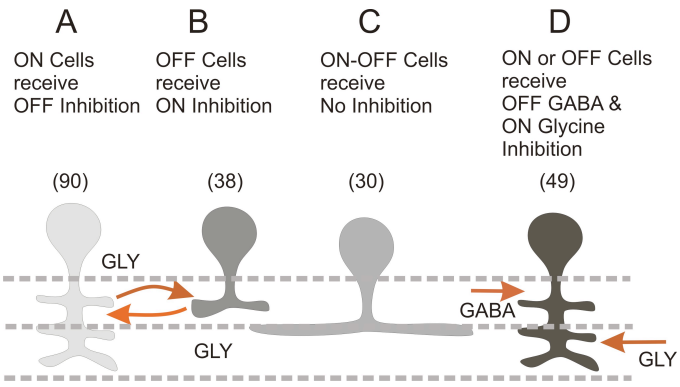

Figure 2-12. Inhibitory inputs for the main forms of excitation/inhibition interactions. A) ON amacrine cell receives OFF crossover inhibition via a glycinergic OFF amacrine cell. B) OFF amacrine cells receive ON crossover inhibition via a glycinergic ON amacrine cell. C) Wide-field monostratified amacrine cells received excitation at ON and OFF, but received no measurable inhibition. D) ON-OFF inhibition to some classes of amacrine cell is derived from two separate sources: a GABAergic OFF inhibition and a glycinergic ON inhibition.

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 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, Pow et al. 1998; Weiss, O'Sullivan et al. 2008). We have found this to be true in our study: OFF inhibition to ON cells and ON inhibition to OFF cells is 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, Wassle 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 that GABA to mediate the lateral ON inhibition and that glycine mediates 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 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, while 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 to 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 in order to examine this hypothesis.

Asymmetries between the ON and OFF channels in the inner retina

The significant majority of ON amacrine cells (excluding AII amacrine cells) receive purely OFF inhibition, while 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, less than 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, while 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 while most OFF cells receive ON inhibition (Roska, Molnar 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. 2-13.

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 towards ON channels (Pang, Gao et al. 2002). In guinea pig retina, ON brisk transient (Y) cells exhibit contrast sensitivity differently from OFF brisk transient (Y) cells (Zaghloul, Boahen 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 (Volgyi, Abrams et al. 2005). Very recent work on a class of wide-field ON-OFF amacrine cells reveal that they have different spatial properties: the ON phase of the response has a larger receptive field than the OFF phase (Bloomfield and Volgyi 2007). The role of these striking asymmetries in processing the visual signal are not yet fully understood.

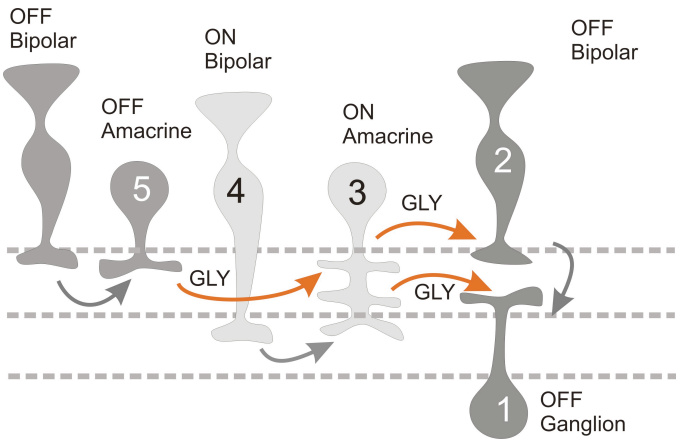

Figure 2-13. The crossover pathways leading to the dominant ON crossover inhibition to the OFF bipolar and ganglion cells. OFF ganglion cells at the right (1) receive rectified OFF excitation from the OFF bipolar cells (2). Crossover inhibition from ON amacrine cells (3)

compensates for this rectification. But the ON amacrine cells themselves receive rectified excitation from the ON bipolar cell to its left (4). The rectified output from this ON bipolar cell is compensated by crossover inhibition from the OFF amacrine cell to its left (5). Glutamate shown in dark gray; glycinergic inhibition shown in red.

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 more than 250µm 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 (150µm) to wide for alpha cells (approximately 1mm) (Peichl, Buhl et al. 1987; Rockhill, Daly 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 comprised of the GlyRα1 subunit display fast kinetics, while receptors with GlyRα2 are slow by comparison (Weiss, O'Sullivan et al. 2008). Even among the different subunits that comprise 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 only be revealed 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 upon 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, Pflug 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 AII amacrine cells, which are ON-center cells). Furthermore, A2 cells are believed to contain GABA (Pourcho and Goebel 1983). Our data confirms 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, Gao 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, while in rabbit, ON-OFF cells are monostratified 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), while 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, while 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; Sterling 1983; Roska, Molnar et al. 2006) 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, Carandini 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.

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Chapter 3

Spatial Characteristics of Amacrine-to-Amacrine Cell Inhibition

Amacrine cells whose somas are not displaced to the ganglion cell layer have been studied using the retinal slice preparation (Pang, Gao et al. 2002; Pang, Gao et al. 2007; Hsueh, Molnar et al. 2008). In these studies, the extent to which the circuitry could be studied was limited by the thickness of the retinal slice, which was between 250-300 μm. In a patch clamp experiment, it would be difficult to study the contribution of neurons more than 250 μm away to the amacrine cell of interest. While local circuits have been elucidated with the retinal slice preparation, the contributions of faraway inhibitory circuits have been truncated.

The wholemount retinal preparation would be ideal to study the interactions between amacrine cells in space, however, this preparation has primarily been used to study displaced amacrine cells, such as starburst amacrine cells (Fried, Munch et al. 2005; Lee and Zhou 2006) and A1 amacrine cells (Davenport, Detwiler et al. 2007). Here, we use the wholemount retinal preparation to study the amacrine cells whose somas reside in the inner nuclear layer. In this preparation, we are able to record the excitatory and inhibitory currents that interact in both time and space to characterize the interactions between amacrine cells. Similar to the findings in retinal slice, amacrine cells interact with one another in a few major forms, but there is an added layer of complexity in how they interact in space. Interestingly, we found that glycine played an important role in amacrine to amacrine inhibition, and it would appear that GABA is mostly involved in

feedback or feedforward inhibition. Our findings suggest that amacrine cells interact with one another using primarily glycine in a local fashion, and it is likely that they interact with bipolar and ganglion cells both locally and globally, with glycine and GABA,.

Methods

Electrophysiology

See the previous chapter for detailed steps concerning dissection and solution preparation, as well as light adaptation of the retina prior to measurements. Unlike the slice preparation, after the retina was removed from the sclera, it was mounted on a 3 mm by 4 mm rectangle of filter paper (Millipore), with a small rectangular (2 mm by 2.5mm) window. The retina was mounted with the photoreceptor layer facing downwards, taking special caution that the region of the retina over the rectangular window was not perturbed. After centering the retina over the stimulus field, a glass pipette was brought down with a micromanipulator under visual guidance and a small tear was made in the inner limiting membrane, which now allowed access to the inner plexiform and inner nuclear layers. The glass pipette was removed and a glass electrode with cesium-based intracellular solution (see Methods in Chapter 3) was lowered towards the inner nuclear layer with light positive pressure. A soma in the inner nuclear was targeted and the patch electrode was brought alongside of it, positive pressure released to establish a gigaohm seal, and with depolarizing voltage steps, we were able to break into and dialyze the neuron. All successive steps in electrophysiology (with the additional light stimuli described below) are described in Chapter 2.

Mapping spatial parameters

The cell was stimulated with a series of light or dark spots of increasing size (100, 200, 300, 400, 500, 700, 1000 μm diameter, as well as full-field stimulation). The receptive field size of the cell was measured by noting which diameter spot the cell responded most strongly to. For the sake of simplicity, cells that responded to a full-field stimulus more strongly than a 1000 μm stimulus were binned with cells that responded most strongly to 1000 μm spots (but responded more weakly to a full-field stimulus). The receptive field size was measured for both excitatory and inhibitory inputs.

In addition to stimulating the cells with different size spots, we also used the light or dark raster stimulus to examine the space-time characteristics of the cell we measured from. The raster stimulus consisted of a 600 μm light or dark square that was marched in 60 μm increments across the retina. The raster plot represents the response of a population of cells of the same type to a 600 μm square (which is essentially a large edge). Based on the raster response, cells were grouped as narrow, medium, or wide. A wide cell was one that received excitatory synaptic inputs from a light or dark square whose edge was more than 600 μm away from its soma. A medium cell was one that responded to the edge of a light or dark square between 300 and 600 μm away from its soma. A narrow cell was one that only responded to a light or dark flash positioned directly over its receptive field.

Pharmacology

We applied a variety of pharmacological blockers to determine the neurotransmitter that mediated the inhibition we measured, as well as determine the contribution of either GABA or glycine to the spatial characteristics of inhibition. To block glycine receptors,

we used 2 μM strychnine, and to block $GABA_A$ receptors, we used 5 μM SR95531. The response during wash steps was obtained for these experiments whenever possible (SR95531 consistently washed out in a timely manner, but strychnine often took at least 10-15 minutes to wash and in some cases, did not wash out at all). We computed the percent change in light-elicited current responses, and used the paired t-test to determine if the pharmacological agent had a significant effect. This computation is described in detail in Chapter 2.

Morphology

At the end of cell recording, live grid confocal z-stacks were taken of the cell to determine its dendritic spread as well as stratification. Cells were grouped by their dendritic spread: narrow (<200 μm), medium (200-400 μm), and wide (>400 μm). For most cells, we could identify monostratified vs. multiply-stratified cells, but in some cases, the dispersion across z-planes were prohibited us from determine any further subdivisions.

Results

We were able to characterize the light responses of 115 amacrine cells in the flatmount rabbit retina. Of these amacrine cells, 15 were morphologically identified as AII amacrine cells and will be omitted from this analysis.

ON amacrine cells that receive OFF inhibition

We measured from 35 amacrine cells that received an increase in excitatory input at light ON. Of these 35 ON amacrine cells, 15 cells received an increase in inhibitory input at light OFF, 18 cells received inhibitory input at both light ON and OFF, and 2 cells received inhibitory input at light ON. Figure 3-1 shows a sample light response from an ON amacrine cell that received OFF inhibition. The stimulus is a light flash that is mapped to the receptive field size of the cell, which is shown for 2.5 seconds. The excitatory currents measured by clamping the cell at -60mV are shown on the left column, and the inhibitory currents are shown on the right column. The response under control conditions is shown in Figure 3-1A, B.

Strychnine did not significantly affect the ON excitatory input, shown in Figure 3-1C. However, the OFF inhibitory current is blocked, as shown in Figure 3-1D (stats), which indicates that the OFF inhibitory current was mediated by glycine.

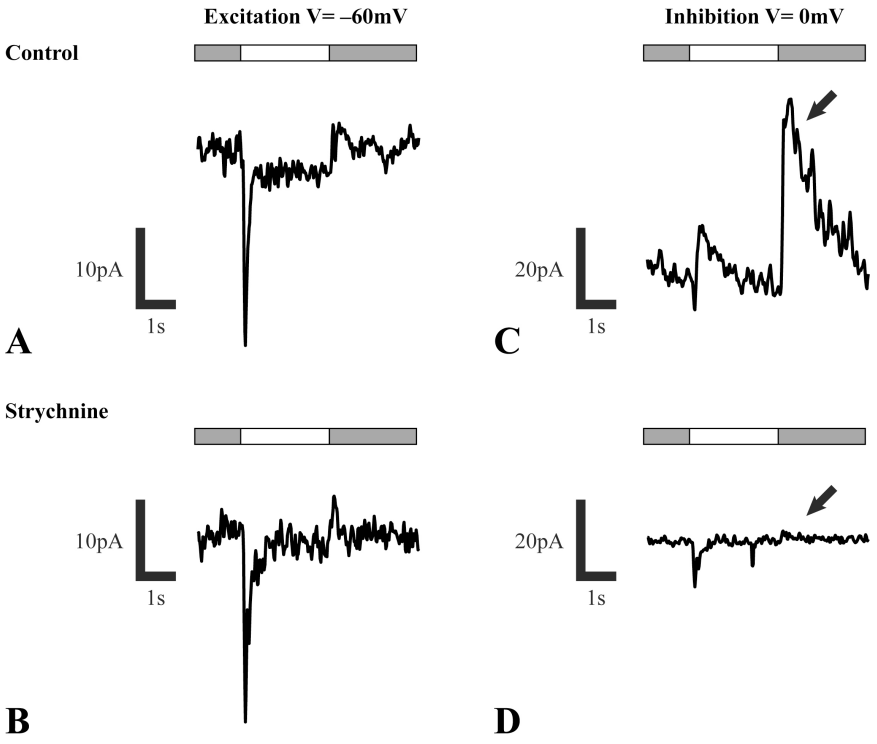
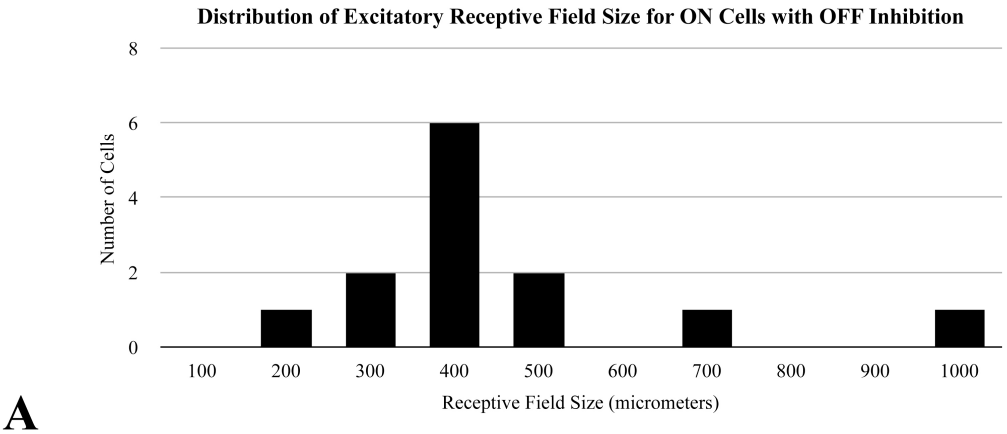


Figure 3-1. ON amacrine cells receive glycinergic OFF inhibition. In this and future figures, the top bar represents the time course of the 2.5 second light stimulus. The light region represents an increment in light intensity, and dark region represents a decrement in light intensity. A) ON excitatory input measured by clamping the amacrine cell at -60mV. B) ON excitation persists in the presence of 2µm strychnine. C) OFF inhibitory input measured by clamping the amacrine cell at 0mV. D) OFF inhibition is blocked by strychnine, which indicates it is glycinergic.

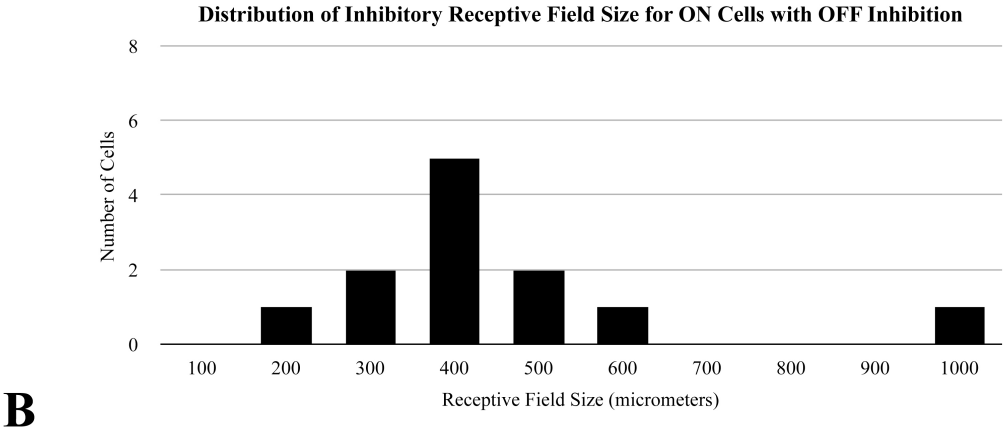
We mapped the receptive field for both excitatory and inhibitory inputs for ON cells that received OFF inhibition and found that the receptive field for both excitation and inhibition had similar spatial characteristics, as shown in Figure 3-2. Most cells whose excitatory receptive fields were wide also received inhibition on the same scale. The receptive field for excitation ranged from 200 µm to 1000 µm (n=15, 583.33µm ±

280.73μm, mean receptive field size ± standard deviation), while the receptive field for inhibition spanned a similar range, though generally somewhat smaller by comparison (n=12, 458.33μm ± 198.67μm).

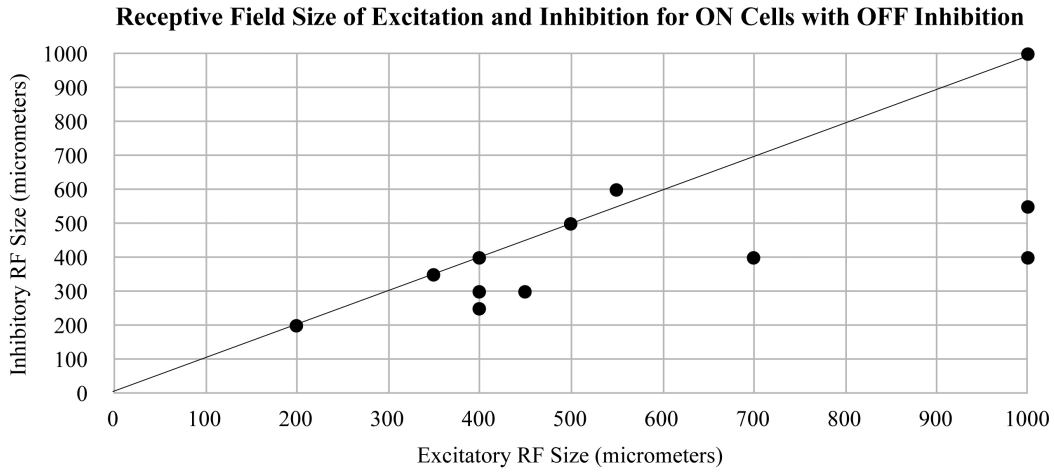
The raster responses for excitation were quite similar to the raster responses for inhibition: a cell responded strongly only when the stimulus was positioned directly over the cell, and not when the stimulus was more than 300 μm away. In some cases, the excitatory receptive field for the cell was greater than the 600μm square, and yet was able to discern the edge of the stimulus; that is, it responded only to the region *within* the 600 μm stimulus, and not to the region *outside* the stimulus. Examples of a typical raster plot for both excitation and inhibition are shown in Figure 3-3.



A



B



C

Figure 3-2. Receptive field for ON excitatory and OFF inhibitory inputs. A) The receptive field for the excitatory input was measured by clamping the cell at -60mV, the

reversal potential for chloride-mediated current. Across the 15 ON cells that received OFF inhibition, the receptive field for excitation varied from 200µm to 1000µm, with most cells' receptive field size at 600µm. This suggests that we measured from a variety of amacrine cell types. B) The receptive field for inhibitory input was measured by clamping the cell at 0mV. The distribution of receptive field sizes was similar to the distribution in A, which indicates that the inhibitory input to a cell was of the same spatial scale as its excitatory input. It is noteworthy that the receptive field for inhibition was typically somewhat smaller than excitation. C) A scatterplot where each point represents a cell, and the x-variable is the receptive field size for excitation, and the y-variable is the receptive field for inbibition. The solid line is a unity line, y=x. Most cells are either on the unity line, or below it, indicating that the inhibitory receptive field is typically somewhat smaller than the excitatory receptive field.

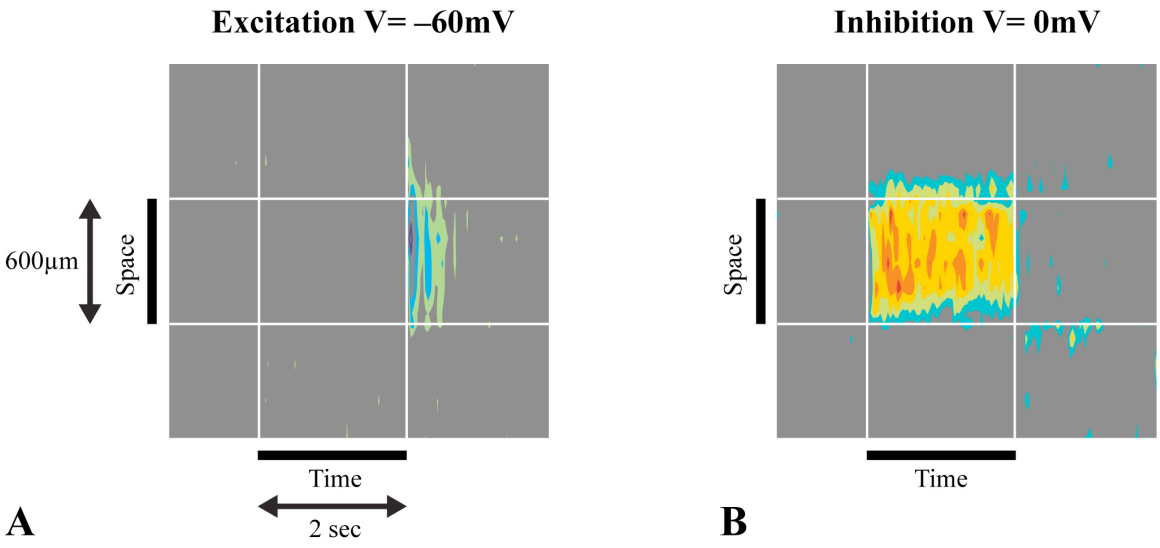


Figure 3-3. Excitatory and inhibitory dark raster plot for ON cells that received OFF inhibition. This is an example raster plot measured from a different ON amacrine cell. The x-axis represents the time course of the stimulus, while the y-axis represents the region in space that the stimulus covers. The stimulus is a 600µm dark square presented to the retina for 2 seconds. Cool colors (i.e. green, blue) represent inward currents, while hot colors (i.e. orange, red) represent outward currents. A) An excitatory raster response measured by clamping the cell at -60mV. The strongest inward currents occur at the termination of the dark flash, which is an ON response. This response is confined within the boundaries of the flash. B) An inhibitory raster response measured by clamping the cell at 0mV. The strongest outward currents occur at the initiation of the dark flash, and are confined between the boundaries of the flash. Both the responses described in A and B show that this cell type only responds to a stimulus that is situated directly over its dendritic field, and is capable of responding to the edge of a 600 µm flash.

ON cells that receive ON-OFF inhibition

Approximately half of the ON cells we measured from (18/33) received ON-OFF inhibition. Examples of this ON-OFF inhibition is shown for two ON cells in Figure 3-4A, B. When we held the cell at 0mV, there were outward currents at light onset and offset.

When we blocked $GABA_A$ receptors with SR95531, the ON-OFF currents were not significantly affected, as shown in Fig. 3-4C (n=4). However, the ON-OFF currents were strongly suppressed in the presence of strychnine, as shown in Figure 3-4D. These experiments demonstrate that these ON amacrine cells received glycinergic inhibition, either from a single ON-OFF amacrine cell, or two amacrine cells: one ON and another OFF cell. Pharmacological experiments do not allow us to discern if there are one or two pre-synaptic amacrine cells.

The receptive field of this cell type is shown in Fig. 3-5. The excitatory receptive field varied from 200µm to over 1000µm (n=18, 572.22µm ± 304.00µm), however, the inhibitory receptive field was comparably more narrow (n=18, 402.78µm ± 213.84µm). We can further characterize the spatial response of this cell type using the raster stimulus. An example raster response is shown in Fig. 3-6. The response of this cell type for both excitatory and inhibitory inputs was strongest at the area underneath the flash. There was little or no response when the cell was 300µm away from the edge.

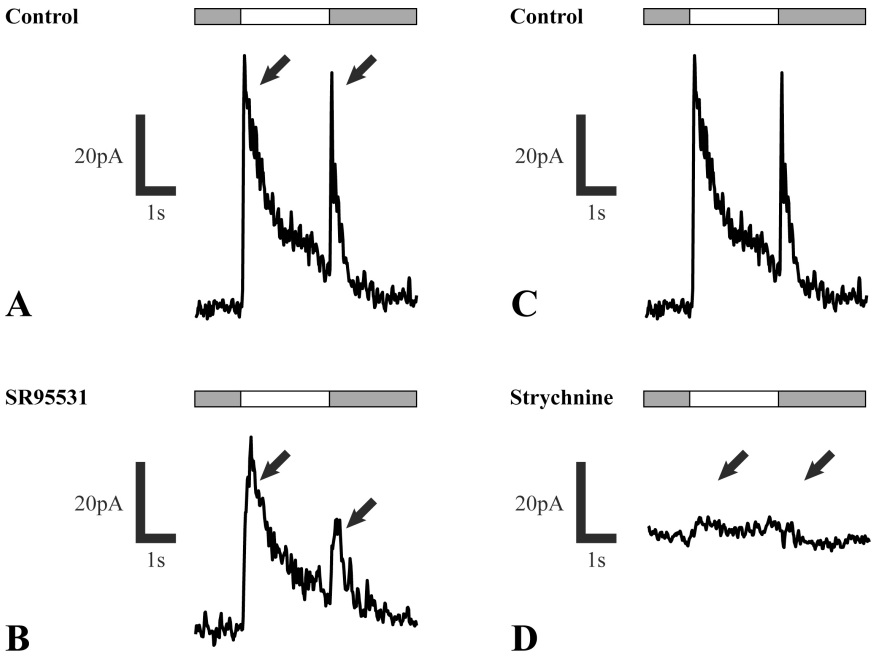
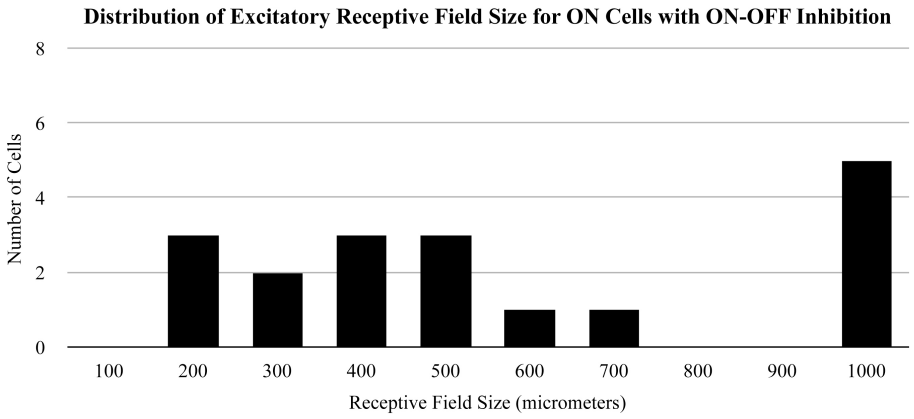
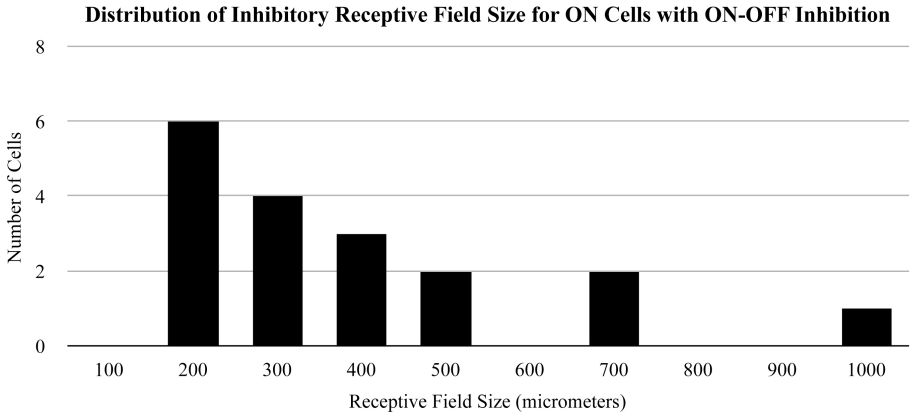


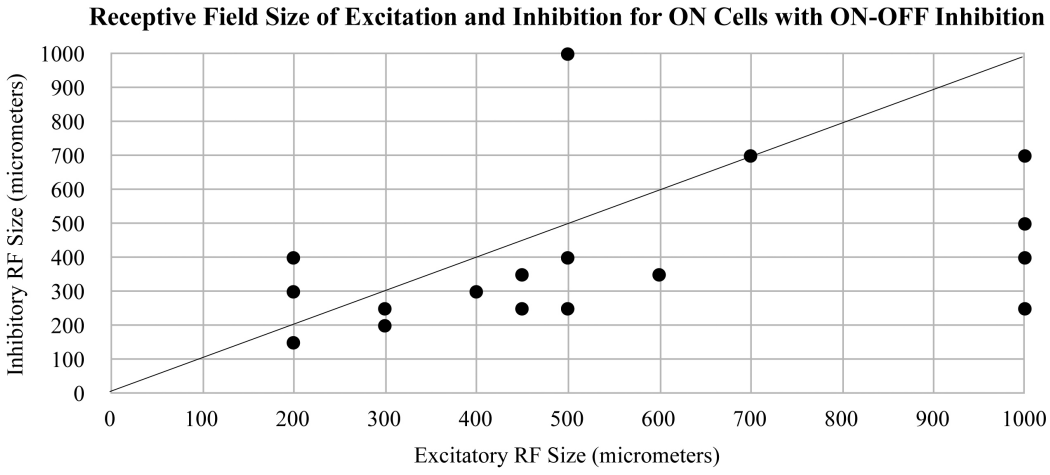
Figure 3-4. ON-OFF inhibition to ON amacrine cells was mediated primarily by glycine. Example inhibitory traces measured from an ON amacrine cell voltage-clamped at 0mV. A) In control conditions, we measured outward currents are light ON and OFF. B) In the presence of 5μM SR95531, the ON-OFF inhibitory currents were not significantly affected. C) ON-OFF outward inhibitory currents in control conditions. D) In the presence of 2μM strychnine, the ON-OFF inhibition was blocked, which indicates that the ON-OFF inhibition was mediated primarily by glycine and not GABA.



A



B



C

Figure 3-5. Distribution of receptive field size for ON amacrine cells that received ON-OFF inhibition. A) The excitatory receptive field size varied from 200µm to

1000µm, distributed relatively evenly across all sizes. This variety suggests that we measured from diverse amacrine cell types, from narrow to wide. B) The inhibitory receptive field size also varied from 200µm to 1000µm, but in contrast to the distribution of receptive field sizes for excitation, the majority of the cells received relatively narrow inhibition, with most cells (13/18) receiving ON-OFF inhibition that was ≤ 400µm. This suggests that glycinergic ON-OFF inhibition between amacrine cells was relatively narrow. C) A scatterplot where each point represents a cell, and the x-variable is the receptive field size for excitation, and the y-variable is the receptive field for inbibition. The solid line is a unity line, y=x. Many cells are either on the unity line, or below it, indicating that the inhibitory receptive field is typically somewhat smaller than the excitatory receptive field.

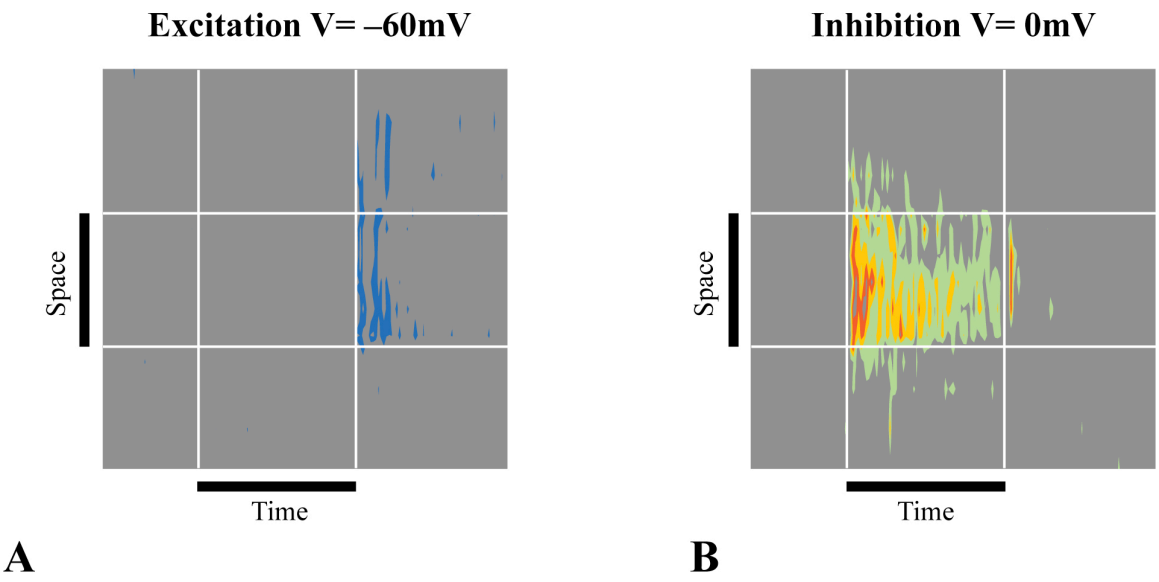


Figure 3-6. Excitatory and inhibitory dark raster plot for ON cells that received ON-OFF inhibition. Example raster plots from an ON amacrine cell that received ON-OFF inhibition. A) Excitatory raster plot. The majority of the response was in the region under the dark flash, and occurred transiently at light onset (regions in blue). This cell also responded in regions outside of the flash. B) Inhibitory raster plot. Most of the activity occurred within the boundaries of the flash, at both the flash onset and offset (regions in orange). The majority of glycinergic ON-OFF inhibition we measured had relatively small receptive fields and narrow raster responses like this one.

ON cells with ON inhibition

A minority of ON cells received ON inhibition (2/33). Because these cells were encountered so rarely, we could not characterize the pharmacology of their inhibition, nor their morphology.

Morphology of ON amacrine cells

We were able to characterize the stratification and dendritic spread of 13 ON amacrine cells, shown in Fig. 3-7. ON cells that received OFF inhibition had processes that spanned across the proximal layers of the inner plexiform layer, usually extending deep into the ON sublamina, as seen in Fig. 3-7A. The spread of these processes varied from very small (~100µm) to large (>400µm), which corresponded to the large range in receptive field size for excitation. With our current dataset, it appears that at least a few morphological types of ON amacrine cells receive OFF inhibition.

ON cells that received ON-OFF inhibition appear to fall into two morphological classes, as shown in Fig. 3-7B. The first type is wide, and less diffuse, stratifying deeply in the ON sublamina. The second type is narrow, with processes that are diffuse, terminating in all layers of the inner plexiform layer.

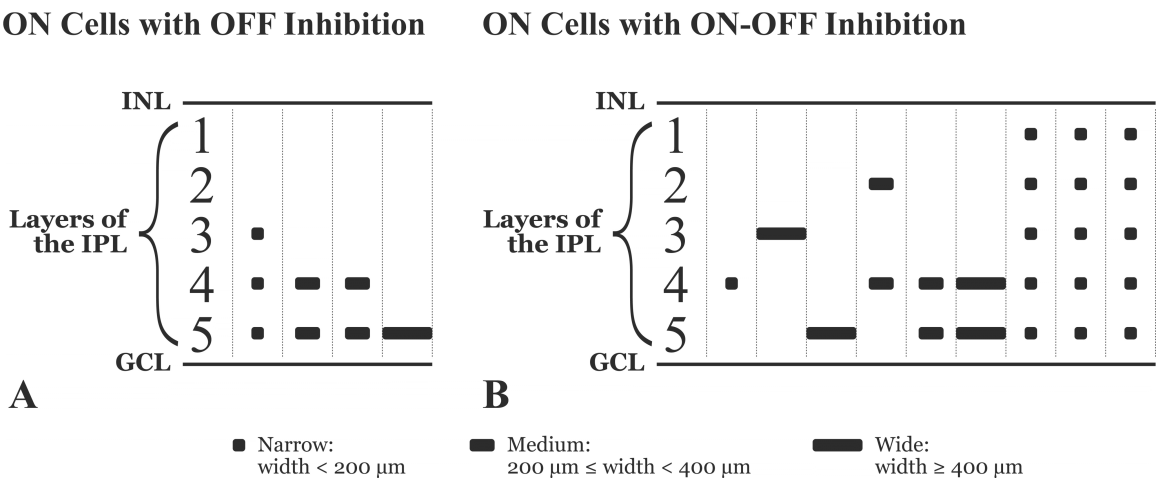


Figure 3-7. Morphology of ON amacrine cells. Each column represents one cell, with the location of the bar indicating the layers in which the processes stratify, and the width of the bar indicating the size of the dendritic spread. A) ON cells that received OFF inhibition stratified deeply in the inner plexiform layer, and had dendritic spreads that

varied from narrow to wide. B) ON cells that receive ON-OFF inhibition fell into two major types. The first type consists of medium to wide cells that stratified deeply in the inner plexiform layer, such as the cells on the left. The second type consists of narrow cells that stratified throughout all the layer of the inner plexiform layer, and had narrow dendritic spread.

OFF amacrine cells with ON inhibition

We measured from 23 amacrine cells that received OFF excitation. The majority of these OFF cells (15/23) received ON inhibition. This ON crossover inhibition was blocked by 10μM strychnine, as shown in Fig. 3-8. Thus, like OFF crossover inhibition to ON cells, ON crossover inhibition was mediated primarily by glycine.

We mapped the receptive field size of both the OFF excitation and ON inhibition and found that while the receptive field size for excitation varied from narrow to wide (n=18, 544.44μm ± 334.26μm), the ON inhibition was generally narrower (n=18, 411.11μm ± 162.30μm), as shown in Fig. 3-9. We further characterized the inhibitory spatial response using the raster stimulus, and found that the cell responded most strongly in the region underneath the flash, as shown in Fig. 3-10.

Thus, like crossover OFF inhibition, crossover ON inhibition was glycinergic and relatively narrow-field.

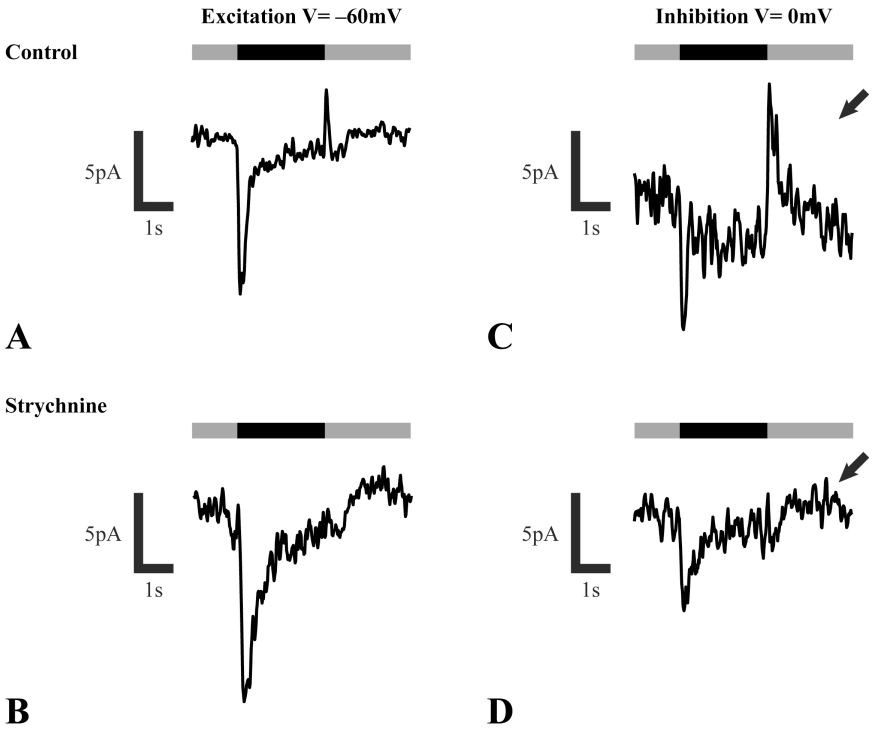
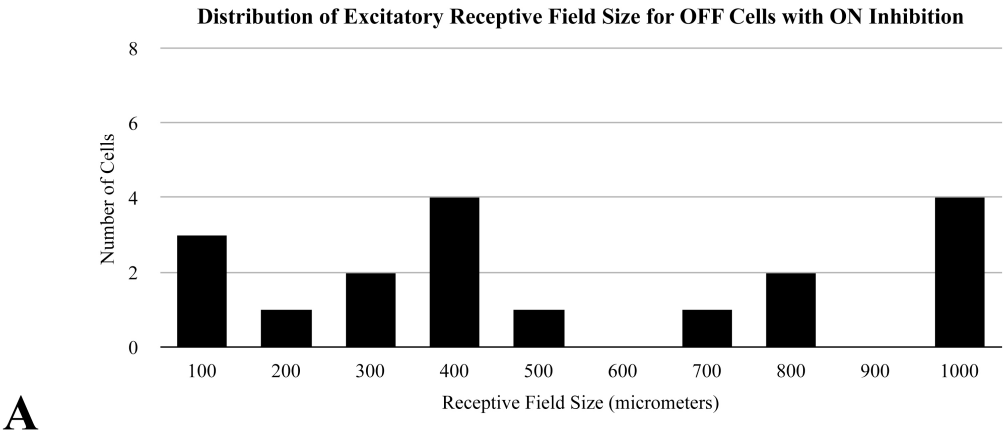
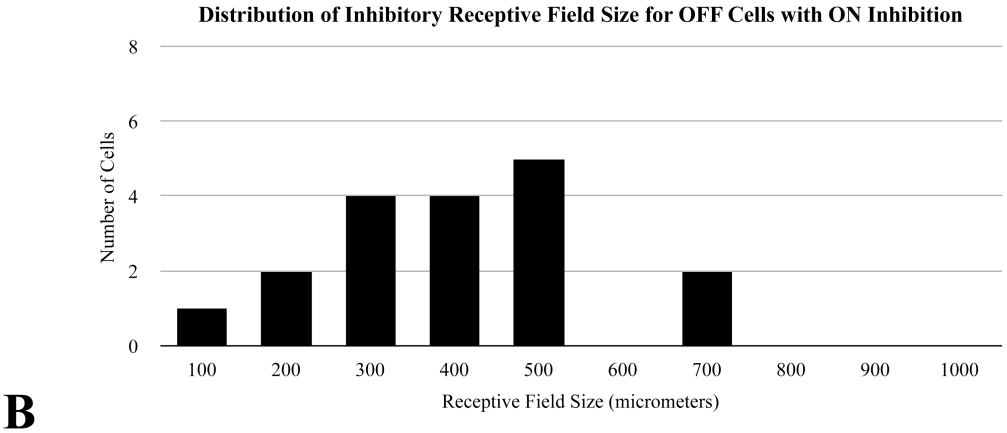


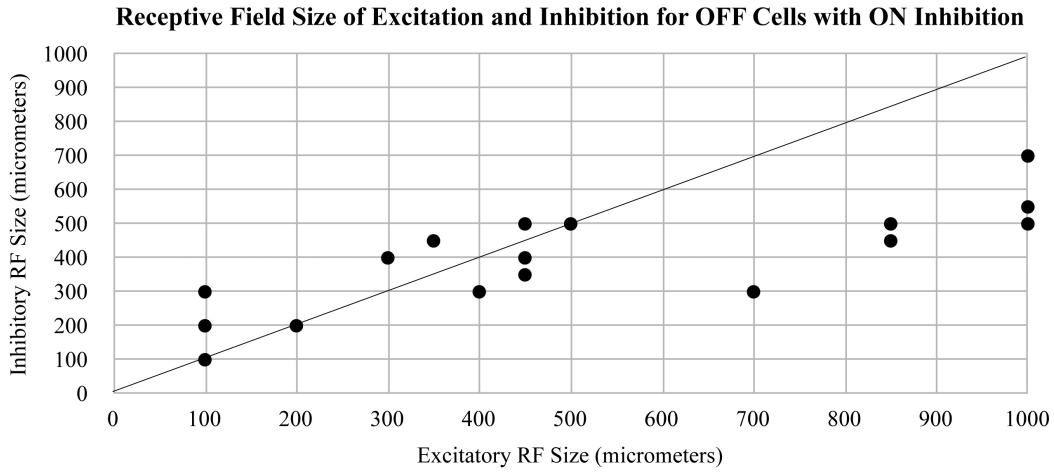
Figure 3-8. ON inhibition to OFF cells is mediated by glycine. Example traces of an OFF amacrine cell that received ON inhibition in response to a dark flash. A) OFF excitatory input is maximum at light OFF. B) OFF excitation persists in the presence of 2µm strychnine. C) ON inhibitory input is maximum at light ON. D) ON inhibition is blocked by strychnine, which indicates it is mediated by glycine.



A



B



C

Figure 3-9. Histograms showing the receptive field for OFF excitatory and ON inhibitory input. A) The receptive field for OFF excitation varied considerably from

100µm to 1000µm, which likely indicates the large range of dendritic spread in the various morphological types of OFF amacrine cells that received ON inhibition. B) The distribution of receptive field size for ON inhibition tended to be smaller, between 300-500µm, and suggests that a smaller subset of amacrine cells provided the ON inhibition to these OFF cells. C) A scatterplot where each point represents a cell, and the x-variable is the receptive field size for excitation, and the y-variable is the receptive field for inbibition. The solid line is a unity line, y=x. Most cells are either on the unity line, or below it, indicating that the inhibitory receptive field is typically somewhat smaller than the excitatory receptive field.

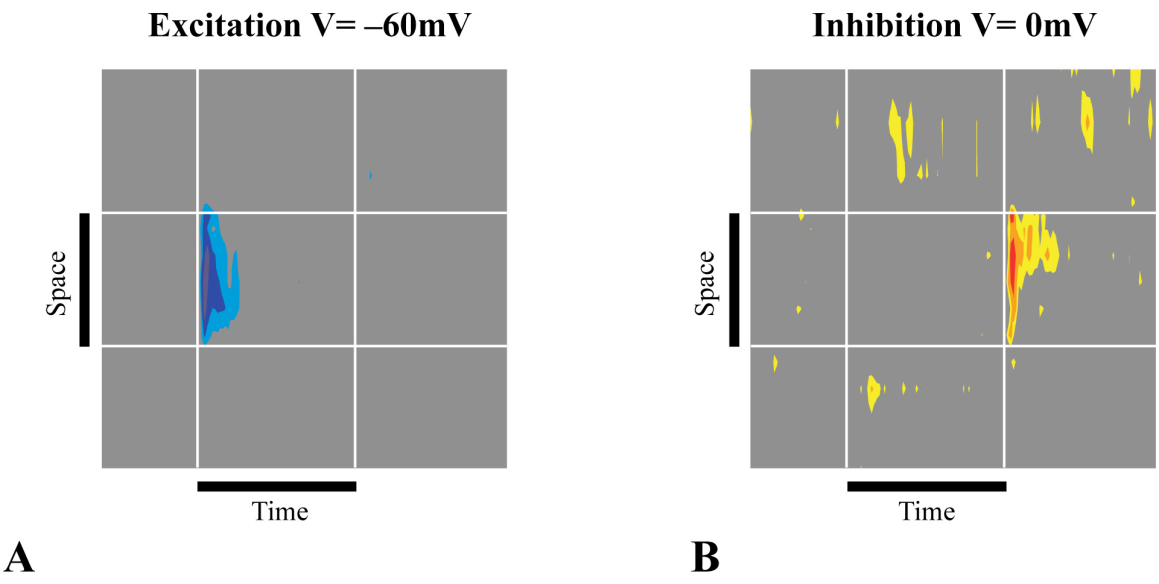


Figure 3-10. Excitatory and inhibitory dark raster plot for OFF cells that received ON inhibition. This is an example raster plot measured from a single OFF amacrine cell that received ON inhibition. A) Excitatory raster plot measured by clamping at -60mV. The inward excitatory current response was confined to the region underneath the dark flash, however, for other OFF cells with ON inhibition, the response extended outside the region of the flash. B) Inhibitory raster plot measured by clamping at 0mV. The outward inhibitory current response was largely confined to the region occupied by the dark flash (with some responses extending outside of the flash), and occurred at the termination of the dark flash (ON response). In most cases, the inhibitory response remained constrained to the region beneath the flash, reflecting the relatively limited size of the receptive field for inhibition.

OFF amacrine cells that receive ON-OFF inhibition

A few OFF amacrine cells received ON-OFF inhibition (5/23). We were able to characterize the pharmacology of two of these cells, and in both cases, the ON-OFF

inhibition was strongly blocked in the presence of 2μM strychnine, which suggests that the ON-OFF inhibition was primarily mediated by glycine, as shown in Fig. 3-11. The inhibitory receptive field and raster response of these cells is relatively narrow (n=5, excitatory receptive field: 500μm ± 291.54μm; inhibitory receptive field: 420μm ± 356.37μm), which is consistent with our other data suggesting that glycine mediates more local inhibition. The receptive field and raster responses for both excitation and inhibition are shown in Fig. 3-12.

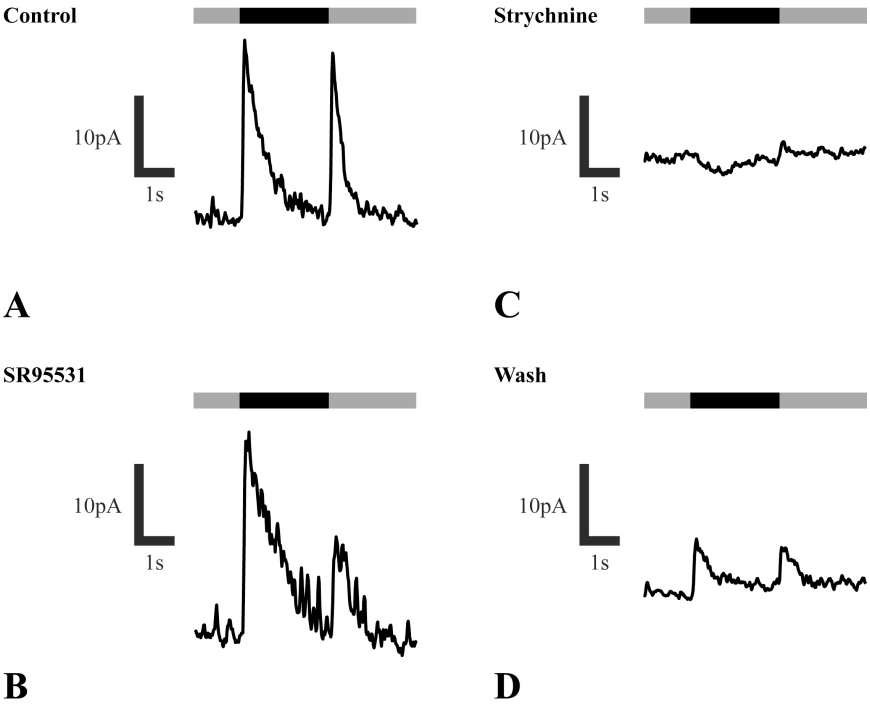
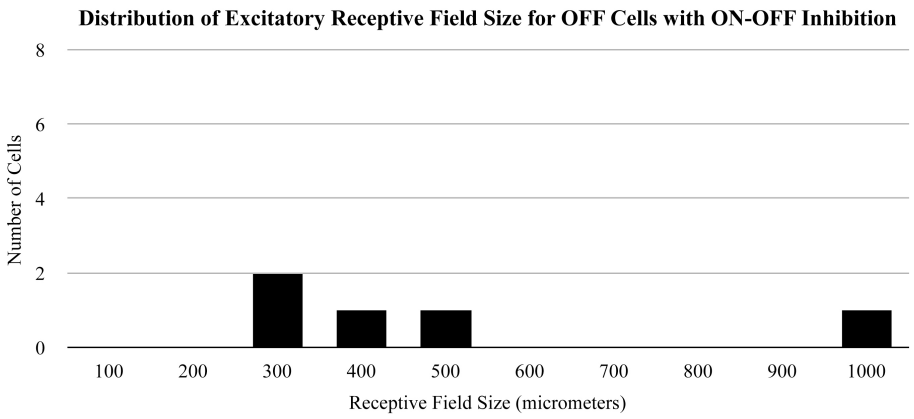
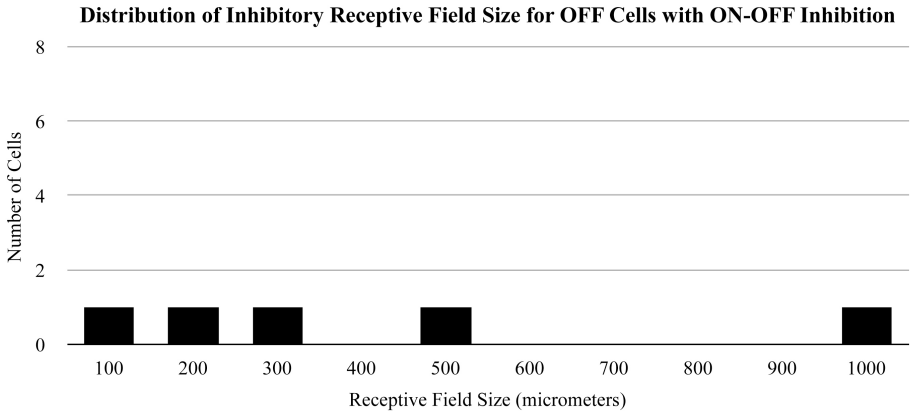


Figure 3-11. ON-OFF inhibition to OFF amacrine cells is mediated primarily by glycine. Inhibitory input to an OFF amacrine cell measured by clamping the cell at 0mV for the entire experiment. A) Outward inhibitory currents occurred at both light decrement and increment – an ON-OFF inhibition – in control conditions. B) After the application of SR95531, the ON-OFF inhibition became somewhat sluggish, but

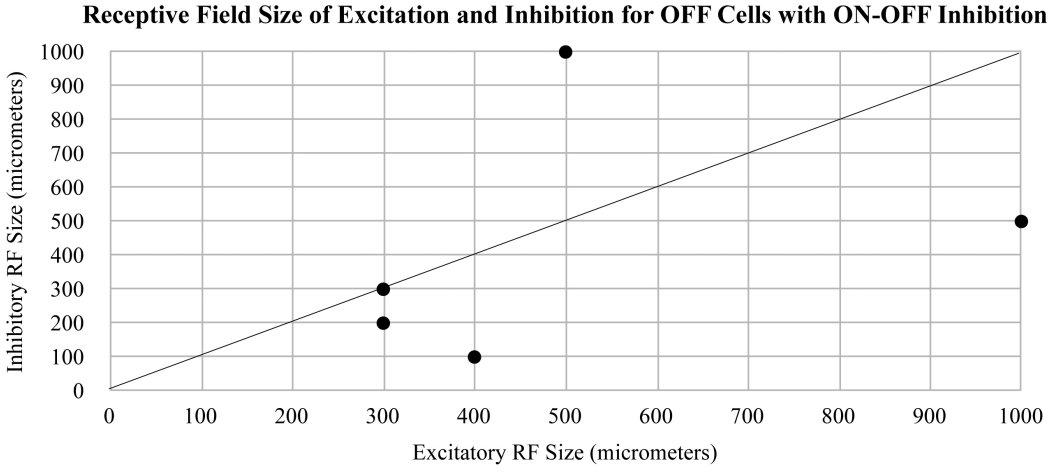
persisted, indicating that GABA was not the main neurotransmitter mediating this inhibition. C) In the presence of strychnine, the ON-OFF inhibition was blocked, which indicates that glycine was the major neurotransmitter that mediated the inhibition. D) In this case, we were able to wash the strychnine out (required a 10-15 minute long wash), and while the ON-OFF inhibitory response is greatly attenuated, it was restored after strychnine washed out.


Distribution of Excitatory Receptive Field Size for OFF Cells with ON-OFF Inhibition
Number of Cells
0
2
4
6
8
100
200
300
400
500
600
700
800
900
1000
Receptive Field Size (micrometers)

A


Distribution of Inhibitory Receptive Field Size for OFF Cells with ON-OFF Inhibition
Number of Cells
0
2
4
6
8
100
200
300
400
500
600
700
800
900
1000
Receptive Field Size (micrometers)

B


Receptive Field Size of Excitation and Inhibition for OFF Cells with ON-OFF Inhibition
Inhibitory RF Size (micrometers)
0
100
200
300
400
500
600
700
800
900
1000
Excitatory RF Size (micrometers)

C

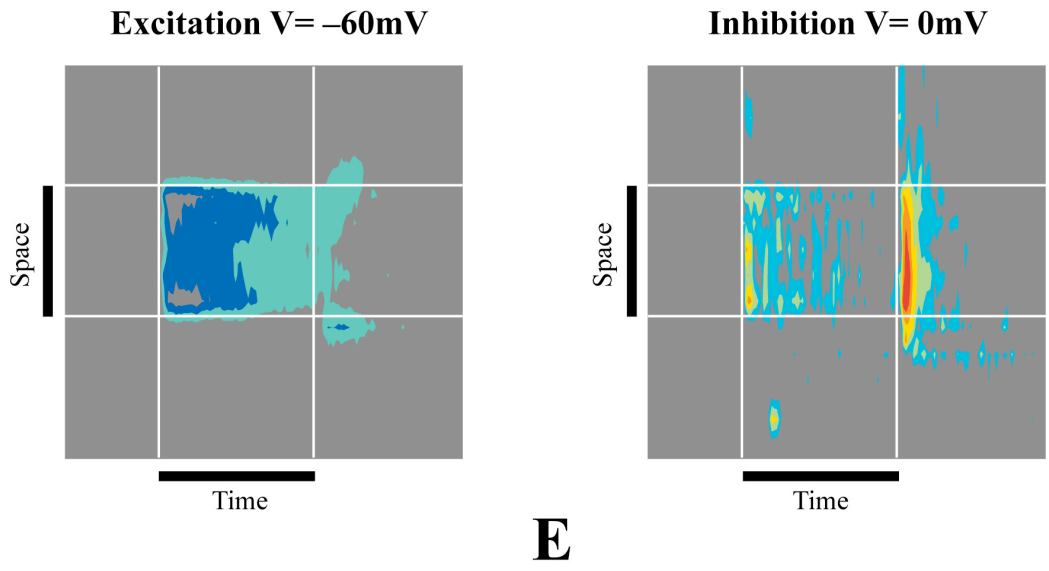


Figure 3-12. Receptive field distribution and raster response of OFF cells that received ON-OFF inhibition. A) The receptive field for OFF excitation was mostly medium sized, around 300-500µm, with one cell whose receptive field was large, at 1000µm. B) The receptive field for ON-OFF inhibition was relatively smaller in comparison with the excitatory receptive field: most cells received inhibition from amacrine cells with narrow receptive fields. C) A scatterplot where each point represents a cell, and the x-variable is the receptive field size for excitation, and the y-variable is the receptive field for inbibition. The solid line is a unity line, y=x. D) An example of an excitatory raster plot from one OFF amacrine cell that received ON-OFF inhibition. In this cell, the inward excitatory current response occurred underneath the region of the dark flash. E) The inhibitory raster for the same cell, which showed transient outward currents at both the initiation and termination of the dark flash, with the majority of the response confined to the region underneath the stimulus.

OFF amacrine cells that received OFF inhibition

In two cases, we measured from OFF cells that received OFF inhibition. We were unable to characterize the pharmacology of this OFF inhibition. The morphological properties of these two cells suggest that they were not of the same type: one was narrow and the other was wide. One would expect based on past work that this OFF inhibition would be GABAergic, however we did not encounter these cells frequently enough to test this hypothesis.

Morphology of OFF amacrine cells

We were able to image 18 of the OFF amacrine cells we measured from, and they varied from narrow to wide, indicating that we had measured from a diversity of amacrine cell types, summarized in Fig. 3-13.

OFF cells that received crossover ON inhibition were diverse, ranging from 300µm to 1000µm dendritic spread, with most of their processes terminating in the OFF sublamina, as shown in Fig. 3-13A. Wide cells typically had their processes in the distal portion of the inner plexiform layer, comprising of the OFF sublamina. Narrow cells tended to be diffuse, with processes even extending into the ON sublamina. It appears that narrow cells were more diffusely stratified than monostratified cells.

OFF cells that received OFF inhibition were confined to the OFF sublamina, as shown in Fig. 3-13B. The sample size is currently too small to determine if more than one morphological type comprised this physiological type.

OFF amacrine cells that received ON-OFF inhibition had more diffuse processes, and spanned both the ON and OFF sublamina, shown in Fig. 3-13C. Their dendritic

spread varied from narrow to wide, and suggests that multiple morphological types comprised this physiological type.

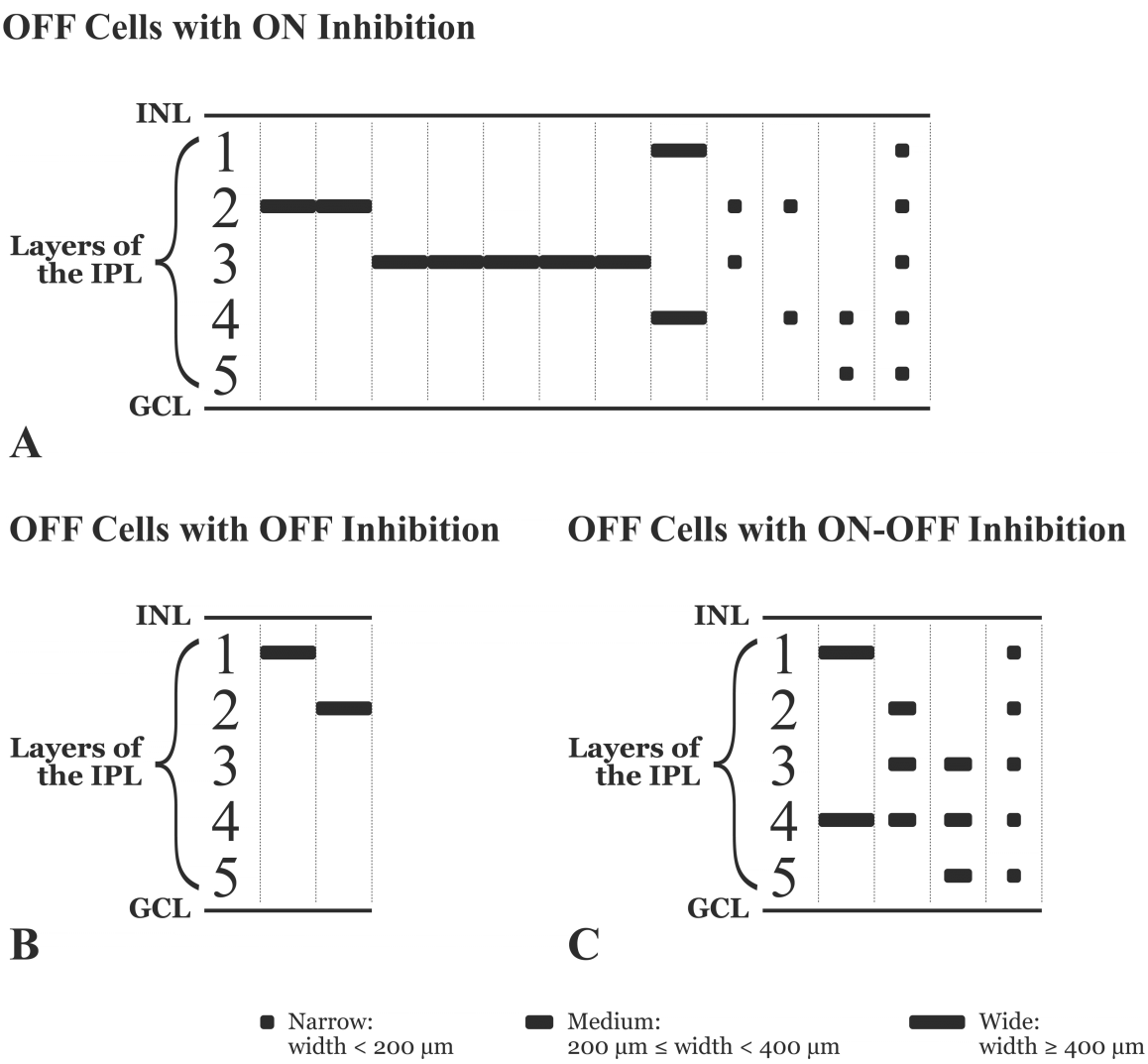


Figure 3-13. Morphology of OFF amacrine cells. A) OFF cells that received ON inhibition fell into two major types: monostratified and diffusely stratified. The monostratified cells stratified in the OFF sublamina and were wide-field. The processes of diffusely stratified cells extended into both the ON and OFF layers, and were narrow-field. B) OFF cells that received OFF inhibition were wide-field, and stratified

exclusively in the OFF sublamina. C) OFF cells that received ON-OFF inhibition were diffusely stratified, spanning both the ON and OFF layers, and varied in size from narrow to wide.

ON-OFF amacrine cells with little inhibition

We measured from 38 amacrine cells that received excitation at both light ON and OFF. When we clamped ON-OFF cells at 0mV to measure inhibitory input, many of these cells (15/39) showed inward currents at light ON and OFF, and no outward currents. Since these inward currents were smaller than the inward currents we measured when we clamped the cell at -60mV, it appeared that we did not space clamp these cells adequately (i.e. the soma was voltage-clamped, but the far dendrites were sitting at their native membrane potential), and thus any currents we measured would be some combination of excitatory and inhibitory inputs. Excitatory inputs would open cation ion channels, tending to generate inward currents, and inhibitory inputs would open chloride channels, tending to generate outward currents. What we have been able to measure is potentially a mixture of these inputs, with a net inward current. If the inward excitatory current were masking an inhibitory input, we would expect that if we blocked the inhibitory input, the inward currents would increase in magnitude.

We tested 9 of the 15 ON-OFF amacrine cells that were difficult to space-clamp, and measured their currents in the presence of strychnine or SR95531. As shown in Fig. 3-14B, the application of strychnine did not increase the magnitude of the inward current. Likewise, as shown in Fig. 3-14D, the application of SR95531 did not increase the magnitude of the inward current. This suggests that if there were any direct inhibitory

input to these cells, they are small and difficult to measure under our experimental conditions. In both the strychnine and SR95531 experiments, we saw a notable *decrease* in the magnitude of the inward current. This decrease was most likely due to the gradual run-down of the light response, and not a result of the inhibitory receptor blockers because we saw similar rates of run-down in the pure excitatory input that we measured at -60mV, and this reduction in current magnitude was not recovered in the wash step. These results suggest that this type of ON-OFF amacrine cell does not receive significant direct inhibitory input.

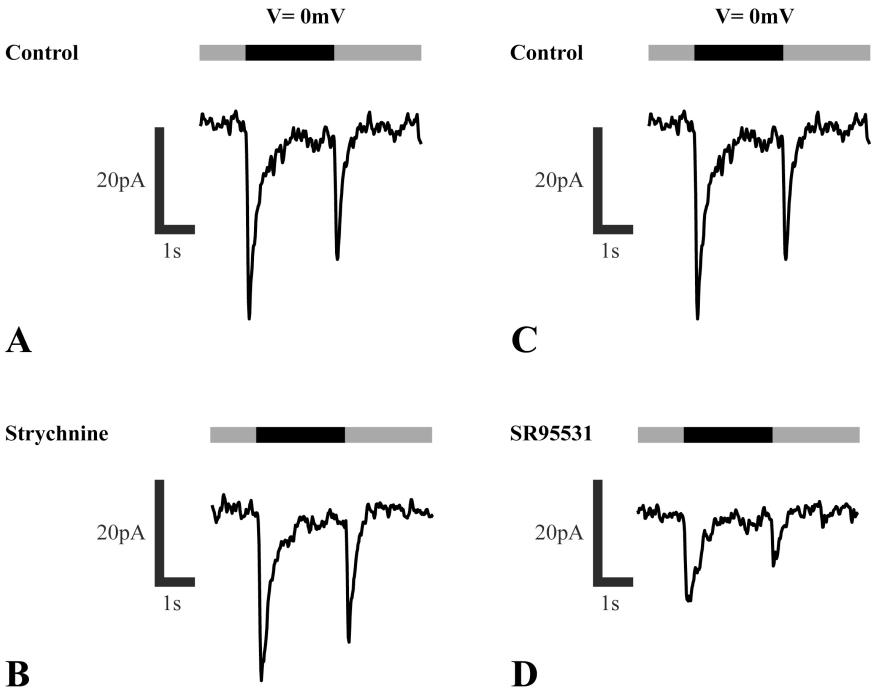


Figure 3-14. ON-OFF cell responses in the presence of strychnine and SR95531. Currents measured from an ON-OFF amacrine cell. A) ON-OFF inward currents measured when the cell is held at 0mV. B) In the presence of 2μM strychnine, the magnitude of the inward currents do not increase, but decrease slightly. Currents

measured from a second ON-OFF amacrine cell. C) ON-OFF inward currents measured when the cell is held at 0mV, similar to the cell in part A and B. D) In the presence of 5μM SR95531, the magnitude of these inward currents are not increased, but rather decrease.

Because the light responses of these ON-OFF cells were so similar, we expected that they would be of the same amacrine cell type. Surprisingly however, their spatial responses were quite varied. The receptive field for excitation ranged from narrow-field to wide-field, and this could be measured in the spot responses, as well as the raster plots, shown in Fig. 3-15. Physiologically, even though the basic connectivity of these cells were similar (they received excitatory input from both ON and OFF bipolar cells, without significant inhibitory from other amacrine cells), these ON-OFF cells may have different roles in spatial processing.

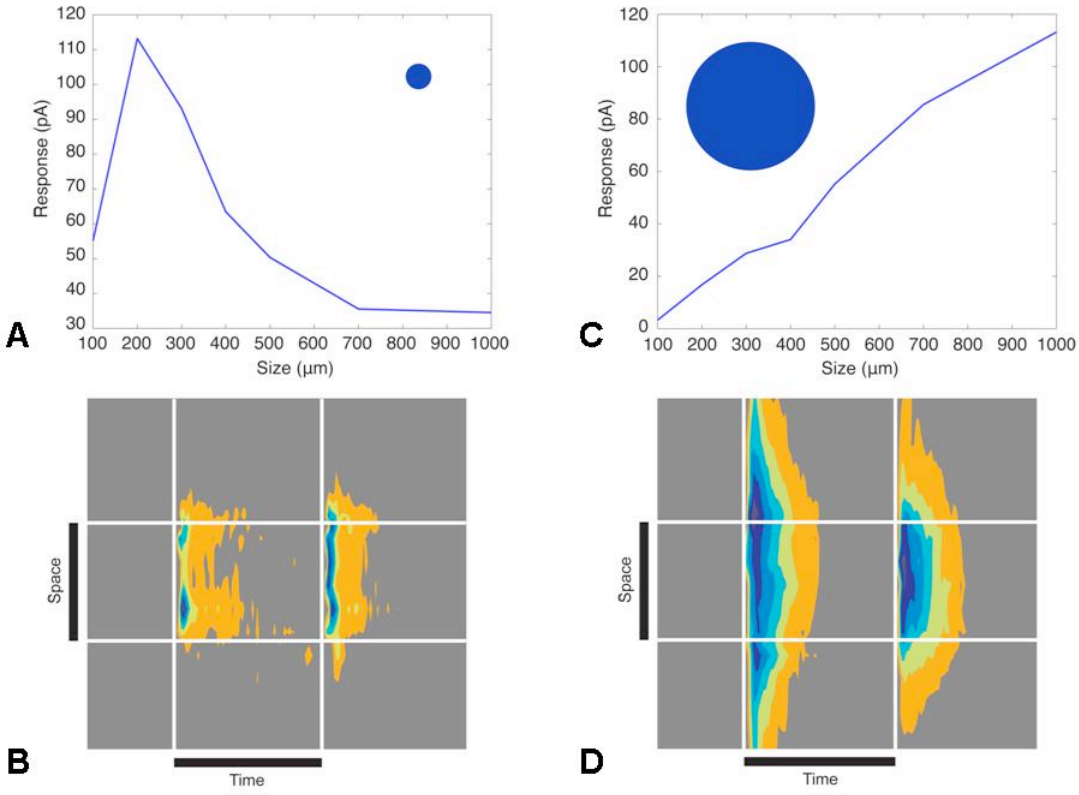


Figure 3-15. ON-OFF cells without significant direct inhibitory inputs have different spatial characteristics. A) Receptive field mapping for an ON-OFF cell that does not receive significant inhibitory input. Peak current response to 200µm spot. B) Same cell as A. Raster plot shows that the strongest excitatory response occurs in the area underneath the 600µm flash. Both the receptive field mapping and the raster plot show that this cell has a narrow receptive field. C) Receptive field mapping for an ON-OFF cell that does not receive significant inhibitory input. The peak response that we measured was at 1000µm, but actual receptive field size could be larger than 1000µm. D) Same cell as C. Raster plot shows the cell responds strongly, even in areas that are far outside the boundaries of the flash. Both the receptive field mapping and the raster plot show that this cell has a wide receptive field.

ON-OFF amacrine cells that receive ON-OFF inhibition

We measured from 12 ON-OFF amacrine cells that also received ON-OFF inhibition. We were able to characterize the pharmacology of 7 cells. In one case, we applied strychnine which strongly suppressed the ON-OFF inhibition, as shown in Fig. 3-16. In the other six cases, we applied SR95531 and found that while SR95531 altered the response kinetics, it did not strongly block the ON-OFF inhibition. The ON-OFF inhibitory responses become more sustained when we applied SR95531, and lost any transient components that were present in control conditions. Additionally, SR95531 blocked wide-field responses, while leaving local responses relatively unaffected, as shown in Fig. 3-17. Taken together, the results suggest that local ON-OFF inhibition is not mediated by GABA, but likely mediated primarily by glycine. Wide-field ON-OFF inhibition may be mediated primarily by GABA.

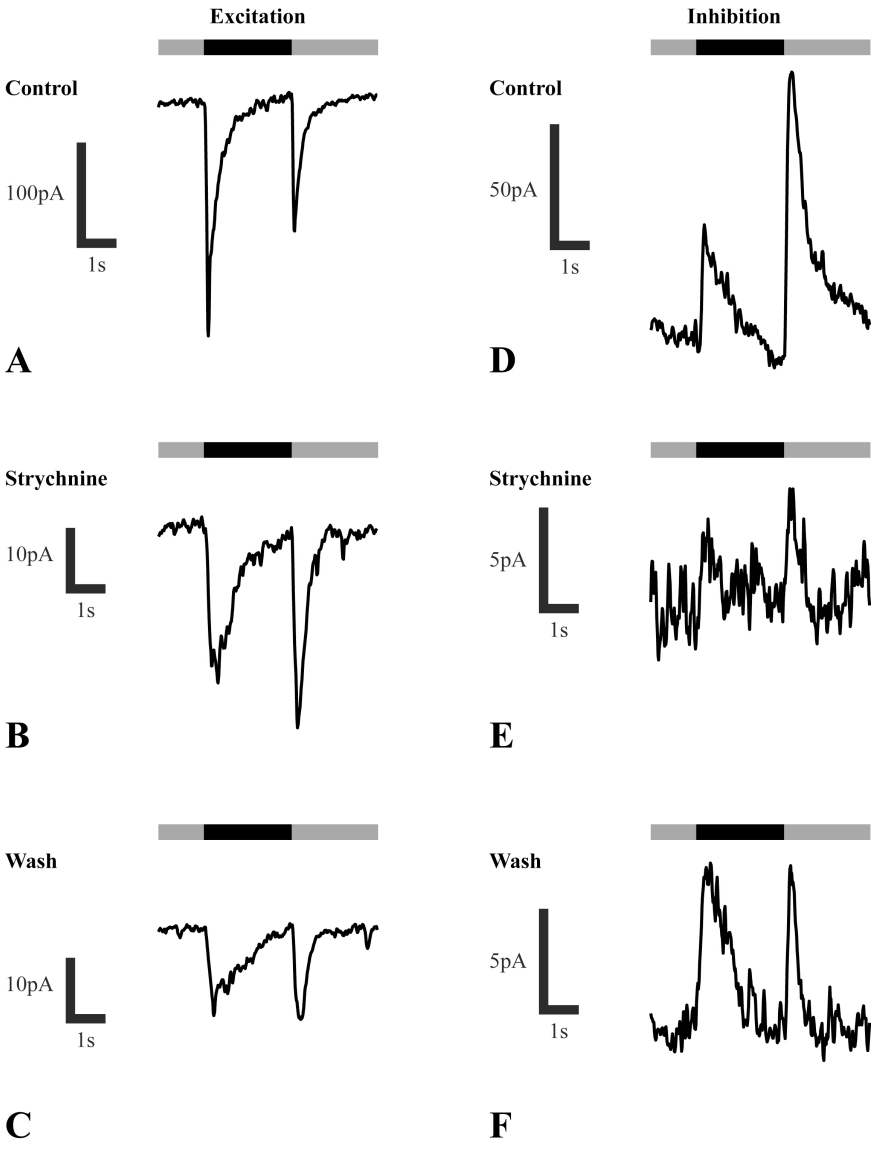


Figure 3-16. Strychnine suppresses ON-OFF inhibition. Excitatory and inhibitory inputs measured in an ON-OFF cell that received ON-OFF inhibition. A) Inward excitatory currents at light ON and OFF under control conditions. B) Excitatory currents were relatively unaffected in the presence of strychnine. C) ON-OFF excitatory currents were weaker (due to prolonged experiment), but persisted in the wash step. D) Outward inhibitory currents at light ON and OFF under control conditions. E) In the presence of strychnine, the ON-OFF inhibition is greatly attenuated, indicating that a large portion of

this inhibition is mediated by glycine. F) We were able to wash strychnine (10-15 minute long wash step) and the ON-OFF inhibition currents recovered.

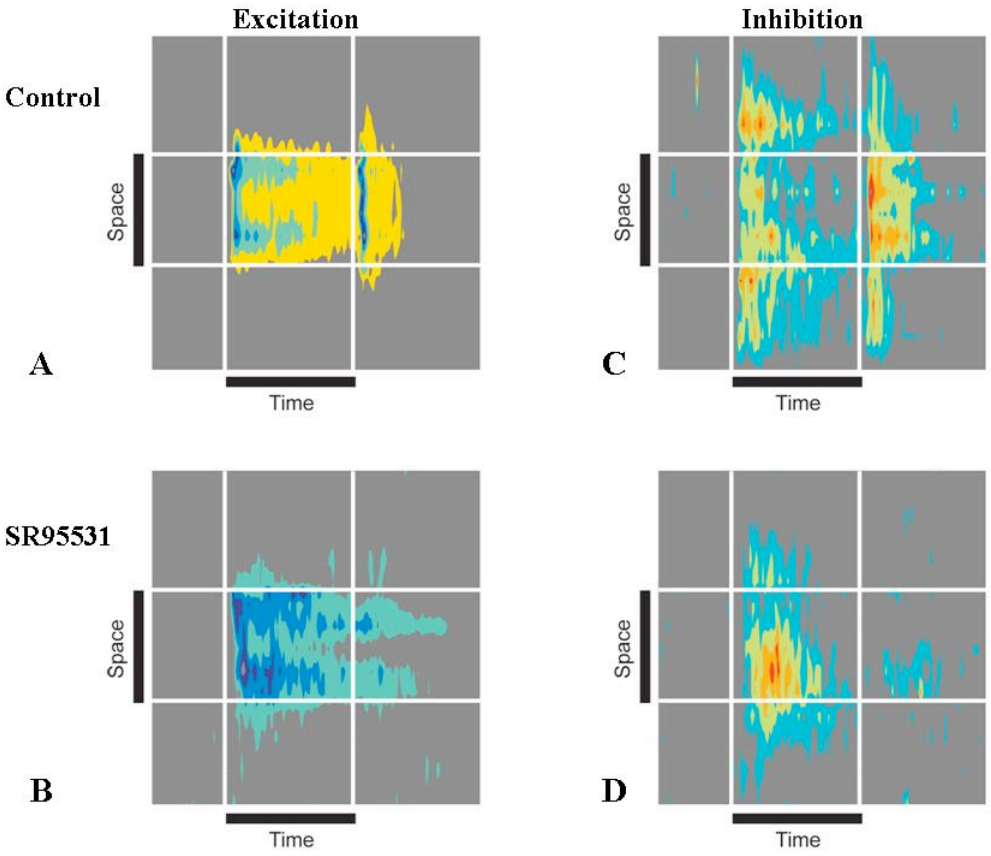


Figure 3-17. SR95531 blocks wide-field ON-OFF inhibition. These are raster plots recorded from a single ON-OFF amacrine cell. A) Excitatory raster, showing ON-OFF activity beneath the area of the flash. B) Excitatory raster in the presence of SR95531. ON-OFF excitation was more sustained. C) Inhibitory raster, showing wide-field ON-OFF activity. D) Inhibitory raster in the presence of SR95531. Local activity in the area underneath the flash was relatively maintained, while wide-field activity was mostly suppressed.

ON-OFF amacrine cells with ON inhibition

A small subset (10/38) of ON-OFF amacrine cells received ON inhibition. We were able to characterize the pharmacology of 5 of these cells. In four cells, strychnine significantly blocked the ON inhibition, as shown in Fig. 3-18. In the fifth cell, the wide lobes of inhibition were blocked by SR95531, but the cells located at the center of a raster plot still responded, as shown in Fig. 3-19. Taken together, it seems that the local ON inhibition to ON-OFF cells was primarily mediated by glycine, but the more wide-field inhibition was mediated by GABA.

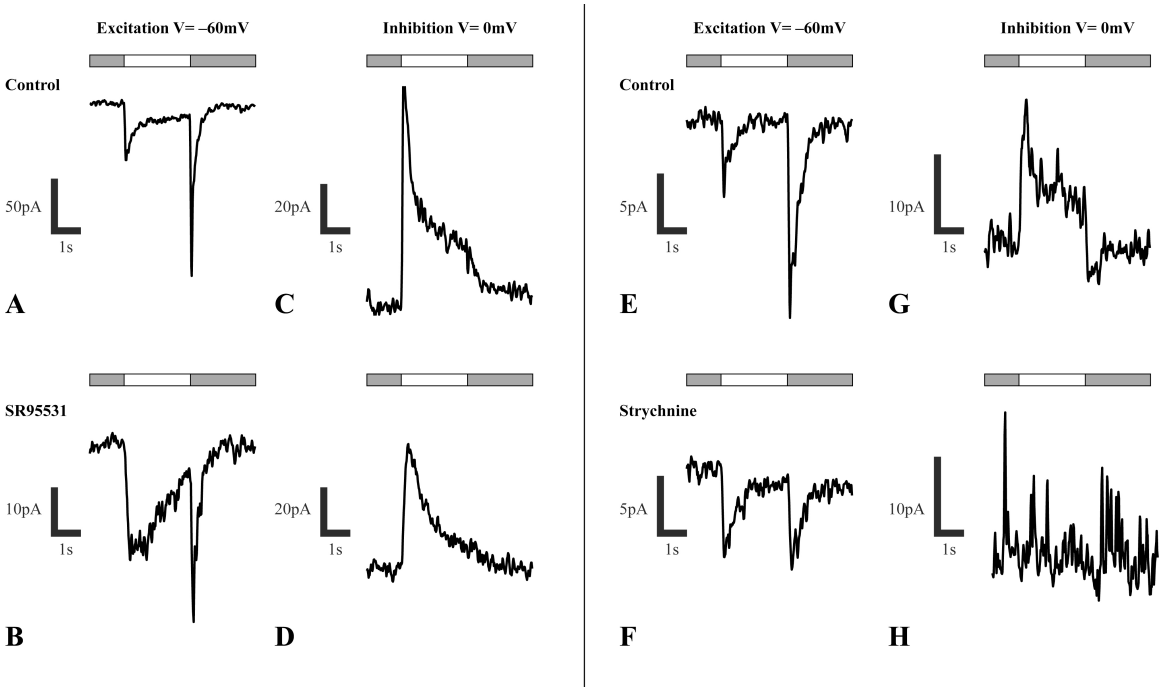


Figure 3-18. ON inhibition to ON-OFF cells is glycinergic, not GABAergic. Parts A-D were measured from one amacrine cell, and parts E-H were measured from a second amacrine cell. Both were ON-OFF cells that received ON inhibition. A) ON-OFF excitatory input under control conditions. B) In the presence of SR95531, the ON phase of the excitatory input became more sluggish, but the response was relatively unchanged.

C) The ON inhibitory input under control conditions. D) The ON inhibition persisted even in the presence of SR95531. E) ON-OFF excitatory input under control conditions for a second amacrine cells. F) In the presence of strychnine, the ON-OFF excitation was somewhat smaller, but largely unaffected. G) ON inhibitory input under control conditions. H) In the presence of 2μM strychnine, the ON inhibition was blocked. Taken together, these experiments indicate that ON inhibition to ON-OFF cells was glycinergic and not GABAergic.

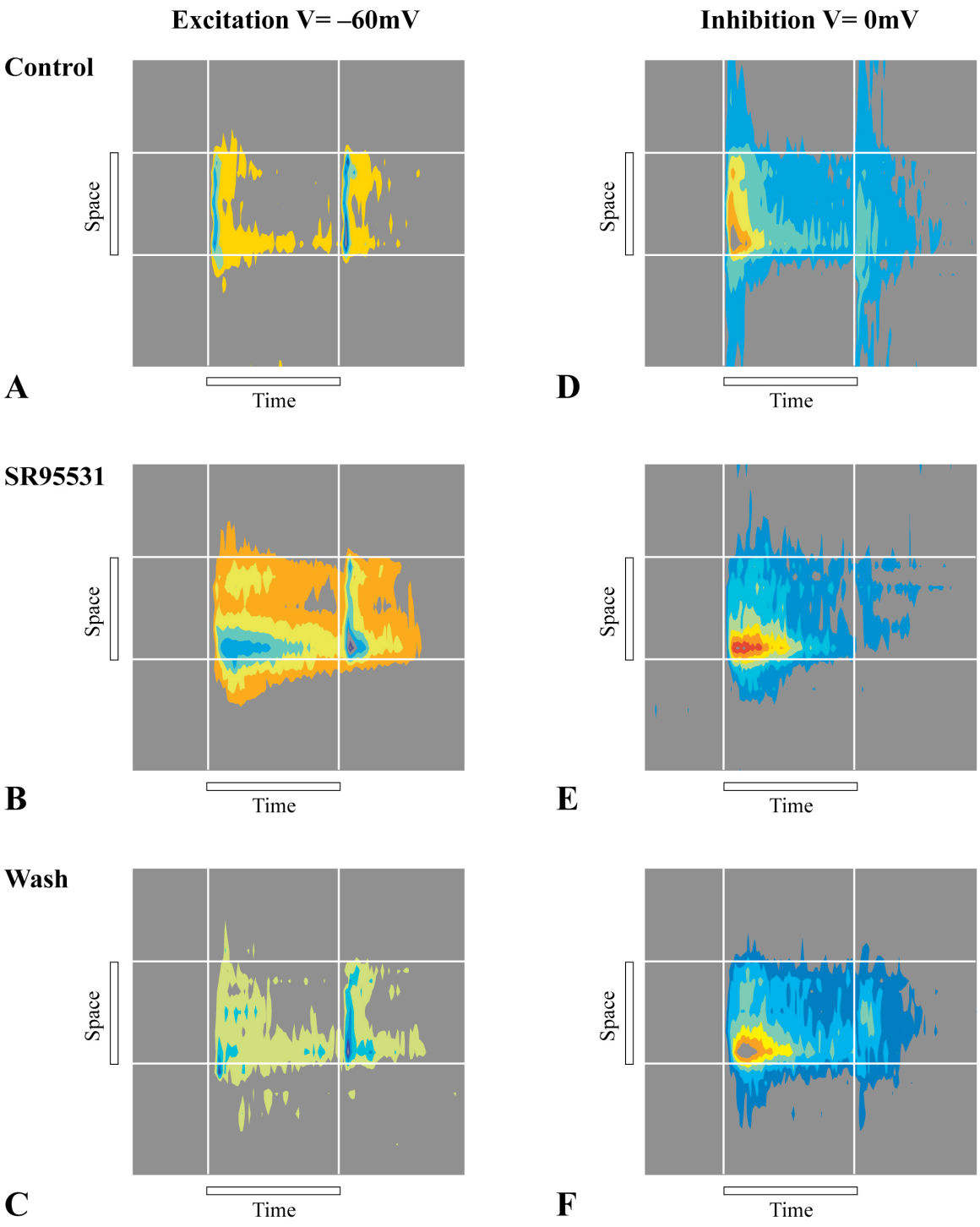


Figure 3-19. Wide-field ON inhibition is GABAergic. Light raster plots from an ON-OFF amacrine cell that receive ON inhibition. A) Excitatory raster plot in control conditions showed that the response to a 600 µm light square was confined to the region

underneath the stimulus, and occurred at both light onset and offset. B) In the presence of SR95531, the ON-OFF excitatory response became more sustained, but confined in space. C) After washing SR95531, the ON-OFF excitatory response became more transient. D) Inhibitory raster plot in control conditions showed that while the strongest response was in the region of the light flash, cells that were far away and not under the area of the flash responded. This is an example of a pre-synaptic wide-field inhibition. E) In the presence of SR95531, the wide-field inhibition was blocked, but the local response persisted. F) Unfortunately, we were unable to wash out the effects of SR95531 to show the recovery of the wide-field lobes of inhibition.

Morphology of ON-OFF amacrine cells

ON-OFF cells that did not receive significant inhibitory input were typically wide-field and monostratified in the middle of the inner plexiform layer, as shown in Fig. 3-20A. However, within these general morphological characteristics, the branch patterns varied considerably. In some cases, the processes were radial, and in other cases, they were polar, with the main stalks extending from the soma at 180 degree angles. Also, some cells had processes with many branches, while other cells had processes that had sparse branch patterns. It would seem that ON-OFF cells that did not receive significant inhibitory input were of a few morphological types. This was consistent with our finding that the spatial characteristics of this cell type was also varied.

ON-OFF cells that received ON-OFF inhibition were almost all narrow-field, usually under 300μm in diameter, as shown in Fig. 3-20B. While some were

monostratified, most of these cells were diffusely stratified across both ON and OFF sublamina.

ON-OFF cells that received ON inhibition were typically narrow-field, with even smaller dendritic fields, no more than 200μm across. About half of these cells were diffusely stratified, extending into the ON sublamina in many cases. The other half of these cells were bistratified, some crossing both ON and OFF layers and some remaining in just one functional layer or the other.

In a few cases, dye-coupling was observed between the cell that we measured from and cell bodies in the ganglion cell layer. While noteworthy, these dye-coupled cells were not encountered frequently, so we were unable to further characterize its physiological and morphological properties.

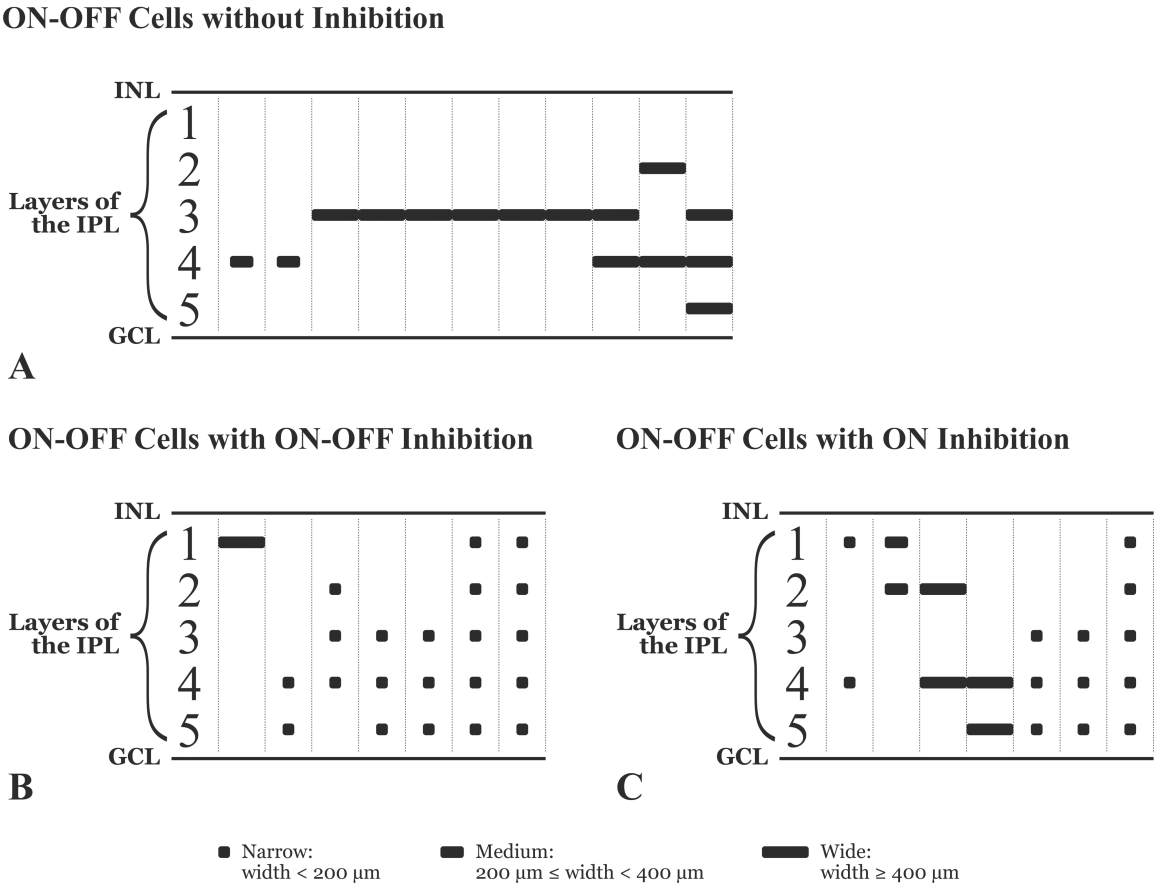


Figure 3-20: Morphology of ON-OFF amacrine cells. A) ON-OFF cells that did not receive significant inhibitory input were typically monostratified in the middle of the inner plexiform layer. In some cases, the cells were bistratified, between both the ON and OFF layers. B) ON-OFF cells with ON-OFF inhibition were diffusely stratified, crossing both functional sublamina, and were almost all narrow-field. C) ON-OFF cells with ON inhibition were more varied, consisting of two broad type: bistratified relatively wider cells, and diffusely stratified narrow cells.

Discussion

Glycingeric crossover inhibition was the most common interaction in amacrine cells

Almost all ON amacrine cells and OFF amacrine cells received glycinergic crossover inhibition: 33/35 ON cells received OFF inhibition, and 23/27 OFF cells received ON inhibition. In some cases, the glycinergic crossover inhibition was combined with other forms of inhibition. Notably, this glycinergic inhibition generally had smaller receptive fields than excitation, suggesting that local interactions between amacrine cells are mediated by glycine. Several studies have shown that amacrine cells that stain positive for glycine content are generally, if not exclusively, narrow-field, and thus are ideal candidates for mediating this glycinergic inhibition (Menger, Pow et al. 1998). A similar result has been found in amacrine cells in the slice preparation, where the majority of ON cells received OFF inhibition and OFF cells received ON inhibition (Hsueh, Molnar et al. 2008). In this study, we were able to show that this crossover inhibition acted locally, and usually smaller than the receptive field for excitation. Similar crossover inhibition has been observed for bipolar cells and ganglion cells (Roska, Molnar et al. 2006; Eggers, McCall et al. 2007; Molnar and Werblin 2007). In every stage of processing in the retina, crossover inhibition is prevalent, and may be acting to re-linearize synapses.

Wide-field amacrine cells likely feedback to bipolar cells or feedforward to ganglion cells.

Wide-field amacrine cells were frequently encountered, however, wide-field inhibition was rarely measured. Furthermore, GABAergic inhibition between amacrine cells was far less common than glycinergic inhibition. While it is possible that this may be due to an experimental bias against cells that receive GABAergic inhibition, it does

suggest that inhibition in amacrine cells might be dominated by local glycinergic circuitry. In previous studies, while GABAergic inhibition in amacrine cells was observed with higher frequency, the majority of inhibition measured in amacrine cells was still glycinergic (Hsueh, Molnar et al. 2008). Perhaps in addition to the apparent dichotomy of glycine mediating local interactions and GABA mediating global interactions, glycine may be the dominant neurotransmitter between amacrine cells, while GABA is largely reserved for feedback and feedforward inhibition to bipolar and ganglion cells (Roska and Werblin 2003; Eggers, McCall et al. 2007).

Further directions

In this study, we have characterized the synaptic input to amacrine cells. The excitatory input informs about the input from bipolar cells, while the inhibitory input informs about the input from amacrine cells. In the wholemount retina preparation, interactions both in time and space are kept intact, and with the patch clamp technique, the interaction between excitation and inhibition have been studied. However, the cell we recorded from not only receives inputs, but itself generates an output that impinges on downstream neurons. How can we characterize the output of the cell we are recording from? Electrophysiological recordings allow us to examine its voltage response, which shows (to a first approximation) how synaptic inputs sum across the membrane to depolarize or hyperpolarize the cell. Another aspect of synaptic output is the neurotransmitter that is contained in the cell of interest. The neurotransmitter content of a cell cannot be easily determined using only the cell patch clamp technique; other

techniques need to employed to examine the type of neurotransmitter output that the cell generates.

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Chapter 4

Neurotransmitter Content of Amacrine Cells

The excitatory and inhibitory inputs of amacrine cells can be isolated and studied using the whole cell patch clamp method. In order to examine the post-synaptic neurons of a given cell, we need to use a different set of techniques. In this chapter, the outputs of amacrine cells will be described in terms of their dendritic spread, stratification, and neurotransmitter content. These data will begin to shed light on the output characteristics of a given amacrine cell.

Morphological data can be obtained as a by-product of the whole cell patch clamp technique, since the intracellular solution contains both Alexa Fluor 488 and Neurobiotin. Alexa Fluor 488 staining can be viewed live, while the electrode is still attached to the cell. Since this fluorescent signal quenches quickly, there usually is not enough time to examine the full extent of a cell's morphology, especially when the cell is diffusely stratified, or has extensive branching. Neurobiotin filling can be revealed after fixing the tissue and conjugating with streptavidin-Alexa Fluor 488, at which point the images can be acquired with greater detail on a confocal microscope. The dendritic spread of a cell, along with the spatial characteristics of its excitatory input, will give us an idea if the cell is conveying local information to its dendrites, or if the processing is occurring along the entire length of the dendrites. The stratification of a cell reveals which bipolar, amacrine and ganglion cells it may be inhibiting. Bipolar cells are typically narrowly monostratified, receiving and conveying inputs within a narrow stratum. Ganglion cell dendrites reach into the inner plexiform layer and read out signals from the layers they

terminate in. By correlating amacrine cell stratification with the stratification of known ganglion cell types, we can begin to postulate which ganglion cells an amacrine cell is inhibiting.

Data about the neurotransmitter content of an amacrine cell can be obtained using immunocytochemical staining. Antibodies raised against the major inhibitory neurotransmitters in the retina, GABA and glycine are available both experimentally and commercially (Pourcho and Goebel 1985; Pow and Crook 1993; Connaughton, Behar et al. 1999), which we employed in our study to determine what neurotransmitter a given amacrine cells contained. Such a study has been done in the past with amacrine cells in the salamander retina (Yang, Lukasiewicz et al. 1991) and zebrafish retina (Connaughton, Behar et al. 1999) which we can compare now with our results in mammalian retina. There is a precedent for a correlation between morphological characteristics and neurotransmitter content in mammalian retina (Menger, Pow et al. 1998; Perez De Sevilla Muller, Shelley et al. 2007): amacrine cells with narrow dendritic fields are glycinergic, and cells with wide dendritic fields are GABAergic. We will examine our body of data in the rabbit retina, and determine if these conventions also hold true in this animal model. Integrating the data from electrophysiology, pharmacology, morphology and immunocytochemistry will reveal the fundamental circuit principles that drive the inhibition between amacrine cells that ultimately shapes the inhibition measured in bipolar and ganglion cells.

Methods

Electrophysiology

Detailed description in Chapter 2. Briefly, we measured the excitatory and inhibitory inputs to an amacrine cell in response to light and dark flashes (100µm-1000µm) to determine the receptive field size. The receptive field size of the cell was determined by the size of the spot that induced the largest current amplitude response. We also generated raster plots by stepping a 600 µm flash across the retina as described in Chapter 2. After measuring the light response of the amacrine cell in both control and drug conditions, the electrode was removed from the soma. The soma remained embedded in the intact retina.

Immunocytochemistry

In earlier trials, the retina was first cryoprotected by 15%, 30% sucrose prior to freezing in OCT. The resulting frozen tissue was cryosectioned into 12 µm thick slices, then screened for the cell stained with Neurobiotin. Only this section was immunostained for neurotransmitter content. The reason for the sectioning was to optimize antibody penetration. In later trials, we found that we could effectively stain for neurotransmitter content in wholemount retina, which became the preferred technique. The following protocol was adapted from a few immunocytochemical studies in the retina (Cuenca, Deng et al. 2002; Perez De Sevilla Muller, Shelley et al. 2007).

After electrophysiological measurements, the retina was fixed in 4% paraformaldehyde, 0.3% glutaraldehyde (to trap neurotransmitters within cells, minimizing their extracellular diffusion) for 40-60 minutes at 4 ºC. After washing in phosphate buffered saline (PBS; no calcium or magnesium for all wash steps) for ten minutes thrice, the retina was incubated with Alexa Fluor 488 conjugated with streptavidin (streptavidin

Alexa Fluor 488 conjuate, Molecular Probes) in block solution (10% normal donkey serum, 1% bovine serum albumin, 10% TritonX-100 in PBS) for 24 hours at 4 ºC. The retina was then washed in PBS thrice, then an image of the cell was acquired with a 20X dry objective with a grid confocal imaging system. Following, the retina was incubated in primary antibodies against glycine (raised in rat, obtained from David Pow) and GABA (raised in rabbit, from David Pow and Abcam) for 24-36 hours at 4 ºC. The dilutions were 1:500 for the glycine antibody, and 1:1000 for the GABA antibody, in 10% TritonX-100 and PBS. The retina was washed thrice in PBS, then incubated in secondary antibodies (Jackson ImmunoResearch) rat Cy3 (1:100) and rabbit Cy5 (1:100) for 2 hours at 25 ºC, protected from light. Following a wash step, the retina was mounted on a slide with Hard-set Vectashield (Vector Laboratories, Burlingame, CA), stored at 4 ºC for confocal imaging within one week. For long-term storage, the mounted retinas were frozen at -20 ºC.

Imaging

After we completed the electrophysiological measurements of a cell, we acquired images of the Alexa Fluor 488 staining using a grid confocal microscope (Volocity image acquisition and deconvolution software, Improvision Inc., Waltham, MA). This enabled us to visualize the gross stratification parameters (monostratified vs. multi-stratified, ON vs. OFF sublamina) and determine if the cell was narrow-field (<200µm), medium-field (200µm ≤ dendritic spread < 400µm), or wide-field (≥ 400µm). After we acquired images of the live-cell staining, we fixed the retina as described above.

Kenneth Greenberg was the lead on the confocal imaging. First, we located the cell of interest in the Alexa Fluor 488 channel, then viewed that section of the retina under high magnification. Then we viewed the cell in the Cy3 channel, which revealed glycine staining, and the Cy5 channel, which revealed GABA staining. The final image was acquired at high resolution of the three channels, which were superimposed to determine the neurotransmitter content of the amacrine cell.

In some cases, the morphology of the cell was well-stained, and we could acquire additional confocal images of dendritic spread.

	Antibody description	**Dilution**	**Source of supply**
Primary Antibodies			
Anti-glycine	Rat anti-glycine	1:500	Pow, David
Anti-GABA	Rabbit anti-GABA	1:1000	Pow, David and Abcam (ab43865-50)
Secondary Antibodies			
Cy3-conjugated AffiniPure Donkey Anti-Rat IgG (H+L)	Raised in rat	1:100	Jackson Immuno (712-165-150)
Cy5-conjugated AffiniPure Donkey Anti-rabbit IgG (H+L)	Raised in rabbit	1:100	Jackson Immuno (711-175-152)

Table 1. Antisera and Dilutions

Results

Specificity of primary antibodies

We were able to confirm that the primary antibodies used to stain for glycine and GABA were specific. A sample section is shown in Fig. 4-1. In Fig. 4-1A, we located the somas and processes of glycinergic inner nuclear layer neurons. Based on the proximity of these somas to the inner plexiform layer, these neurons were likely amacrine cells. In Fig. 4-1B, we viewed the GABA-containing neurons in the same retinal slice, where the staining was strongest along the membranes of the neurons. By comparing these two panels, the cells that stained positively for glycine did not stain positively for GABA, and vice versa. The cell that we measured from was stained with Alexa Fluor 488 conjugated with Streptavidin, which binds to the Neurobiotin that is present in the target cell, as shown in Fig. 4-1C. A merge of all panels, shown in Fig. 4-1D, includes DAPI staining to delineate the locations of all somas present in the slice. This shows that glycine positive cells did not co-stain with GABA, and more importantly, the cell that we measured from co-localized with glycine staining. The white arrowhead points to the location of the cell we measured from in all channels. This antibody specificity persisted even when we stained the retina without cryosectioning.

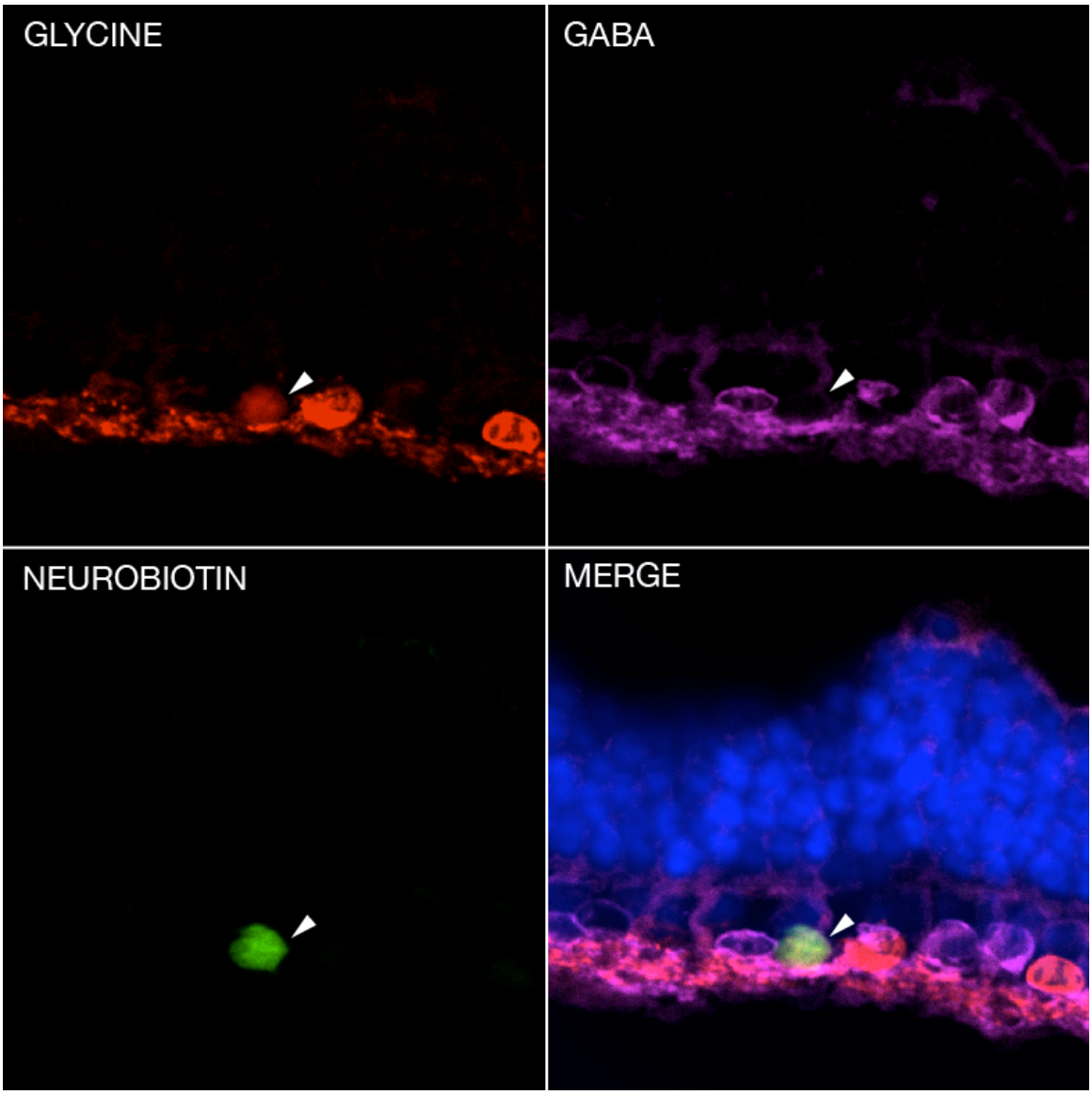


Figure 4-1. Primary antibodies for glycine and GABA are specific. This is an example of a 12µm cryosectioned retina slice, stained with primary antibodies against glycine and GABA. Secondary antibodies Cy3 (red channel) and Cy5 (purple channel) were used to reveal positive glycine and GABA staining, respectively. The slice is orientated with the outer retina towards the top and the ganglion cell later towards the bottom. White arrowhead denotes the location of the cell of interest, based on the location in C. A) Glycine-containing amacrine cell somas and processes were revealed. B) GABA-containing amacrine cell somas and processes were revealed. These cells are not coincident with the cells stained in A. C) The cell that we measured from is stained with Alexa Fluor 488 conjugated with streptavidin, bound to the

neurobiotin present in the interacellular solution. D) A merge of all channels, including DAPI, shows that the Alexa Fluor 488 staining co-localizes with the glycine staining, and not GABA staining. The thick layer of small somas is the outer nuclear layer, consisting of the cell bodies of photoreceptors.

Most narrow-field cells contained glycine

We were able to immunostain 24 cells for neurotransmitter content and we were able to characterize the morphology of 21 of these cells. Of the 21 cells in which we were able characterize both the morphology and neurotransmitter content, 8 of the cells had dendritic field sizes that were less than 200μm. Half of these narrow-field cells (4/8) were OFF cells, a couple were ON-OFF cells (2/8), one was an ON cell, and one was an AII amacrine cell, as shown in Fig. 4-2A. All the OFF cells received ON inhibition. The ON-OFF cells each received different forms of inhibition: one received ON-OFF inhibition, while the other received ON inhibition. The ON cell received ON-OFF inhibition.

When we identified the neurotransmitter content of these narrow-field cells, we found that most of them contained glycine (6/8), an example of which we show in Fig. 4-2B. In two cases, the cells stained positively for GABA, an example which is shown in Fig. 4-2C. Notably, in those two cases, the raster plot responses contained wide-field components, not present in the glycinergic narrow-field cells. The raster for a glycinergic narrow-field cell is shown in Fig. 4-3A, and both the excitatory and inhibitory inputs were narrow compared to the inputs to the GABA-containing cells shown in Fig. 4-3B and 4-3C. For one GABAergic narrow-field cell, the excitatory input was relatively narrow, while the inhibitory input was wide, as shown in Fig. 4-3B. In another cell, both the excitatory and inhibitory raster responses showed wide-field components, as shown in Fig. 4-3C. These results suggest that most narrow-field cells contained glycine, but a few

types of narrow-field cells could contain GABA, especially if either of their excitatory or inhibitory inputs contained wide-field components.

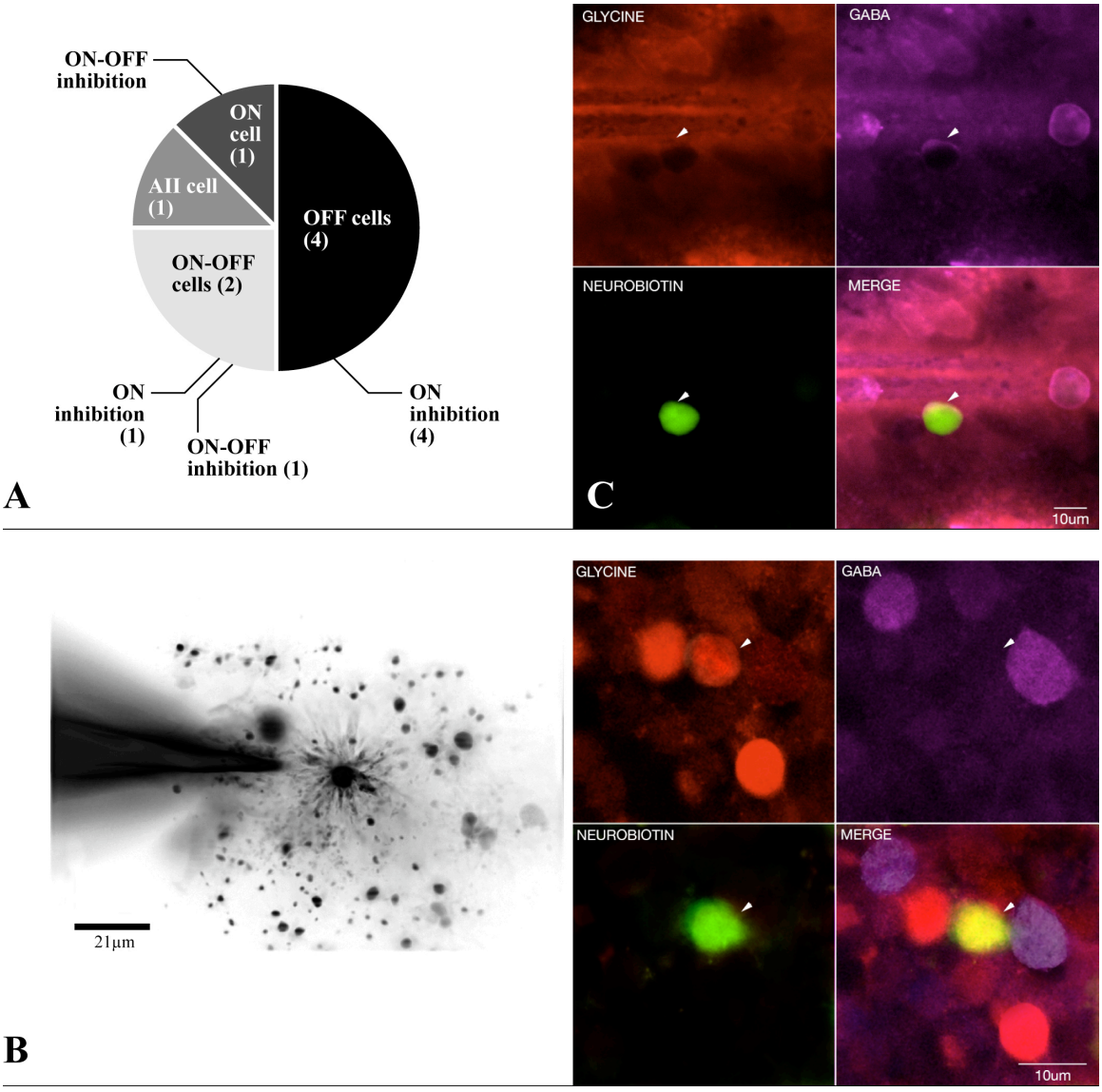
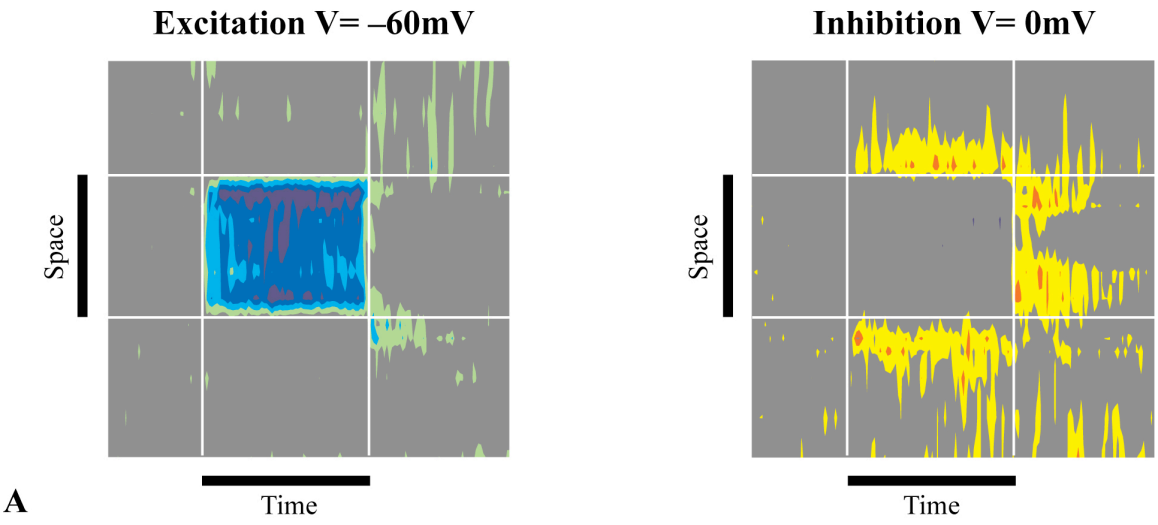


Figure 4-2. Narrow-field amacrine cells. A) We stained 8 amacrine cells whose dendritic spread sizes were less than 200µm. Half of these narrow-field cells were OFF cells that received ON inhibition. Of the two ON-OFF cells that we stained, one received ON inhibition and the other received ON-OFF inhibition. The ON cell received ON-OFF inhibition, and we also stained an AII amacrine cell. B) The morphology of an ON-OFF narrow-field cell revealed by Alexa Fluor 488 staining is shown on the left.

The processes of this cell terminated in the middle of the inner plexiform layer (not shown here in the flattened image of a z-stack), and spanned no more than 200µm. The immunostaining of this cell is shown on the right. The upper left panel shows the glycine staining revealed by the Cy3 fluorophore, the upper right panel shows the GABA staining revealed by the Cy5 fluorophore, and the lower left panel shows the neurobiotin staining that identifies the cell that we measured from. In the merged image on the lower right panel, we can see that this cell is glycine-positive. Most of the narrow-field amacrine cells we stained contained glycine. C) An example of one of the two narrow-field cells that stained positive for GABA. The region stained by Neurobiotin co-localized most closely with positive GABA staining and not glycine.


Excitation V= –60mV
Inhibition V= 0mV
Space
Time
A
Excitation V= –60mV
Inhibition V= 0mV
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Time
B
Excitation V= –60mV
Inhibition V= 0mV
Space
Time
C

Figure 4-3. Excitatory and inhibitory raster responses of narrow-field amacrine cells. The left column shows the excitatory raster plots measured by clamping the cell at -60mV, and the right column shows the inhibitory raster plots measured by clamping the cell at 0mV. Cooler colors represent inward currents and hotter colors represent outward currents. A dark raster was used to generate these responses. A) Raster responses from a glycinergic narrow-field cell. The OFF excitatory response was confined to the region under the flash, and lasted the entire duration of the flash. The ON inhibitory response occurred at the termination of the dark flash, bounded by the 600µm flash region, as well as in the surround regions of the cell. B) Raster responses from a GABAergic narrow-field cell. The ON-OFF excitatory response was confined to the region under the flash, while the ON-OFF inhibitory response extended to regions more than 300µm outside of the flash boundary. This cell received a narrow excitatory input and a comparably wide inhibitory input. C) Raster responses from another GABAergic narrow-field cell. Both the OFF excitatory and ON inhibitory inputs were wide, extending more than 300µm from the flash boundary. This cell whose processes were smaller than 200µm, received excitatory and inhibitory inputs wider than its morphological spread.

Most wide-field cells contained GABA

We measured from and immunostained 9 cells that had dendritic field sizes greater than 400µm. The majority (5/9) of these wide-field cells received ON-OFF excitation, and only a few received either ON excitation (2/9) or OFF excitation (2/9), as shown in Fig. 4-4A. The morphology of one of these wide-field cells is shown in Fig. 4-4B. Of the cells that received ON-OFF excitation, 2 did not receive significant inhibition, 2 received ON-OFF inhibition, and 2 received ON inhibition. One of the wide-field ON cells received ON-OFF inhibition and the other received OFF inhibition. Both OFF cells received ON inhibition.

In all but one cell, the neurotransmitter content of these wide-field cells was GABA, as shown in Fig. 4-4B. However, even though the dendritic spreads of these cells were large, in some cases their receptive field size was significantly smaller. An example of such a cell is shown in Fig. 4-5. The dendritic spread of this cell was at least 600µm in diameter, as shown in Fig. 4-5A. However, the receptive field for this GABA-containing cell was between 400-500µm, and the raster plot shown in Fig. 4-5B was consistent with this, since the majority of the ON-OFF response occurred within the boundaries of the 600µm dark flash. Half of GABAergic wide-field cells (4/8) showed this characteristic, as well as one glycinergic cell, where the receptive field was significantly smaller than the dendritic field. In 4 of these 5 cells, depolarizing voltage steps revealed that these cells may have voltage-gated Na^+ and K^+ channels. Example current responses to the depolarizing voltage steps are shown in Fig. 4-5C. These voltage step responses were measured immediately after cell break-in, prior to fully dialyzing the cell. The cesium in the intracellular solution had not yet blocked the potassium channels. When the cell was depolarized from -60mV to -10mV, the voltage-gated channels were activated. The inward current indicated by the arrow in Fig. 4-5C was likely from the activation of voltage-gated Na^+ channels, and the rebound outward current indicated by the asterisk was from voltage-gated K^+ channels. This outward current was blocked by the cesium in the intracellular solution a few minutes after the cell was fully dialyzed, as shown in Fig. 4-5D. The inward current persisted, but the outward current was blocked, which suggests that the outward current was mediated by voltage-gated K^+ channels. We were unable to confirm that the inward currents were mediated by voltage-gated Na^+ channels using either TTX or QX-314. Taken together, these results suggest that while most wide-field

cells are GABAergic, their receptive field is not always as large as their dendritic field, and they may be capable of generating action potentials.

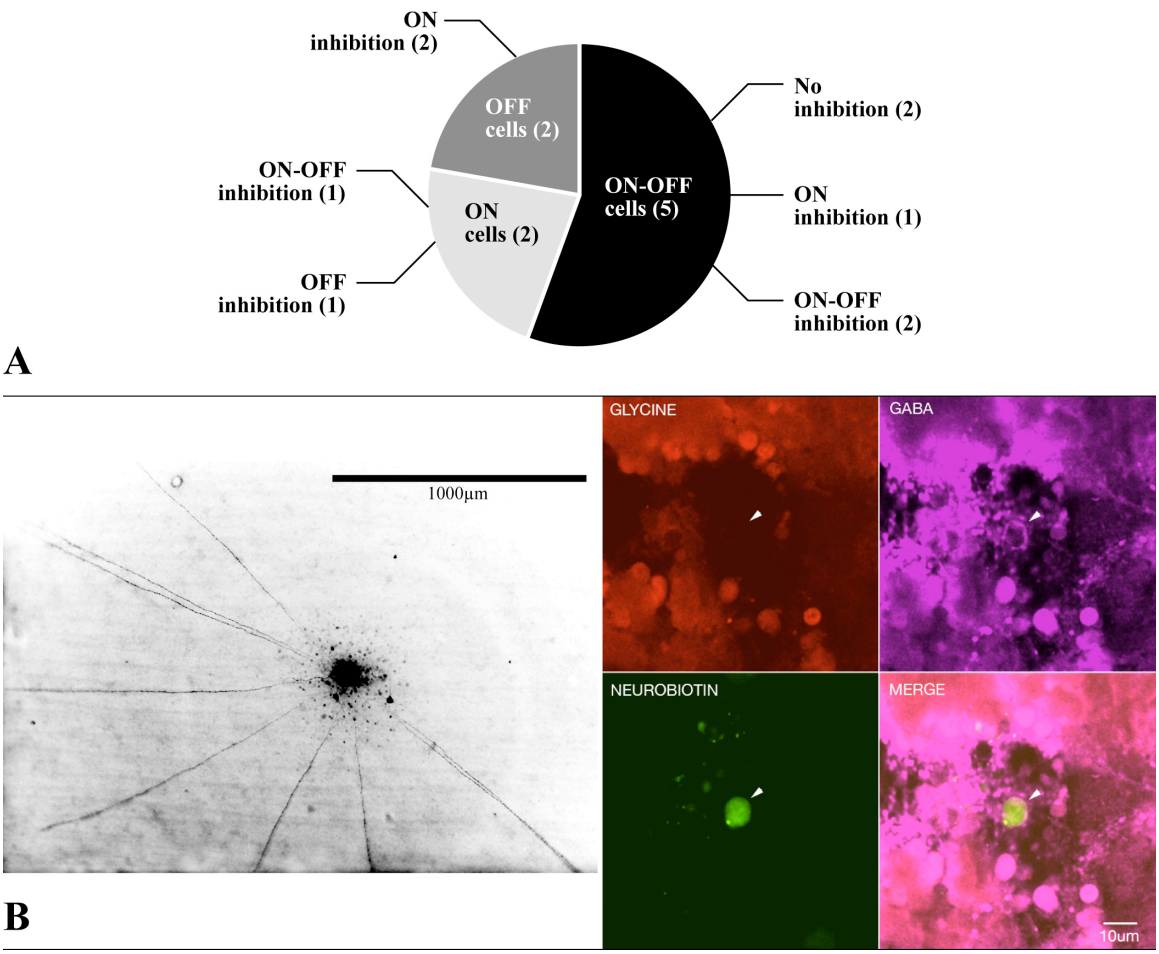


Figure 4-4. Wide-field amacrine cells. A) We stained 9 cells whose dendritic fields were greater than 400µm. The majority of these wide-field cells were ON-OFF cells, and half of the remaining cells received ON excitation and half received OFF excitation. Of the 5 ON-OFF cells, 2 did not receive any measurable inhibition, 2 received ON-OFF inhibition and 1 received ON inhibition. Both of the OFF cells received ON inhibition. Of the 2 ON cells, one received ON-OFF inhibition and the other received OFF inhibition. B) An Alexa Fluor 488 image of a wide-field ON amacrine cell whose spread extended for more than 1000µm. C) Immunostaining of this wide-field cell. The arrowhead indicates the cell we measured from, shown in the lower left panel. The upper left panel shows the glycine staining and the upper right

panel shows the GABA staining. The cell co-localized with the GABA staining, indicating that it contained GABA, and not glycine.

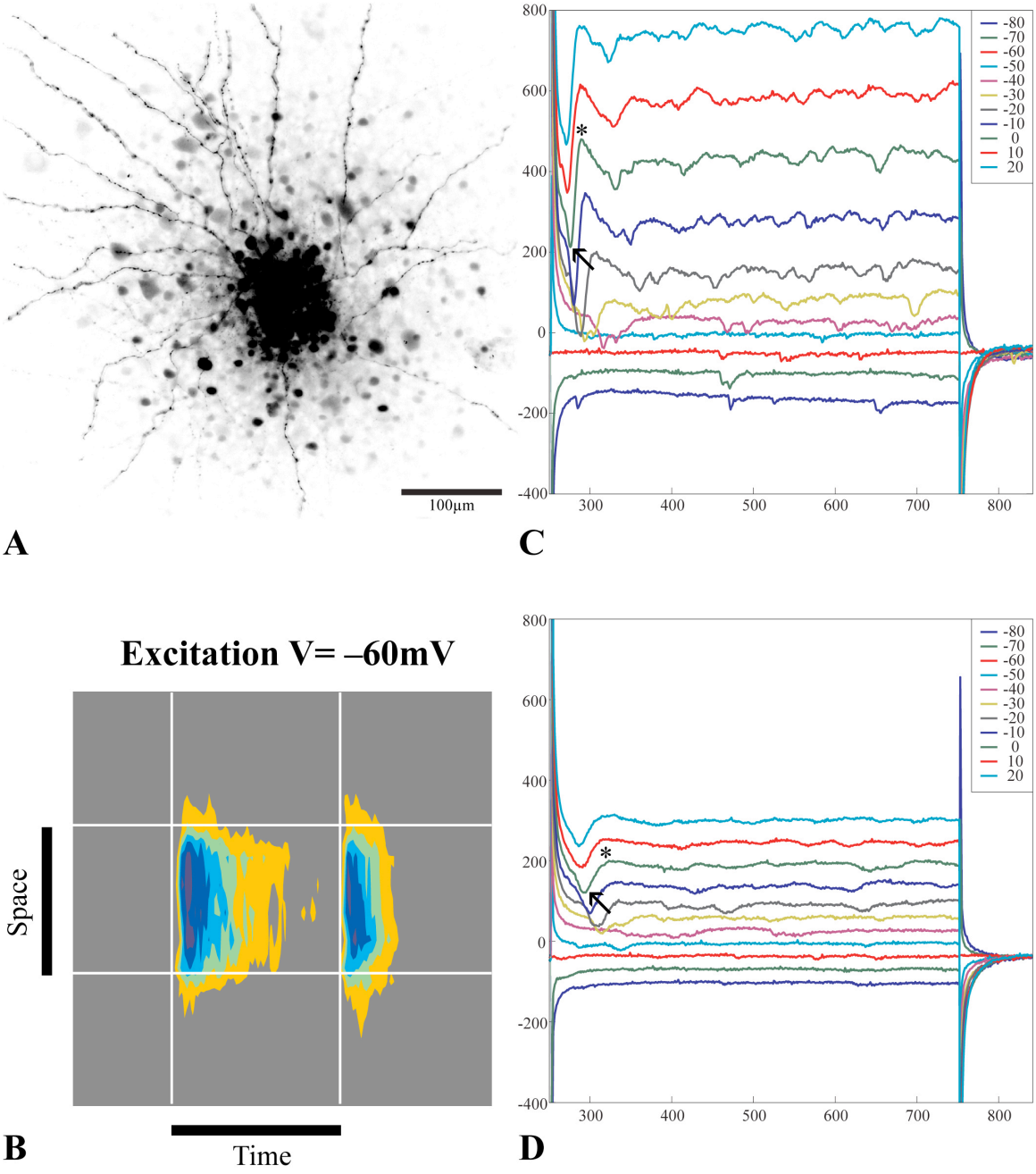


Figure 4-5. Wide-field GABAergic amacrine cells may have smaller receptive fields.

Example morphology, raster plot, and voltage-step responses of an ON-OFF cell with a receptive field of

400-500µm, and a dendritic spread of approximately 600µm. A) Morphology revealed by Alexa Fluor 488 staining shows the extent of the dendritic spread of this cell, which is far larger than its receptive field size of 400µm, and larger than expected for a cell with a narrow raster response. B) The raster plot represents the excitatory response, which is confined to the region underneath the dark flash. This narrow raster response is typical of amacrine cells with smaller dendritic field sizes. C) Current responses to depolarizing voltage steps taken prior to dialyzing the cell, from -80mV to +20mV in 10 mV increments. The arrow indicates the voltage-activated inward current, and the asterisk indicates the voltage-activated outward current. D) Current responses to depolarizing voltage steps taken after dialyzing the cell. The voltage-gated inward currents remain, but the voltage-gated outward current was blocked by cesium, which indicates that the outward current in response to depolarizing voltage steps was mediated by voltage-gated K^+ channels.

Medium-field cells contained either GABA or glycine with equal probability

We stained 4 cells whose dendritic fields ranged between 200µm and 400µm. These medium-field amacrine cells consisted of one ON cell, one OFF cell and one ON-OFF cell. The fourth cell was not well clamped, so we were not able to characterize its physiology. Half of these cells stained positively for glycine and the other half stained positively for GABA. Although more experiments would be required to draw conclusions with higher certainty, these results suggest that medium-field cells have equal probability of containing glycine or GABA.

Our results are summarized in Fig. 4-6. Most narrow amacrine cells contained glycine, though subclasses of narrow cells that receive wide inputs may also contain GABA, as shown in Fig. 4-6A. Almost all wide amacrine cells contained GABA, and in subsets of these cells, their receptive field sizes are smaller than their dendritic field size. Medium-field amacrine cells had an equally likely chance of containing either GABA or

glycine. In Fig. 4-6B, we show that most glycine-positive amacrine cells are narrow-field, with dendritic field sizes around 100µm. No cells had dendritic fields larger than 400µm. GABA-positive cells were both narrow and wide, as shown in Fig. 4-6C. There were a couple of narrow-field cells (<200µm), a couple of medium-field cells (between 200µm and 400µm), but most cells were wide-field (≥400µm). Narrow-field cells typically had narrow receptive fields, but wide-field cells could have either narrow or wide receptive fields.

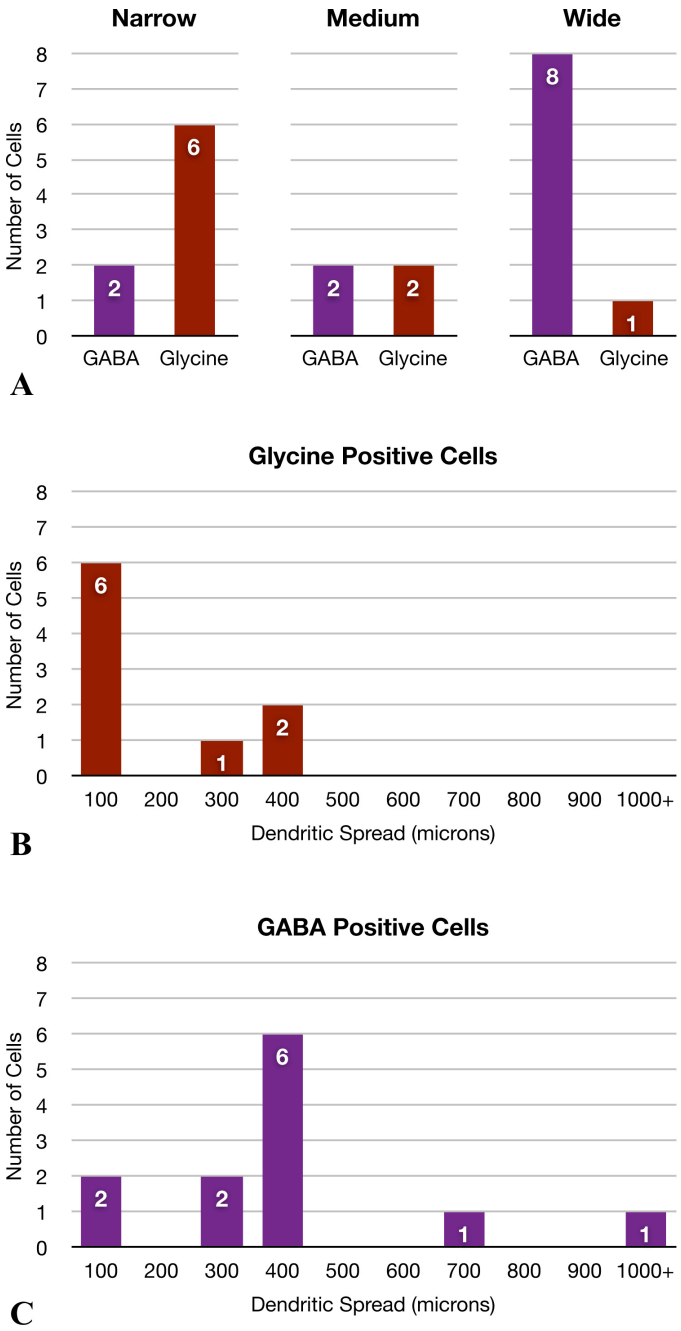


Figure 4-6. Summary of results. We immunostained and characterized the morphology of 21 amacrine cells in the flatmount rabbit retina. A) Most narrow-field (dendritic spread ≤ 20µm) cells contained glycine, and almost all wide-field (dendritic spread > 400µm) cells contained GABA. Medium-field cells (200µm < dendritic spread ≤ 400µm) either contained GABA or glycine in equal frequency. B) Amacrine cells that contained glycine had dendritic fields smaller than 400µm, and most were smaller than

100µm (157.78µm ± 49.52µm, mean ± SEM). C) Amacrine cells that contained GABA had dendritic fields across a much larger range, though most cells had dendritic spreads around 400µm (491.67µm ± 152.17µm).

Discussion

Comparison with other studies in mammalian amacrine cells

The basic finding in this study is that glycinergic amacrine cells are generally narrow-field, while GABAergic cells are almost always wide-field. These results are consistent with other studies in the mammalian retina that examine the morphology of glycinergic or GABAergic amacrine cells. In the rat retina, it has been shown that amacrine cells that stain positive for glycine are narrow-field, with their dendrites typically no wider than 50µm (Menger, Pow et al. 1998). Additionally, these amacrine cells have diffuse processes that span multiple sublamina in the inner plexiform layer. While we have also found that narrow-field cells typically contain glycine, the amacrine cells that we have identified include cells with dendritic spreads larger than 50µm. A possible reason for this discrepancy in dendritic spread size is that we characterized the cell morphology in an intact retina, while the rat retina study characterized amacrine cell morphology after slicing the retina into 100-200µm thick slices, so their dendritic spread dimensions may be an underestimate of the actual size. Slicing the retina makes it difficult to visualize or even detect wide processes, so there may be glycinergic amacrine cells with wide processes that were not detected.

In terms of GABAergic cells, a recent study of displaced amacrine cells in the mouse retina (Perez De Sevilla Muller, Shelley et al. 2007) showed that these cells are typically wide-field and exclusively GABAergic. Our results suggest that a similar

correlation also exists in the non-displaced amacrine cells in the rabbit retina. There is also electrophysiological evidence that wide-field inhibition is mediated by GABA (Roska and Werblin 2003), and these GABAergic amacrine cells may be involved in saccadic suppression.

Comparison with salamander amacrine cell neurotransmitter content

The results in this study suggest that there is a correlation between the dendritic spread size and neurotransmitter content of an amacrine cell. A previous study had examined this correlation in salamander retinal slices (Yang, Lukasiewicz et al. 1991). Their findings report that in the salamander retina, wide-field cells typically contained glycine, while narrow-field cells usually contained GABA. Medium-field cells contained either GABA or glycine with similar frequency. Our results suggest that in the rabbit retina, wide-field cells typically contained GABA, not glycine, and that narrow-field cells usually contained glycine, not GABA. Similar to the study in salamander, our study in the rabbit shows that medium-field amacrine cells were about equally likely to be glycine-positive as GABA-positive. It appears that these differences in findings are a result of species variation, since our findings are consistent with other studies of amacrine cells in mammalian animal models.

Wide-field GABAergic amacrine cells transmit local information globally

We have found that about half the GABAergic cells we recorded had receptive fields that were smaller than their dendritic fields, and that most of these cells likely had voltage-gated K^+ channels and possibly had voltage-gated Na^+ channels. This suggests

that these wide-field amacrine cells might be conveying local information across the retina via action potentials. The ON-OFF GABAergic wide-field cells we have measured here may be Type II polyaxonal amacrine cells characterized in previous studies (Volgyi, Xin et al. 2001): their processes extend well over 600µm across the retina, they do not generate spontaneous action potentials and respond to light by depolarizing at ON and OFF.

Wide-field GABAergic amacrine cells rarely appear to inhibit other amacrine cells, as we have seen in Chapter 3, where most ON-OFF inhibition is narrow and glycinergic. However, many ON-OFF amacrine cells have dendritic spreads wider than their receptive field. Functionally, these wide-field ON-OFF amacrine cells may be taking local visual signals and providing feed forward, wide-field inhibition to ganglion cells. Wide-field GABAergic ON-OFF inhibition has been measured in rabbit ganglion cells (Roska and Werblin 2003).

Glycinergic amacrine cells primarily involved in local processing

Unlike the GABAergic amacrine cells that had receptive fields smaller than their dendritic fields, the receptive and dendritic receptive fields of glycinergic cells were usually on the same spatial scale. The majority of glycinergic amacrine cells had small dendritic spreads, and both their excitatory and inhibitory inputs had similarly-sized receptive fields. These glycinergic cells are involved in local processing, integrating the inputs from nearby bipolar cells and amacrine cells, and providing adjacent ganglion, amacrine and bipolar cells. Many of the glycinergic interactions that we have measured are of the crossover type, where the OFF system is inhibiting the ON, and vice versa. The

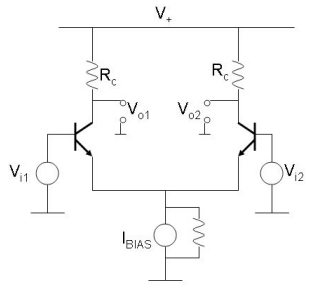
immunocytochemistry confirms that the glycinergic amacrine cells that mediate crossover inhibition are indeed narrow, and morphological studies showed that a subset of these cells stratify across the functional ON/OFF border in the inner plexiform layer.

Crossover is the most common interaction between amacrine cells

In all, we have recorded from 407 amacrine cells in both the retinal slice and flatmount preparations, and 39.6% (161/407) of the inhibition between amacrine cells is the crossover type, where the OFF system inhibits ON cells, and the ON system inhibits OFF cells. In both the slice and flatmount retinal preparations, we have confirmed that glycine mediates crossover inhibition. From the cells recorded in wholemount retina, we were able to determine that this crossover inhibition acts in a relatively local manner: its receptive field is rarely larger than the receptive field for excitation, and most of the time, its receptive field is similar in size to the receptive field for excitation. This implies that the total crossover inhibition to a cell is made up of smaller units that tile the receptive field of an amacrine cell, and that it is likely the amacrine cell that conveys this glycinergic inhibition has a small dendritic field size. Indeed, in Fig. 4-6B, we found that most glycinergic amacrine cells had dendritic field sizes smaller than 100μm; additionally, the ubiquitous glycinergic AII amacrine cell could be implicated as well. Regardless of what class(es) of glycinergic amacrine cells are mediating crossover inhibition, the question is, what is the functional role of this form of inhibition? Crossover inhibition has been measured in bipolar and ganglion cells as well. A suggested function of crossover inhibition in ganglion cells was to accentuate the differences in spiking patterns between adjacent ON ganglion cells and OFF ganglion

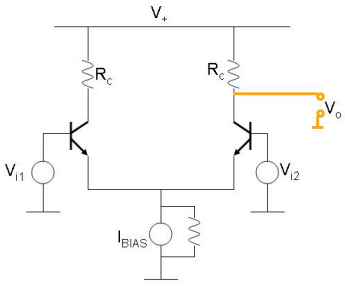
cells (Murphy and Rieke 2008). Others have suggested that crossover inhibition in bipolar cells may serve a "push-pull" function, where inhibition serves to enhance excitation rather than oppose it (Molnar and Werblin 2007). Crossover inhibition in amacrine cells may also serve a "push-pull" function, and though most amacrine cells do not generate action potentials as ganglion cells do, crossover inhibition may serve some function in accentuating the differences in responses between ON and OFF cells.

Notably, this circuit topology is often used in electronics for noise rejection and to retain signal robustness, but it remains yet to be determined if this circuit topology performs the same functions in the retina. In electronic circuits, a typical differential input to differential output circuit allows for common-mode rejection, shown in Fig. 4-7A. However, if a single-ended output is desired, then there is a loss of common-mode rejection capability (by about 50%, Fig. 4-7B). If this differential circuit topology includes a current mirror (which is analogous to including one additional stage of crossover inhibition in the retina), some of the common-mode rejection capability is regained, shown in Fig. 4-7C. However, if the transistors are cascoded, there is an even greater improvement in the common-mode rejection ratio, shown in Fig. 4-7C. While it is not entirely certain that the repeated crossover inhibition in the retina is performing this same function, it is certainly interesting that such a simple circuit topology can be used in a repeated fashion to reject noise. Modeling experiments would need to be conducted to help devise an *ex vivo* experiment to prove this hypothesis.



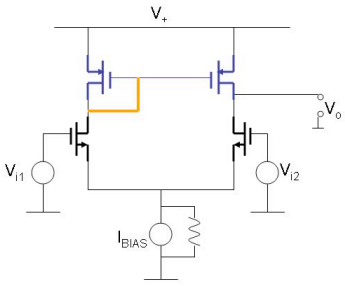
- Differential Mode= V_{i1}-V_{i2}
- Common Mode=(V_{i1}+V_{i2})/2
- Differential Mode Gain=
  A_{dm}=-$g_m R_c$
- Common Mode Gain=
  A_{cm} =- $g_m R_c$ /(1+2$g_m r_{ob}$)
- CMRR=Common Mode Rejection Ratio
  A_{dm} / A_{cm} =1+2$g_m r_{ob}$

A

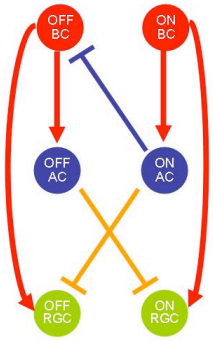


- Differential Mode Gain=
  A_{dm}= V_o/V_{id}= -(1/2)$g_m R_c$
- Common Mode Gain=
  A_{cm} = V_o/V_{ic}= - $g_m R_c$ /(1+2$g_m r_{ob}$)
- CMRR=
  A_{dm} / A_{cm} = (1/2)(1+2$g_m r_{ob}$)
- Compare CMRR(diff-diff)=
  A_{dm} / A_{cm} =1+2$g_m r_{ob}$
- LOSE 50% of common mode rejection capability

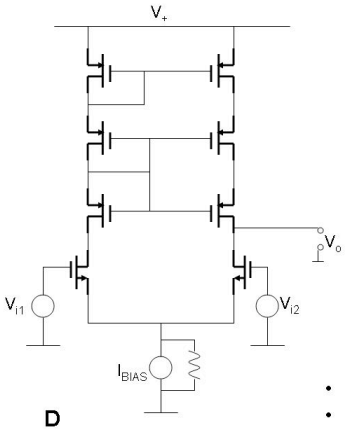
B

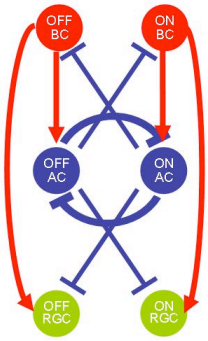


Helps to regain CMRR, approx=2$g_m^2 R_o^3$



C





D

- Now CMRR approx =(1/2)$g_m^3 R_o^3$
- Which is a factor of g_m better than with just one current mirror

Figure 4-7. Differential circuitry in electronics and possibly the retina. Differential circuits and derivations of gain and common-mode rejection ratio obtained from *Microcircuits* by Howe and Sodini (1996). A) Typical differential circuit, where there are two inputs of different polarity and there are two outputs of differential polarity. By subtracting the two differential inputs, noise common to both inputs can be eliminated (common mode rejection). The differential inputs and outputs are analogous to the ON and OFF systems in the retina. B) A differential circuit where there are two differential inputs, but only one output (single-ended). This is analogous to a ganglion cell that receives excitatory input from one system and inhibitory input from the opposite system. This simple topology actually results in a loss of common-mode rejection capability by 50%. C) Adding a current mirror (shown in orange) to the differential to single-ended output circuit increases the common-mode rejection ratio. The proposed analogous retinal circuit topology is on the right. The current mirror may be analogous to an additional stage of crossover inhibition (shown in orange). D) A cascoded configuration yields the best common-mode rejection ratio for a differential to single-ended output, and the repeated crossover inhibition motif in the retina may perform the same function, though further experimentation would be required to show this definitively.

Glycine is the main neurotransmitter used in amacrine-to-amacrine inhibition

In retinal slice, we found that most of the interactions between amacrine cells was glycinergic. Crossover inhibition was glycinergic, as was the ON phase of ON-OFF inhibition. The OFF phase of ON-OFF inhibition was GABAergic, as well as within-layer inhibition (ON inhibition to ON cells, OFF inhibition to OFF cells). In the 292 amacrine cells we recorded in slice, 177 of them (60.6%) received some form of glycinergic inhibition, compared to just 25% of cells that received some form of GABAergic inhibition (the remaining cells were either AII amacrine cells, or did not receive any measurable inhibition).

In retinal wholemount, it was clear that glycinergic inhibition played a larger role than GABAergic inhibition in shaping the responses to light stimuli presented at the center. Again, crossover inhibition was seen to be a common interaction, and shown to be glycinergic. All the ON-OFF inhibition measured in the receptive field center was glycinergic, and together with crossover inhibition, these glycinergic interactions account for 59.1% (68/115) of the interactions we measured in retinal wholemount. We could not confirm that GABAergic inhibition played a major role in the formation of the light response at the center, but our data suggests that GABAergic inhibition may serve a greater contribution in the response to wide-field light stimulation.

From correlating morphological data with neurotransmitter content revealed by immuncytochemistry, we found that most glycinergic cells had small dendritic fields, usually smaller than 100μm. This points to the conclusion that the majority of amacrine cells interact with each other in local, glycinergic circuits. Few amacrine cells receive wide-field GABAergic inhibition, which means that wide-field GABAergic amacrine cells are primarily feeding forward to ganglion cells, or feeding back to bipolar cells. Amacrine cells, generally speaking, do not iteratively process the visual signal over large regions of the retina; information regarding large regions of the retina are directly passed to ganglion cells, or possibly bipolar cells. This study, however, omits the role of gap junctions as a way in which signals can be conveyed from cell to the next. Some classes of amacrine cells are coupled via gap junctions (Mills and Massey 1995; Volgyi, Xin et al. 2001), and while examining these electrical synapses falls outside the scope of this work, these gap junctions act as important conduits of visual information and merit additional study.

Amacrine to amacrine cell interactions

Our findings are summarized in the schematics shown in Fig. 4-8. The goal of this body of research was to determine the basic connectivity and interactions between amacrine cells. We characterized the inputs to amacrine cells using electrophysiology and pharmacology, and characterized their outputs by examining their morphology and neurotransmitter content with immunocytochemistry. Our cumulative results show that glycinergic crossover inhibition is the most common form of inhibition in the inner retina, and the diversity of inhibitory responses measured at bipolar and ganglion cells arises from combinations of inhibition built on top of this crossover inhibition. This top-view of the inhibition between amacrine cells shows that glycine is the major neurotransmitter that carries inhibition between amacrine cells, and GABA plays secondary roles in amacrine-amacrine interactions. GABA appears to more involved in feedback and feedfoward inhibition (Roska and Werblin 2003; Eggers and Lukasiewicz 2006; Molnar and Werblin 2007), though there are a few GABAergic interactions between amacrine cells as well (Hsueh, Molnar et al. 2008). Glycinergic amacrine cells, regardless of their polarity, convey local information, while GABAergic cells convey global information.

Even with all the data we have characterizing the synaptic and input to amacrine cells, some questions still remain. We consistently measured from amacrine cells that received ON-OFF inhibition, and in many cases, these cells *hyperpolarized* in response to light, and could contain either glycine or GABA. What could be the purpose of a hyperpolarizing response? One possible function for ON-OFF hyperpolarizing cells is to

provide push-pull inhibition to ON-OFF ganglion cells. We have shown that crossover inhibition acts in a push-pull manner to enhance the responses of ON or OFF cells. These ON-OFF hyperpolarizing cells may be performing the same kind of function, however our current body of work does not contain direct data to prove this hypothesis. Additional directions for future examination include the role of gap junctions between amacrine cells (and between amacrine and ganglion cells), and determining the exact locations of synaptic release on amacrine cell processes. Additionally with the improvement of circuit-tracing techniques, specific microcircuits involving bipolar, amacrine and ganglion cells can be visually mapped out, and new questions will emerge to challenge and expand the schema we put forth here. As the field continues to grow, linking circuitry with function, we will hopefully be inspired with new approaches to the repair of degenerate retinas, and to restore vision to the blind. While this is yet far off, every circuit that is elucidated, every synapse that is newly understood, every retina prosthetic that is designed based on these findings, brings us one step closer to the best solution.

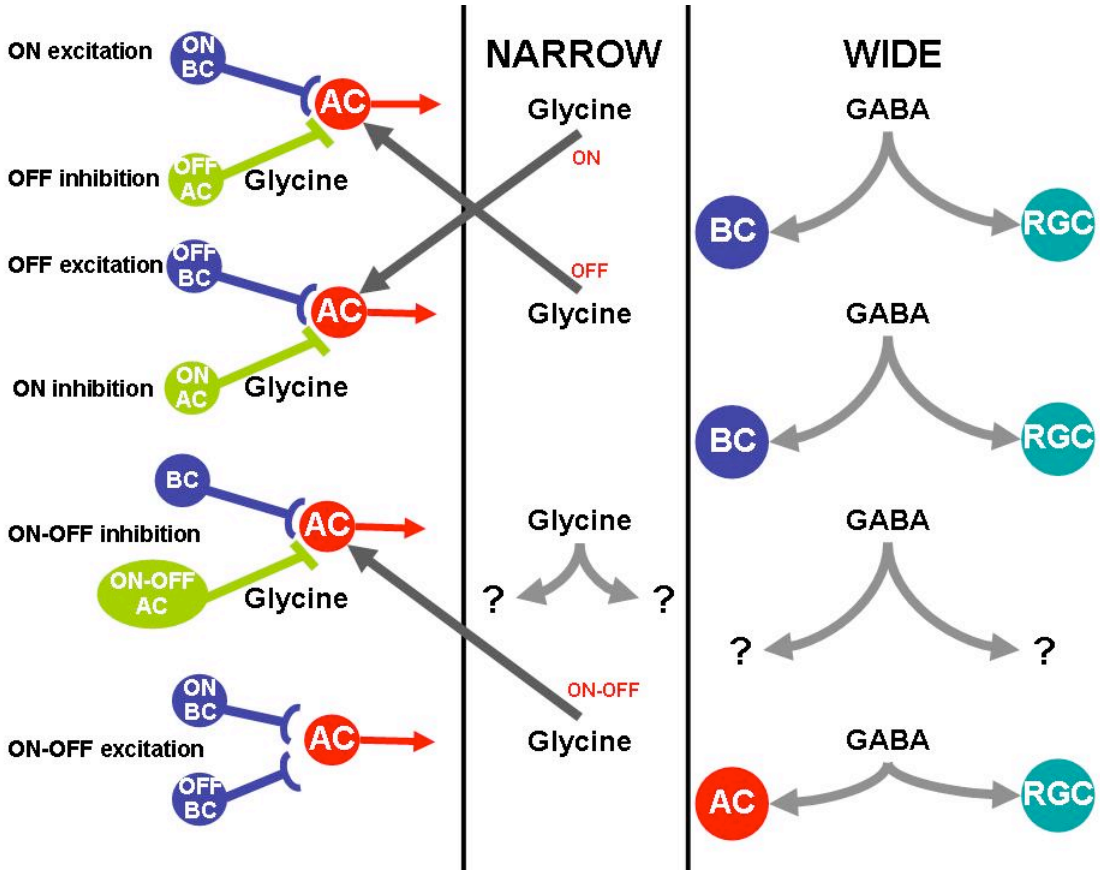


Figure 4-8. Summary of amacrine to amacrine circuits. The leftmost column describes the inputs to the amacrine cells that we recorded from. The neurotransmitter that mediates the inhibitory input can be determined using pharmacological blocker. The top two amacrine cells (in red) receive inhibition from the opposite system (crossover inhibition). ON-OFF inhibition is mediated by glycine (though in sliced retina, the OFF phase is mediated by GABA). The bottommost red amacrine cell does not receive inhibition from other amacrine cells.

The two right columns describe the outputs of the red amacrine cells in the left column. We determined if an amacrine cell was wide or narrow by its morphology and the neurotransmitter it released by immunocytochemistry. In general, glycinergic amacrine cells were narrow and GABAergic amacrine cells were wide. Basic circuitry can be derived from this information. Narrow glycinergic cells inhibited other amacrine cells (and bipolar cells, though not shown here), in either crossover form, or ON-OFF glycinergic inhibition. Wide GABAergic cells likely inhibit bipolar and ganglion cells, and amacrine cells to a lesser degree.

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