MODULATION OF INNER RETINAL INHIBITION WITH LIGHT ADAPTATION

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The retina is able to adjust its signaling over a wide range of light levels. A functional result of this is increased visual acuity at brighter luminance levels, such as during the day, due to changes in the organization of retinal receptive fields. This process is commonly referred to as light adaptation. These organizational changes have been shown to occur at the level of the ganglion cells, the output neurons of the retina, which have shifts in their excitatory center-inhibitory surround receptive fields that increase their sensitivity to small stimuli. Recent work supports the idea that light-adapted changes in ganglion cell spatial sensitivity are due in part to inner retinal signaling changes, possibly including changes to inhibition onto bipolar cells, the interneurons at the center of retinal signal processing. However, it is unknown how inhibition to the bipolar cells changes with light adaptation, how any changes affect the light signal or what mediates the changes to the bipolar cells that have been suggested by previous reports. To determine how light adaptation affects bipolar cell inhibition, the inhibitory inputs to OFF bipolar cells were measured. OFF bipolar cells, which respond to the offset of light, in particular may be involved in retinal adaptation as they bridge dim- and bright-light retinal pathways. Their inputs were compared between dark- and light-adapted conditions to determine how any inhibitory changes affects their output onto downstream ganglion cells. We found that there was a compensatory switch from primarily glycinergic-mediated inhibition to OFF bipolar cells in the dark to primarily GABAergic-mediated inhibition in the light. Since glycinergic and GABAergic inhibition perform very different roles and are mediated by morphologically different cells, it is likely this switch underlies a change in the spatial distribution of inhibition to these cells. We found that the
spatial inhibitory input to OFF bipolar cells became significantly smaller and narrower with light adaptation, translating to smaller inhibitory surrounds of the OFF bipolar cell receptive fields. Through a model, our data suggested that the OFF bipolar cell output to downstream ganglion cells was stronger in the light, due to the narrowing and reduction in the spatial input, to small light stimuli. This would effectively be one way the retina could use to increase visual acuity. Additionally, we found that the inhibitory changes to OFF bipolar cells with light-adaptation are partially mediated by dopamine D1 receptor signaling. Dopamine is released in the light and has been shown to be an important modulator of retinal light-adaptation. However, there are likely other factors involved in mediating inhibitory changes to OFF bipolar cells. Through these studies, we show that light adaptation heavily influences inner retina inhibition and likely plays a prominent role in determining and shaping light signals under different ambient light conditions which may ultimately be one mechanism for increasing visual sensitivity and acuity.
CHAPTER 1: GENERAL INTRODUCTION

Retinal Function

The retina is a thin and highly organized neural tissue located in the back of the eye that is connected directly to the brain. It is the first step in the detection and processing of environmental light which is then further processed by the visual cortex to allow for sight. Light that enters the eye is first focused by the cornea and lens before traversing the vitreous, a gel-like substance that fills the main space of the eye, and the retina itself before being detected by specialized cells called photoreceptors. The photoreceptors are embedded in the retinal pigment epithelium, a layer of specialized cells that nourish the photoreceptors, regenerate the pigments that allow for the detection of light, and absorb stray light so the retina does not detect reflected light (Strauss, 2005). After light is detected by the photoreceptors it is transmitted and processed through numerous specific pathways in the inner retina before being sent to the brain via the optic nerve (Figure 1). The optic nerve is composed all the neuronal axons of retinal ganglion cells, the output neurons of the retina.

Since the retina is the first part of the visual pathway, it performs a number of important functions to allow higher visual centers to combine the information for more complicated visual processing. The first of these is the ability to detect photons of light via two types of photoreceptors: rod photoreceptors associated with night or dim-light vision and cone photoreceptors used in day or bright-light vision. Both photoreceptors have outer segment processes which contain light-sensitive opsin proteins, rhodopsin in the rod photoreceptors and a variety of cone opsins in cone photoreceptors, that absorb photons
of light leading to activation of biochemical pathways which translate the physical signal into a neuronal response (Hubbell and Bownds, 1979). Rod photoreceptor rhodopsins have a very high sensitivity to light and are active in dark environmental conditions while cone opsins require more light to activate (Schneeweis and Schnapf, 1995). The difference in the type of opsin molecules the photoreceptors contain leads to another main retinal function: detection of color. In humans and many primates, cone photoreceptors come in three flavors, short (blue), medium (green), and long (red) wavelength photoreceptors, depending on the opsins peak wavelength sensitivity (Dobelle et al., 1969; Bowmaker and Dartnall, 1980; Baylor et al., 1987). However, this differs between mammalian species. Most non-primate mammals, including the mouse, the mammalian model used in this study, have two cone photoreceptors with different sensitivities containing medium (green) and short (blue/UV) wavelength opsins (Jacobs et al., 1991; Jacobs and Deegan, 1994). Non-mammalian vertebrates and invertebrates, however, utilize many different retinal strategies for color detection that can differ drastically from the mouse model (Ammermuller and Kolb, 1996).

In addition to direct detection of light, the retina also has organizational units responsible for the detection and processing of contrasts and edges in the visual scene as well as directional motion. Detecting changes and differences in contrast is the ability to differentiate an object from a background. Different cells in the retina are tuned to have better contrast sensitivity than others as well as to specific spatial frequencies of stimuli (different sizes of light stimuli) (Enroth-Cugell and Robson, 1966). This is accomplished by signaling the edges around objects to differentiate between the two different contrasts.
(Spitzer et al., 1994) which is combined with additional information in the visual cortex to signal orientation and direction of stimuli in the environment (Reid and Alonso, 1996; Shapley et al., 2003). However, while higher cortical centers relay and process complex motion information, the retina also has a group of distinct cells that allow for direction selectivity. This utilizes unique inhibitory interneurons which provide inhibitory signals to direction selective ganglion cells (Barlow et al., 1964; Demb, 2007; Zhou and Lee, 2008; Vaney et al., 2012). In this way, the retina is the first line in processing motion information before the brain incorporates other sensory information.

Lastly, and the focus of this dissertation, is that the retina is where the phenomenon of light adaptation, or the ability to change signaling across a range of ambient light levels to adjust visual acuity, occurs. As a sensory system, the retina needs to signal under many varying light conditions in order to accurately encode the environment. To do this, the retina adapts to increasing luminance levels as well as differences in contrast, when going from a dark to light environment such as night to day, to avoid saturation and increase visual acuity to relay fine details during the day. The retina can adapt over 10 log units of light intensity throughout the changing environment (Yau, 1994). Adaptation occurs at several levels within the retina. It begins by using dim-light sensing rod and bright-light sensing cone photoreceptors. This creates a system where the rod pathway is active in the dark but as the environment becomes brighter, rod pathway signaling decreases and cone photoreceptor signaling increases, providing a gradual transition in signaling. Photoreceptor adaptation involves a number of signaling mechanism changes (Pugh et al., 1999; Fain et al., 2001) which occur at each ambient luminance. The rod pathway in
particular has been studied as it becomes ‘saturated’ with bright light so that it no longer increases its signaling with more activation due to the depletion of the rhodopsin which only regenerates after a period of darkness (Thomas and Lamb, 1999). However, there is an upper limit to cone photoreceptor light adaptation and when this is reached, visual signaling saturates (Burkhardt, 1994). A large part of retinal light adaptation also occurs in downstream networks, involving the inner retinal interneurons and ganglion cells. Ganglion cell adaptation has been measured and their signaling resets as background luminance increases to a new background threshold so they can continue to increase their firing rate (Figure 2C). The contrast adaptation component in part involves a reduction in synaptic gain with increasing background luminance, effectively resetting retinal baseline so that increases and decreases around the baseline can be signaled (Jarsky et al., 2011). How light adaptation affects inner retinal signaling in the mammalian mouse model is the focus of the following studies.

Retinal Excitatory Circuitry and Signaling

The retina consists of five general types of neurons, organized in two main streams of information, located within specific tissue sub layers (Figure 1). The first stream is the excitatory, or vertical, pathway that acts as the main signal transduction pathway for light information. This pathway consists of cells organized in a vertical path down the height of the retina and ends by sending the information out the optic nerve. The first group of neurons, as mentioned previously, are the photoreceptors. Photoreceptor neurons traverse three sub layers the retina: their outer segments that contain the light detection complexes, the photoreceptor cell nuclei located in the outer nuclear layer (ONL), and
their terminals (called rod spherules and cone pedicles) located in the outer plexiform layer (OPL). The two types of photoreceptors, rods and cones, are responsibly for detecting dim- and bright-light information respectively. This physical stimulus is converted to a neurochemical stimulus by the photoreceptor and ends with the release of the excitatory neurotransmitter glutamate (Ehinger et al., 1988; Marc et al., 1990; Van Haesendonck and Missotten, 1990; Kalloniatis and Fletcher, 1993; Yang and Yazulla, 1994; Jojich and Pourcho, 1996). Glutamate release is high in the dark and when there is a light stimulus, the photoreceptor hyperpolarizes and glutamate release ceases (Stryer, 1991; Yau, 1994).

The released glutamate binds to glutamate receptors on the dendrites of next class of neurons: the bipolar cell interneurons. The photoreceptor-bipolar cell synapse along with the photoreceptors are referred to as the outer retina. Rod photoreceptors give input to a single type of bipolar cell called the rod bipolar while cone photoreceptors contact multiple types of cone bipolar cells each (Figure 1). There are 11 distinct types of cone bipolar cells, split into two main groups: OFF cone bipolar cells (OFF bipolar cell) that respond to decrements in light, or hyperpolarize with light, and ON cone bipolar cells (ON bipolar cell) that respond to increments in light, or depolarize with light (Werblin and Dowling, 1969; Kaneko, 1970; Nelson and Kolb, 1983; Masland, 2012). The bipolar cell difference in response to light is determined by type of glutamate receptors expressed by OFF and ON bipolar cells (Miller and Slaughter, 1986). OFF cone bipolar cells have traditional AMPA and/or kainate ligand gated cation channels so that they have a sign-conserving synapse and follow the glutamate release from the photoreceptors (Puthussery
et al., 2014). In the dark when the photoreceptor glutamate release is high, the cation channels on OFF bipolar cells are open, depolarizing the bipolar cell, and they close with the decrease in glutamate during a light stimulus (Saito and Kaneko, 1983). In this way, OFF bipolar cells respond when the light turns off, or to a dark flash. ON bipolar cells on the other hand do not have traditional glutamate receptors. Instead they express the metabotropic glutamate receptor 6 (mGluR6), a G-protein coupled receptor (Slaughter and Miller, 1981; Nawy and Copenhagen, 1987). In the dark, with high glutamate release, glutamate binds to the mGluR6 protein activating a signaling cascade to close the non-selective cation channel TRPM1 (Vardi and Morigiwa, 1997; Morgans et al., 2009). With a light stimulus, cone glutamate release decreases and shuts off the inhibitory signaling cascade so the TRPM1 channels open and the ON bipolar cell depolarizes (Werblin and Dowling, 1969). This is referred to as a sign-inverting synapse.

The OFF and ON pathways create parallel streams of information which signal different aspects of the visual scene allowing for strong signal computation, fast signaling, and high contrast sensitivity (Schiller et al., 1986; Asari and Meister, 2012). In the mouse there are 5 types of OFF cone bipolar cells called OFF type 1, 2, 3a, 3b, and 4 and 6 types of ON cone bipolar cells called ON type 5a, 5b, 6, 7, 8, and 9 (Ghosh et al., 2004; Mataruga et al., 2007; Wassle et al., 2009; Euler et al., 2014). All these different bipolar cells contain different types of ionotrophic glutamate receptors (DeVries, 2000; Puller et al., 2013), receive input from different cone photoreceptors (Mataruga et al., 2007; Haverkamp et al., 2008; Wassle et al., 2009; Breuninger et al., 2011; Dunn and Wong, 2012; Tsukamoto and Omi, 2013), terminate in different synaptic inner plexiform layers...
of the retina (Pignatelli and Strettoi, 2004; Wassle et al., 2009; Breuninger et al., 2011; Helmstaedter et al., 2013), have different temporal responses to light stimuli (Cui and Pan, 2008; Saszik and Devries, 2012; Baden et al., 2013; Ichinose et al., 2014; Puthussery et al., 2014), and are contacted by different classes of modulator neurons (Euler and Wässle, 1998; Ivanova et al., 2006; Eggers et al., 2007; Mazade and Eggers, 2013).

Bipolar cell somas are located in the inner nuclear layer (INL) and axon terminals stratify various sublaminae of the inner plexiform layer (IPL) where they synapse onto ganglion cell dendrites. The INL and IPL together are referred to as the inner retina. Like photoreceptors, bipolar cells release glutamate from their terminals.

The light signal of the excitatory pathway is next sent to ON, OFF, and ON/OFF ganglion cells, whose cell bodies are located in the ganglion cell layer closest to the interior of the eye, and whose dendrites extend into the areas of the inner plexiform layer where ON and OFF bipolar cells stratify (Hartline, 1938; Nelson et al., 1978; Peichl and Wässle, 1981; Amthor et al., 1989) (Figure 1). ON and OFF bipolar cells synapse onto ON and OFF ganglion cells respectively, while both ON and OFF bipolar cells synapse onto ON/OFF ganglion. The ganglion cells themselves do not determine whether they are activated at the onset or offset of light as they contain traditional AMPA/kainite glutamate receptors, but it is the connections to the bipolar cells that determines their activation (Massey and Miller, 1988). It is at the level of the ganglion cell that the ultimate retinal processing is completed before being sent to the brain. Though all ganglion cells use glutamate receptors to receive the light signal, the expression of
specific receptors differs between ganglion cell subtypes (Marc, 1999). From here, the processed light information is transmitted to the thalamus and then the visual cortex.

Retinal Inhibitory Circuitry and Signaling

The vertical excitatory pathway is modulated by the second stream of information that carries inhibitory signals laterally across the retina. In the outer retina, at the photoreceptor – bipolar cell synapse, this inhibition is mediated by horizontal cells. There is some contention on the exact mechanism that horizontal cells use to shape photoreceptor output and bipolar cell dendritic light signals, but previous studies have shown that they can perform ephaptic transmission, voltage changes across two separate neuronal membranes due to extremely close proximity (Verweij et al., 1996; Kamermans et al., 2001; Kamermans and Fahrenfort), inhibit with pH changes (Davenport et al., 2008) and even may utilize GABAergic mechanisms though this is likely species dependent (Marc et al., 1978; Malchow and Ripps, 1990; Cammack and Schwartz, 1993; Dong et al., 1994; Nelson et al., 2008). In the inner retina, at the bipolar cell – ganglion cell synapse, amacrine cells are responsible for regulating, shaping, and modulating bipolar cell signals to ganglion cells as well as ganglion cell dendritic transmission (Eggers and Lukasiewicz, 2006a, b; Sagdullaev et al., 2006; Eggers et al., 2007; Eggers and Lukasiewicz, 2010) (Figure 1). Amacrine cells are divided into two major groups: those that release glycine or those that release gamma-aminobutyric acid (GABA) as their neurotransmitters. Bipolar cell terminals and ganglion cells dendrites sense these signals using combinations of glycine, GABA\textsubscript{A} and GABA\textsubscript{C} receptors, which are all ligand-gated
chloride channels, the composition of which depends on each bipolar cell or ganglion cell subtype (Sassoe-Pognetto et al., 1994; Haerkamp et al., 2003).

The morphologies of glycinergic and GABAergic amacrine cells are also an important factor in retinal circuitry. Glycinergic amacrine cells are narrow-field, meaning their processes do not span long lengths of the retina (Pourcho and Goebel, 1985; Kolb, 1997; Menger et al., 1998). Rather, they often span vertically through multiple sublaminae of the IPL often contributing to crossover inhibition between ON and OFF pathways (Molnar et al., 2009; Werblin, 2010; Mazade and Eggers, 2013). Certain subtypes of glycinergic amacrine cells in multiple animal models have been shown to be extensively electrically coupled, especially the AII amacrine cell, extending the extent of signal transmission (Vaney, 1991; Vardi and Smith, 1996; Bloomfield et al., 1997; Xin and Bloomfield, 1997; Bloomfield and Volgyi, 2004; Veruki et al., 2010; Marc et al., 2014). GABAergic amacrine cells on the other hand are wide-field cells whose processes extend hundreds of microns laterally across the retina, uniform in all directions (Pourcho and Goebel, 1983; Vaney, 1990; Kolb, 1997; Wright and Vaney, 2004). Additionally, their processes often stay within the same sublamina of the IPL and may be coupled to other GABAergic amacrine cells, but this is not always the case (Xin and Bloomfield, 1997; Li et al., 2002; Greschner et al., 2014). Amacrine cells are activated via glutamate release from ON and OFF bipolar cells and thus can provide inhibition during luminance increases or decreases as well as mediate crossover inhibition between the two pathways (Ackert et al., 2009) (Figure 1). Inhibitory amacrine cells, which combine their signals to shape retinal excitatory signaling at the bipolar cell-ganglion cell synapse (Eggers and
Lukasiewicz, 2006a, b; Sagdullaev et al., 2006; Eggers et al., 2007; Eggers and Lukasiewicz, 2010) are important for network circuitry adaptation by modulation retinal gain control (Green et al., 1975; Naka et al., 1979; Green and Powers, 1982; Shapley and Enroth-Cugell, 1984; Page-McCaw et al., 2004; Dunn et al., 2006; Dunn et al., 2007). The interactions between the excitatory and inhibitory pathways along with the many subtypes of bipolar, amacrine, and ganglion cells provides the retina with many unique but parallel pathways that are responsible for detection and processing of certain properties of the visual stimulus such as color, size, location, and timing.

Though many retinal pathways follow the prototypical linear signal transduction from photoreceptor to ganglion cell, one important pathway, including the subset of bipolar cells investigated in this study, does not. Light onset signals detected by the rod photoreceptor are sent to the rod bipolar cell via glutamate. However, instead of synapsing onto a ganglion cell, the rod bipolar cell activates two amacrine cells: the A17 and the AII (McGuire et al., 1984; Strettoi et al., 1990) (Figure 1). The GABAergic A17 amacrine cell acts as a reciprocal feedback inhibitor which has been extensively studied, at both the synaptic and functional levels, and shown to shape rod bipolar cell signaling (Nelson and Kolb, 1985; Hartveit, 1999; Menger and Wässle, 2000; Dong and Hare, 2003; Singer and Diamond, 2003; Chavez et al., 2006; Eggers and Lukasiewicz, 2006a; Grimes et al., 2009; Grimes et al., 2010; Grimes et al., 2015; Moore-Dotson et al., 2015). Activation of the AII amacrine cell causes release of glycine to inhibit the OFF bipolar cells and OFF ganglion cells (Strettoi et al., 1994; Grunert and Wässle, 1996; Haverkamp et al., 2003) In this way rod pathway signals are sent via inhibition to the OFF cone.
pathway, which in turn transmits this signal to the OFF ganglion cells. AII amacrine cells also contain voltage-gated Na\(^+\) channels which work to accelerate rod pathway signals in dark conditions (Tian et al., 2010). In addition to this crossover synapse, AII amacrine cells are also electrically coupled to ON cone bipolar cells via connexin 36/45 gap junctions (Strettoi et al., 1992; Chun et al., 1993; Trexler et al., 2001; Deans et al., 2002). Rod excitatory signals sent through the AII amacrine cell excite the ON bipolar cell terminals causing glutamate release onto ON ganglion cells. Lastly, these AII-ON bipolar cell gap junctions are bidirectional, so ON cone-activated signals can utilize rod pathway AII inhibitory input onto OFF bipolar cells (Trexler et al., 2001; Veruki and Hartveit, 2002; Trexler et al., 2005; Beaudoin et al., 2008; Manookin et al., 2008; Munch et al., 2009; Mazade and Eggers, 2013) and OFF ganglion cells (Manookin et al., 2008; Murphy and Rieke, 2008; Munch et al., 2009; Arman and Sampath, 2012). In this way, rod pathway circuitry uses existing cone pathway circuitry with the nexus of both pathways involving inhibitory signaling being the OFF cone bipolar cells.

Retinal Receptive Field Organization

As mentioned previously, one main function of the retina is to detect contrast and edges to provide the brain with high resolution information for clear vision of fine details in the environment. In addition to the many excitatory and inhibitory signaling pathways that contribute to these functions, the overall spatial organization of retinal circuitry plays a critical role. This is especially true in how the retina adapts to new luminance levels which is the focus of this dissertation. Ganglion cells, as well as bipolar cells, have spatial receptive fields (Hare and Owen, 1996), which refers to the retinal area (all the
photoreceptors and/or bipolar cells) above the dendrites of the respective cell that elicits a response when that area is stimulated. This has been modeled using ON bipolar cell arrays which were shown to simulate the spatial properties of the downstream ganglion cell receptive field (Freed et al., 1992).

Ganglion cell excitatory center and antagonistic surround receptive fields were first discovered and characterized in classical experiments in the cat (Kuffler, 1953; Wiesel, 1959) (Figure 2A). It was found that when recording action potentials from individual neurons, a small spot of light presented over the retinal area above the dendrites of the ganglion cell (receptive field center) caused an increase in spiking frequency. It was concluded that the circuity in place over the center led to excitation of the ganglion cell. However, when an annular light stimulus was presented to the area surrounding the dendritic arbor, there was a hyperpolarization of the ganglion cell and a cessation in spiking frequency. It was found that the area surrounding the dendrites led to inhibition of the ganglion cell and was noted as the inhibitory surround. Lastly, a large spot of light that covered the entire excitatory center and much of the inhibitory surround caused only a small increase in spiking in the ganglion cell, suggesting that activation of surround inhibition decreases the excitatory center drive of the ganglion cell. Another way that retinal receptive field responses are represented are with 2D Gaussian distributions (Figure 2B). In this representation, separate center and surround Gaussian distributions can be subtracted, called difference-of-Gaussians, to create a center-surround receptive field distribution. These distributions provide information on the strength of the response when a stimulus is presented on part of the receptive field. For example, a spot of light
that covers the center of the cell’s receptive field will activate a large proportion of excitatory input but also a smaller proportion of inhibitory input (Figure 2B). However, the overall response would be an increase in the cell’s firing as shown by the center-surround distribution. However, if a stimulus is presented further away from the cell’s center, more inhibitory inputs are stimulated than excitatory inputs, and the cell is inhibited.

These classical experiments showed a receptive field organization of ganglion cells where the interplay and balance between the center and surround determine how the ganglion cell can respond to spatially distinct light stimuli. In this way ganglion cells are spatially tuned to certain sizes of light (Flores-Herr et al., 2001; Schwartz et al., 2012; Di Marco et al., 2013). It has been shown through many studies that the creation of center-surround receptive fields of ganglion cells is not only determined by the extent of the cell’s dendrites, but the direct and indirect connections the cell has between the various amacrine cells (Cook and McReynolds, 1998; Flores-Herr et al., 2001; Sinclair et al., 2004; Buldyrev and Taylor, 2013; Di Marco et al., 2013; Protti et al., 2014; Venkataramani et al., 2014). These inhibitory connections define the inhibitory surround of the receptive field and modulation of these inputs can shift the center/surround balance.

In simplistic terms, the center size of a ganglion cell is related directly to the size of that cell’s dendritic arbor (Peichl and Wassle, 1983; Wassle et al., 1983a, b; Nelson et al., 1993; Pu, 1999, 2000). Under this definition, receptive field center sizes vary drastically
with the different subtypes of ganglion cells. In the mouse, ganglion cell dendritic arbors have a large range of lengths, extending up to 400 µm in diameter (Wässle et al., 1981; Doi et al., 1995). However, this does not completely explain the size of the excitatory center. Other work has shown that the excitatory center can extend farther than the particular cell’s dendrites, likely due to coupling between upstream photoreceptors, or be smaller than the ganglion cell’s dendritic field (Fukuda et al., 1984). Thus, the size of the center is a fluid parameter that depends on a several different circuity connections.

Additionally, many previous studies have investigated the composition of the inhibitory surround. The main conclusions of a number of studies is that the surround size is determined by wide-field GABAergic amacrine cell input (Cook and McReynolds, 1998; Flores-Herr et al., 2001; Sinclair et al., 2004). Many of these amacrine cells are spiking cells and may be coupled to extend the surround size (Xin and Bloomfield, 1997; Li et al., 2002; Greschner et al., 2014). In this way, lateral GABAergic signals carrying information across the retina to define and shape the border of the excitatory center of ganglion cell receptive fields.

Center-surround receptive fields have also been reported previously at the bipolar cell level (Dacey et al., 2000). For bipolar cells, the excitatory center consists of the photoreceptors that give inputs to their dendrites however this may include neighboring photoreceptors, that are likely coupled to the photoreceptors that have direct synaptic connections with the bipolar cells, to increase center extent, improve sensitivity, and increase signal-to-noise ratio (DeVries et al., 2002; Hornstein et al., 2005; Li et al., 2012). Like ganglion cells, bipolar cell surrounds are created via lateral inhibition
through glycinergic and GABAergic inputs providing spatial information (O'Brien et al., 2003; Sinclair et al., 2004; Ichinose and Lukasiewicz, 2005). The balance between the excitatory center and inhibitory surround of both the bipolar cells and ganglion cells themselves allows ganglion cells fine spatial tuning to specific sizes of light, vital for visual resolution. It reasons that any condition which affects the size and strength of the center-surround receptive fields modulates the retina’s ability to discriminate between stimuli of different spatial frequencies.

Changes in Ganglion Cell Receptive Fields with Light Adaptation

It is known that the retina must signal over a wide range of light intensities and avoid signal saturation. The start of this process is the utilization of two types of photoreceptors that are active under dim or bright conditions, as discussed, but it also follows that downstream circuitry of the photoreceptors would also change in different light conditions. Under dark-adapted conditions, there is very little light entering the eye so the retinal circuitry must try to use all light information possible, sacrificing spatial resolution for signal sensitivity. This would be reflected in the receptive field organization and signaling as this organization ultimately determines retinal output. In this case ganglion cells have large centers with little surround and large signal convergence (Barlow et al., 1957; Sterling et al., 1988) in addition to utilizing specific bipolar cell and photoreceptor pathways for signaling to different types of ganglion cells (Wu et al., 2004). Under light-adapted conditions there is ample amount of light entering the eye. The retina would no longer care about high sensitivity for detection but now can ‘focus’ on separating out fine details in the light stimuli from the background.
illumination recruiting large inhibitory surrounds for fine spatial tuning (Barlow et al., 1957). In addition to background luminance, the light stimulus itself can alter receptive fields as shown in in vivo mouse ganglion cell experiments (Sagdullaev and McCall, 2005). Thus, is it logical that there would be a modulation of the retinal center-surround receptive fields to allow for the detection of more distinct light information for higher spatial resolution.

The concept of dynamic changes in receptive fields has been partially studied at the level of the ganglion cell receptive field. Ganglion cells have been shown to increase their spatial sensitivity and visual acuity when transitioning to a lighted environment, effectively able to signal smaller light stimuli with greater strength (Barlow et al., 1957; Dedek et al., 2008; Farrow et al., 2013). The increase in high spatial frequency signaling is vital for increasing visual acuity under conditions of sufficient background light. These ganglion cell changes first involve an adjustment of retinal gain, or the strength of signaling to a certain stimulus, beginning with adaptation at the photoreceptor level by changing rod photoreceptor gain and activating cone photoreceptors (Wu and Yang, 1992; Dunn et al., 2006). Activation of cone photoreceptors with increasing illumination then activates specific signaling pathways which when combined at the ganglion cell level could begin to change the center-surround balance.

Downstream retinal circuity also plays a critical role in influencing center and/or surround properties of ganglion cells. Previous studies have shown that ganglion cell sensitivity to small lights increased due to a decrease in excitatory center size, fine tuning
the ganglion cell output to smaller stimuli (Merwine et al., 1995; Troy et al., 1999). Changes in the center may be a result of changes in the outer retina, inner retina, or both. With light adaptation, in addition to activation of cone photoreceptors and saturation of rod photoreceptors, the coupling state between photoreceptors also changes (Ribelayga et al., 2002; Ribelayga et al., 2008; Li et al., 2009; Li et al., 2012; Li et al., 2013). The coupling state depends on which photoreceptors are coupled but these differences can change the way excitatory signals are spread throughout the ganglion cell’s center. Additionally, the horizontal cells of the outer retina, long thought to be the only player in determining retinal receptive fields, become uncoupled with light adaptation (Xin and Bloomfield, 1999b). As a result this decreases the spatial extent of horizontal cell inhibitory input at the photoreceptor-bipolar cell synapse which may play a role in decreasing the excitatory center size of ganglion cells. However, a recent study found that blocking connections between horizontal cells had no effect on ganglion cell receptive field centers (Dedek et al., 2008) so the full extent of horizontal cell contribution needs further investigation. Lastly, one study found that coupling between ganglion cells increases with light adaptation which may also be an important contributor to receptive field changes (Hu et al., 2010).

Changes in the inner retina, most importantly, could also contribute to the excitatory center changes observed in ganglion cells. Bipolar cells receive spatial information from photoreceptors so that changes at the photoreceptor level may affect the signal strength the bipolar cells would be giving to different areas of the ganglion cell receptive field. Modulation of the amacrine cells in the inner retina also may change the spatial extent of
bipolar cells signaling onto the ganglion cells thus determining ganglion cell center size. At least some amacrine cells are highly coupled, such as the AII amacrine cell of the rod pathway, which become uncoupled with bright light likely changing the spatial extent of their signaling (Xin and Bloomfield, 1999a; Bloomfield and Volgyi, 2004; Hartveit and Veruki, 2012). The contribution of inner retinal inhibition to changes in receptive fields is one focus of this dissertation and will be addressed further in Chapter 3.

The ganglion cell center is not the only portion of the receptive field that changes with light adaptation. Other work has reported that there are decreases in the ganglion cell surround size and/or strength which also works to increase ganglion cell spatial sensitivity in the light (Troy et al., 1993; Merwine et al., 1995; Troy et al., 1999; Dedek et al., 2008; Farrow et al., 2013). As with changes of the ganglion cell center, surround changes are likely due to a combination of inner and outer retinal mechanistic changes. Inputs to the surround portions of the ganglion cell could also be affected by the coupling states of the photoreceptors and horizontal cells mentioned above which work to decrease the size of the center. These changes could be responsible for activating and then modulating the surround in the light. However, a likely target for determining changes in the surround are the direct amacrine cell connections onto ganglion cells. Ganglion cells receive both GABAergic and glycinergic inputs and several studies have shown that abolishing certain amacrine cell pathways eliminates the ability of the ganglion cells to have a spatially tuned surround (Cook and McReynolds, 1998; Taylor, 1999; Flores-Herr et al., 2001; Sinclair et al., 2004). Since it is known that light adaptation modulates the coupling state of certain amacrine cells, it is therefore likely that other unknown amacrine
cells determining surround input to ganglion cells are also coupled and affected by light. However, much more work needs to be done to have a better understanding of the role of direct inhibition onto ganglion cells to determine receptive field surrounds. While ganglion cell receptive field changes with light adaptation have been partially characterized, the inner retinal mechanisms preceding these changes are not well understood.

_Changes in Inhibition to Bipolar Cells with Light Adaptation_

Ganglion cell receptive field changes rely heavily on upstream bipolar cell signaling and processing of the light signal. Bipolar cells themselves have receptive fields so changes in the center and surround of bipolar cell receptive fields would translate to changes at the ganglion cell level. The outer retina may play a role in changing bipolar cell receptive fields (Shen et al., 2003). However, the inner retina may be important as well in ganglion cell acuity changes with light which one study suggested is due to processing at the inner retinal level (Dedek et al., 2008). Additionally, the connections between bipolar and ganglion cells have been shown to be important for light adaptation (Dunn et al., 2007; Oesch and Diamond, 2011; Farrow et al., 2013). Other reports show that both total retinal inhibition (Flores-Herr et al., 2001; O'Brien et al., 2003; Sinclair et al., 2004; Ichinose and Lukasiewicz, 2005) and inhibition to bipolar cells specifically (Flores-Herr et al., 2001; Russell and Werblin, 2010; Buldyrev and Taylor, 2013; Protti et al., 2014), are important for determining the spatial resolution and receptive field properties of ganglion cells. Modulation of bipolar cell inhibition by light is therefore a potential mechanism for the increase in visual acuity that has long been reported in ganglion cells.
The modulation of signals to the OFF pathway in particular may be important in retinal network adaptation, as inhibitory inputs switch between rod and cone photoreceptor sources (McGuire et al., 1984; Strettoi et al., 1990; Strettoi et al., 1992; Chun et al., 1993; Trexler et al., 2001; Deans et al., 2002; Mazade and Eggers, 2013). OFF bipolar cells contain glycine, GABA\textsubscript{A}, and GABA\textsubscript{C} receptors (Sassoe-Pognetto et al., 1994; Haverkamp et al., 2003) and so receive glycine, GABA\textsubscript{A}, and GABA\textsubscript{C}R-mediated inhibitory input (Euler and Wässle, 1998; Ivanova et al., 2006; Eggers et al., 2007). In dark-adapted conditions, OFF bipolar cells receive significant glycinergic input in response to light (likely from AII amacrine cells) which may change with light adaptation since rod pathway signaling decreases (Mazade and Eggers, 2013). Although OFF bipolar cells respond to light offset, light stimuli or luminance increases from a dark background, still elicit evoked responses due to the cessation of the light stimulus and can be effectively used to test light-evoked responses in OFF bipolar cells as done in previous reports (Eggers et al., 2007; Mazade and Eggers, 2013). Since OFF bipolar cells consist of 4 main subtypes (Ghosh et al., 2004) there may be connectivity difference in inhibitory input between them. Morphological and later physiological studies showed that one subtype (type 3) does not receive input from the AII amacrine cell (Tsukamoto et al., 2001; Mazade and Eggers, 2013). Despite this, all subtypes changed the source of their inhibition with light adaptation. Spatial inhibitory changes to OFF bipolar cells therefore, may play a vital role in determining spatial center-surround changes seen in ganglion cells with light adaptation, however this has not yet been tested.
Role of Dopamine in Light Adaptation

There are many potential mechanisms for the ganglion cell and bipolar cell changes with light adaptation reported in many previous studies. The most likely candidate is the neuromodulator dopamine. Dopamine is considered a main ‘molecule of light adaptation’ and is released from specialized dopaminergic amacrine cells in the light (Witkovsky, 2004), during the day, and plays a key role in in modulating many retinal pathways (Godley and Wurtman, 1988; Boatright et al., 1989; Doyle et al., 2002). Dopaminergic amacrine cells have ON bipolar cell synaptic contacts (Contini et al., 2010) and have a basal tonic release during the night which increases with light, as well as stimulated released by light, to follow a loose circadian rhythm (Doyle et al., 2002). The dopaminergic amacrine cell body itself is located in the INL and has processes that extend throughout the IPL as well as the OPL and photoreceptor layer (Zhang et al., 2004; Keeley and Reese, 2010). The D1 receptor, which is a G-protein coupled receptor, acts to increase cyclic AMP (cAMP) and protein kinase A (PKA) and is located on bipolar, amacrine, horizontal and ganglion cells (Veruki and Wässle, 1996; Nguyen-Legros et al., 1997) and modulates OFF ganglion cell responses in the light-adapted retina (Yang et al., 2012). D2 receptors, which are Gi-protein coupled receptors, act to decrease cAMP and are located on the dopaminergic amacrine cells themselves and are thought to act as negative feedback for dopamine release (Nguyen-Legros et al., 1999). Lastly D4 receptors, which are part of the D2-like receptor family and are Gi-protein coupled receptors, are expressed on the photoreceptors affecting their protein phosphorylation (Pozdeyev et al., 2008; Jackson et al., 2009). The myriad of dopamine

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receptors located throughout the retina modulate many different aspects of signaling and thus retinal adaptation.

Dopamine has been shown to be important for light and contrast adaptation (Witkovsky, 2004), and modulates OFF ganglion cell receptive fields and sensitivity in mammals possibly through changes in bipolar cell inhibition (Jensen and Daw, 1984; Maguire and Smith III, 1985; Jensen and Daw, 1986; Jensen, 1989; Jensen, 1991, 1992; Maguire and Hamasaki, 1994). Studies have confirmed that activation of dopamine D1 receptors leads to potentiation of GABA_A receptor currents in cultured rat amacrine cells and cultured rat striatal neurons (Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995; Hoerbelt et al., 2015) and decreased GABA_C receptor-mediated currents in catfish horizontal cells, tiger salamander bipolar cells and cultured rat bipolar cells (Dong and Werblin; Feigenspan and Bormann, 1994b; Wellis and Werblin).

Additionally, other studies have shown dopamine modulation of neurotransmitter release onto amacrine cells directly (Pycock and Smith, 1983; Kato et al., 1985; O'Brien and Dowling, 1985; Calaza et al., 2001) and dopamine uncoupling of gap junctions between AII amacrine cells as well as other retinal neurons (Hampson et al., 1992; Mills and Massey, 1995; Kothmann et al., 2009). Dopaminergic modulation of the coupling state of the retina is therefore a very likely mechanism for light-adapted retinal changes in inhibition.

All of this evidence suggests that D1 receptors especially, due to their expression on bipolar and amacrine cells, play a role in retinal inhibitory network adaptation to
luminance level although the magnitude, timing, and localization of release to D1 receptors is not known. This supports the idea that dopamine is playing a role in shaping bipolar cell signals, especially OFF bipolar cells due to their involvement in both rod and cone pathways and their inhibitory changes in the light. However, the role of dopamine modulation of inhibitory inputs in retinal pathways is not fully understood and this potential mechanism is part of the current investigation of this dissertation and covered in Chapter 4.

Specific Aims
The ultimate goal of this dissertation is to investigate how retinal OFF pathway signaling changes under different light conditions. Specifically, the role that inhibition plays in mediating signaling changes will be examined as inhibitory amacrine cells are responsible for shaping and tuning retinal output. Modulation of these connections under brighter light conditions could drastically alter the output of this pathway, thus enhancing or depressing visual responses.

In summary off previous findings, inhibition via amacrine cells is now thought to be responsible for establishing the unique center-surround organization of ganglion cells which is crucial in high acuity vision (Flores-Herr et al., 2001; Schwartz et al., 2012; Di Marco et al., 2013). Thus changes in bipolar cell inhibition could be a major mechanism in mediating light adaptation to allow for stable and high acuity vision in the light, but this is currently unknown. The following studies will determine if light adaptation modulates the source and spatial inhibitory input to OFF pathway neurons and if any
changes are due to the direct actions of the neuromodulator dopamine, which has been implicated as a molecule of light adaptation. These experiments are the first to look at bipolar cell inhibition as a potential driver and site of the signaling sensitivity changes with light adaptation that is seen in ganglion cells.

In Chapter 2 the changes in inhibition to the OFF bipolar cells when rod vs cone pathways are active will be inspected. Previous work has suggested that the large amount of glycinergic input to OFF bipolar cells in the dark is from the rod bipolar cell-AII amacrine cell pathway. It is unknown how this changes in the light, when the rod system is suppressed. The magnitude and kinetics of light-evoked and spontaneous currents due to glycine and GABA receptors in OFF bipolar cells in response to short light stimuli in both dark- and light-adapted conditions will be measured. It is predicted there will be a decrease in OFF bipolar cell inhibitory input with light adaptation due to a decrease in rod pathway signaling.

Chapter 3 examines how the spatial inhibitory input to OFF bipolar cells changes with light adaptation. Ganglion cell spatial sensitivity increases when moving from dim- to bright-light conditions, possibly mediated at the bipolar cell level. The data from Chapter 2 suggests that the changes in inhibition may affect the inhibitory surrounds of the OFF bipolar cells. To address this, light-evoked currents of OFF bipolar cells in response to narrow stimuli will be measured in order to map the spatial inhibitory input to these cells. This will be compared between both dark- and light-adapted conditions as well as while isolating GABA and glycine receptors to determine differences in specific receptor and
amacrine cell input. Additionally, a model will be constructed to simulate OFF bipolar cell signal output onto downstream ganglion cells.

Lastly, Chapter 4 looks at whether dopamine is directly involved in mediating inhibitory changes to the OFF pathway. Dopamine is released during the day and affects almost every point of retinal signaling cascades, binding to D1 receptors on multiple cell types. However, dopamine was shown to specifically potentiate GABA\textsubscript{A} and reduce GABA\textsubscript{C} receptor currents so there may be significant effects on inner retinal inhibition. To address this aim, total and glycinergic light-evoked and spontaneous currents from OFF bipolar cells will be recorded while mapping spatial inhibitory input under dark-adapted conditions with application of a D1 receptor agonist. This will allow the direct testing of whether activation of D1 receptors in the inner retina is sufficient to elicit light-adapted changes in inhibition.
Figure 1. Schematic of basic retinal circuitry connections.
Cone photoreceptors (cone) are activated by brighter light and release glutamate onto ON and OFF cone bipolar cells. These cells then release glutamate to activate ON and OFF ganglion cells, respectively. Activation of these bipolar cells also release glutamate onto inhibitory amacrine cells which can have inhibitory synaptic contacts onto OFF and/or ON bipolar and ganglion cells. Rod photoreceptors (rod) are activated by dim light and release glutamate onto rod bipolar cells which release glutamate onto amacrine cells. These amacrine cells then have connections with OFF and ON cone pathways, where they inhibit the OFF cone pathways and excite ON cone pathways through gap junction connections to specific amacrine cells. Additionally, amacrine cells can also inhibit other amacrine cells.
Figure 2. Ganglion cell center-surround receptive field and light adaptation response curves.

A, adapted from (Wiesel, 1959), a small spot of light presented over the center of the ganglion cell caused an increase in spiking frequency. When an annular light stimulus was presented to the area surrounding the ganglion cell dendrites was a decrease in spiking frequency. Lastly, a large spot of light that covered the entire ganglion cell and the area around the cell, there was only a moderate increase in spiking in the ganglion cell.

B, adapted from (Rowe, 2002), center and surround Gaussian distributions of retinal neurons. Stimuli presented over the center of the cell elicit the largest excitatory and inhibitory inputs to that cell whereas stimuli presented far from the cell only activate inhibitory input to the cell. The center and surround Gaussian distributions can be subtracted (difference-of-Gaussians) to create the total center-surround receptive field of the retinal cell. This curve tells the overall response of the cell due to a stimulus presented at any location in the cells receptive field.

C, adapted from (Laming, 2013), the ganglion cell response curves as a function of stimulus intensity over a range of background adapting luminances. The adapting luminance is indicated by the arrows in order of the individual curves. As background intensity increases, the retinal network laterally shifts the ganglion cell response curves to brighter intensities, creating a “new” baseline for ganglion cell signaling of brighter light.
CHAPTER 2: LIGHT ADAPTATION ALTERS THE SOURCE OF INHIBITION TO
THE MOUSE RETINAL OFF PATHWAY

Introduction:

Sensory systems must avoid signal saturation to encode a wide dynamic range of incoming information, for example when stepping outside into the bright sunlight from a dimly lit room. This occurs in the retina partly by using two photoreceptors with different sensitivities – rod photoreceptors that sense dim light and cone photoreceptors that sense brighter light. Light information sent to bipolar cells forms three primary pathways. ON and OFF cone bipolar cells respond to the onset and offset of light signals from cones respectively, while rod bipolar cells respond to the onset of light signals from rods. ON and OFF cone bipolar cells synapse onto ON and OFF ganglion cells respectively, which are the output neurons of the retina. Rod bipolar cells are unique however in that they do not contact ganglion cells directly, instead they synapse onto the AII amacrine cell (McGuire et al., 1984; Strettoi et al., 1990).

Rod bipolar cells and AII amacrine cells are part of a specialized rod pathway which makes use of existing cone circuitry (Figure 1A). Light onset signals from rods are relayed from rod bipolar cell to AII amacrine cells, which have electrical connections with ON bipolar cells (Strettoi et al., 1992; Chun et al., 1993; Trexler et al., 2001; Deans et al., 2002). These electrical connections can also be bi-directional, to relay cone light information to the AII amacrine cell, but are more robust in the AII amacrine cell → ON bipolar cell direction (Trexler et al., 2001; Veruki and Hartveit, 2002; Trexler et al.,
2005; Beaudoin et al., 2008; Manookin et al., 2008; Munch et al., 2009). Light offset signals from rods are also relayed through the AII amacrine cell, which has anatomical connections with OFF bipolar cells and OFF ganglion cells (Strettoi et al., 1994; Grunert and Wässle, 1996; Haverkamp et al., 2003). As the AII amacrine cell releases glycine, it inhibits the OFF pathway at light onset and the removal of inhibition at light offset serves as an “Off” signal. OFF bipolar cells may also receive rod input through direct connections or rod-cone coupling providing alternate routes for light offset signals (DeVries and Baylor, 1995; Tsukamoto et al., 2001; Volgyi et al., 2004).

A recent paper (Arman and Sampath, 2012) suggested that the AII amacrine cell-OFF ganglion cell connection determines the response to very dim light stimuli, signaling rod threshold responses. However, OFF bipolar cells have a prominent localization of glycine receptors (Sassoe-Pognetto et al., 1994; Haverkamp et al., 2003) showing large spontaneous (Eggers and Lukasiewicz, 2006b; Ivanova et al., 2006) and light-evoked (Eggers et al., 2007) glycine receptor-mediated currents compared to rod bipolar cells. Although glycnergic inputs to OFF bipolar cells may not be crucial for determining the scotopic threshold of the retina (Arman and Sampath, 2012), there are clearly significant AII amacrine cell inputs to OFF bipolar cells that are likely mediating the significant glycnergic input that OFF bipolar cells receive at brighter rod and potentially cone light intensities, which have not previously been tested. Additionally OFF bipolar cells are divided into at least 4 main subtypes (Ghosh et al., 2004) and previous anatomical work has suggested that at least one subtype of OFF bipolar cell does not receive input from the AII amacrine cell (Tsukamoto et al., 2001). The strength and importance of this
connection therefore likely varies across OFF bipolar cell pathways, but this has not previously been determined physiologically.

OFF bipolar cell signaling to ganglion cells is also shaped by inhibition from other amacrine cells, which are roughly divided into narrow-field glycinergic and wide-field GABAergic groups (Pourcho and Goebel, 1983, 1985; Vaney, 1990; Menger et al., 1998). Amacrine cell inhibitory inputs combine their signals onto bipolar cells which has been shown to significantly modulate the output of bipolar cells (Eggers and Lukasiewicz, 2006a, b; Sagdullaev et al., 2006; Eggers et al., 2007; Eggers and Lukasiewicz, 2010). Since rod and cone pathways signal at different light intensities, applying a bright background light that causes rod saturation allows the two pathways to be investigated separately (Dacheux and Raviola, 1986; Xin and Bloomfield, 1999a). It is unknown how glycinergic input to OFF bipolar cells changes when switching between rod and cone pathways or if there are functional differences in glycinergic inputs to multiple OFF bipolar cell subtypes (Figure 1B) with rod vs. cone activation (Euler and Wässle, 1995; Ghosh et al., 2004; Pignatelli and Strettoi, 2004).

Here we investigated how inhibition to OFF bipolar cell subtypes changes with light adaptation. In order to elucidate how light adaptation is affecting the different OFF bipolar cell circuits, we examined the physiological connections that the rod pathway has with the different OFF bipolar cell subtypes. We found that one type of OFF bipolar cell is not contacted by the rod bipolar cell–AII amacrine cell while the other three types are. A decrease in AII amacrine cell activity, due to a decrease in rod pathway signaling with
light adaptation, might result in a decrease in inhibitory input to OFF bipolar cells.

Surprisingly, we found that a compensatory switch from glycine to GABA-mediated OFF bipolar cell inhibition occurs with light adaptation that may alter OFF bipolar cell spatial inhibition and sensitivity.

Methods:

Mouse retinal slice preparation

Animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). As described previously (Eggers and Lukasiewicz, 2006a; Eggers et al., 2013), male mice (C57BL/6J strain, Jackson Laboratories, Bar Harbor, ME, USA) 35-60 days of age were euthanized using carbon dioxide. Their eyes were enucleated and the cornea and lens removed. The eyecup was incubated for 20 min in cold extracellular solution (see Solution and Drugs) with 800 units/mL of hyaluronidase to dissolve the remaining vitreous humor. The hyaluronidase solution was then replaced with ice cold, oxygenated extracellular solution and the retina was dissected out of the eyecup. Following removal, the retina was trimmed down into one large flat rectangle by removing the peripheral retina and leaving only the central retina surrounding the optic disc. A nitrocellulose membrane filter paper (0.45 μm pore size, Millipore, Ireland) was placed on the retina section which was transferred to a hand chopper. An average of six 250 μm slices were cut, rotated 90°, and mounted onto glass cover slips using vacuum grease. Cells used from these slices were never more than 700 μm away from the center of the retina and only cells near the center of each slice were used for recordings. In this way, much of the differential input due to the dorsal-ventral cone opsin gradient reported
in mice was mitigated (Applebury et al., 2000; Haverkamp et al., 2005). The tissue was maintained in oxygenated extracellular solution at room temperature. All dissection procedures were performed under infrared illumination to preserve the light sensitivity of the preparations.

_Solutions and drugs_

The extracellular recording solution used for dissection and to examine light-evoked currents contained (in mM) 125 NaCl, 2.5 KCl, 1 MgCl2, 1.25 NaH2PO4, 2 CaCl2, 20 glucose, and 26 NaHCO3 and was bubbled with 95% O2-5% CO2. The intracellular solution contained (in mM) 120 CsOH, 120 gluconic acid, 1 MgCl2, 10 HEPES, 10 TEA-Cl, 10 phosphocreatine-Na2, 4 Mg-ATP, 0.5 Na-GTP, 10 EGTA and 50 μM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. To isolate the inhibitory receptor inputs, SR-95531 (SR, 20 μM) to block GABA_A receptors, (1,2,5,6-tetrahydropyridine-4yl) methyphosphinic acid (TPMPA, 50 μM) to block GABA_C receptors, and strychnine (1 μM) to block glycine receptors were used. Strychnine was washed on first, followed by TPMPA and finally SR-95531 to prevent serial inhibitory effects (Eggers and Lukasiewicz, 2006a, 2010). To block synapse-activated inhibition, CNQX (25 μM) was used to block AMPA and Kainate receptors and AP-5 (50 μM, Santa Cruz Biotechnology, Santa Cruz, CA) was used to block NMDA receptors. All drug solutions were washed on the slice for 5 min before recordings began using a gravity-driven superfusion system (Cell Microcontrols, Norfolk, VA) at a rate of ~1-2 mL/minute. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Whole-cell recordings

Whole cell patch clamp recordings, sampled at 10 kHz, were made from bipolar cells and amacrine cells from retinal slices. Light-evoked inhibitory post synaptic currents (L-IPSCs) and spontaneous (s)IPSCs were recorded from retinal bipolar cells voltage clamped to 0 mV, the reversal potential of nonselective cation channel currents. Bipolar cell recordings were stable and no rundown of the light response was observed over the recording period. Light-evoked excitatory post synaptic currents (L-EPSCs) and sEPSCs were recorded from amacrine cells voltage clamped to -60 mV, the reversal potential for chloride channel-mediated currents. Liquid junction potentials of 20 mV were corrected at the beginning of each recording. Electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) on a P97 Flaming/Brown puller (Sutter Instruments, Novato, California, USA) and had resistances of 5-7 MΩ. Mice were dark-adapted overnight, and all recording procedures were performed in the dark under infrared illumination to preserve the light sensitivity of the slices. Recordings were made in extracellular solution heated to 32°C, using thin stage and inline heaters (Cell Microcontrols, Norfolk, VA). Responses were filtered at 6 kHz with the four-pole Bessel filter on a Multi-clamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, California, USA) and digitized with a Digidata 1140 data acquisition system (Molecular Devices, Sunnyvale, California, USA).
Morphological identification of cell subtypes

Alexa 488 included in the recording pipette was used to label OFF bipolar cell and amacrine cell subtypes. They were classified as either type 1/2, 3, 4 OFF bipolar cells or AII amacrine cells based on their axonal morphologies and stratification within the inner plexiform layers and the position of their somas in the inner nuclear layer (Ghosh et al., 2004). Type 1 and 2 bipolar cells were difficult to distinguish based on morphology, so their results were pooled. Additionally, although subtypes of the OFF type 3 bipolar cell have been described, (Mataruga et al., 2007; Wassle et al., 2009) their morphologies were quite similar so all OFF type 3 cells were pooled. The cells were imaged with Nikon Digital Sight camera with Elements software using a Nikon Intensilight C-HGF1E Fluorescent lamp (Nikon Instruments, Tokyo, Japan). Photoshop (Adobe, Seattle, WA) and Elements software were used to measure and trace the cells. Detailed analysis of axon terminal morphology was performed on a subset of all recorded OFF bipolar cells (n=24) to determine anatomical differences between OFF bipolar cell types, and these criteria were subsequently used to identify OFF bipolar cell. Cell tracings are provided for easy quantification as well as visualization since the fluorescent images of OFF bipolar cell terminal morphologies were often at multiple depths vertically in the slice preparation.

Light stimuli

Full-field light stimuli were evoked using a light-emitting diode (LED, Agilent HLMP-3950, λpeak = 525 nm, Palo Alto, CA) projected through the camera port of the microscope, which elicited a strong response in both dark- and light-adapted conditions.
We focused our recordings on cells located within the regions of mixed green/UV cone opsin input (Applebury et al., 2000; Haverkamp et al., 2005) where there was little difference between bipolar cell or ganglion cell responses to green light (Breuninger et al., 2011; Wang et al., 2011). Thus, 525 nm light should stimulate both rods (peak sensitivity at 500 nm) and cones robustly. The stimulus intensity (max of $9.5 \times 10^5$ photons/$\mu m^2$/sec), background rod-saturating light (950 photons/$\mu m^2$/sec) and duration (30 ms) were controlled with Clampex software by varying the current through the LED. The background intensity was chosen as it was shown to maximally activate rods (Wang and Kefalov, 2009). A long light stimulus (500 ms) was used to determine the type of inhibition to all recorded BCs (ON or OFF) as well as the type of excitation of the amacrine cell. A rod saturating background light was applied for 5 min to light-adapt the retina slice and was sustained throughout all light-adapted recordings.

**Data analysis and statistics**

L-IPSC and L-EPSC traces from a given response condition were averaged using Clampfit software (Molecular Devices, Sunnyvale, California, USA) and the charge transfer (Q), peak amplitude and decay to 37% of the peak (D37) were measured in each condition. Due to the significant amount of spontaneous activity, it was difficult to measure a peak from OFF bipolar cell L-IPSCs. Therefore to estimate the peak and D37, average traces were decimated (100 fold) and each point was replaced with the average of those data points to limit variations due to spontaneous activity. To determine changes in total current, the Q was measured, which represents the magnitude of the response. Q was measured in Clampfit over the length of the response, typically 1-2 seconds, using the
same time parameters in each condition for the same cell. All example response traces show responses to the max light flash intensity of $9.5 \times 10^5$ photons/µm²/sec. For intensity response curves, light-evoked responses were normalized to the maximal response in the dark-adapted condition. The normalized data were plotted against the log₁₀ of the stimulus intensity.

Spontaneous inhibitory (sIPSC) and excitatory (sEPSC) post-synaptic current data were analyzed using Clampfit software. A sIPSC or sEPSC template was calculated for each data file, using the average of about 10 prototypical events from the recording. The software used this template to automatically detect spontaneous events which were manually accepted or rejected based on strict criteria: events used to calculate the frequency were rejected if they appeared to be noise and events used to calculate the average peak amplitude were rejected if they appeared to be noise or consisted of two or more overlapping events. Frequency was calculated by dividing the number of events by the recording time. Peak amplitude and inter-event interval histogram distributions were normalized to the number of events. The signal-noise-ratio (SNR), which gives a measurement of the actual response signaled over the background noise, was calculated by dividing peak amplitude of the L-IPSC by the variance of the baseline.

Student’s $t$-test (2-tailed, paired) was used to compare response characteristics before and after drug application or light adaptation. Standard student’s $t$-test (2-tailed, unequal variance) was used to compare values between different cells. Differences were considered significant when $p < 0.05$ ($*,\dagger,\#$) and $p < 0.01$ (**,††). All averaged data are
reported as means ± SEM. The distributions of sIPSC and sEPSC amplitude and inter-event interval values were compared using the Kolmogorov-Smirnov test (K-S) with significance being \( p < 0.05 \).

**Results:**

*OFF Bipolar cell subtypes receive different sources of inhibition in the dark*

Previous studies have shown that OFF bipolar cells receive glycine, GABA\(_A\), and GABA\(_C\)-R-mediated inhibition (Euler and Wässle, 1998; Ivanova et al., 2006; Eggers et al., 2007), that likely includes both rod pathway connections from rod bipolar cell-AII amacrine cell inputs and cone pathway amacrine cell input driven by ON and OFF cone bipolar cells (Figure 3A). However, these previous studies did not determine if OFF bipolar cells receive rod bipolar cell-AII amacrine cell mediated inputs or if the light-evoked inhibitory inputs vary between OFF bipolar cell subtype. To resolve this, we identified 3 OFF bipolar cell subtypes by the stratification of their axon terminals within the inner plexiform layer (IPL) (Ghosh et al., 2004). We quantified the percentage of the IPL in which OFF bipolar cell axon terminals ramify (0% = outer nuclear layer/IPL border) and found that type 4 OFF bipolar cells have terminals that ramify in 1±1-38±8\% (n = 4) of the IPL, type 3 OFF bipolar cells have terminals that cover 17±2-50±4\% (n = 11) of the IPL and both type 1 and type 2 OFF bipolar cells have terminals that cover 1±1-16±4\% of the IPL (n = 9) (Figure 3B). Because of their similar morphology, type 1 and type 2 OFF bipolar cells were difficult to differentiate and their data were pooled together in these measurements.
It is not known if all of these OFF bipolar cell subtypes receive input from the rod-rod bipolar cell-AII amacrine cell circuit. A previous study suggested that one type of OFF bipolar cell (likely type 4) had significant anatomical contacts from AII amacrine cells, while another OFF bipolar cell (likely type 3) did not (Tsukamoto et al., 2001). To test for physiological differences in potential AII amacrine cell inputs, we recorded L-IPSCs from dark-adapted retinas, where rod pathways are active, in response to long light stimuli to categorize the inhibition as ON, OFF or ON/OFF. If an OFF bipolar cell only receives inhibition at the offset of light, then it could not be receiving rod bipolar cell-AII amacrine cell mediated inhibition that is activated at light onset. However, if an OFF bipolar cell received ON inhibition, then it may be due in part to the rod pathway and coming from the AII amacrine cell. We found that the responses of the OFF bipolar cell subtypes to long light stimuli differ. OFF bipolar cell types 1, 2, and 4 (OFF1,2,4) have an ON inhibitory response while OFF bipolar cell type 3 (OFF3) has an OFF inhibitory response (Figure 3C). This suggests that OFF3 bipolar cells do not receive rod pathway input from AII amacrine cells. To quantify this, we measured the charge transfer (Q) 500 ms before, during, and after the long light stimulus. OFF1,2,4 bipolar cells had a significantly higher Q only during the light stimulus (normalized to the baseline Q, Figure 3D). OFF3 bipolar cells had a significantly higher normalized Q only after light offset (Figure 3D). OFF1,2,4 responses during light offset and OFF3 responses during light onset were not different than the baseline (p > 0.05). We found no significant difference between the light responses of OFF bipolar cell types 1 and 2 and OFF bipolar cell type 4, so these data have been pooled as OFF bipolar cell types that all receive potential input from AII amacrine cells.
To confirm our results on connections between AII amacrine cells and OFF bipolar cells, we used the fact that AII amacrine cells are activated both by rod pathway activation and by ON cone bipolar cell activation through gap junction connections. Since the ON bipolar cell-AII activation requires only metabotropic glutamate receptors, we recorded OFF bipolar cell L-IPSCs in light-adapted conditions with CNQX and AP-5 to block AMPA/Kainate and NMDA receptors. Blocking these receptors blocks both OFF bipolar cell activation as well as all chemical synapse-activated inhibition so that only ON bipolar cell pathways are active. We found that OFF3 bipolar cells are not contacted by the AII amacrine cells as their inhibition is abolished in light-adapted conditions with CNQX and AP-5 (Fig 3E,F, n = 5). In contrast, OFF1,2,4 bipolar cells had significant inhibition remaining after blocking all ionotropic glutamate receptors (Fig 3E,F, 42±19% of total inhibition at the maximum light intensity), suggesting significant contribution from ON bipolar cell-activated AII amacrine cells (n = 4), similar to the input from AII amacrine cells to OFF ganglion cells shown previously (Manookin et al., 2008; Murphy and Rieke, 2008; Munch et al., 2009). This inhibition was blocked by strychnine (n = 2, data not shown), showing that it was coming from glycinergic amacrine cells, presumably the AII. From these results we can confirm that only OFF1,2,4 bipolar cells are contacted by an ON pathway mediated by the rod-AII amacrine cell. The remaining inhibition in OFF1,2,4 bipolar cells also suggests that cones are being robustly stimulated with a 525 nm LED in a light-adapted condition.

As seen in the examples in Figure 3C and 3D, the sIPSCs from OFF 1,2,4 and OFF3 bipolar cells also appear to have different amplitudes and frequency, with OFF 1,2,4
bipolar cells receiving much more spontaneous input. If inhibitory input to the OFF1,2,4 and OFF3 bipolar cells is coming from different sources we would expect that the spontaneous activity might also be different, as spontaneous activity varies between neurons. We measured sIPSCs in the absence of light stimuli from dark-adapted retinas. The sIPSC peak amplitude was significantly lower in OFF3 bipolar cells (16±1 pA, n = 11) than in OFF1,2,4 bipolar cells (38±7 pA, n = 13) (p < 0.01). Additionally, the sIPSC frequency was also significantly lower in OFF3 bipolar cells (7±3 Hz, n = 12) than OFF1,2,4 bipolar cells (25±6 Hz, n = 15) (p < 0.05). The lower amplitude and frequency of sIPSCs suggests that OFF3 and OFF1,2,4 bipolar cell are receiving inputs from distinct sources.

**Dark-adapted L-IPSCs are mostly glycinergic in OFF1,2,4 but not OFF3 bipolar cells**

The differences in OFF1,2,4 and OFF3 bipolar cell L-IPSCs also suggest that they are receiving distinct inhibitory inputs from different sources. The majority of the dark-adapted inhibition to OFF 1,2,4 bipolar cells likely comes from the rod bipolar cell-AII amacrine cell pathway, leading to the large glycinergic light-evoked currents previously observed by Eggers et al. (2007), since the rod pathway should be the most activated in the dark-adapted state. However, our results from Figure 3 suggest that the OFF3 bipolar cells are not receiving AII input and thus may not have large glycinergic inputs. To test this, we recorded L-IPSCs and sIPSCs from dark-adapted OFF bipolar cells in control conditions and with application of strychnine, a glycine receptor antagonist, to separate glycinergic and GABAergic contributions (Eggers et al., 2007). There was a significant decrease in OFF1,2,4 bipolar cell L-IPSCs (n = 6) with application of strychnine (Figure
4 A,C) at all light intensities used (p < 0.01). In contrast, OFF3 bipolar cells (n = 4) only had significant decreases in L-IPSCs at the brighter light intensities (Figure 4 B,D), while at the two dimmest light intensities, glycine receptor blockade produced no significant change. In dark-adapted conditions, OFF1,2,4 L-IPSCs were dominated by glycine (80±5% of the total inhibition at the maximum light intensity) in contrast to OFF3 bipolar cell responses (51±4%, p < 0.05). These results suggest that in the dark, the main inhibition to OFF1,2,4 bipolar cells is glycinergic mainly mediated by the rod bipolar cell-AII amacrine cell pathway while only half of the inhibition to OFF3 bipolar cells was due to glycine input from OFF cone activated amacrine cells. Since glycinergic and GABAergic inhibition have distinct time courses (Eggers and Lukasiewicz, 2006b; Eggers et al., 2007), this could contribute to further differences between signaling of OFF bipolar cell subtypes.

Dark-adapted sIPSCs are primarily glycinergic in both OFF1,2,4 and OFF3 bipolar cells

As may be seen in Figures 3 and 4, OFF bipolar cells have a significant amount of background spontaneous activity, which reflect their inputs and may affect signal transmission of light-evoked responses. Although the light-evoked inputs to the classes of OFF bipolar cells differ, sIPSCs for both OFF1,2,4 and OFF3 bipolar cells in the dark were mostly due to glycine receptor activation. Strychnine abolished sIPSCs in half of the OFF1,2,4 and a quarter of the OFF3 bipolar cells. Of the spontaneous events that remained, there was a significant reduction in the sIPSC peak amplitude for OFF1,2,4 (K-S p < 0.01, n = 3) and OFF3 bipolar cells (K-S p < 0.01, n = 3) (Figure 5 A,B,C,D) but no
change in the decay $\tau$ of the sIPSCs in either group (OFF1,2,4, $n = 3$, $p = 0.88$ or OFF3 bipolar cells, $n = 3$, $p = 0.79$, data not shown). Although small differences in the decay of GABA$_A$ and glycine receptor-mediated sIPSCs have previously been shown (Eggers et al., 2007), the lack of a significant change in the kinetics of the sIPSCs that remain after strychnine, presumably due to GABA$_A$ receptor input (Eggers et al., 2007), could be due to the very small sample of sIPSCs that remained in only a handful of cells. There was also a significant decrease in sIPSC frequency with strychnine application for both OFF1,2,4 ($n = 6$) and OFF3 ($n = 4$) bipolar cells ($p < 0.01$). These results suggest that in the dark, most of the tonic spontaneous inhibition to OFF bipolar cells is due to glycinergic mediated currents likely coming from both the AII amacrine cell and other ON amacrine cells for OFF1,2,4 bipolar cells and only OFF glycinergic amacrine cells for OFF3 bipolar cells.

**Light adaptation alters the timing and charge transfer of AII amacrine cell L-EPSCs**

We have found that dark-adapted inhibition to OFF1,2,4 and OFF3 bipolar cells varies, likely due to AII amacrine cell versus non-AII amacrine cell inputs. However, since OFF bipolar cells function in both rod and cone dominant conditions, switching from rod to cone pathways could modulate inhibition to the OFF bipolar cell subtypes differently. Light adaptation has been used to separate the rod and cone pathways by applying a maximally rod activating background light to the retina (Xin and Bloomfield, 1999a). If the rod pathway is saturated via light adaptation, then the amount of excitation the rod bipolar cell gives to the AII amacrine cell should decrease. However, AII amacrine cells can also be directly activated in cone-dominant conditions by ON bipolar cells through
electrical connections (Strettoi et al., 1994; Trexler et al., 2001; Volgyi et al., 2004). To confirm that activation of AII amacrine cells declines when switching from rod to cone activation with our light stimulus, we recorded light-evoked excitatory post synaptic currents (L-EPSCs) from AII amacrine cells in dark- and light-adapted (maximal rod pathway activation of 950 photons/μm²) (Wang and Kefalov, 2009) conditions.

AII amacrine cells were identified prior to recording by their characteristic soma shape and large proximal dendrite and labeled with a fluorescent dye, Alexa 488, during the recording to quantify their morphology after the recording. We found that AII amacrine cell processes covered between 10±2 and 97±4% of the IPL (n = 6) (Figure 6B). With light adaptation, there was a significant decrease in the Q of the AII L-EPSCs at each light intensity (n = 9, Figure 6A,C). In addition, there was a significant decrease in the normalized peak amplitude (to 43±7%) and D37 (to 69±8%) of the L-EPSC (Figure 4D). The remaining excitatory input is most likely due to cone activation via electrical connections of the AII amacrine cell and ON bipolar cells which is smaller than the high gain rod bipolar cell-AII amacrine cell synapse (Xin and Bloomfield, 1999a; Pang et al., 2004; Volgyi et al., 2004). Although previous studies show that AII amacrine cells do function during light-adapted cone dominant conditions by receiving rod input via rod-cone coupling and ON bipolar cell-AII amacrine cell electrical coupling (Manookin et al., 2008; Munch et al., 2009; Demb and Singer, 2012), our results, in combination with Figure 3E,F and other studies, show that this drive is less robust than AII amacrine cell activation in the dark. AII amacrine cell sEPSCs were also recorded under both dark- and light-adapted conditions. With light adaptation, the sEPSC peak amplitude significantly
decreased from 22±3 pA to 14.5±2 pA (n = 8) (Figure 6E,F,G). In addition, the normalized sEPSC frequency significantly decreased to 45±10% of control with light adaptation (n = 9) and the inter-event interval significantly increased (487% of dark-adapted inter-event interval average) (Figure 6H). These data suggest that AII amacrine cell inhibition to OFF bipolar cells decreases with our light adapting stimulus.

*The magnitudes of OFF bipolar cell L-IPSCs are unaffected by switching from rod to cone circuits*

We have shown that in the dark, OFF1,2,4 bipolar cells receive mostly glycinergic input, likely from the AII amacrine cells, while only about half of OFF3 bipolar cell L-IPSCs consist of glycine receptor-mediated currents from other amacrine cell types. However, it is unknown how inhibition to either OFF1,2,4 or OFF3 bipolar cells will change when the cone pathways are dominant. To determine if there are changes in inhibition to OFF bipolar cells with light adaptation, L-IPSCs were recorded from OFF1,2,4 and OFF3 bipolar cells under a rod-adapting background light. If OFF1,2,4 bipolar cells are receiving primarily rod-mediated inhibition we would expect there to be a significant reduction in L-IPSCs after light adaptation, while we might not expect OFF3 bipolar cells L-IPSCs to significantly change since they are receiving cone pathway inhibition. Surprisingly, under these conditions, there was no significant difference in the Q in either OFF1,2,4 (n = 13, p > 0.05) or OFF3 (n = 12, p > 0.05) bipolar cells with light adaptation at any stimulus intensity (Figure 7A,B,C). The absence of any change in the Q of the L-IPSC in OFF1,2,4 bipolar cells suggests cone-pathway mediated inhibition is
compensating for the reduction in rod-pathway mediated inhibition from the AII amacrine cell.

A change in the source of inhibition to OFF1,2,4 bipolar cells from AII amacrine cells to cone pathway amacrine cells was also suggested by the significant decrease in the normalized peak amplitude (63±9%, p < 0.01) and a significant increase in the normalized D37 (193±30%, p < 0.05) (Figure 7D). In dark-adapted conditions there was no significant difference in the D37 between the OFF1,2,4 bipolar cell L-IPSCs and the AII amacrine cell L-EPSCs (AII D37 of 147±40 ms, OFF1,2,4 bipolar cell D37 of 142±45 ms, p > 0.05), while in the light they significantly differed (AII D37 of 104±29 ms, OFF1,2,4 bipolar cell D37 of 195±34 ms, p = 0.05), further suggesting different sources of input. There was no difference in the time-to-peak between dark- and light-adapted conditions for either OFF bipolar cell group (p > 0.05, data not shown). In contrast, the lack of change in the Q or timing parameters (D37 107±27, p=0.8) of OFF3 bipolar cells agrees with our previous conclusion that their inhibition comes from cone pathways.

Additionally, there were differences in L-IPSC timing parameters between OFF1,2,4 and OFF3 bipolar cells in the dark and light. There was a significant difference in the time-to-peak of L-IPSCs between OFF1,2,4 and OFF3 bipolar cells in the dark (OFF1,2,4 average of 125±24 ms, OFF3 average of 213±23 ms, p < 0.05) and in the light (OFF1,2,4 average of 124±33 ms, OFF3 average of 222±21 ms, p < 0.05). It makes sense for OFF3 bipolar cells to have longer response delays as they begin to receive inhibitory input only
when the light turns off. Lastly, the L-IPSC D37 of OFF1,2,4 bipolar cells was significantly longer than OFF3 bipolar cells only in the light (OFF1,2,4 average of 195±38 ms, OFF3 average of 90±16 ms, p < 0.05). The timing differences of L-IPSCs between the two groups of bipolar cells highlight their different circuitry connections and pathways.

Light adaptation differentially modulates OFF1,2,4 and OFF3 bipolar cell sIPSCs

sIPSCs in OFF bipolar cells form the background inhibition they are receiving from all amacrine cell inputs with no external stimulus involved. Thus, any modulation of the sIPSCs would change the baseline level of inhibition the cells are receiving and in turn, alter their glutamate release onto ganglion cells. In contrast to the L-IPSCs, the sIPSCs of OFF1,2,4 and OFF3 bipolar cells were differentially changed with a switch to cone pathways under light-adapted conditions. The sIPSC peak amplitude of OFF1,2,4 bipolar cells decreased significantly (38±9 to 21±4 pA, K-S p < 0.01, Figure 8A,C,E). This is consistent with the idea that a majority of these glycinergic sIPSCs are coming from the rod bipolar cell-AII amacrine cell mediated pathway, as we show in Figure 6 that spontaneous AII amacrine cell activity decreases significantly with light. There was no change in the sIPSC peak amplitude in OFF3 bipolar cells (13.58±1.54 to 15±3 pA, K-S p > 0.05, Figure 8B,D,F) further suggesting that the sIPSCs of OFF3 bipolar cells are coming from cone-dependent OFF pathways. The decay τ of the sIPSCs did not significantly change with light adaptation in OFF1,2,4 (n = 8, p = 0.68) or OFF3 bipolar cells (n = 7, p = 0.93) (data not shown). However, the sIPSC frequency normalized to the dark-adapted condition significantly decreased in the light in both OFF1,2,4 (to 69±11%,
n = 10, p < 0.05) and OFF3 bipolar cells (to 51±10%, n = 9, p < 0.01). As a result, the inter-event intervals of the sIPSCs also significantly increased in both OFF bipolar cell groups (119% of dark-adapted inter-event interval average in OFF1,2,4 bipolar cells and 166% of dark-adapted inter-event interval average in OFF3 bipolar cells) (Figure 8G,H). Taken together, these results suggest that with light adaptation, OFF1,2,4 bipolar cells are losing a main source of spontaneous inhibition (rod bipolar cell-AII amacrine cell pathway) due to a decrease in both peak amplitude and frequency whereas OFF3 bipolar cells may just be receiving fewer overall sIPSCs from the same amacrine cells.

Changes in the signal-to-noise ratio suggest distinct sources of input
Changes in the background activity of OFF bipolar cell inhibition with light adaptation suggested that the strength of signal over background noise might also be changing. This can be estimated by calculating the signal-to-noise ratio (SNR) of a response. The SNR was calculated by dividing the peak amplitude of the L-IPSC by the variance of 100 ms of baseline, which accurately represented baseline variation for the given cell. Given that OFF1,2,4 bipolar cells have significantly more inhibitory spontaneous activity than OFF3 bipolar cells, we predicted that the SNR for their responses might be different as well, which would affect the efficiency of OFF bipolar cell signal transmission to OFF ganglion cells. For this reason, we calculated the SNR of OFF1,2,4 (n = 13) and OFF3 bipolar cells (n = 11) in both dark- and light-adapted conditions to determine if there was a change in the relationship between the L-IPSC and the baseline sIPSCs. The SNR significantly increased for both OFF1,2,4 and OFF3 bipolar cells with light adaptation (p < 0.05) suggesting that the decrease in the sIPSCs helps to magnify the L-IPSC (Figure 9
In the light, when the cone pathways are active, the light-evoked inhibitory input to OFF bipolar cells is larger relative to the dark-adapted state as a result of the decrease in background noise. However, the SNR was significantly larger in OFF3 bipolar cells in both dark- and light-adapted conditions (Figure 9C). This is most likely a result of the significantly fewer sIPSCs in OFF3 bipolar cells (Figure 3). Our results further support that OFF1,2,4 and OFF3 bipolar cells are receiving inhibitory input from distinct sources, and suggest that in general, cone-mediated inputs have a higher signal-to-noise ratio than rod-mediated inputs. A previous study (Arman and Sampath, 2012) showed that glycinergic inputs do not significantly modulate the SNR (calculated using the unequal variance model of $d'$ or $d_0$), of very dim light voltage responses in OFF bipolar cells. Our results however suggest that at brighter rod and cone light intensities this may be an important mechanism for determining the gain of inhibition to OFF bipolar cells, possibly allowing for larger light-evoked modulation of the OFF bipolar cell excitatory signal.

Dark-adapted AII amacrine cells receive a large amount of spontaneous activity (Figure 6), which sets the background for light-evoked signals. Thus, any changes in the sEPSCs could change the light signal to postsynaptic OFF bipolar cells. As a result of the decrease in the peak amplitude and frequency of spontaneous activity, the SNR of the AII amacrine cell did not change ($n = 9$, $p = 0.18$, Figure 9D). The increase in SNR of OFF bipolar cells with light adaptation, contrasted to the unchanged SNR of the AII amacrine cell suggests that OFF1,2,4 bipolar cells that receive primarily AII amacrine cell input in the dark must be getting input from other sources in the light. The smaller AII amacrine cell signal, presumably due to both less quantal release from rod bipolar cells and rod
saturation, could not be providing the drive for the increase of the OFF bipolar cell signal relative to spontaneous noise. Taken together, these findings indicate that light adaptation increases the SNR, allowing for more sensitive higher visual responses in the light-adapted condition.

*Increased GABAergic input provides compensatory light-adapted inhibition to OFF bipolar cells*

In dark-adapted conditions, the L-IPSCs and sIPSCs in OFF1,2,4 bipolar cells were due mostly to glycinergic input, presumably from AII amacrine cells. However, because there was no change in the L-IPSCs of OFF1,2,4 bipolar cells with light adaptation, while the AII amacrine cell activation significantly decreases, it is necessary to determine what input is mediating the compensatory inhibition. To investigate the inhibition OFF bipolar cells are receiving in the light, strychnine was applied to the preparation to block glycine receptors followed by TPMPA and finally SR-95531 to block GABA_C and GABA_A receptors respectively. In OFF1,2,4 bipolar cells, strychnine significantly decreased the L-IPSCs at all light intensities (n = 11) and application of SR-95531 and TPMPA abolished all light-evoked responses (n = 5) (Figure 10A,C). There was no significant reduction in L-IPSCs after TPMPA application (data not shown, 37±7% after strychnine to 50±25% after TPMPA of the total initial Q, p > 0.05) but the additional application of SR-95531 abolished the light responses in all cells tested (n = 4). This suggests that light-adapted OFF bipolar cells, like dark-adapted OFF bipolar cells (Eggers et al., 2007), receive very little GABA_C receptor-mediated input. The OFF1,2,4 bipolar cell L-IPSCs switched to significantly more GABA input mediated by GABA_A receptors (43±10%
glycine, 57±10% GABA, p < 0.05) when going from dark- to light-adapted conditions (Figure 10E). The decrease in glycinergic input to OFF1,2,4 bipolar cells (37±11%) with light adaptation correlates with the decrease in excitatory input to AII amacrine cells (54±10%) in the light, suggesting the majority of the glycinergic input comes from this connection (Welch’s t-test, p = 0.08).

Although OFF3 bipolar cells receive no input from AII amacrine cells and have much less glycinergic input in the dark than the OFF1,2,4 bipolar cells (Figure 10E, p < 0.05), the rod pathway input eventually is distributed across at least some cone bipolar cell pathways via electrical synapses. This could mean that the activation of the OFF cone bipolar cells and OFF amacrine cells that send input to the OFF3 bipolar cells could vary between rod and cone dominant conditions. In OFF3 bipolar cells, strychnine had no significant effects on the light-adapted L-IPSCs (n = 8) and application of SR95531 and TPMPA abolished all light-evoked responses (n = 5) (Figure 10B,D). Similar to OFF1,2,4 bipolar cells, TPMPA application had no effect (data not shown, 93±14% after strychnine to 79±19% after TPMPA of the total initial Q, p > 0.05) but the additional application of SR-95531 virtually eliminated the light responses in all cells tested (n = 4, 12±4% after SR-95531, p < 0.05). The OFF3 bipolar cell L-IPSCs switched to solely GABA\textsubscript{A} receptor mediated input (p < 0.05) in the light. There was a significantly smaller percentage of light-evoked glycine receptor input to OFF3 than OFF1,2,4 bipolar cells in both dark- and light-adapted conditions (Figure 10E, p < 0.05), likely due to remaining AII amacrine cell inputs to OFF 1,2,4 bipolar cells in the light. These results suggest cone pathway activation significantly alters the proportions of inhibition to switch in favor of
GABAergic input to OFF1,2,4 and OFF3 bipolar cells, effectively altering the type and source of inhibition that the OFF bipolar cells receive.

Unlike the L-IPSCs, the sIPSCs in light-adapted conditions were mostly mediated by glycinergic input for both OFF1,2,4 and OFF3 bipolar cells (data not shown). For most cells, application of strychnine eliminated all sIPSCs, causing a significant decrease in frequency in OFF1,2,4 (to 20±16% of total frequency, n = 8) and OFF3 (to 21±16% of total frequency, n = 6) bipolar cells (p < 0.01). For the cells where some sIPSCs remained, there was no significant difference in the sIPSC peak amplitude of OFF1,2,4 (n = 3, p = 0.25) and OFF3 (n = 2, p = 0.54) bipolar cells. The decay τ of the sIPSCs did not significantly change in the light when glycine receptors were blocked in OFF1,2,4 (n = 3, p = 0.14) or OFF3 (n = 3, p = 0.14) bipolar cells. Despite the switch of the L-IPSCs to larger GABAergic input, the sIPSCs of both OFF1,2,4 and OFF3 bipolar cells in the light are still mediated by glycine release, suggesting that glycinergic amacrine cells high basal rate of neurotransmitter release plays a role of setting background inhibition to OFF bipolar cells.

Discussion:

In natural visual situations, light levels may activate both rods and cones simultaneously and changes in background light levels may emphasize rod or cone activation. Several studies have analyzed retinal signaling across different background light levels and have shown that gain and noise in the rod pathway changes in turn (Dunn et al., 2006; Dunn and Rieke, 2008). Here we show that the ambient light state modulates the source and
noise level of OFF bipolar cell inhibition when switching between rod and cone pathways. When a rod-saturating background was applied, glycinergic input to OFF bipolar cells was reduced, but this reduction was compensated by an increase in GABAergic input. In the dark, OFF1,2,4 bipolar cells are receiving substantial glycinergic input from the rod-AII amacrine cell pathway while in the light they receive equal amounts of glycine and GABAergic input from ON cone pathways (Figure 11A,C). In contrast, OFF 3 bipolar cells, which do not receive input from the rod pathway AII amacrine cell, receive much more GABAergic input in both the dark- and light-adapted conditions from OFF cone pathways (Figure 11B,D). Although the total amount of inhibition to OFF bipolar cells did not change, the SNR increased with light adaptation due to a decrease in spontaneous baseline inhibition to OFF bipolar cells and was different between the rod and cone activated pathways. Collectively, these suggest a specialization among OFF bipolar cell pathways for light information from the rods and cones.

*All amacrine cells target specific OFF bipolar cell pathways*

The AII amacrine cell-OFF bipolar cell synapses are prominent morphologically (McGuire et al., 1984; Strettoi et al., 1990), but had not been directly investigated physiologically. A previous report concluded that AII amacrine cell-OFF ganglion cell connections, but not AII amacrine cell-OFF bipolar cell connections, signal the threshold of rod responses (Arman and Sampath, 2012) and did not find large glycinergic inputs in response to the dim light intensities tested or changes in the SNR when blocking glycinergic inputs. However, our data show that when using brighter rod light stimuli,
OFF bipolar cells receive robust AII amacrine cell input, that is targeted to 3 subtypes (OFF 1,2,4) of OFF bipolar cells (Figure 3). This supports a previous morphological study that showed OFF4 bipolar cells, but not OFF3 bipolar cells, received AII amacrine cell inputs (Tsukamoto et al., 2001). The potential differences in sensitivity between OFF bipolar cells and OFF ganglion cells to AII input may be due to differential synapse structure between the AII and OFF bipolar cells and OFF ganglion cells.

*Increased GABAergic inhibition with light adaptation may modulate the spatial sensitivity of the OFF pathway*

We show that decreases in glycinergic OFF bipolar cell inhibition are compensated by increases in GABAergic input under light-adapted conditions. Glycinergic amacrine cells such as the AII amacrine cell are narrow-field cells and thus may not mediate wide spatial inhibition, whereas GABAergic amacrine cell processes span long lengths of the retina (Pourcho and Goebel, 1983, 1985; Vaney, 1990; Menger et al., 1998). Although, in the dark AII amacrine cells are connected via gap junctions that increase their spatial range to about 200-400 µm in very dim light, they are uncoupled with light adaptation and their spatial spread is much lower than potentially coupled wide-field GABAergic amacrine cells (1000-4000 µm) (Bloomfield et al., 1997; Xin and Bloomfield, 1997, 1999a). Thus the switch from glycinergic to GABAergic inhibition has the potential to widen the spatial inhibitory surrounds of OFF bipolar cells which may change their spatial sensitivity.
Many previous studies have shown that ganglion cell spatial sensitivity increases when moving from dim to bright light conditions (Kuffler, 1953; Barlow et al., 1957; Maffei et al., 1971; Troy et al., 1999), which has been suggested to be due to changes in inner retinal circuitry (Dedek et al., 2008). Our findings suggest that changing inhibition to bipolar cells may be one signaling mechanism to downstream ganglion cells to enable more differentiation between spatially distinct light signals. It may be more important to have narrow-field inhibition in the dark where there is no need for high spatial sensitivity, as shown by the large convergence of rod signals (Vaney, 1991). In light-adapted conditions, cone vision mediates the highest spatial acuity in order to differentiate novel light signals from the background luminance. This is formed from GABAergic input modulating center-surround organization which is an important feature for ganglion cell spatial tuning (Cook and McReynolds, 1998; Flores-Herr et al., 2001; Volgyi et al., 2002; O'Brien et al., 2003). Increasing GABAergic inhibition to OFF bipolar cells could be one way of adjusting the inhibitory surrounds of bipolar cells to affect the spatial center signals that ganglion cells receive.

One potential mechanism for modulation of OFF bipolar cell inhibition is the increase in dopamine levels in the light (Doyle et al., 2002). Dopamine was found to potentiate GABAAR currents possibly through phosphorylation by the cAMP-PKA signaling cascade (Feigenspan and Bormann, 1994a). This mechanism could cause circuitry changes such that cone activated GABAergic amacrine cells are potentiated in the light while glycinergic amacrine cell outputs are decreased. A logical progression of this idea is that the increase in GABAergic inhibition could be partly inhibiting AII amacrine cell
glycinergic release in addition to inhibiting OFF bipolar cells. However, several studies have shown that AII amacrine cells in the light receive primarily glycine-mediated inhibition and not GABAergic inhibition (Gill et al., 2006; Weiss et al., 2008). Thus it is likely that inhibition of the AII amacrine cell is not the direct cause of the inhibitory switch and dopamine may be acting through other means. Additionally, the increase in the inhibitory SNR could modulate the relative output of OFF bipolar cells to filter out unneeded background light information. Further examination is necessary to determine what role the increase in the SNR plays in OFF bipolar cell signaling and whether there is a change in the spatial signaling to these cells. This would be an interesting future expansion of the present study.

**OFF bipolar cell subtypes are receiving and sending different types of signals**

In this study we have investigated the potential role of the primary rod pathway which transfers dim light information through the rod bipolar cell-AII amacrine cell circuit to OFF bipolar cells (McGuire et al., 1984; Strettoi et al., 1990; Chun et al., 1993; Sassoe-Pognetto et al., 1994; Trexler et al., 2001; Deans et al., 2002). However, there are two other rod pathways: the secondary pathway, activated by moderate light which signals OFF bipolar cells through electrical connections between rods and cones (DeVries and Baylor, 1995; Volgyi et al., 2004) and the tertiary pathway where brighter light causes direct rod activation of OFF bipolar cells (Tsukamoto et al., 2001; Volgyi et al., 2004). Since there are multiple OFF bipolar cell pathways that convey distinct portions of the light signal, it is possible that the inputs from these three rod pathways will vary among OFF bipolar cell types. Our conclusions in this study support previous anatomical work
showing that OFF3 bipolar cells do not have AII amacrine cell connections (Tsukamoto et al., 2001). Several studies have shown that OFF3-like bipolar cells contact both rods and cones and may be the bipolar cell that mediates the alternative rod pathway from rods directly to bipolar cells (Tsukamoto et al., 2001; Mataruga et al., 2007). Furthermore it has been recently shown that OFF bipolar cells (types 1 and 2) may receive rod information via rod-cone coupling in scotopic conditions (Pang et al., 2012) whose input may also activate cone inhibitory circuits which provide inhibition to OFF bipolar cells. This pathway may be responsible for additional inhibition to OFF bipolar cells but requires a different set of investigations to elucidate specific connections and its role in the inhibitory switch with light adaptation. We have shown here that OFF1,2,4 subtypes may receive input from the primary rod pathway (rod bipolar cell-AII amacrine cell-OFF bipolar cell) while OFF3 bipolar cells instead may be getting brighter light information from the secondary and tertiary rod pathways. Our data support the idea that portions of rod pathway signals are specialized to different OFF bipolar cell pathways, although more work is required to determine how the dynamic range and timing of rod pathway input to OFF bipolar cells varies.

In summary, we found that L-IPSCs in OFF bipolar cells change from primarily glycine-mediated to GABA-mediated between dark- and light-adapted conditions. This occurs in a pathway specific manner that may serve to help retinal fine-tuning of spatial sensitivity during daytime vision so that the visual scene can be accurately signaled. This type of switch allows the retina to make efficient use of available neurons and to use them in multiple signaling modes, a mechanism that may be common across many brain systems.
Figure 3. Inhibition to OFF bipolar cells in the dark-adapted retina.

A, schematic of the rod pathway circuitry. Rod photoreceptors (R) activated by dim light release glutamate onto rod bipolar cells (RB) which release glutamate onto AII amacrine cells (AII). AII amacrine cells release glycine onto OFF cone bipolar cells (OFF) while transmitting the depolarizing signal to ON cone bipolar cells (ON) via gap junctions. Cone photoreceptors (C) are activated by brighter light and release glutamate onto OFF and ON bipolar cells. Activation of these bipolar cells in turn releases glutamate onto other GABAergic (GABA) and glycinergic (Gly) amacrine cells which also have inputs onto OFF bipolar cells. Additionally, activation of ON bipolar cells can depolarize AIIIs through electrical gap junctions. INL = inner nuclear layer, IPL = inner plexiform layer.

B, example cell morphology traces of #4, #3, and #1/2 OFF bipolar cells. #4 OFF bipolar cells have terminals that ramify in 1-38% (n = 4) of the IPL, #3 OFF bipolar cells have terminals that ramify in 17-50% (n = 11) of the IPL and #1/2 OFF bipolar cells have terminals that ramify in 1-16% of the IPL (n = 9). #1 and #2 OFF bipolar cells are difficult to morphologically differentiate and so have been pooled.

C, example L-IPSCs recorded from an OFF4 (representing the OFF 1,2,4 group that we have pooled together) and an OFF3 bipolar cell to a 500 ms stimulus (dark gray bar). OFF1,2,4 bipolar cells show an inhibitory response with the onset of light while OFF3 bipolar cells show an inhibitory response with the offset of light. The recording paradigm is shown to the left.

D, OFF1,2,4 (n = 12) cells have a significantly higher charge transfer during light onset while OFF3 (n = 10) bipolar cells have significantly higher charge transfer during light offset. The dashed gray line represents the normalized baseline responses. OFF1,2,4 during light offset and OFF3 during light onset were not different than the baseline (p > 0.05).

E, example L-IPSCs recorded from an OFF4 and OFF3 bipolar cell to a 30 ms stimulus (dark grey bar) in the light and with the application of CNQX and AP5. L-IPSCs remained in OFF1,2,4 bipolar cells but were abolished in OFF3 bipolar cells with CNQX and AP5.

F, intensity response curves of charge transfer normalized to the max light-adapted L-IPSC in OFF1,2,4 (n = 4) and OFF3 (n = 5) light-adapted bipolar cells. CNQX and AP5 significantly reduced OFF1,2,4 L-IPSCs (*) at the brighter light intensities but abolished OFF3 bipolar cell L-IPSCs at all light intensities. (* = p < 0.05, ** = p < 0.01)
Figure 4. Dark-adapted L-IPSCs are mostly mediated by glycinergic input in OFF1,2,4 but not OFF 3 bipolar cells. 

A,B, example L-IPSCs recorded from an OFF1/2 and OFF3 bipolar cell, respectively, in response to a 30 ms flash of light (dark gray bar below L-IPSC). The dark-adapted L-IPSC (black trace) was greatly reduced by application of strychnine (gray trace). The recording paradigm is shown to the left with glycine receptors blocked (Ø).

C,D, intensity response curves of normalized charge transfer of OFF1,2,4 (n = 6) and OFF3 bipolar cells (n = 4), respectively, to the max dark-adapted L-IPSCs. Strychnine significantly reduced OFF1,2,4 L-IPSCs at all light intensities while only the three highest light intensities were reduced in OFF3 bipolar cells. Glycinergic inhibition in OFF3 bipolar cells appears to be a smaller component than in OFF1,2,4 bipolar cells (p<0.05). (* = p < 0.05, ** = p < 0.01)
Figure 5. Dark-adapted sIPSCs are mediated mainly by glycinergic input in both OFF1,2,4 and OFF3 bipolar cells.

A,B, example traces showing the sIPSCs from an OFF4 and OFF3 bipolar cells, respectively, in dark adapted conditions (black trace) and with application of strychnine (gray trace). The recording paradigm is shown to the left with glycine receptors blocked (Ø).

C,D, sIPSC peak amplitude histogram distributions (normalized to number of events) of the OFF1,2,4 and OFF3 bipolar cell from panel A and B respectively. Application of strychnine significantly reduced the sIPSC peak amplitude for OFF1,2,4 bipolar cells (K-S p < 0.01) and OFF3 bipolar cells (K-S p < 0.01). Arrows show the average amplitude of the sIPSC. Insets are averages of dark-adapted (black line) and strychnine application (gray line) sIPSCs. Scale bars are the same for both insets. (**) = p < 0.01)
Figure 6. Light adaptation alters the timing and the charge transfer of the AII amacrine cell L-EPSC and the magnitude and frequency of sEPSCs.

A, L-EPSCs from an AII amacrine cell in response to a 30 ms flash of light (dark gray bar above the L-EPSC). There was a significant reduction of the dark-adapted L-EPSC (black trace) with light adaptation (gray trace). The recording paradigm is shown to the left.

B, example cell morphology traces of an AII amacrine cell. AII amacrine cells have terminals that cover 10 - 97% (n = 6) of the IPL. They have lobular terminals in the OFF bipolar cell sublamina of the IPL, responsible for glycine release, and longer thinner processes in the ON sub lamina which make gap junction connections to ON bipolar cells.

C, intensity response curves of the charge transfer normalized to the max dark-adapted L-EPSC in AII dark- and light-adapted conditions (n = 9). There was a significant difference in the charge transfer with light adaptation at all intensities measured.

D, normalized change in L-EPSC response parameters of AII (n = 9) amacrine cells with light adaptation. In the light, the peak amplitude and decay time (D37) significantly decreased.

E, example traces showing the sEPSCs of AII amacrine cells in dark-adapted (black trace) and light-adapted conditions (gray trace). The recording paradigm is shown to the left.

F, sEPSC peak amplitude histogram distribution (normalized to number of events) of the AII amacrine cell seen in panel E. Light adaptation significantly reduced the sEPSC peak amplitude for the AII amacrine cell (K-S P < 0.01). Arrows show the average amplitude of the sEPSCs. The inset is an average of dark-adapted (black line) and light-adapted (gray line) sEPSCs.

G, the peak amplitude of sEPSCs was significantly decreased in AII amacrine cells following light-adaptation (n = 8).

H, sEPSC inter-event interval histogram distribution (normalized to number of events) of the AII amacrine cell seen in panel E. Light adaptation significantly increased the sEPSC inter-event interval for the AII amacrine cell (K-S P < 0.01). Arrows show the average interval between the sEPSCs. (* = p < 0.05, ** = p < 0.01)
Figure 7. Light adaptation alters the timing of the L-IPSC in only OFF1,2,4 bipolar cells while the magnitude of L-IPSCs remain unaffected for each bipolar cell group.

A,B, example L-IPSCs from an OFF1/2 and OFF3 bipolar cell, respectively, in response to a 30 ms flash of light (dark gray bar below L-IPSC trace). There was no significant reduction of the dark-adapted L-IPSC (black trace) with light adaptation (gray trace). The recording paradigm is shown to the left.

C, intensity response curves of charge transfer normalized to the max dark-adapted L-IPSC in OFF1,2,4 (n = 13) and OFF3 (n = 12) dark- and light-adapted L-IPSCs. There was no significant difference in the charge transfer between OFF1,2,4 and OFF3 bipolar cells with light adaptation.

D, normalized change in L-IPSC response parameters of OFF1,2,4 (n = 13) and OFF3 (n = 10) bipolar cells to max dark-adapted response with light adaptation. The peak amplitude was significantly reduced while the decay time was significantly increased in OFF1,2,4 bipolar cells. In OFF3 bipolar cells, neither the peak amplitude nor the decay time significantly changed. (* = p < 0.05, ** = p < 0.01)
**Figure 8.** Light adaptation decreased sIPSC peak amplitude in OFF1,2,4 bipolar cells but not OFF3 bipolar cells.  

*A,B*, example traces showing the sIPSCs from an OFF1/2 and OFF3 bipolar cell, respectively, in dark-adapted (black trace) and light-adapted conditions (gray trace). The recording paradigm is shown to the left. The sIPSC peak amplitude histogram distributions (normalized to number of events) of the OFF1,2,4 and OFF3 bipolar cells are shown in panels *C* and *D* respectively. Light adaptation significantly reduced the sIPSC amplitude for OFF1,2,4 bipolar cells (K-S *p* < 0.01) but not OFF3 bipolar cells (K-S *p* > 0.05). Arrows show the average peak amplitude of the sIPSCs. Insets are averages of dark-adapted (black line) and light-adapted (gray line) sIPSCs. Scale bars are the same for both insets.  

*E,F*, the peak amplitude of sIPSCs was significantly decreased in OFF1,2,4 bipolar cells (*E*, *n* = 10) following light adaptation and did not change in OFF3 bipolar cells (*F*, *n* = 8). *G,H*, sEPSC inter-event interval histogram distributions (normalized to number of events) of the OFF1/2 and OFF3 bipolar cells seen in panel *A* and *B*, respectively. Light adaptation significantly increased the sIPSC inter-event interval for both cell groups (K-S *P* < 0.01). Arrows show the average interval between the sIPSCs. (* = *p* < 0.05, ** = *p* < 0.01)
Figure 9. The signal-to-noise ratio (SNR) increased in both OFF bipolar cell groups with light adaptation, but did not change in AII amacrine cells.

A, B, SNRs in OFF1,2,4 bipolar cells (n = 13) and OFF3 cells (n = 11), respectively. There was a significant increase in the SNR in the OFF1,2,4 as well as OFF3 bipolar cells with light adaptation.

C, the SNR comparing OFF1,2,4 (n = 13) and OFF3 (n = 11) bipolar cells in dark- and light-adapted conditions. In both instances, there was a significantly higher SNR in OFF3 bipolar cells. The SNR ratio was calculated by dividing the peak amplitude of the L-IPSC by the variance of 100 ms of the baseline current. (* = p < 0.05)

D, the SNR of AII amacrine cell light responses in dark- and light-adapted conditions. There was no significant difference between the conditions (n = 9). (* = p < 0.05, ** = p < 0.01)
**Figure 10.** With light adaptation, both OFF bipolar cell groups received compensatory GABAergic inhibition.

*Figure 10.* With light adaptation, both OFF bipolar cell groups received compensatory GABAergic inhibition. A,B, example light-adapted L-IPSCs from an OFF1/2 and OFF3 bipolar cell, respectively, in response to a 30 ms flash of light (dark gray bar below the L-IPSC). The light-adapted L-IPSC (black trace) was unchanged with isolation of GABAergic input (light gray strychnine trace) in OFF3 bipolar cells only. OFF1,2,4 light-adapted L-IPSCs decreased when GABAergic input was isolated. All L-IPSCs were absent when both glycine and GABA receptors were blocked. The recording paradigm is shown to the left with GABA and glycine receptors blocked (Ø).

*C,D,* intensity response curves of charge transfer normalized to the max light-adapted L-IPSC in OFF1,2,4 (n = 11) and OFF3 (n = 8) bipolar cells, respectively. Addition of strychnine had no effect on the L-IPSCs of OFF3 bipolar cells but significantly decreased the L-IPSCs of OFF1,2,4 bipolar cells. An * indicates a significant difference between the light-adapted control and strychnine application while a † indicates a significant difference between the strychnine treatment and the GABAR blockade (SR95531 + TPMPA).

*E,* the relative proportions of glycinergic and GABAergic input to OFF bipolar cells under dark- and light-adapted conditions for OFF1,2,4 (n = 5, n = 11 respectively) and OFF3 (n = 5, n = 8, respectively) subtypes. With light adaptation, OFF1,2,4 bipolar cells had a significant decrease in glycinergic input and OFF3 bipolar cells had virtually no glycine response. OFF1,2,4 bipolar cells had significantly more glycinergic input than OFF3 bipolar cells in both light conditions. An * indicates a significant difference between the dark- and light-adapted conditions within an OFF bipolar cell group where as a # indicates a significant difference between OFF1,2,4 and OFF3 bipolar cells in each light condition. (*, †, # = p < 0.05, **, †† = p < 0.01)
Figure 11. Summary schematics showing inhibitory input to OFF1,2,4 and OFF3 bipolar cells under dark- and light-adapted conditions.

A, under dark-adapted conditions, OFF1,2,4 bipolar cells receive large light-evoked glycinergic inhibition from the rod-AII amacrine cell pathway in addition to other glycinergic and GABAergic inputs. This is done through crossover inhibition via excitation of the AII amacrine cell with dim light and serves as an ON inhibitory signal to the OFF pathway.

B, under dark-adapted conditions, OFF3 bipolar cells receive large light-evoked glycinergic and GABAergic inhibition from OFF cone pathways activated by brighter light intensities.

C, under light-adapted conditions OFF1,2,4 bipolar cells receive compensatory GABAergic and glycinergic inhibition from cone activated amacrine cells. The AII amacrine cell may receive depolarizing signals through gap junction connections with ON bipolar cells, and partly mediate inhibitory currents in the OFF1,2,4 bipolar cells (Fig. 3E,F). However, there is an overall higher proportion of GABAergic input in the light.

D, in light-adapted conditions, OFF3 bipolar cells receive compensatory inhibition solely mediated by GABAergic input from OFF cone activated amacrine cells.
CHAPTER 3: LIGHT ADAPTATION ALTERS INNER RETINAL INHIBITORY SURROUNDS TO INCREASE OFF RETINAL PATHWAY SIGNALING

Introduction:

The retina must signal under many light conditions to accurately encode the environment. To do this, the retina adapts to increasing luminance levels to avoid saturation and increase visual acuity. The retina signals multiple light levels by using both dim light rod and bright light cone photoreceptors as well as adaptation of both the photoreceptors themselves (Tamura et al., 1991; Woodruff et al., 2008) and downstream networks (Green et al., 1975; Naka et al., 1979; Green and Powers, 1982; Shapley and Enroth-Cugell, 1984; Page-McCaw et al., 2004; Dunn et al., 2006; Dunn et al., 2007).

Photoreceptor light information is sent to bipolar cells that synapse onto ganglion cells, the output neurons of the retina, and onto inhibitory amacrine cells which shape bipolar cell-ganglion cell signaling (Dong and Werblin, 1998; Eggers and Lukasiewicz, 2006b; Sagdullaev et al., 2006; Eggers et al., 2007; Eggers and Lukasiewicz, 2010). Amacrine cells release either glycine or GABA (Pourcho and Goebel, 1983, 1985; Vaney, 1990; Kolb, 1997; Menger et al., 1998) where GABAergic amacrine cells are thought to play a primary role in carrying wider lateral information for center-surround receptive fields (Dacey et al., 2000; Sinclair et al., 2004; Ichinose and Lukasiewicz, 2005; Zhang and Wu, 2009) as opposed to glycinergic amacrine cells that contribute to inhibition between different pathways (Molnar et al., 2009; Werblin, 2010; Mazade and Eggers, 2013). The balance between the excitatory center and inhibitory surround allows ganglion cells fine spatial tuning to specific sizes of light, vital for visual resolution.
Ganglion cells increase their spatial sensitivity and visual acuity after ambient illumination is increased (Barlow et al., 1957; Dedek et al., 2008; Farrow et al., 2013) and retinal network adaptation is thought to be crucial for this process (Wu and Yang, 1992; Dunn et al., 2006). Previous studies have shown that ganglion cell sensitivity to small lights increased due to a decrease in excitatory center size (Merwine et al., 1995; Troy et al., 1999) or decreases in surround size and strength (Troy et al., 1993; Merwine et al., 1995; Troy et al., 1999; Dedek et al., 2008; Farrow et al., 2013). Inner retinal processing likely plays an important role in ganglion cell spatial resolution increases with light (Dedek et al., 2008) as the connections between bipolar and ganglion cells are important for light adaptation (Dunn et al., 2007; Oesch and Diamond, 2011; Farrow et al., 2013). Given other reports that show total retinal inhibition (Flores-Herr et al., 2001; O'Brien et al., 2003; Sinclair et al., 2004; Ichinose and Lukasiewicz, 2005) and inhibition to bipolar cells specifically (Flores-Herr et al., 2001; Russell and Werblin, 2010; Buldyrev and Taylor, 2013; Protti et al., 2014) are important for determining the spatial resolution and receptive field properties of ganglion cells, modulation of bipolar cell inhibition by light is a potential mechanism for increasing visual acuity.

Modulation of inhibitory inputs to the pathway that responds to the offset of light (OFF) in particular may be important in retinal network adaptation, as inhibitory inputs switch between rod and cone sources (McGuire et al., 1984; Strettoi et al., 1990; Strettoi et al., 1992; Chun et al., 1993; Trexler et al., 2001; Deans et al., 2002; Mazade and Eggers, 2013). This change from rod- to cone-mediated input could change the distribution of inhibitory input to OFF (cone) bipolar cells and affect ganglion cell center-surround
organization, but this is not known. Here we investigated how the spatial extent of inhibition to OFF bipolar cells changes with light adaptation and used our findings to model the strength of OFF bipolar cell output to downstream ganglion cells. We found that the spatial inhibition to OFF bipolar cells became narrower with light adaptation and that this change is important for determining stronger signal output to ganglion cells for small light stimuli.

Methods:

Mouse retinal slice preparation

All animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). As described previously (Eggers and Lukasiewicz, 2006a; Eggers et al., 2013; Mazade and Eggers, 2013), male mice (C57BL/6J strain, Jackson Laboratories, Bar Harbor, ME, USA) 35-60 days of age were euthanized using carbon dioxide and their eyes enucleated. The cornea and lens were removed and the eyecup was incubated for 20 min in cold extracellular solution (see Solution and Drugs) with 800 units/mL of hyaluronidase to dissolve the remaining vitreous humor. The hyaluronidase solution was then replaced with ice cold, oxygenated extracellular solution and the retina was dissected out of the eyecup. Following removal, the retina was trimmed into one large flat rectangle by removing the peripheral retina and leaving only the central retina surrounding the optic disc. A nitrocellulose membrane filter paper (0.45 μm pore size, Millipore, Ireland) was placed on the retina section which was transferred to a hand chopper. An average of six 250 μm slices were cut, rotated 90°, and mounted onto glass cover slips using vacuum grease. Cells used from these slices were never more
than 700 μm away from the center of the retina and only cells near the center of each slice were used for recordings in order to ensure accurate spatial distances. The tissue was maintained in oxygenated extracellular solution at room temperature. All dissection procedures were performed under infrared illumination to preserve the light sensitivity of the preparations.

Solutions and drugs

The extracellular recording solution used for dissection and to examine light-evoked currents contained (in mM) 125 NaCl, 2.5 KCl, 1 MgCl2, 1.25 NaH2PO4, 2 CaCl2, 20 glucose, and 26 NaHCO3 and was bubbled with 95% O2-5% CO2. For voltage clamp recordings, the intracellular solution contained (in mM) 120 CsOH, 120 gluconic acid, 1 MgCl2, 10 HEPES, 10 TEA-Cl, 10 phosphocreatine-Na2, 4 Mg-ATP, 0.5 Na-GTP, 10 EGTA and 50 μM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. To isolate the inhibitory receptor inputs, SR-95531 (SR, 20 μM) to block GABA\(_A\) receptors, (1,2,5,6-tetrahydropyridine-4yl) methyphosphinic acid (TPMPA, 50 μM) to block GABA\(_C\) receptors, and strychnine (1 μM) to block glycine receptors were used. For current clamp recordings, the intracellular solution contained (in mM) 120 KOH, 120 Gluconic Acid, 1 MgCl2, 10 HEPES, 10 EGTA, 10 TEA-Cl, 10 phosphocreatine-Na2, 4 Mg-ATP, 0.5 Na-GTP, 50 μM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. All drug solutions were washed on the slice for 5 min before recordings began using a gravity-driven superfusion system (Cell Microcontrols, Norfolk, VA) at a rate of ~1-2
mL/minute. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Whole-cell recordings

Whole-cell patch-clamp recordings, sampled at 10 kHz, were made from bipolar cells in retinal slices. Light-evoked inhibitory post synaptic currents (L-IPSCs) were recorded from retinal bipolar cells voltage clamped to 0 mV, the reversal potential of nonselective cation channel currents. Bipolar cell recordings were stable and no rundown of the light response was observed over the recording period. Light-evoked excitatory post synaptic currents (L-EPSCs) were recorded from bipolar cells voltage clamped to ~60 mV, the reversal potential for chloride channel-mediated currents. Resting membrane potentials were recorded passively in current clamp (I-clamp) mode. Liquid junction potentials of 20 mV were corrected for at the beginning of each recording. Electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) on a P97 Flaming/Brown puller (Sutter Instruments, Novato, California, USA) and had resistances of 5-7 MΩ. Mice were dark-adapted overnight, and all recording procedures were performed in the dark under infrared illumination to preserve the light sensitivity of the slices. Recordings were made in extracellular solution heated to 32°C, using thin stage and inline heaters (Cell Microcontrols, Norfolk, VA). Responses were filtered at 6 kHz with the four-pole Bessel filter on a Multi-clamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, California, USA) and digitized with a Digidata 1140 data acquisition system (Molecular Devices, Sunnyvale, California, USA).
Morphological identification of cell subtypes

Alexa 488 included in the recording pipette was used to label OFF bipolar cell subtypes. They were classified as either type 1/2, 3, or 4 OFF bipolar cells based on their axonal morphologies and stratification within the inner plexiform layers and the position of their somas in the inner nuclear layer (Ghosh et al., 2004). All OFF bipolar cells were pooled due to the results from Chapter 2. The cells were imaged with Nikon Digital Sight camera with Elements software using a Nikon Intensilight C-HGFIIE Fluorescent lamp (Nikon Instruments, Tokyo, Japan). Detailed analysis of axon terminal morphology and response properties was performed previously on all OFF bipolar cell subtypes (Mazade and Eggers, 2013) and these same criteria were used to identify OFF bipolar cells in the current study.

Light stimuli

Bar stimuli (25 µm wide) were presented using a white organic light-emitting diode (OLED Microdisplay, eMagin EMA-100503 SXGA Monochrome White XL, Bellevue, WA) projected through the camera port of the microscope, which elicited strong responses in both dark- and light-adapted conditions (Figure 12B). Recordings were from cells located within the regions of mixed green/UV cone opsin input (Applebury et al., 2000; Haverkamp et al., 2005) which ensured that all possible pathways were present. The stimulus intensity (7.83 x 10^4 photons/µm^2/sec) and the background rod-saturating light (1150 photons/µm^2/sec) were controlled with custom MatLab software by controlling the intensity, size, location, and duration of the bar stimuli through the OLED screen. The background intensity was chosen as it was shown to maximally activate rods.
(Wang and Kefalov, 2009) and produced significant changes in inner retinal inhibition (Mazade and Eggers, 2013). For similar intensity-step experiments, a dim background of 158 photons/μm²/sec was used in the dark-adapted condition with a stimulus intensity of $7.65 \times 10^4$ photons/μm²/sec. This was calculated by subtracting the background from the stimulus and matched so that under these conditions, the increase in light intensity of the stimulus from the background was similar between dark- and light-adapted conditions. A long light stimulus (1 s) was used to determine the type of inhibition and excitation to all recorded bipolar cells as well as to illicit robust responses with a very small stimulus. An adapting background light was applied for 5 min to light-adapt the retina slice and was sustained throughout all light-adapted recordings.

**Data analysis and statistics**

L-IPSC and L-EPSC traces from a given response condition were averaged using Clampfit software (Molecular Devices, Sunnyvale, California, USA) and the charge transfer (Q) and peak amplitude were measured in each condition. Due to the significant amount of spontaneous activity, it was difficult to measure a peak from OFF bipolar cell L-IPSCs. Therefore to estimate the peak, the sampling rate of averaged traces was reduced (50 fold) and each point was replaced with the average of those data points to limit variations due to spontaneous activity. To determine changes in total current, the Q, the magnitude of the response, was measured in Clampfit over the length of the response, typically 1-2 seconds, using the same time parameters in each condition for the same cell. The baseline Q was added to the Q standard deviation which was subtracted from all raw Q measurements to negate any current due to baseline or spontaneous events. All
example response traces show responses to the center bar stimulus directly over the recorded cell or 200 μm away from the cell.

For spatial distribution curves, light-evoked Q’s were normalized to the maximal response in the dark-adapted condition and peak amplitude raw data were used. Normalized Q’s were used to control for variability between bipolar cell L-IPSCs so that spatial extent could be accurately compared and visualized between light conditions. Raw peak amplitude were used as a reliable response magnitude value. The normalized and raw data were plotted against the distance the stimulus was from the cell. In order to compare between the dark- and light-adapted conditions at any given stimulus distance as well as to average out any spatial variation since there should be equal inhibitory inputs from stimuli equidistant from the cell, both sides of the spatial distributions at equal distances were averaged and plotted as bar graphs.

To measure timing differences between light conditions, the transient and sustained components of center L-IPSCs were measured. The transient L-IPSC component was measured as the first 20% Q of the response based on the 1 sec light stimulus, similar to Nobles et al., 2012 for ganglion cell responses to long light stimuli (Nobles et al., 2012). Sustained L-IPSC components were measured by subtracting the transient Q from the total Q of the each center L-IPSC within each light conditions. Proportions were calculated by dividing the transient and sustained values by the total Q. For the I-clamp experiments, baseline voltage was averaged over ~200 seconds of stable baseline in each condition to calculate the resting membrane potential.
2-way Analysis of Variance (ANOVA) with Student-Newman-Keuls (SNK) posthoc test was used to compare spatial distributions before and after light adaptation as well as between response characteristics at each stimulus distance. Student’s t-test (2-tailed, paired) was used to compare resting membrane potentials and timing parameters before and after light adaptation. Differences were considered significant when $p < 0.05$ (*) and $p < 0.01$ (**). All averaged data are reported as mean ± SEM.

Spatial Inhibition Model

A model of inputs to a ganglion cell was constructed based on the spatial changes reported in this study. OFF bipolar cell spatial inhibitory extent changes and average peak amplitude values were used from both dark- and light-adapted conditions. Average spatial inhibition curves were fitted with a Gaussian curve from which standard deviations were obtained for both dark- and light-conditions. The standard deviations were then used as a base for constructing model OFF bipolar cell inhibitory spatial distributions. The same method was used for excitatory spatial distributions. These distributions were then normalized and multiplied by a scaled peak amplitude ($I_{sc}$, Eq.1) which was calculated using measured peak amplitudes ($I_{measured}$) at holding potentials ($V_{hold}$) of either 0 mV (L-IPSC) or -60 mV (L-EPSC),

Eq.1: $I_{sc} = (V_{rest} - V_{rev}) \times (G)$

where $V_{rest}$ is the resting membrane potential of dark- or light-adapted OFF bipolar cells, $V_{rev}$ is the reversal potential for either cation or chloride channels, $V_{hold}$ is the holding voltage for measuring excitatory or inhibitory currents and $G = ((I_{measured} / (V_{rev} - V_{hold})))$. 

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Excitatory and inhibitory spatial Gaussian distributions based on the scaled currents were constructed. A difference of Gaussians was applied by subtracting the inhibitory from the excitatory Gaussian distributions. To account for the fact that glutamate release is necessary for bipolar cell signals to ganglion cells, bipolar cell scaled currents were used to calculate real membrane voltage values ($V_{\text{real}}$, Eq.2) by converting scaled currents ($I_{sc}$) to the amount of voltage change they would cause using an average input resistance ($R$) measured from OFF bipolar cells and adding the value to the resting membrane potential ($V_{\text{rest}}$) in the dark- and light-adapted conditions.

$$\text{Eq.2: } V_{\text{real}} = (I_{sc} \times R) + V_{\text{rest}}$$

Bipolar cell voltage values in the dark and light were then used as a baseline for whether the bipolar cells would signal to downstream ganglion cells, and thus the bipolar cell signal output strength.

**Results:**

*The spatial extent of total OFF bipolar cell inhibition is narrower after light adaptation*

OFF bipolar cells receive glycine, $\text{GABA}_A$, and $\text{GABA}_C$ receptor-mediated inhibition including glycinergic inputs from the rod pathway and glycinergic and GABAergic inputs from cone pathways (Euler and Wässle, 1998; Ivanova et al., 2006; Eggers et al., 2007) (Figure 12A) that change with light adaptation (Mazade and Eggers, 2013). Previous work showed changes in the excitatory center size (Merwine et al., 1995; Troy et al., 1999) and inhibitory surround size and strength (Troy et al., 1993; Merwine et al., 1995; Troy et al., 1999; Dedek et al., 2008; Farrow et al., 2013) of ganglion cells with light adaptation which may be a result of spatial changes at the bipolar cell level (Dedek
et al., 2008). To test this, we mapped the spatial extent of OFF bipolar cell inhibition using a long light stimulus (1 s) that was flashed across a retinal slice every 100 μm (Figure 12B).

We predicted that the spatial distribution of inhibition to OFF bipolar cells would become wider with light adaptation due to the larger proportion of inputs from wide GABAergic amacrine cells (Mazade and Eggers, 2013). Surprisingly, we found that total inhibition became significantly narrower and smaller under light-adapted conditions (Figure 13). OFF bipolar cell L-IPSCs in the dark were robust with inhibition activated at distances more than 200 μm away from the recorded cell (Figure 13A). After light adaptation, most OFF bipolar cells did not receive any inhibition if stimuli were presented more than 200 μm away from the cell (Figure 13B). The spatial distribution of Q’s normalized to the center bar became significantly narrower with light adaption (p < 0.01) (Figure 13C). To compare the normalized Q between light conditions at each stimulus distance, both sides of the spatial distribution curves were averaged and values plotted. There was a significantly smaller proportion of inhibition in the light-adapted than in the dark-adapted case from 200–800 μm away from the recorded cell (p < 0.05), with little to no inhibition remaining at the furthest distances (Figure 13D). OFF bipolar cell center L-IPSCs also became significantly more transient, with a decrease in sustained response with light adaptation (p < 0.05) (Figure 13E). The L-IPSC peak amplitude spatial distribution also became significantly smaller and narrower in light-adapted conditions (p < 0.05) (Figure 11F). There was a significantly smaller proportion of inhibition in light- than in the dark-adapted conditions from 0–200 μm away from the recorded cell (p < 0.05), with no
inhibition remaining from stimuli farther than 300 µm (Figure 13G). The average peak amplitude for the center stimulus in the dark was 21.8±7.9 pA which significantly decreased to 8.2±1.6 pA after light-adaptation (p < 0.05).

Previous studies determined that although OFF bipolar cell subtypes receive input from distinct pathways (Tsukamoto et al., 2001; Mazade and Eggers, 2013), all OFF bipolar cells had similar glycine/GABA inhibitory proportion changes with light adaptation (Mazade and Eggers, 2013). Despite this, there may still be differences in spatial inhibitory input between the subtypes. To resolve this, we compared the spatial distribution of inhibition between OFF cone bipolar cell types 1,2, and 4, previously shown to receive inhibition at the onset of light from the rod pathway, and OFF cone bipolar cell type 3, which receives inhibitory input from cone activated pathways at the offset of light (Mazade and Eggers, 2013). There was no significant difference between OFF1,2,4 (n = 6) and OFF3 (n = 9) bipolar cell spatial inhibitory Q, normalized to the largest center L-IPSC (data not shown, p = 0.06), or normalized peak amplitude distribution of the L-IPSCs in the dark (p = 0.37). As expected from previous work, Mazade and Eggers, 2013, OFF1,2,4 bipolar cells had significantly greater L-IPSC peak amplitudes than OFF3 bipolar cells (center OFF1,2,4 bipolar cell = 23.6±8.7 pA, OFF3 bipolar cells = 10.1±3.9 pA, p < 0.01). Larger L-IPSC peak amplitude in the dark is likely due to the large glycinergic AII amacrine cell input from the rod pathway (Mazade and Eggers, 2013). However, since all OFF bipolar cell subtypes showed similar spatial distributions, results from all types were pooled for the remainder of this study. Taken together, these results suggest that circuitry connections are in place to provide wide
spread inhibition to OFF bipolar cells in the dark, but changes with light adaptation act to shorten the spatial extent of inhibitory signaling as well as the L-IPSCs themselves.

*Isolated GABAergic spatial inhibitory input to OFF bipolar cells narrows with light adaptation*

The narrowing of OFF bipolar cell spatial inhibition (Figure 13) could be caused by changes in GABAergic inhibitory inputs, glycinergic inhibitory inputs, or both. To determine this, we first investigated how pharmacologically isolated GABAergic spatial input (in strychnine) to OFF bipolar cells changes with light adaptation. GABAergic amacrine cells are wide-field and have processes that can span hundreds of microns laterally across the retina potentially mediating very wide spatial signaling (Pourcho and Goebel, 1983, 1985; Vaney, 1990; Kolb, 1997; Menger et al., 1998). As expected, dark-adapted isolated GABAergic input, consisting of both GABA$_A$ and GABA$_C$-mediated inhibition, was significantly wider than total inhibition (Figure 13C, p < 0.01). Bar stimuli centered on the cell elicited large L-IPSCs which remained even when the stimulus was 200 µm away (Figure 14A). However, after light-adaptation, L-IPSCs were smaller in the center with decreasing responses farther from the cell (Figure 14B). The spatial distribution of normalized Q’s became significantly narrower after light-adaptation (p < 0.01) (Figure 14C). At each stimulus distance from the cell, the proportion of GABAergic inhibition was smaller after light-adaptation than in the dark with significant decreases beginning at 100 µm away (Figure 14D). OFF bipolar cell center L-IPSCs also shifted to being significantly more transient, with a large decrease in sustained portion of the response with light adaptation (p < 0.01) (Figure 14E).
GABAergic peak amplitude distribution of L-IPSCs also became smaller and narrower with light adaptation (p < 0.05) (Figure 14F). The peak amplitudes compared between dark- and light-adapted conditions for the majority of stimuli outside the center were smaller (Figure 14G). There were GABAergic L-IPSCs 800 µm away from the recorded cell on average in the dark, however after light-adaptation spatial inhibitory input shortened significantly on average from 300 µm away. The average GABAergic peak amplitude at the center did not significantly decrease with light adaptation (dark, 8.1±1.8 pA; light, 5.0±1.8 pA, p = 0.22). Overall total GABAergic inhibition, consisting of GABA\textsubscript{A} and GABA\textsubscript{C} receptor-mediated inputs narrowed with light adaptation. These results suggest that GABAergic inhibition helps to increase spatial inhibitory width in the dark and also works to shorten inhibitory L-IPSCs in light-adapted conditions.

*Isolated GABA\textsubscript{C} spatial inhibitory input to OFF bipolar cells is abolished with light adaptation*

We found that light adaptation caused a significant narrowing of spatial GABAergic input to OFF bipolar cells. Under light-adapted conditions, this spatially narrow input presents an initial challenge; how could wide-field cells whose processes span hundreds of microns laterally across the retina, many of which use action potentials, change to only provide spatially close inhibition? However, previous work has shown that there are inhibitory connections between GABAergic amacrine cells which decrease inhibition from these amacrine cells onto downstream bipolar cells (Eggers et al., 2007). These amacrine cell-amacrine cell serial inhibitory connections, mediated by GABA\textsubscript{A} receptors, are activated when large light stimuli excite circuitry distant from the recorded bipolar
cell (Eggers et al., 2007; Eggers et al., 2013). It is possible that under light-adapted conditions, stimuli distant from the target cell more strongly activate this serial inhibition which would decrease the amount of inhibition that GABAergic amacrine cells are able to provide to OFF bipolar cells. To investigate this, we pharmacologically isolated GABA_C receptor-mediated inhibitory input (in SR and strychnine), thereby blocking GABA_A receptor-mediated serial connections, and measured spatial input and peak amplitude of the L-IPSCs. Under dark-adapted conditions, OFF bipolar cells received robust GABA_C receptor-mediated inhibition at the center bar and smaller L-IPSCS 200 µm away (Figure 15A). With light adaptation, OFF bipolar cells received no GABA_C receptor-mediated inhibition at any stimulus distance (Figure 15B). The normalized GABA_C receptor-mediated spatial inhibitory Q and peak amplitude in the dark was moderately wide (Figure 15C,D), similar to total inhibition (Figure 11C, p = 0.08), with no inhibition remaining after light adaptation (p < 0.01). GABA_C receptor-mediated spatial input was significantly narrower than isolated GABAergic input (p < 0.01) (Figure 15E). The magnitudes of GABA_C receptor-mediated inhibition are likely partially inflated, as blocking inhibitory GABA_A receptor-mediated inhibition on the amacrine cells themselves, will increase their signal output. Given that GABA_C receptor-mediated inhibition was eliminated in the light-adapted retina, we could not test our hypothesis about serial connections. However, serial inhibitory input could still likely be playing a role in GABAergic narrowing.
Isolated glycineergic spatial inhibitory input to OFF bipolar cells narrows with light adaptation

Previous studies have shown that OFF bipolar cells receive proportionally larger glycineergic input in the dark (Eggers and Lukasiewicz, 2006b; Mazade and Eggers, 2013). Since GABAergic spatial inhibition becomes narrower in light-adapted conditions we wanted to determine whether glycineergic amacrine cells were also contributing to OFF bipolar cell spatial changes with light adaptation. Using the same stimulus parameters described above, pharmacologically isolated glycineergic spatial inhibitory input (in SR and TPMPA) was wide in the dark and was not significantly different than the total inhibitory spatial input (Figure 13C, p = 0.30). Center bar stimuli elicited robust L-IPSCs with smaller but still robust L-IPSCs at 200 µm away (Figure 16A). This was surprising since the narrow morphology of glycineergic amacrine cells suggest that they would not send inhibitory signals over long lateral distances. However, light-adapted L-IPSCs were smaller in the center and absent in most cells at 200 µm away (Figure 16B). The spatial distribution of normalized Q’s became significantly more narrow in the light-adapted condition (p < 0.01) (Figure 16C), similar to total inhibition. There was a significantly smaller proportion of glycineergic inhibition in the light- than in the dark-adapted condition, with little inhibition at large distances from the recorded cell (Figure 16D). OFF bipolar cell center L-IPSCs became significantly more transient, with a large decrease in sustained portion of the response, with light adaptation (p < 0.01) (Figure 16E). The peak amplitude distribution of glycineergic input became significantly smaller and narrower in the light-adapted retina (p < 0.01) (Figure 16F). When comparing between dark- and light-adapted conditions at each stimulus distance, the peak amplitude
was significantly smaller as well, with no light-adapted inhibition remaining from stimuli farther than 400 µm (Figure 16G). The average peak amplitude for the center significantly decreased to 4.6±0.8 pA after light-adaptation from 12.8±3.8 pA in the dark (p < 0.05). These results suggest that narrowing of glycinergic spatial inhibition to OFF bipolar cells and reduction in peak amplitude at least in part contributes to the total light-adapted narrowing of spatial inhibition.

GABAergic spatial input is wider than glycinergic spatial input in dark- but not light-adapted conditions

While GABAergic spatial inputs to OFF bipolar cells were quite wide in dark-adapted conditions, we found that glycinergic spatial input was also wide. Since these two neurotransmitters come from distinct sources, and wide glycinergic spatial input in the dark is unexpected, we compared the spatial inputs between glycinergic and GABAergic connections. In dark-adapted conditions, the spatial distribution of isolated glycinergic Q’s (normalized to the center bar) was significantly narrower than isolated GABAergic responses (p < 0.01) (Figure 17A). With light adaptation, both isolated glycinergic and isolated GABAergic spatial inputs to OFF bipolar cells became significantly narrower and were not different from each other (p = 0.52) (Figure 17B). The normalized peak amplitude distributions followed the same trend as the normalized Q’s, isolated glycinergic spatial input was narrower than isolated GABAergic spatial input in dark-adapted but not light-adapted conditions (dark p < 0.01, light p = 0.22) (Figure 17C,D). Isolated glycinergic responses had a significant decrease in magnitude at the center bar with light adaptation (~ 64%, Figure 5), whereas GABAergic input did not (~38%, Figure...
3), so that the proportion of GABA and glycine input to OFF bipolar cells changes, similar to our previous findings, suggesting the change from full-field stimuli is still present with small stimuli (Mazade and Eggers, 2013). This dark-adapted relationship was not unexpected as wide-field GABAergic amacrine cells would mediate wider spatial signals than glycinergic amacrine cells. However, since glycinergic spatial input still extend hundreds of microns across the retina, there must be circuitry organization to extend the extent of their signaling. Despite the dark-adapted differences, both distinct inputs have similar spatial extents with light-adaptation.

Light adaptation narrowed total spatial inhibition under similar step intensity conditions

We have found that the total spatial inhibition, whether coming from either glycinergic or GABAergic sources, becomes significantly narrower in light-adapted conditions (Figures 13-17). These experiments were conducted under true dark-adapted and light-adapted conditions, in response to the same intensity bar stimulus (7.83 x 10^4 photons/μm^2/sec). Thus, it is possible that some of the spatial changes in L-IPSC magnitude may be due to contrast adaptation in addition to light adaptation. To see if there were any differences when the light intensity step was the same in dark- and light-adapted conditions, we mapped the total spatial inhibition to OFF bipolar cells under dark-adapted conditions by applying a dim background light (see Methods) which was not enough to light adapt the preparation. In the dark, bar stimuli produced similar L-IPSCs as in the previous experiments with the largest inhibitory response at the center bar, and smaller responses 200 μm away (Figure 18A). After light-adaptation, the center bar stimulus was smaller with little to no response remaining at 200 μm away from the recorded cell (Figure 18B).
The normalized spatial inhibitory Q distribution in the dark was significantly different than the dark-adapted spatial distribution in Figure 4 (p < 0.01), likely due to the slight adaptation to the dim background light, but also became significantly more narrow after light-adaptation (n = 4, p < 0.01) (Figure 18C). The normalized Q averaged on both sides of the spatial curve compared between light conditions revealed that the proportion of total inhibition was significantly smaller in the light-adapted state (n = 4) at 100 – 600 µm away, beyond which there was little remaining inhibition (Figure 18D). Center bar L-IPSCs became significantly more transient, with a small decrease in the sustained portion of the response, in the light-adapted condition (n = 4, p < 0.05), similar to Figure 4 L-IPSC changes (Figure 18E). The peak amplitude distribution of L-IPSCs also became narrower with light adaptation (center stimulus: dark, 9.8±2.7 pA; light, 4.1±1.0 pA; p < 0.05) and no inhibition remained from stimuli more than 400 µm from the recorded cell (Figure 18F,G). The center bar L-IPSC peak amplitude was not different between the step-controlled and complete darkness conditions in dark- or light-adapted states (dark p = 0.68, light p = 0.56). On average, step-controlled conditions reduced distant L-IPSCs less than complete darkness conditions but the overall trend of decreasing spatial input was consistent in both cases. The changes we have shown in inhibitory spatial signaling at the bipolar cell level would be transmitted to downstream ganglion cells which form the main center-surround organizational units for determining visual acuity. To determine if the changes in the spatial extent of inhibitory signaling to bipolar cells contribute to increased sensitivity of ganglion cells to small stimuli after light-adaptation, we sought to create a model for how changes in bipolar cell inhibition change the strength of bipolar cell signal output.
Light adaptation changes OFF bipolar cell $V_{\text{rest}}$ and L-IPSCs without changing L-EPSCs

In order to create a model of how bipolar cell signaling changes with light adaptation affect bipolar cell output to ganglion cells, we measured several different OFF bipolar cell response parameters under dark- and light-adapted conditions. First, we measured the amounts of inhibition and excitation in step-controlled dark- and light-adapted conditions within the same cell. This is important in determining the output from OFF bipolar cells to OFF ganglion cells which depends on the balance of these inputs. L-IPSCs and L-EPSCs were recorded from OFF bipolar cells in response to a small 25 µm bar of light flashed over the center of the recorded cell, similar to the central bar of the intensity step-controlled spatial mapping experiments. Using the small bar provides more accurate information on input magnitudes to the OFF bipolar cells from small light stimuli. As expected from the spatial experiments, peak amplitude of the L-IPSCs was significantly decreased from 21.6±5.8 pA under dark-adapted conditions to 12.0±3.4 pA in light-adapted conditions ($n = 12$, $p < 0.05$) (Figure 19A,C). In contrast, peak amplitudes of the L-EPSCs were not significantly different between dark- (16.95±4.4 pA) and light-adapted (17.0±2.6 pA, $n = 8$, $p = 0.92$) (Figure 19B,D). With a large decrease in inhibition and no change in excitation, the balance of excitatory and inhibitory input to OFF bipolar cells shifted in the light-adapted state. Under dark-adapted conditions, the proportion of total synaptic input to OFF bipolar cells was composed of 56.0±15.0% inhibition and 44.0±12.0% excitation, whereas in the light-adapted conditions, the proportion of total input was reversed, trending towards significance, 41.8±12.0% inhibition and 58.2±9.1% excitation ($p = 0.07$) (Figure 19E). Additionally, we measured the spatial extent of excitatory input to OFF bipolar cells under dark- and light-adapted conditions (data not
shown). This was performed by using a 25 µm bar of light and stimulating every 50 µm away from the cell for 150 µm in each direction. There was no difference between dark- and light-adapted states (dark n = 7, light n = 3) (spatial Q p = 0.38, spatial peak amplitude p = 0.30) and cells typically only had robust L-EPSCs in response to the 25 µm bar when it was directly over the cell.

Next, we measured the resting membrane potential (V_{rest}) using current-clamp recordings of OFF bipolar cells in dark- and light-adapted conditions. In the dark, OFF bipolar cells have a relatively depolarized resting potential likely due to the large amount of constant glutamate release from the highly active cone photoreceptors. The dark-adapted OFF bipolar cell V_{rest} was on average −44.4±1.9 mV, similar to a previous study (Arman and Sampath, 2012) (Figure 20A,B). With light adaptation, there is less release from photoreceptors (Choi et al., 2005) suggesting that the OFF bipolar cells should become hyperpolarized relative to dark-adapted conditions. However, we found that in light-adapted conditions, the OFF bipolar cell V_{rest} became even more depolarized, averaging −37.6±2.8 mV (total range from −30 to −45 mV) (Figure 20A,B), which was significantly more depolarized than in dark-adapted conditions (p < 0.05). The more depolarized V_{rest} is likely due to a decrease in inhibitory inputs to the OFF bipolar cells as a result of light adaptation (Mazade and Eggers, 2013), despite the reduction in glutamate release. Determining the resting state of OFF bipolar cells in the dark- and light-adapted conditions allows for the incorporation of OFF bipolar cell activation in the construction of the spatial model.
Light adaptation increases the signal output strength of small light stimuli from bipolar cells

The experimental findings of this study show that under dark-adapted conditions, OFF bipolar cells had wide spatial inhibition and thus a large surround in dark-adapted conditions. Under light-adapted conditions however, there is no change in the excitatory center size, but the narrowing of spatial inhibition would imply a smaller surround (Figure 21A). To use these parameters in a model, Gaussian distributions were calculated using standard deviations obtained from fitting Gaussian distributions to the averaged step-controlled total spatial inhibitory and excitatory data in both light conditions (Figure 18). The excitatory center and inhibitory surround standard deviations of the Gaussian distributions were used to construct prototypical Gaussian receptive field curves in dark- and light adapted conditions (Table 1). The normally distributed spatial curves in dark- and light-adapted conditions were normalized to the central response and multiplied by the conductance-scaled peak amplitude of the L-IPSCs and L-EPSCs (see Methods Eq.1). There is little difference between the bipolar excitatory center distributions while the inhibitory surround is much wider and larger in the dark-adapted condition than in the light-adapted state, as seen in our data (Figure 21B).

The difference of excitatory and inhibitory Gaussian distributions was then used to produce center-surround receptive field curves (Figure 21C). Inhibition is a stronger component of dark-adapted center-surround receptive fields than in light-adapted receptive fields. Given that neurotransmitter release is required for a bipolar cell to signal to a downstream ganglion cell, linear transfer of bipolar cell currents to ganglion cells
cannot be assumed. To estimate what signals would be sent to a downstream cell, we calculated the voltage change that each current level would cause at each point in the bipolar cell center-surround distributions using our average measured input resistance of $1.94 \pm 0.18 \ \Omega$ for all OFF bipolar cells in control, dark-adapted conditions (see Methods Eq.2). The calculated voltage change was added to the OFF bipolar cell resting membrane potential ($V_{rest}$) in the dark- and light-adapted conditions to obtain the real peak voltage a stimulus at each distance would elicit (Figure 21D). For example, the peak current at the center stimulus in Figure 13C would depolarize the cell to -30.8 mV in the dark and to -25.3 mV after light adaptation. However, OFF bipolar cells are more depolarized at rest by ~ 6 mV on average (Figure 20) which may act to decrease input resistance due to voltage-gated channel openings and thus affect calculated voltages in light-adapted state. A depolarization to ~38 mV (light-adapted $V_{rest}$) from -50 $V_{hold}$ (close to our measured dark-adapted $V_{rest}$) in OFF bipolar cells causes a 20 pA $Ca^{2+}$ current, values taken from (Cui et al., 2012) Figure 4D, with a calculated input resistance of 1.9 G$\Omega$ (Eq.2). Since there was no significant change from dark-adapted conditions, a 1.9 G$\Omega$ input resistance was used for voltage calculations in each light state.

The real voltages were then used to determine whether the OFF bipolar cell at the given voltage would initiate glutamate release onto the OFF ganglion cell, using the activation voltage of bipolar cell $Ca^{2+}$ channels of -50 mV (Singer and Diamond, 2006). Any voltage below -50 mV was assumed to cause no glutamate release, so a minimum threshold was applied to the currents in Figure 21C so that any current that would cause a hyperpolarization of more than -50 mV was set to the value that would hyperpolarize to -
50 mV (-3 pA). After this nonlinear component was added, the new threshold-corrected center-surround bipolar cell current distribution was constructed (Figure 21E). Seven bipolar cells (OFF bipolar cell dendritic diameter = ~ 30 µm) were chosen as the number of bipolar cells that cover a large percentage of the excitatory center (dendrites, ~300 µm) of an OFFα ganglion cell in a single 2D plane in the mouse (Ghosh et al., 2004; Thyagarajan et al., 2010). Seven threshold-corrected bipolar cell center-surround current distributions were constructed with the center peak of each cell being 25 µm apart, the size of the bipolar cell center (Figure 21F). Finally, the center-surround receptive fields were summed to create a total amount of relative bipolar cell current that would cause glutamate release onto the excitatory center of the downstream ganglion cell (Figure 21G). These spatial curves represent the signal strength, or the probability that the ganglion cell will receive stronger output from the bipolar cells at its excitatory center, of all bipolar cell outputs to the ganglion cell.

Given that the peak of this simulated ganglion cell response was higher in the light adapted conditions, narrowing spatial inhibition after light adaptation, with no change in excitation, allows for the bipolar cells to provide strong output to spatially small and distinct light stimuli. To quantify this change, currents from stimuli extending 50 µm in either direction from the center from Figure 19G were averaged in dark- and light-adapted conditions. Dark-adapted relative signal strength was 1.66±0.07 pA which increased to 6.42±0.04 pA with light adaptation (Figure 21H). To determine if the reduction in L-IPSC peak amplitude or inhibitory spatial input is necessary or sufficient to increase signal strength, the model was tested by including only differences in peak
amplitude (PA) or surround size. When only the surround size became smaller with light adaptation, the signal strength was reduced from baseline, decreasing by 14.37±0.19 pA. The change in surround size alone was not sufficient to increase signal strength. When only peak amplitude became smaller with light adaptation, the signal strength remained near dark-adapted levels at 1.69±0.17 pA, and was not itself sufficient to equal total light-adapted signal strength. However, the model suggest that both decreases in bipolar cell surround size and L-IPSC peak amplitude are necessary for maximum signal output strength to ganglion cells. Our model predicts that light adaptation increases the strength of bipolar cell output in which the interactions between both the reduction in surround size and L-IPSC magnitude are necessary and vital in determining the signal strength.

Discussion:

A key aspect of retinal adaptation is increasing visual acuity, or increasing signal strength to small light stimuli, when there is abundant background illumination. This allows for a new signaling threshold for the comparison of distinct light stimuli to detect more subtle differences in contrast in the visual scene. Here we show for the first time that spatial inhibitory input to the inner retina becomes narrower, smaller and more transient with increasing background light and that these changes affect bipolar cell output strength. Our results suggest that the increase in ganglion cell sensitivity to small light stimuli with light adaptation may be in part due to changes in inhibition to OFF bipolar cells.
Dark-adapted spatial inhibition relies on extending the spread of inhibitory signaling

Our data show that OFF bipolar cell inhibition in dark-adapted conditions consists of relatively large, wide, and sustained responses to small light stimuli. This inhibitory input consists of both widely distributed glycinergic and GABAergic inhibition (Figure 22A). The wide GABAergic inhibition in the dark is expected due to the long processes of GABAergic amacrine cells, some of which can be coupled and/or fire action potentials to further extend their spatial signaling (Cook and Werblin, 1994; Heflin and Cook, 2007; Volgyi et al., 2009). Isolated GABAergic input was wider than total input (Figures 13 and 14), likely because glycinergic inputs dominate OFF bipolar cell L-IPSCs (Mazade and Eggers, 2013). However, it is also possible that blocking potential glycinergic inputs to GABAergic amacrine cells could be widening the spatial extent of GABAergic inhibition, as has been shown previously when blocking GABAergic connections between amacrine cells (Eggers and Lukasiewicz, 2010).

Although dark-adapted spatial inputs from glycinergic amacrine cells were significantly narrower than inputs from GABAergic amacrine cells, they were not as different as expected by amacrine cell morphology (Figure 17). Glycinergic inputs come from two distinct sources: rod pathway-mediated AII amacrine cell synaptic contacts and cone-activated amacrine cell inputs from both ON and OFF pathways (Mazade and Eggers, 2013), all of which should be narrow-field amacrine cells. How then could dark-adapted glycinergic inhibition to OFF bipolar cells be activated from stimuli presented hundreds of microns away? One way this could be achieved is through electrical gap junction coupling between amacrine cells that could increase their excitatory spread. AII amacrine
cells, which give input to most OFF bipolar cells (OFF 1,2,4) (Tsukamoto et al., 2001; Mazade and Eggers, 2013) are highly coupled in the very dim light conditions shown from labeling studies (Bloomfield et al., 1997; Xin and Bloomfield, 1997, 1999a) which mediated signal spread through the network (Veruki et al., 2010). This glycinergic signal spread has been shown to extend far beyond the dimensions of the dendritic arbor further suggesting the use of gap junctions in narrow-field amacrine cell networks (Chen et al., 2011). It is probable that other types of glycinergic amacrine cells could also be coupled to extend non-AII glycinergic signaling to OFF type 3 bipolar cells, like the A3 amacrine cell in the macaque (Klump et al., 2009). In fact, labeling studies have shown that there are a myriad of gap junctions located within the IPL in distinct bands, many of which likely connect amacrine cells (Marc et al., 1988). Additionally, glycinergic spatial inhibition was not different than total inhibition, suggesting that we are not altering glycinergic spatial input by blocking GABA\(_A\)Rs mediating serial inhibition. All these data suggest that the retina attempts to match these two different circuits so that all OFF bipolar cells receive uniform spatial signaling, creating a defined and wide inhibitory receptive-field surround in the dark.

*Light-adaptation shortens the extent of inhibitory spatial signaling*

While spatial inhibitory input to OFF bipolar cells is relatively wide in the dark, increasing the background luminance causes a dramatic shift (Figure 13). Both the glycinergic and GABAergic inhibitory input to OFF bipolar cells became significantly narrower, smaller and more transient after light adaptation (Figures 14, 16, 17, and 22B). Previous reports have shown that AII amacrine cell gap junctions are uncoupled
(Bloomfield et al., 1997; Xin and Bloomfield, 1997, 1999a) and activation is decreased (Dacheux and Raviola, 1986; Mazade and Eggers) after light adaptation, which would narrow and reduce glycinergic inhibition. Uncoupling of other glycinergic amacrine cells may contribute as well. Additionally, horizontal cells in the outer retina, which also contribute to surround receptive fields of bipolar cells (Zhang and Wu, 2009), become uncoupled with light adaptation (Xin and Bloomfield, 1999b), although amacrine cells likely play a larger role in light adaptation of spatial signaling (Dedek et al., 2008).

Lastly, there may be serial inhibitory connections between glycinergic amacrine cells that become activated in light-adapted conditions which also act to narrow OFF bipolar cell inhibition (Eggers and Lukasiewicz, 2010; Eggers et al., 2013). Glycinergic inhibition in light-adapted slices is similar to what we would expect given the narrow morphology of glycinergic amacrine cells.

The total GABAergic spatial inhibition also decreased in light-adapted conditions and consisted solely of GABA_A receptor-mediated input. Since GABA_C receptor-mediated currents are abolished, GABA_A and GABA_C receptor-mediated input to OFF bipolar cells may come either from different amacrine cells (Moore-Dotson et al., 2015) or light adaptation could preferentially decrease GABA_C receptor-mediated input. The narrowing of spatial inhibition was initially unexpected, as GABAergic amacrine cells have wide-field morphologies, whose processes can span hundreds of microns laterally across the retina (Kolb, 1997). However, previous reports have shown that there are GABA_A receptor-mediated inhibitory connections between amacrine cells that narrow spatial inhibition to bipolar cells (Zhang et al., 1997; Roska et al., 1998; Eggers and
Lukasiewicz, 2006a, 2010) and may be more active after light adaptation (Eggers et al., 2013). Additionally, both glycinergic and GABAergic L-IPSCs became significantly more transient, with a loss of the sustained component. This suggests that inhibition provides a sharper, more finely tuned input mediated by cone circuitry for faster modulation of stimuli in a changing environment. Overall, both glycinergic and GABAergic spatial input to OFF bipolar cells decreased with light adaptation suggesting the retina works to match the spatial signaling of distinct pathways.

*Narrower spatial inhibition to the inner retina increases signal output strength*

We found that several OFF bipolar cell signaling and cellular properties change with light adaptation, in addition to spatial changes. OFF bipolar cells are more depolarized at rest in dark-adapted conditions than rod bipolar cells ($V_{\text{rest}} = -50.3$ mV) and ON cone bipolar cells ($V_{\text{rest}} = -66.5$ mV) (Veruki et al., 2006; Oesch and Diamond, 2011; Saszik and Devries, 2012). This is likely due to the high glutamate release from the cone photoreceptors and the ionotropic glutamate receptors on OFF bipolar cells. In light-adapted conditions, OFF bipolar cells become more depolarized and the balance of excitatory and inhibitory input shifts to favoring excitation (Figure 19). With these changes, and the narrowing spatial inhibition, our results predict that light adaptation would have the overall effect of increasing the bipolar cell signal output to downstream ganglion cells (Figure 121). In dark-adapted conditions, OFF bipolar cells have large inhibitory surrounds so that if two small light stimuli are presented on the retina, one light (A), stimulates the center of the bipolar cell receptive field while the other light (B) is close enough to stimulate the wide surround of the same bipolar cell (Figure 22C). As a
result, inhibition would be stimulated, decreasing bipolar cell excitatory output. In light-adapted conditions, the inhibitory surrounds of the OFF bipolar cells shrink so that if two spatially close light stimuli are presented, light B no longer stimulates the inhibitory surround. Thus, excitatory bipolar cell output would increase and the downstream ganglion cell would see a stronger input. This concept was supported by our spatial model which predicts that total OFF bipolar cell current output to the OFF ganglion cell excitatory center will be stronger in light-adapted conditions (Figure 21). Both reduced L-IPSCs and narrower surrounds are necessary to obtain maximum output strength. Our results add support to growing evidence (Dedek et al., 2008; Protti et al., 2014) that inner retinal inhibition is important for determining ganglion cell spatial sensitivity to determine visual acuity.

In summary, we show for the first time that the spatial inhibition to OFF bipolar cells changes from widely distributed in dark-adapted conditions to a narrowly distributed in light-adapted conditions due to the narrowing spatial extent of both glycinergic and GABAergic amacrine cell input. These data predict that the output of OFF bipolar cells onto downstream ganglion cells is stronger with light adaptation, thereby fine-tuning the spatial sensitivity of ganglion cells to smaller light stimuli. Bipolar cell inhibition may be one main mechanism for controlling the gain of ganglion cell center strength under different light conditions to ultimately determine visual acuity and resolution.
**Figure 12.** OFF bipolar cell inhibition.

*Figure A.* schematic of OFF bipolar cell inhibitory input pathways. Rod photoreceptors (R) activated by dim light release glutamate onto rod bipolar cells (RB) which release glutamate onto AII amacrine cells (AII). AII amacrine cells release glycine onto OFF cone bipolar cells (OFF) (black pathway). Cone photoreceptors (C) are activated by brighter light and release glutamate onto OFF and ON cone (ON) bipolar cells. Activation of these bipolar cells in turn releases glutamate onto other wide-field GABAergic (GABA) and narrow-field glycinergic (Gly) amacrine cells which also have inputs onto OFF bipolar cells (white pathways). INL = inner nuclear layer, IPL = inner plexiform layer.

*Figure B.* diagram of experiment. Example cell morphology of an Alexa 488 filled OFF type 3 bipolar cell in a retinal slice preparation. To examine spatial inhibitory input to OFF bipolar cells, 25 µm bars of white light were presented to the retinal slice for 1 sec, as shown, using a white OLED screen mounted on the microscope. Bars of light were presented every 100 µm from the recorded cell, extending in both directions. *Scale bar = 25 µm.*
Figure 13. Light adaptation narrows the total spatial inhibitory input to all OFF bipolar cells.

A, B, example L-IPSCs recorded from an OFF type 3 bipolar cell in dark- and light-adapted conditions, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -200, 0, and 200 µm away from the recorded cell. Light-adaption greatly reduced L-IPSCs 200 µm away from the OFF bipolar cell and decreased the center L-IPSC. *Light stimulus = gray bar under L-IPSC, OFF type 3 bipolar cells respond at the offset of light.*

C, spatial inhibition curves of Q normalized to the center bar stimulus, in dark- (n = 8, black trace) and light- (n = 5, gray trace) adapted conditions. The spatial inhibitory distribution became significantly narrower with light adaptation.

D, normalized L-IPSC Q, to the center L-IPSC, compared between dark- (0–300 µm n = 17, 400–800 µm n = 8, black trace) and light- (0–300 µm n = 14, 400–800 µm n = 5, gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller after light adaptation starting at 100 µm away from the cell after which there was little to no inhibition present.

E, the average proportion of center L-IPSC response that is transient or sustained in dark- and light-adapted conditions (n = 14). L-IPSCs became significantly more transient with light adaptation.

F, spatial inhibition curves of peak amplitude in the dark- (black trace) and light- (gray trace) adapted condition. The peak amplitude distribution was significantly narrower and smaller with light adaptation.

G, L-IPSC peak amplitude compared between dark- (black trace) and light- (gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller in the light at 0-200 µm away from the cell after which there was no inhibitory input present. (* = p < 0.05, ** = p < 0.01)
Figure 14. Light adaptation narrows the isolated GABAergic spatial inhibitory input to all OFF bipolar cells. A, B, example GABAergic L-IPSCs recorded from an OFF type 3 bipolar cell in dark- and light-adapted condition, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -200, 0, and 200 µm away from the recorded cell. Light-adaption greatly reduced L-IPSCs 200 µm away from the OFF bipolar cell and decreased the center L-IPSC. Light stimulus = gray bar under L-IPSC, OFF type 3 bipolar cells respond at the offset of light.

C, spatial inhibition curves of GABAergic Q normalized to the center bar stimulus, in dark- (n = 5, black trace) and light- (n = 4, gray trace) adapted conditions. The spatial inhibitory distribution became significantly narrower with light adaptation.

D, normalized GABAergic L-IPSC Q, to the center L-IPSC, compared between dark- (0–200 µm n = 10, 300–800 µm n = 4, black trace) and light- (0–200 µm n = 7, 300–800 µm n = 4, gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller at multiple locations with no inhibition present after 600 µm.

E, the average proportion of center GABAergic L-IPSC response that is transient or sustained in dark- and light-adapted conditions (n = 7). L-IPSCs became significantly more transient with light adaptation.

F, spatial inhibition curves of GABAergic peak amplitude in the dark- (black trace) and light- (gray trace) adapted condition. The peak amplitude distribution was significantly narrower and smaller with light adaptation.

G, GABAergic L-IPSC peak amplitude compared between dark- (black trace) and light- (gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller at multiple locations with no inhibition present after 300 µm. (* = p < 0.05, ** = p < 0.01)
Figure 15. Light adaptation abolishes the isolated GABA\textsubscript{C} spatial inhibitory input to OFF bipolar cells.

\(A, B\), example GABA\textsubscript{C} receptor-mediated L-IPSCs recorded from an OFF type 4 bipolar cell in the dark- and light-adapted condition, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -200, 0, and 200 µm away from the recorded cell. Light-adaptation abolished all GABA\textsubscript{C} R-mediated input. \textit{Light stimulus = gray bar under L-IPSC, OFF type 4 bipolar cells respond at the onset of light}\n
\(C, D\), spatial inhibition curves of GABA\textsubscript{C} receptor-mediated Q normalized to the center bar stimulus, and of peak amplitude in the dark- (black trace) and light- (gray trace) adapted condition. There was no GABA\textsubscript{C} receptor-mediated inhibition in the light (\(n = 6\)).

\(E\), dark-adapted spatial distribution curves of normalized GABA\textsubscript{C} receptor-mediated (\(n = 6\), closed circles) and total GABAergic (\(n = 4\), open circles) spatial input. GABA\textsubscript{C} receptor-mediated spatial input was significantly narrower than total GABAergic input. (\(* = p < 0.05\), \(** = p < 0.01\)
Figure 16. Light adaptation narrows the isolated glycinergic spatial inhibitory input to OFF bipolar cells.

A,B, example glycinergic L-IPSCs recorded from an OFF type 1/2 bipolar cell in dark- and light-adapted condition, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -200, 0, and 200 µm away from the recorded cell. Light-adaption significantly reduced L-IPSCs 200 µm away from the OFF bipolar cell and decreased the center L-IPSC. Light stimulus = gray bar under L-IPSC, OFF type 1/2 bipolar cells respond at the onset of light.

C, spatial inhibition curves of glycinergic Q normalized to the center bar stimulus, in dark- (n = 10, black trace) and light- (n = 4, gray trace) adapted conditions. The spatial inhibitory distribution became significantly narrower with light adaptation.

D, normalized glycinergic L-IPSC Q, to the center L-IPSC, compared between dark- (0–300 µm n = 20, 400–800 µm n = 10, black trace) and light- (0–300 µm n = 9, 400–800 µm n = 4, gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller at multiple locations with little to no inhibition remaining after 500 µm away.

E, the average proportion of center glycinergic L-IPSC response that is transient or sustained in dark- and light-adapted conditions (n = 9). L-IPSCs became significantly more transient with light adaptation.

F, spatial inhibition curves of glycinergic peak amplitude in the dark- (black trace) and light- (gray trace) adapted condition. The peak amplitude distribution was significantly narrower and smaller with light adaptation.

G, glycinergic L-IPSC peak amplitude compared between dark- (black trace) and light- (gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller in the light at multiple locations with no little to no inhibition beginning at 500 µm away. (* = p < 0.05, ** = p < 0.01)
Figure 17. GABAergic spatial inhibition was wider that glycinergic input in dark- but not light-adapted conditions.

A, spatial inhibition curves of Q normalized to the center bar stimulus in the dark-adapted retina of isolated GABAergic (n = 4, closed circles) and isolated glycinergic (n = 10, open circles) bipolar cell L-IPSCs. GABAergic spatial input was significantly wider than glycinergic spatial input.

B, spatial inhibition curves of Q normalized to the center bar stimulus in the light-adapted retina of isolated GABAergic (n = 4) and isolated glycinergic (n = 4) bipolar cell L-IPSCs. There was no difference between the two groups (p = 0.52).

C, spatial inhibition curves of normalized peak amplitude, to the center bar stimulus, in the dark-adapted retina of isolated GABAergic and isolated glycinergic bipolar cell L-IPSCs. GABAergic spatial peak amplitudes were significantly wider than glycinergic L-IPSC peak amplitudes.

D, spatial inhibition curves of normalized peak amplitude in the light-adapted retina of isolated GABAergic and isolated glycinergic bipolar cell L-IPSCs. There was no difference in peak amplitude between the two groups (p = 0.22). (** = p < 0.01)
Figure 18. Light adaptation narrows the total spatial inhibitory input to OFF bipolar cells under similar intensity step conditions. 

A,B, example L-IPSCs recorded from an OFF type 3 bipolar cell in dark and light conditions with similar stimulus intensity steps, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -200, 0, and 200 µm away from the recorded cell. Light-adaptation greatly reduced L-IPSCs 200 µm away from the OFF bipolar cell and decreased the center L-IPSC. Light stimulus = gray bar under L-IPSC, OFF type 3 bipolar cells respond at the offset of light. 

C, spatial inhibition curves of Q normalized to the center bar stimulus, in dark- (black trace) and light- (gray trace) adapted conditions (n = 4). The spatial inhibitory distribution became significantly narrower with light adaptation. 

D, normalized L-IPSC Q, to the center L-IPSC, compared between dark- (black trace) and light- (gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller in the light starting at 100-600 µm away from the cell with little inhibition remaining after 200 µm. 

E, the average proportion of center L-IPSC response that is transient or sustained in dark and light conditions (n = 4). L-IPSCs became significantly more transient with light adaptation. 

F, spatial inhibition curves of peak amplitude in the dark- (black trace) and light- (gray trace) adapted condition. The peak amplitude distribution was significantly narrower and smaller with light adaptation. 

G, L-IPSC peak amplitude compared between dark- (black trace) and light- (gray trace) adapted conditions at each stimulus distance. The proportion of inhibition was significantly smaller in the light at 0-500 µm away from the cell with no inhibition present after 300 µm. (* = p < 0.05, ** = p < 0.01)
Figure 19. Light adaptation decreases inhibition to OFF bipolar cells in response to a small light stimulus without changing excitatory input.

A, example L-IPSC from an OFF type 3 bipolar cell in response to a 1 s flash of a 25 µm bar of light, presented directly over the cell. The L-IPSC decreased with light adaptation. Light stimulus = gray bar under L-IPSC, OFF type 3 bipolar cells respond at the offset of light.

B, example L-EPSC from the same cell in panel A in response to a 1 s flash of a 25 µm bar of light, presented directly over the cell. There was no change in the excitatory response with light adaptation.

C, peak amplitude of the L-IPSCs in dark- and light-adapted conditions (n = 12). There was a significant decrease in the magnitude of the response with light adaptation.

D, peak amplitude of the L-EPSCs in the dark- and light-adapted conditions (n = 8). There was no change in the magnitude of the response.

E, the proportion of excitatory and inhibitory input to OFF bipolar cells in dark- and light-adapted conditions. In the dark, OFF bipolar cells receive relatively equal amounts of excitatory (44.0±12.0%) and inhibitory (56.0±15.0%) input. In the light, OFF bipolar cells receive a larger proportion of excitatory input (58.2±9.1%). (* = p < 0.05)
Figure 20. OFF bipolar cells become more depolarized under light-adapted conditions. 
A, example current-clamp recordings from OFF bipolar cells under dark- (black trace) and the light-adapted (gray trace) conditions from an OFF type 1/2 bipolar cell. 
B, the mean and individual resting membrane potentials of OFF bipolar cells in the dark- and light-adapted conditions. The resting membrane voltage is significantly more depolarized in the light, from $-44.4\pm1.9 \text{ mV}$ to $-37.6\pm2.8 \text{ mV}$ ($n = 6$). (* = $p < 0.05$)
Figure 21. Light-adapted narrowing and reduction of spatial inhibition increases the strength of OFF bipolar cell output.

A, OFF bipolar cell spatial properties. In dark-adapted intensity-step controlled conditions, the OFF bipolar excitatory center is 25 µm wide and the inhibitory surround radius extends 600 µm wide. In light-adapted contrast-controlled conditions, the OFF bipolar cell center remains 25 µm wide whereas the surround radius decreases to 200 µm wide.

B, OFF bipolar cell excitatory center (upper) and inhibitory surround (lower) Gaussian distributions using conductance-adjusted scaled peak amplitudes. There is no significant change in excitatory center spatial distributions with light adaptation however, the light-adapted inhibitory spatial distribution (gray trace) became smaller and narrower.

C, difference of excitatory and inhibitory Gaussian distributions. Inhibition has less impact on the OFF bipolar cell center-surround distribution in the light than in the dark.

D, calculated real potentials from the EPSPs caused by a current from the center-surround distributions. Under light-adapted conditions, the combination of excitatory and inhibitory current inputs depolarizes the OFF bipolar cell more at a given stimulus distance than in the dark.

E, threshold-corrected center-surround distributions in the dark- (black trace) and light-adapted (gray trace) conditions.

F, seven threshold-corrected bipolar cell center-surround current distributions constructed with the center peak of each cell being 25 µm apart, summed to obtain total bipolar cell output

G, model of the total current output of seven summed threshold-corrected OFF bipolar cell center-surround receptive field distributions (correlated to input the OFF ganglion cell would receive). This bipolar cell signal strength represents the probability that the collective OFF bipolar cell group comprising the center receptive field of the OFF ganglion cell would cause release leading to a voltage change in the ganglion cell. Light adaptation increases the strength of the input to the OFF ganglion cell excitatory center to small stimuli.

H, relative signal output strength under several model manipulations. Dark-adapted signal strength largely increased with light adaptation (dark gray bar). When only bipolar cell surround size changes were included in the model, signal strength decreased from dark-adapted conditions (white bar). When only bipolar cell L-IPSC peak amplitudes were included in the model, signal strength did not change from dark-adapted conditions (light gray bar). The interaction between the surround size and peak amplitude changes are necessary to increase signal strength in the light. *Error bars = SEM of signal strength values across 100 µm of ganglion cell center.*
Figure 22. Spatial circuitry models in dark- and light-adapted conditions.

A, in dark-adapted conditions, OFF bipolar cells receive wide spatial inhibition from wide-field GABAergic amacrine cells. Coupling between both AII and other glycinergic amacrine cells likely contribute to increasing the wide spatial spread of glycinergic signals to OFF bipolar cells.

B, in light-adapted conditions, OFF bipolar cells receive spatially narrow glycinergic input likely due to uncoupling of AII and other glycinergic amacrine cells. Light stimuli distant from the bipolar cell likely active serial inhibitory connects between GABAergic amacrine cells which would shorten spatial GABAergic signals to OFF bipolar cells.

C, functional schematic of changing bipolar cell center-surround sizes. In dark-adapted conditions, OFF bipolar cells receive wide inhibition so their inhibitory surrounds are large. If two small spots of light are presented to the retina, spot A stimulates excitatory output from the center of the bipolar cell while spot B stimulates surround inhibitory connections to the OFF bipolar cell. Overall output is reduced in this instance due to the addition of inhibitory input. In light-adapted conditions, OFF bipolar cells receive narrow inhibition so their inhibitory surrounds are small. Now, spot B does not stimulate inhibitory surround and there is no reduction in excitatory bipolar cell output from spot A. Thus, the strength of the bipolar cell output in the light-adapted case is stronger.
Table 1: Spatial Model Parameters

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Dark-adapted</th>
<th>Light-adapted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory Fitted Gaussian distribution $\sigma$</td>
<td>213.15 µm</td>
<td>83.88 µm</td>
</tr>
<tr>
<td>Excitatory Fitted Gaussian distribution $\sigma$</td>
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<td>30.00 µm</td>
</tr>
<tr>
<td>Average Real Inhibitory Peak Amplitude</td>
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<td>11.99±3.43 pA</td>
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<tr>
<td>Average Real Excitatory Peak Amplitude</td>
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<td>16.67±2.59 pA</td>
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<td>Conductance-scaled Inhibitory Peak Amplitude</td>
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<td>4.20 pA</td>
</tr>
<tr>
<td>Conductance-scaled Excitatory Peak Amplitude</td>
<td>12.47 pA</td>
<td>11.05 pA</td>
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Data are average values where applicable.
CHAPTER 4: DOPAMINE D1 RECEPTORS ARE SUFFICIENT TO ELICIT LIGHT-DEPENDENT CHANGES IN SPONTANEOUS INHIBITION TO OFF BIPOLAR CELLS BUT NOT LIGHT-EVOKED INHIBITION

Introduction:

In order to accurately encode visual signals under different light conditions, the retina changes its signaling to highlight the important aspects of the visual scene. Under dim light conditions, retinal signaling works to maximize detection and sensitivity as opposed to under bright conditions where visual acuity is maximized. To move between these two states, the retina adapts at both the photoreceptor level (Tamura et al., 1991; Woodruff et al., 2008) and inner retinal network level, consisting of excitatory bipolar cell and inhibitory amacrine cell connections (Green et al., 1975; Naka et al., 1979; Green and Powers, 1982; Shapley and Enroth-Cugell, 1984; Page-McCaw et al., 2004; Dunn et al., 2006; Dunn et al., 2007). Photoreceptor light information, consisting of dim light sensed by rods and bright light sensed by cones, is sent to bipolar cells that synapse onto ganglion cells, the output neurons of the retina, and onto inhibitory amacrine cells which shape bipolar cell-ganglion cell signaling (Dong and Werblin, 1998; Eggers and Lukasiewicz, 2006b; Sagdullaev et al., 2006; Eggers et al., 2007; Eggers and Lukasiewicz, 2010).

The change of retinal circuitry and receptive fields with light adaptation has been studied at the ganglion and bipolar cell level. Ganglion cells increase their spatial sensitivity and signaling strength to small stimuli with light (Barlow et al., 1957; Dedek et al., 2008; Farrow et al., 2013). This increased acuity is due to decreases in ganglion cell excitatory center size (Merwine et al., 1995; Troy et al., 1999) or decreases in ganglion cell and
bipolar cell surround size and strength (Troy et al., 1993; Merwine et al., 1995; Troy et al., 1999; Dedek et al., 2008; Farrow et al., 2013). Inhibition at the bipolar cells is especially important in retinal spatial resolution changes of the ganglion cells (Flores-Herr et al., 2001; Russell and Werblin, 2010; Buldyrev and Taylor, 2013; Protti et al., 2014). One likely mechanism for mediating the bipolar and ganglion cell receptive field changes with light adaptation is dopamine.

Dopamine is released from dopaminergic amacrine cells in the light (Witkovsky, 2004) and plays a key role in in modulating many retinal pathways (Godley and Wurtman, 1988; Boatright et al., 1989; Doyle et al., 2002). Dopamine signaling is important for light adaptation (Witkovsky, 2004), and modulates OFF ganglion cell receptive field sensitivity possibly through changes in bipolar cell inhibition (Jensen and Daw, 1984; Maguire and Smith III, 1985; Jensen and Daw, 1986; Jensen, 1989; Jensen, 1991, 1992; Maguire and Hamasaki, 1994) while controlling the coupling state of the retina, especially the rod pathway AII amacrine cell (Hampson et al., 1992; Mills and Massey, 1995; Kothmann et al., 2009). Dopamine activates D1 receptors located on bipolar, amacrine, and ganglion cells (Veruki and Wässle, 1996; Nguyen-Legros et al., 1997) which can modulate inhibitory inputs. Specifically, it was shown that dopamine potentiates GABA_A receptor currents (Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995), decreases GABA_C receptor-mediated currents (Feigenspan and Bormann, 1994b; Wellis and Werblin, 1995), and modulates amacrine cell release (Pycock and Smith, 1983; Kato et al., 1985; O'Brien and Dowling, 1985; Calaza et al., 2001). These studies suggest that dopamine receptors play a role in
retinal inhibitory network adaptation to luminance level although the magnitude, timing, and localization of release to D1 receptors on bipolar cells is not known.

One specific pathway that dopamine may modulate with light adaptation is the retinal OFF pathway, principally, the inhibitory inputs to the OFF bipolar cells. This in particular may be important in retinal network adaptation, as inhibitory inputs switch between rod and cone sources at the OFF bipolar cell nexus (McGuire et al., 1984; Strettoi et al., 1990; Strettoi et al., 1992; Chun et al., 1993; Trexler et al., 2001; Deans et al., 2002; Mazade and Eggers, 2013). Activation of D1 receptors on the OFF bipolar cells or amacrine cells may be the mechanism for the changes seen in the source and spatial extent of inhibition to the OFF pathway with light adaptation, which increased the strength of OFF bipolar cell output to ganglion cells to small light stimuli (Mazade and Eggers, 2013; Mazade and Eggers, 2015 Chapter 3). Dopaminergic modulation may be an important contribution to bipolar cell signaling changes thought to drive ganglion cell receptive field sensitivity changes with light adaptation.

Here we investigated how the activation of D1 receptors affects both spatial extent of light-evoked inhibition and the spontaneous inhibitory inputs to OFF bipolar cells. If D1 receptors are directly mediating the changes in OFF bipolar cell inhibition with light, then activation of dopaminergic pathways should mimic light-adapted changes. We found that activation of D1 receptors was not sufficient to fully elicit light-adapted inhibitory changes to OFF bipolar cells in response to a light stimulus, instead only partially mediated spatial changes. However, the decreases in spontaneous inhibitory activity to
OFF bipolar cells seen in previous reports was obtained by activation of D1 receptors. These data support the idea that dopamine is playing a role in shaping OFF bipolar cell receptors changes and partially mediating light-evoked spatial changes with light adaptation.

**Methods:**

*Methode retinal slice preparation*

All animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). As described previously (Eggers and Lukasiewicz, 2006a; Eggers et al., 2013; Mazade and Eggers, 2013), male mice (C57BL/6J strain, Jackson Laboratories, Bar Harbor, ME, USA) 35-60 days of age were euthanized using carbon dioxide and their eyes enucleated. The cornea and lens were removed and the eyecup was incubated for 20 min in cold extracellular solution (see Solution and Drugs) with 800 units/mL of hyaluronidase to dissolve the remaining vitreous humor. The hyaluronidase solution was then replaced with ice cold, oxygenated extracellular solution and the retina was dissected out of the eyecup. Following removal, the retina was trimmed into one large flat rectangle by removing the peripheral retina and leaving only the central retina surrounding the optic disc. A nitrocellulose membrane filter paper (0.45 μm pore size, Millipore, Ireland) was placed on the retina section which was transferred to a hand chopper. An average of six 250 μm slices were cut, rotated 90º, and mounted onto glass cover slips using vacuum grease. Cells used from these slices were never more than 700 μm away from the center of the retina and only cells near the center of each slice were used for recordings in order to ensure accurate spatial distances. The tissue
was maintained in oxygenated extracellular solution at room temperature. All dissection procedures were performed under infrared illumination to preserve the light sensitivity of the preparations.

Solutions and drugs

The extracellular recording solution used for dissection and to examine light-evoked currents contained (in mM) 125 NaCl, 2.5 KCl, 1 MgCl2, 1.25 NaH2PO4, 2 CaCl2, 20 glucose, and 26 NaHCO3 and was bubbled with 95% O2-5% CO2. For voltage clamp recordings, the intracellular solution contained (in mM) 120 CsOH, 120 gluconic acid, 1 MgCl2, 10 HEPES, 10 TEA-Cl, 10 phosphocreatine-Na2, 4 Mg-ATP, 0.5 Na-GTP, 10 EGTA and 50 μM Alexa Fluor 488 (Invitrogen, Carlsbad, California, USA) and was adjusted to pH 7.2 with CsOH. To isolate the inhibitory receptor inputs, SR-95531 (SR, 20 μM) to block GABA\textsubscript{A} receptors, (1,2,5,6-tetrahydropyridine-4yl) methyphosphinic acid (TPMPA, 50 μM) to block GABA\textsubscript{C} receptors, and strychnine (1 μM) to block glycine receptors were used. To test dopamine D1 receptors, the D1 agonist SKF 38393 (20μM, Tocris) was used. All drug solutions were washed on the slice for 5 min before recordings began using a gravity-driven superfusion system (Cell Microcontrols, Norfolk, VA) at a rate of ~1-2 mL/minute. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Whole-cell recordings

Whole cell patch clamp recordings, sampled at 10 kHz, were made from OFF bipolar cells in retinal slices. Light-evoked inhibitory post synaptic currents (L-IPSCs) and
spontaneous (s)IPSCs were recorded from retinal bipolar cells voltage clamped to 0 mV, the reversal potential of nonselective cation channel currents. Bipolar cell recordings were stable and no rundown of the light response was observed over the recording period. Liquid junction potentials of 20 mV were corrected at the beginning of each recording. Electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) on a P97 Flaming/Brown puller (Sutter Instruments, Novato, California, USA) and had resistances of 5-7 MΩ. Mice were dark-adapted overnight, and all recording procedures were performed in the dark under infrared illumination to preserve the light sensitivity of the slices. Recordings were made in extracellular solution heated to 32°C, using thin stage and inline heaters (Cell Microcontrols, Norfolk, VA). Responses were filtered at 6 kHz with the four-pole Bessel filter on a Multi-clamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, California, USA) and digitized with a Digidata 1140 data acquisition system (Molecular Devices, Sunnyvale, California, USA).

*Morphological identification of cells*

Alexa 488 included in the recording pipette was used to label OFF bipolar cell subtypes. They were classified as OFF bipolar cells based on their axonal morphologies and stratification within the inner plexiform layers and the position of their somas in the inner nuclear layer (Ghosh et al., 2004). The cells were imaged with Nikon Digital Sight camera with Elements software using a Nikon Intensilight C-HGFIE Fluorescent lamp (Nikon Instruments, Tokyo, Japan). Detailed analysis of axon terminal morphology and response properties was performed previously on all OFF bipolar cell subtypes (Mazade
and Eggers, 2013) and these same criteria were used to identify OFF bipolar cells in the current study.

*Light stimuli*

Bar stimuli (25 µm wide) were presented using a white organic light-emitting diode (OLED Microdisplay, eMagin EMA-100503 SXGA Monochrome White XL, Bellevue, WA) projected through the camera port of the microscope, which elicited strong responses in both dark- and light-adapted conditions (See *Chapter 3*). Recordings were from cells located within the regions of mixed green/UV cone opsin input (Applebury et al., 2000; Haverkamp et al., 2005) which ensured that all possible pathways were present. The stimulus intensity (7.83 x 10⁴ photons/µm²/sec) was controlled with custom MatLab software by controlling the intensity, size, location, and duration of the bar stimuli through the OLED screen. A long light stimulus (1 s) was used to determine the type of inhibition to all recorded bipolar cells as well as to illicit robust responses with a very small stimulus.

*Data analysis and statistics*

L-IPSC traces from a given response condition were averaged using Clampfit software (Molecular Devices, Sunnyvale, California, USA) and the charge transfer (Q) and peak amplitude were measured in each condition. Due to the significant amount of spontaneous activity, it was difficult to measure a peak from OFF bipolar cell L-IPSCs. Therefore to estimate the peak, the sampling rate of averaged traces was reduced (50 fold) and each point was replaced with the average of those data points to limit variations.
due to spontaneous activity. To determine changes in total current, the $Q$, the magnitude of the response, was measured in Clampfit over the length of the response, typically 1-2 seconds, using the same time parameters in each condition for the same cell. The baseline $Q$ was added to the $Q$ standard deviation which was subtracted from all raw $Q$ measurements to negate any current due to baseline or spontaneous events. All example response traces show responses to the center bar stimulus directly over the recorded cell or 200 or 400 μm away from the cell.

For spatial distribution curves, light-evoked $Q$’s were normalized to the maximal response in the dark-adapted condition and peak amplitude raw data were used. Normalized $Q$’s were used to control for variability between bipolar cell L-IPSCs so that spatial extent could be accurately compared and visualized between light conditions. Raw peak amplitude were used as a reliable response magnitude value. The normalized and raw data were plotted against the distance the stimulus was from the cell. In order to compare before and after drug application at any given stimulus distance as well as to average out any spatial variation since there should be equal inhibitory inputs from stimuli equidistant from the cell, both sides of the spatial distributions at equal distances were averaged and plotted as bar graphs.

To measure timing differences between treatments, the transient and sustained components of center L-IPSCs were measured. The transient L-IPSC component was measured as the first 20% $Q$ of the response based on the 1 sec light stimulus, similar to Nobles et al., 2012 for ganglion cell responses to long light stimuli (Nobles et al., 2012).
Sustained L-IPSC components were measured by subtracting the transient Q from the total Q of the each center L-IPSC within each light conditions. Proportions were calculated by dividing the transient and sustained values by the total Q.

Spontaneous inhibitory post-synaptic current (sIPSC) data were analyzed using Clampfit software. A sIPSC template was created for each data file, using the average of more than 10 prototypical events from the recording. The software used this template to automatically detect spontaneous events which were manually accepted or rejected based on strict criteria: events used to calculate the frequency were rejected if they appeared to be noise and events used to calculate the average peak amplitude were rejected if they appeared to be noise or consisted of two or more overlapping events. Frequency was calculated by dividing the number of events by the recording time. Peak amplitude and inter-event interval histogram distributions were normalized to the number of events.

2-way Analysis of Variance (ANOVA) with Student-Newman-Keuls (SNK) posthoc test was used to compare spatial distributions before and after D1 receptor activation as well as between response characteristics at each stimulus distance. All averaged data are reported as mean ± SEM. The distribution of sIPSC peak amplitude and inter-event interval values were compared using the Kolmogorov-Smirnov test (K-S) with significance being $p < 0.05$. Student’s $t$-test (2-tailed, paired) was used to compare before and after drug application for averaged sIPSC parameters. All differences were considered significant when $p < 0.05$ (*) and $p < 0.01$ (**).
Results:

*D1 receptors are not sufficient to elicit OFF bipolar cell spatial inhibitory changes with light adaptation*

Inner retinal neurons, consisting of bipolar and amacrine cells, express dopamine D1 receptors on their terminals (Veruki and Wässle, 1996; Nguyen-Legros et al., 1997) (Figure 21). OFF bipolar cells in particular not only receive dopaminergic input but also receive glycine, GABA\textsubscript{A}, and GABA\textsubscript{C} receptor-mediated inhibition from glycinergic connections from the rod pathway and glycinergic and GABAergic connections from cone pathways (Euler and Wässle, 1998; Ivanova et al., 2006; Eggers et al., 2007) (Figure 23). These inhibitory inputs to OFF bipolar cells have been shown to change with light adaptation in a source, magnitude, and spatial input manner (Mazade and Eggers, 2013). Since both OFF bipolar cells and the amacrine cells that give input to the OFF bipolar cells express D1 receptors, and dopamine is known to be released under light-adapted conditions (Witkovsky, 2004), then activation of D1 receptors may be playing a direct role in the inhibitory changes to OFF bipolar cells with light adaptation. Though it is known that dopamine signaling can modulate inhibitory currents (Pycock and Smith, 1983; Kato et al., 1985; O'Brien and Dowling, 1985; Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995; Calaza et al., 2001) and affect ganglion cell receptive fields likely through bipolar cell inhibition (Jensen and Daw, 1984; Maguire and Smith III, 1985; Jensen and Daw, 1986; Jensen, 1989; Jensen, 1991, 1992; Maguire and Hamasaki, 1994), it is unknown how activation of dopamine receptors directly affects bipolar cell surround inhibition as well as specific inhibitory inputs. To investigate this, we mapped the spatial extent of OFF bipolar cell inhibition
using a long light stimulus (1 s) that was flashed across a retinal slice every 100 µm (see *Chapter 3*).

We predicted that spatial distribution of inhibition to OFF bipolar cells would become narrower with activation of D1 receptors with the D1 receptor agonist SKF 38393 due to the narrowing of spatial inputs with light adaptation shown in *Chapter 3*. We found that total inhibition became significantly narrower and smaller under SKF conditions (Figure 24). OFF bipolar cell L-IPSCs in the dark were robust with inhibition activated at distances more than 400 µm away from the recorded cell (Figure 24A). After SKF application, most OFF bipolar cells did not receive any inhibition if stimuli were presented more than 400 µm away from the cell (Figure 24B). The spatial distribution of Q’s normalized to the center bar became significantly narrower with light adaption (p < 0.01) (Figure 24C). To compare the normalized Q between light conditions at each stimulus distance, both sides of the spatial distribution curves were averaged and values plotted. There was a significantly smaller proportion of inhibition in the SKF condition than in the dark-adapted case at 400 away from the recorded cell (p < 0.01), with little to no inhibition remaining at the furthest distances with SKF (Figure 22D). OFF bipolar cell center L-IPSCs also became significantly more transient, with a decrease in sustained response with SKF (p < 0.05) (Figure 24E), which was not different than the decrease in sustained portion of the response with light adaptation (data not shown, p = 0.15). The L-IPSC peak amplitude spatial distribution also became significantly smaller and narrower in SKF conditions (p < 0.01) (Figure 24F). There was a significantly smaller proportion of inhibition in SKF conditions than in the dark-adapted conditions at 0 and 400 µm away.
from the recorded cell ($p < 0.05$), with reduced inhibition on average remaining from stimuli presented 100 – 300 µm, after which, little inhibition remained (Figure 24G). The average peak amplitude for the center stimulus in the dark was $29.6\pm13.0$ pA which significantly decreased to $7.6\pm2.2$ pA after SKF application ($p < 0.05$).

Next, to determine if activation of D1 receptors caused changes equivalent to light-adapted changes in spatial input, we compared the spatial Q and peak amplitude distributions between light-adapted conditions from Chapter 3 and SKF conditions. This allowed us to verify whether D1 receptor signaling is sufficient to produce inhibitory changes with light adaptation. Although there was a significant decrease in spatial inhibitory input to OFF bipolar cells with SKF application (Figure 24C), the spatial distribution was still significantly wider than under light-adapted conditions ($n = 5$) (Figure 25A). Additionally, the spatial inhibitory peak amplitude distribution was also significantly larger under SKF than light-adapted conditions (Figure 25B). The average peak amplitude for the center stimulus in the light was $4.35\pm1.52$ pA which significantly different from the peak amplitude with SKF application ($8.40\pm2.14$ pA). Therefore, D1 receptor activation was only able to partially elicit light-adapted changes in spatial inhibitory distribution to OFF bipolar cells. Taken together, these results suggest that there may be other factors involved in narrowing of spatial inhibitory input to OFF bipolar cells in the light that may be independent of dopaminergic signaling through D1 receptors.
D1 receptor activation reduces sIPSC peak amplitude and frequency resembling light-adapted changes

Though SKF did not fully mimic light-adapted changes in OFF bipolar spatial inhibition, it did significantly modulate the spatial extent of inhibition. We next wanted to determine if activation of D1 receptors could be affecting sIPSCs to the OFF bipolar cells. Previous work has reported that light adaptation decreases OFF bipolar sIPSC peak amplitude and frequency (Mazade and Eggers 2013, Chapter 2). Since dopamine is released with light, it may be playing a role in modulating the inhibitory receptors on OFF bipolar cells.

Dopaminergic signaling has been shown to modulate GABA$_A$ and GABA$_C$ receptor-mediated currents (Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995) so it is likely that SKF activation of D1 receptors could reduce sIPSC parameters in a similar manner as light. sIPSCs were recording from OFF bipolar cells under dark-adapted conditions and with SKF application. The sIPSC peak amplitude distribution decreased significantly (averaged peak amplitude from 27±4 to 17±5 pA, K-S p < 0.01, Figure 26A,B,C). This is consistent with and not significantly different than the decrease in sIPSC peak amplitude with light-adaptation (normalized SKF decrease = 63.7±12.9%, n = 6, normalized light-adapted decrease = 82.0±7.4%, n = 18) (Figure 26E). The sIPSC frequency normalized to the dark-adapted condition significantly decreased with SKF application to 48.2±10.0% (n = 7, p < 0.05) and was not significantly different from the light-adapted decrease (62.1±8.8%, n = 15) (Figure 26E). As a result, the inter-event intervals of the sIPSCs also significantly increased with SKF application (24.64 ms under dark-adapted conditions to 96.90 ms with SKF application) (Figure 26D). Taken together, these results suggest that activation of the D1 receptors, may be
one of the main mechanisms for the decreased spontaneous inhibitory events to OFF bipolar cells with light adaptation. This also implies that D1 receptors may be important for modulating the background spontaneous currents in these cells with light, which works to increase the inhibitory signal-to-noise ratio of OFF bipolar cells, as shown in Mazade and Eggers, 2013, Chapter 2.

**D1 receptors are sufficient to elicit OFF bipolar cell spatial glycinergic changes with light adaptation of L-IPSC charger transfer but not peak amplitude**

Our previous work has shown that glycinergic input to the OFF bipolar cells decreases with light adaptation likely due to reduced rod pathway signaling (Mazade and Eggers 2013) as well as that glycinergic spatial input becomes narrower and smaller (Mazade and Eggers 2015, Chapter 3). Since activation of D1 receptors causes significant narrowing of total spatial inhibitory input to OFF bipolar cells (Figure 23), we wanted to determine whether dopaminergic signaling acts on glycinergic amacrine cell inputs to contribute to these changes. Using the same stimulus parameters described above, pharmacologically isolated glycinergic spatial inhibitory input (in SR and TPMPA to block GABA receptors) was measured in the dark and with SKF application. Center bar stimuli elicited robust L-IPSCs with smaller but robust L-IPSCs at 400 µm away (Figure 27A). This was expected from recordings performed in Chapter 3. However, SKF application reduced all L-IPSCs with absent responses after 400 µm away (Figure 27B). The spatial distribution of normalized glycinergic Q’s became significantly more narrow in the SKF condition (p < 0.01) (Figure 27C), similar to total inhibition. Though the proportion of glycinergic inhibition with SKF was on average smaller than the proportion
in the dark-adapted state, the pairwise comparisons between conditions were not
significant at any stimulus distance (Figure 27D). OFF bipolar cell center glycine
gicergic L-IPSCs did not become significantly more transient unlike the total inhibitory responses
from Figure 21 (p = 0.61) (Figure 27E). The peak amplitude distribution of glycine
gicergic input became significantly narrower in the SKF treated retina (p < 0.01) (Figure 27F).
However, when comparing between dark-adapted and SKF conditions at each stimulus
distance, the peak amplitude was on average smaller but not significant (Figure 27G).
The average peak amplitude for the center slightly decreased to 11.04±3.90 pA after SKF
application from 11.04±3.90 18.16±9.36 pA in the dark (p > 0.05).

These results suggest that narrowing of glycine
gicergic spatial inhibition to OFF bipolar cells
may at least in part be a result of dopaminergic signaling in the light. However, to
determine if the values between light-adapted and SKF-applied responses correlate, we
compared the spatial Q and peak amplitude distributions between the glycine
gicergic light-adapted conditions from Chapter 3 and glycine
gicergic SKF conditions. We found a
significant decrease in spatial inhibitory input to OFF bipolar cells with SKF application
(Figure 27C), which was not significantly different from the reduced glycine
gicergic spatial input under light-adapted conditions (n = 4) (Figure 28A). Although the data suggests that
activation of D1 receptors is sufficient to mediate light-adapted narrowing of spatial
gicergic Q to OFF bipolar cells, the spatial inhibitory peak amplitude distribution was
still significantly larger under SKF than light-adapted conditions (Figure 28B). The
average peak amplitude for the center stimulus in the light was 3.46±0.31 pA which was
significantly different from the peak amplitude with SKF application (11.04±3.90 pA).
These results suggest that there may be other factors involved in reducing the magnitude of glycinergic inhibition in the light while dopamine may be more responsible for spatial changes.

*D1 receptor activation reduces glycinergic sIPSC peak amplitude and frequency*

While studies have shown that dopaminergic signaling can affect GABA-mediated currents, the effects of dopamine receptor action on glycinergic currents and receptors is unknown. Since we have previously shown that glycinergic inhibition, as well as sIPSC peak amplitude and frequency, significantly decreases with light adaptation we wanted to investigate whether D1 receptors are responsible for modulating glycinergic sIPSCs (Mazade and Eggers 2013, *Chapter 2*). OFF bipolar cell sIPSCs consist of mostly glycine receptor-mediated currents, in both dark- and light-adapted conditions (Mazade and Eggers 2013, *Chapter 2*), so that light-adapted decreases in sIPSCs are likely due to decreases in glycinergic sIPSCs that may be a result of D1 receptor activation.

Glycinergic sIPSCs were isolated and measured under dark-adapted and SKF conditions. The glycinergic sIPSC peak amplitude decreased significantly (25±4 to 10±2 pA, K-S p < 0.01, Figure 29A,B,C,E). This is consistent with the decrease in dark-adapted sIPSC peak amplitude with light-adaptation (Mazade and Eggers 2013). This reduction is not significantly different than the decrease in glycinergic sIPSC peak amplitude with light-adaptation (normalized SKF decrease = 41.6±6.3%, n = 5, normalized light-adapted decrease = 66.4.0±17.3%, n = 3) (Figure 29E). The glycinergic sIPSC frequency normalized to the dark-adapted condition significantly decreased with SKF application to 57.2±10.6% (n = 6, p < 0.05) and was not significantly different from the light-adapted
decrease (58.4±16.9%, n = 3) (Figure 29E). Thus, the inter-event intervals of the glycinergic sIPSCs also significantly increased with SKF application (33.74 ms in dark-adapted conditions to 47.96 ms in SKF conditions) (Figure 29D). Taken together, these results suggest that activation of the D1 receptors, may be one of the main mechanisms for the decreasing the glycinergic spontaneous events which greatly contribute to decreasing the total sIPSCs to OFF bipolar cells with light adaptation. This also implies that D1 receptors may be important for modulating both release from glycinergic amacrine cells or the glycine receptors themselves located on the OFF bipolar cell terminals.

*D1 receptors are not responsible for abolishing GABA\textsubscript{C} receptor-mediated input to OFF bipolar cells in light-adapted conditions*

D1 receptor activation affects both total and glycine-mediated spatial inhibitory input to OFF bipolar cells, providing at least part of a mechanism for light-adapted inhibitory changes. We previously found another unique change to OFF bipolar cell inhibition with light adaptation, a complete abolishment of GABA\textsubscript{C} receptor-mediated inputs (Mazade and Eggers 2013; Mazade and Eggers, 2015 *Chapter 3*). Since GABAergic amacrine cells that release GABA onto GABA\textsubscript{C} receptors still have synaptic contacts with the OFF bipolar cell terminals, it follows that there must be something modulating the strength of the input, principally eliminating GABA\textsubscript{C} input. We wanted to investigate if activation of D1 receptors is directly involved in decreasing GABA\textsubscript{C} receptor light-evoked currents, since it plays a role in mediating other inhibitory changes. To do this, we recorded isolated GABA\textsubscript{C} receptor-mediated L-IPSCs, by blocking glycine and GABA\textsubscript{A} receptors,
in response to a bar of light presented over the center of the OFF bipolar cell, similar to the methods above, in dark and SKF conditions. We found that SKF application did not significantly reduce GABA\(_C\) L-IPSC charge transfer or peak amplitude (n = 4, p = 0.66 and p = 0.31 respectively) (Figure 30A,B). This data implies that D1 receptors are not directly involved in reducing OFF bipolar cell GABA\(_C\) receptor currents, and another factor may be playing a dominant role in this change with light adaptation.

**Discussion:**
Dopamine has been shown to be an important modulator of the retina’s ability to adapt to brighter light levels for increased visual acuity. The D1 receptors specifically are a likely candidate for changes at the OFF bipolar cell level; possibly mediating the changes reported in the spatial inhibitory surround and spontaneous currents. Here we show for the first time that D1 receptors modulate inhibition to the OFF bipolar cells which were able to partially mimic light-adapted changes of spatially evoked inhibitory responses. While inhibitory input to OFF bipolar cells became smaller and narrower, D1 receptor activation did not elicit light-adapted magnitudes of the changes. However, the spontaneous current decreases with D1 receptor activation did follow light-adapted changes. Our results suggest that dopamine acting through D1 receptors may only be part of the story for OFF bipolar cell inhibitory changes with light adaptation.

*D1 receptors partially mediate light-evoked spatial inhibition to OFF bipolar cells*

Our data show that dopamine signaling through D1 receptors partially accounts for light-adapted inhibitory changes in OFF bipolar cells. Previous studies have shown that in
dark-adapted conditions OFF bipolar cell spatial inhibitory input is wide and strong (Mazade and Eggers 2015, Chapter 3). However, with light adaptation, OFF bipolar cell inhibitory surrounds shrink and the strength of the inhibitory input decreases (Mazade and Eggers 2015, Chapter 3) leading to a shift in the excitatory-inhibitory input balance to these cells. We show that the narrowing of total spatial inhibition to these cells is partially mediated by D1 receptors (Figure 24). While the spatial input does not decrease as much as with light-adaptation (Figure 25), it is clear that D1 receptor activation can be one mechanism for decreasing OFF bipolar spatial inhibitory input. Our work supports and expands previous work that suggests dopamine is an important mediator of light-evoked signaling (Witkovsky, 2004). Additionally, it has been known that inhibition is modulated by dopamine at both the ganglion cell receptive field surround level as well as at the bipolar cell input and receptive field level (Jensen and Daw, 1984; Maguire and Smith III, 1985; Jensen and Daw, 1986; Jensen, 1989; Jensen, 1991, 1992; Maguire and Hamasaki, 1994). A recent studied looked at dopamine modulation of the OFF pathway in the light-adapted retina (Yang et al., 2012). The authors found that D1 receptors were responsible for modulating signaling to OFF ganglion cells from upstream pathways so there are likely other modulatory network effects on the OFF retinal pathway that are not flushed out in the present study. Taken together, the previous work combined with our data suggests it is highly likely that the increase in dopamine release in the light has modulatory effects on inhibition, which several studies have investigated (Pycock and Smith, 1983; Kato et al., 1985; O'Brien and Dowling, 1985; Calaza et al., 2001). Our results add to this growing body of work suggesting that dopamine signaling through the D1 receptor can contribute to bipolar cell inhibitory changes with light-adaptation.
Since the activation of D1 receptors reduced OFF bipolar inhibition, it was important to specifically test which pathways dopamine signaling may be working through to modulate the spatial inhibition to OFF bipolar cells. Previous work in Chapter 3 showed that the narrowing of spatial inhibitory input to OFF bipolar cells was due to a combination of both glycinergic and GABAergic pathway input becoming narrower and weaker (Figures 14, 16). Dopamine signaling could be acting through either pathway to elicit these changes. Our results show that dopamine signaling acting through glycinergic amacrine cell pathways also partially contributes to the changes seen with total inhibition (Figure 27). However, it appears that dopamine may be more important for modulating the spatial extent of glycinergic distributions, which were significantly smaller with SKF application, but not the magnitude of glycinergic synaptic input at each individual stimulus distance, which were not reduced to light-adapted levels (Figure 27).

Additionally, D1 receptor activation does not appear to be sufficient to modulate GABA\(c\) receptor-mediated input at all, which was unexpected since light-adaptation completely abolishes all GABA\(c\) receptor input to OFF bipolar cells (Chapter 3). While glycinergic inhibition may partially rely on dopamine signaling for modulation with light, GABA\(c\)-mediated input appears to rely on a completely different mechanism altogether.

The changes seen in the spatial surround with D1 receptor activation support our initial predication that dopaminergic signaling in the inner retina is a mechanism for light-adapted changes in OFF bipolar cell receptive field surrounds. Dopamine is known to have significant effects on electrical gap junction coupling throughout the retina, especially between the glycinergic AII amacrine cells which provide large inhibitory
input to the OFF bipolar cells (Bloomfield et al., 1997; Xin and Bloomfield, 1997, 1999a). This coupling may work to extend the spatial spread of the glycinergic signal, leading to the wide glycinergic spatial input in the dark (Veruki et al., 2010). This may be one way that the activation of D1 receptors in the dark mimics light-adapted changes, although the magnitude of the change suggest other factors are involved. Investigating the role D1 receptors play in modulating the GABAergic amacrine cells and how this affects the inhibitory input to OFF bipolar cells would be an interesting future study as dopamine has been shown to modulate GABA receptor-mediated currents and GABAergic amacrine cells mediated wide spatial input to bipolar cells and largely compose retinal inhibitory surrounds (Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995; Cook and McReynolds, 1998; Flores-Herr et al., 2001; Sinclair et al., 2004; Hoerbelt et al., 2015). Additionally, local application of D1 receptor modulators would be required for the specific study of dopamine effects on direct amacrine cell input to the bipolar cells to elucidate specific glycinergic and GABAergic modulation with dopamine signaling. However, bath application as done in the current study, or the activation of all D1 receptors, may be more representative of systemic dopamine release with light.

**OFF bipolar cell spontaneous activity is modulated by D1 receptor activation**

Contrary to the light-evoked inhibitory results, the spontaneous inhibitory currents to OFF bipolar cells were significantly modulated by activation of D1 receptors. We found that both the total and glycinergic sIPSCs became smaller and less frequent with application of the D1 receptor agonist SKF (Figures 26 and 29). These results support the
hypothesis that dopamine signaling is an important mediator of light-adapted signaling changes in the retina. Previous work has shown that spontaneous inhibitory currents to the OFF bipolar cells significantly decrease, in both peak amplitude and frequency, with light adaptation (Mazade and Eggers 2013, Chapter 2). If dopaminergic signaling is being activated under light-adapted conditions then it would follow that activation of the D1 receptors on bipolar and amacrine cell processes would mimic light-adapted changes, as seen by our results.

Activation of dopamine signaling has been shown to affect both GABA<sub>A</sub> and GABA<sub>C</sub> receptor-mediated currents, however, until now, there has been no evidence of effects on retinal glycine receptors (Dong and Werblin, 1994; Feigenspan and Bormann, 1994a; Wellis and Werblin, 1995). We found that activation of D1 receptors decreases both the peak amplitude and frequency and increases the inter-event-interval of glycinergic spontaneous inputs to the OFF bipolar cells (Figure 29D,E). Since we have not blocked action potentials and voltage-gated calcium channels, these are not true miniature events, especially as OFF bipolar cell sIPSCs can be quite large and might be multi-vesicular. This means a decrease in the amplitude could be interpreted as a change in receptor activity or release. The frequency decrease though likely represents a decrease in presynaptic release of glycine. Activation of D1 receptors may be affecting glycinergic amacrine cell release probability, due to the decrease in sIPSC frequency. Several studies have shown that dopamine modulated neurotransmitter release in the retina from GABAergic amacrine cells (O'Brien and Dowling, 1985; Calaza et al., 2001) so there may be a similar effect on glycinergic amacrine cells. Additionally, rod and cone
photoreceptor calcium currents are modulated by dopamine (Stella and Thoreson, 2000) and dopamine has even been shown to regulate synaptic transmission in non-retinal systems (Yeh et al., 1984). Since dopamine has been shown to have effects on neurotransmitter release then it is likely that the decrease in glycinergic sIPSC frequency with activation of D1 receptors reflects modulation at the presynaptic amacrine cell. There does not however appear to be solely a change in the presynaptic cell with D1 receptor activation. The peak amplitude of the glycinergic sIPSCs significantly decreased as well (Figure 29B,C,E). This suggests that the glycine receptors on the post synaptic bipolar cell are being regulated as well by D1 receptors located on the bipolar cells terminals. This could include changes in the open probability or glycine receptor number, though more work is need to distinguish this. We have shown that D1 receptors are sufficient to cause the light-adapted changes in OFF bipolar cell sIPSCs and that glycine receptors and release are modulated by dopaminergic pathways.

Non-D1 receptor factors may also contribute to OFF bipolar cell inhibitory changes with light-adaptation

Overall, the results of this study suggest that dopamine signaling through D1 receptors is only one mechanism for mediating light-adapted changes to OFF bipolar cells. There are however, multiple dopamine receptors expressed in the retina such as D4 receptors on photoreceptors and D2 receptors on the dopaminergic amacrine cells themselves (Nguyen-Legros et al., 1999; Pozdeyev et al., 2008; Jackson et al., 2009). Thus it is likely that it is the combined activation of all dopamine receptors by light that has network effects causing the inhibitory changes which are only being partially activated in our
experimental system by stimulating a single dopamine receptor subtype. Likewise, in parallel with increased dopamine release, light-adaptation by definition involves a switch between specific retinal circuits. In the dark, signaling is mediated by the rod pathway which switches to cone pathways in the light. The balance of rod and cone pathway inputs during light adaptation may in itself have network effects, combined with dopaminergic signaling, that lead to the inhibitory changes reported in earlier work (Mazade and Eggers 2013, Chapter 2; Mazade and Eggers 2015, Chapter 3). This could cause circuitry changes such that interactions between cone activated bipolar cells and amacrine cells modulate the strength of inhibitory inputs to the OFF bipolar cells. There is likely a combinatory effect between pathway activation and dopamine release which may be sufficient for causing the inhibitory changes. This would be an interesting future expansion of the present study.

Lastly, dopamine may not be the full story behind light adaptation in the retina. There are several other retinal neuromodulators that are released under varying light conditions. However, two of these may be more specifically important for light adaptation: melatonin and nitric oxide (NO). First, melatonin levels are highest in darkness and melatonin is produced by the photoreceptors and diffuses throughout the retina (Bubenik et al., 1978; Pang et al., 1980; Vivien-Roels et al., 1981; Wiechmann, 1986; Cahill and Besharse, 1992). Melatonin receptors are expressed on inner retinal neurons, including bipolar and amacrine cells with different subtype expression on different retinal cell subtypes (Wiechmann and Smith, 2001; Wiechmann and Sherry, 2013). Several studies have shown that melatonin signaling affects retinal signals, including potentiating rod pathway
signals and glycinergic inputs to ganglion cells and inhibiting dopamine signals (Dubocovich, 1983; Tosini and Dirden, 2000; Ribelayga et al., 2004; Zhao et al., 2010; Huang et al., 2013). Additionally, melatonin modulates GABA_A receptor-mediated currents in other neural systems (Boatright et al., 1994; Wan et al., 1999) and there may be similar mechanisms in the retina. NO has also been recently implicated in playing a role in retinal adaptation. NO is largely released from specialized amacrine cells and its synthesis increases in a light-dependent manner (Neal et al., 1998; Kim et al., 1999; Kim et al., 2000; Pang et al., 2010). NO has been shown to modulate light responses in many retinal cell types, but specifically was shown to regulate the temporal glutamate response properties of OFF bipolar cells (Snellman and Nawy, 2004; Vielma et al., 2012; Vielma et al., 2014). There is also evidence that NO affects the coupling state of the retina (Daniels and Baldridge, 2011). The data from these studies is highly suggestive of a role for melatonin and NO in mediating inhibitory changes with light adaptation, however, more work needs to be done to determine how they effect inner retinal signal processing.

In summary, we show that dopaminergic signaling through D1 receptors is not, on its own, sufficient to cause spatial inhibitory changes to OFF bipolar cells with light adaptation. However, activation of D1 receptors is a driving factor in the light-adapted changes in inhibitory spontaneous currents to OFF bipolar cells. Additionally, we provide the first evidence that glycine receptors and release from glycinergic amacrine cells is modulated by dopaminergic signaling pathways. These data suggest that dopamine signaling, including activation of other dopamine receptors expressed in the retina as well as other neuromodulators, are worth further investigation for their role in retinal light
adaptation and changes in inhibitory signaling. Lastly, these results further suggest that the inhibitory differences between adaptation states is likely due to multifactorial inputs involving many distinct pathways.
Figure 23. Schematic of dopaminergic circuitry connections in the inner retina. Rod photoreceptors (R) activated by dim light release glutamate onto rod bipolar cells (RB) which release glutamate onto AII amacrine cells (AII). AII amacrine cells release glycine onto OFF cone bipolar cells (OFF) and are coupled to other AII amacrine cells (black pathways). Cone photoreceptors (C) are activated by brighter light and release glutamate onto OFF and ON cone (ON) bipolar cells. Activation of these bipolar cells in turn releases glutamate onto other wide-field GABAergic (GABA) and narrow-field glycineric (Gly) amacrine cells which also have inputs onto OFF bipolar cells (white pathways). Dopaminergic amacrine cells (DA, dotted pathway) have wide-field processes and release dopamine onto D1 receptors located on bipolar and amacrine cells. INL = inner nuclear layer, IPL = inner plexiform layer.
Figure 24. D1 receptor activation narrows the total spatial inhibitory input to all OFF bipolar cells.

A,B, example L-IPSCs recorded from an OFF type 1/2 bipolar cell in dark-adapted and SKF conditions, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -400, 0, and 400 µm away from the recorded cell. SKF application greatly reduced L-IPSCs 400 µm away from the OFF bipolar cell and decreased the center L-IPSC. *Light stimulus = gray bar under L-IPSC, OFF type 1/2 bipolar cells respond at the onset of light.*

C, spatial inhibition curves of Q normalized to the center bar stimulus, in dark-adapted (n = 7, black trace) and SKF (n = 7, gray trace) conditions. The spatial inhibitory distribution became significantly narrower with light adaptation.

D, normalized L-IPSC Q, to the center L-IPSC, compared between dark-adapted (n = 8, black trace) and SKF (n = 7, gray trace) conditions at each stimulus distance. The proportion of inhibition was significantly smaller after light adaptation starting at 400 µm away from the cell after which there was little to no inhibition present.

E, the average proportion of center L-IPSC response that is transient or sustained in dark-adapted and SKF conditions (n = 7). L-IPSCs became significantly more transient with SKF application.

F, spatial inhibition curves of peak amplitude in the dark-adapted (black trace) and SKF (gray trace) conditions. The peak amplitude distribution was significantly narrower and smaller with light adaptation.

G, L-IPSC peak amplitude compared between dark-adapted (black trace) and SKF (gray trace) conditions at each stimulus distance. The proportion of inhibition was significantly smaller in the light at 0 and 400 µm away from the cell after which there was no inhibitory input present. (* = p < 0.05, ** = p < 0.01)
Figure 25. Activation of D1 receptors is not sufficient to elicit light-adapted spatial inhibition changes to OFF bipolar cells.

A, spatial inhibition curves of L-IPSC Q normalized to the center bar stimulus in the SKF (n = 7, dark gray) and light-adapted (n = 5, light gray) conditions. Light-adapted spatial input was significantly narrower than with SKF application.

B, spatial inhibition curves of peak amplitude in the SKF (dark gray trace) and light-adapted (light gray trace) conditions. The peak amplitude distribution was significantly narrower and smaller with light adaptation. (** = p < 0.01)
Figure 26. Activation of D1 receptors reduces OFF bipolar sIPSC peak amplitude and frequency.

A, example traces showing the sIPSCs from an OFF4 bipolar cell in dark-adapted (black trace) and SKF conditions (gray trace).

B, the sIPSC peak amplitude histogram distributions (normalized to number of events) of the OFF4 bipolar cell in panel A. SKF significantly reduced the sIPSC peak amplitude (K-S p < 0.01). Arrows show the average peak amplitude of the sIPSCs.

C, the peak amplitude of sIPSCs was significantly decreased in most OFF bipolar cells (n = 6) following SKF application.

D, sIPSC inter-event interval histogram distributions (normalized to number of events) of the OFF4 bipolar cell seen in panel A. SKF significantly increased the sIPSC inter-event interval (K-S P < 0.01). Arrows show the average interval between the sIPSCs.

E, peak amplitude and frequency data (normalized to control dark-adapted conditions) compared between SKF (dark gray) and light-adapted (light gray) conditions. Both conditions significantly reduced sIPSC peak amplitude and frequency and were not significantly different from each other. (* = p < 0.05, ** = p < 0.01)
Figure 27. D1 receptor activation significantly narrows and reduces the isolated glycinergic spatial inhibitory input to all OFF bipolar cells. 

A, B, example glycinergic L-IPSCs recorded from an OFF type 4 bipolar cell in dark-adapted and SKF conditions, black and gray traces respectively, in response to a 1 s flash of a 25 µm bar of light, presented at -400, 0, and 400 µm away from the recorded cell. SKF application reduce overall response 400 µm away from the OFF bipolar cell. Light stimulus = gray bar under L-IPSC, OFF type 4 bipolar cells respond at the onset of light.

C, spatial inhibition curves of glycinergic Q normalized to the center bar stimulus, in dark-adapted (n = 5, black trace) and SKF (n = 5, gray trace) conditions. The spatial inhibitory distribution was significantly narrower with SKF application.

D, normalized glycinergic L-IPSC Q, to the center L-IPSC, compared between dark-adapted (n = 5, black trace) and SKF (n = 5, gray trace) conditions at each stimulus distance. The proportion of inhibition was not significantly smaller after SKF application at any stimulus distance from the cell, only the overall distribution was significantly smaller.

E, the average proportion of center glycinergic L-IPSC response that is transient or sustained in dark-adapted and SKF conditions (n = 5). L-IPSCs timing did not significantly change with SKF application.

F, spatial inhibition curves of glycinergic peak amplitude in the dark-adapted (black trace) and SKF (gray trace) conditions. The peak amplitude distribution was significantly narrower with SKF application.

G, glycinergic L-IPSC peak amplitude compared between dark-adapted (black trace) and SKF (gray trace) conditions at each stimulus distance. The proportion of inhibition was on average smaller but not significant after SKF application at any stimulus distance from the cell. (* = p < 0.05, ** = p < 0.01)
Figure 28. Activation of D1 receptors is sufficient to elicit light-adapted glycineergic spatial inhibition changes in charge transfer to OFF bipolar cells.  
A, spatial inhibition curves of glycineergic L-IPSC Q normalized to the center bar stimulus in the SKF (n = 5, dark gray) and light-adapted (n = 4, light gray) conditions. SKF spatial input was not significantly narrower than light-adapted conditions.  
B, spatial inhibition curves of peak amplitude in the SKF (dark gray trace) and light-adapted (light gray trace) conditions. The peak amplitude distribution was significantly narrower and smaller with light adaptation. (** = p < 0.01)
A. Isolated Glycine

B. Normalized Frequency vs. sIPSC Peak Amplitude (pA)

C. sIPSC Peak Amplitude (pA)

D. Normalized Frequency vs. sIPSC Inter-event-interval (ms)

E. Change Normalized to Control

- Dark-adapted
- +SKF 38393 (20 μM)
- Light-adapted
**Figure 29.** Activation of D1 receptors reduces OFF bipolar glycinergic sIPSC peak amplitude and frequency.

A, example traces showing the glycinergic sIPSCs from an OFF4 bipolar cell in dark-adapted (black trace) and SKF conditions (gray trace). *Due to high frequency sIPSCs, a dotted line was included to mark the baseline current of the recording.*

B, the glycinergic sIPSC peak amplitude histogram distributions (normalized to number of events) of the OFF4 bipolar cell in panel A. SKF significantly reduced the sIPSC peak amplitude (K-S p < 0.01). Arrows show the average peak amplitude of the sIPSCs.

C, the peak amplitude of glycinergic sIPSCs was significantly decreased in all OFF bipolar cells (n = 5) following SKF application.

D, glycinergic sIPSC inter-event interval histogram distributions (normalized to number of events) of the OFF4 bipolar cell seen in panel A. SKF significantly increased the sIPSC inter-event interval (K-S P < 0.01). Arrows show the average interval between the sIPSCs.

E, peak amplitude and frequency data (normalized to control dark-adapted conditions) compared between SKF (dark gray) and light-adapted (light gray) conditions. Glycinergic sIPSC peak amplitude and frequency were significantly reduced with SKF and were not significantly different from light-adapted reductions (n = 3). (* = p < 0.05, ** = p < 0.01)
**Figure 30.** Activation of D1 receptors has no significant effect on GABA<sub>C</sub> receptor-mediated input to OFF bipolar cells.

A, example GABA<sub>C</sub> receptor-mediated L-IPSCs recorded from an OFF type 4 bipolar cell in dark-adapted (black trace) and SKF (gray trace) conditions in response to a 1 s flash of a 25 µm bar of light, presented directly over the recorded cell. *Light stimulus = gray bar under L-IPSC, OFF type 4 bipolar cells respond at the onset of light*

B, charge transfer and peak amplitude normalized to the dark-adapted condition of isolated GABA<sub>C</sub> receptor-mediated input to OFF bipolar cells (n = 4). There was no significant change in either light-evoked response parameter with SKF.
CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE STUDIES

The retina is a unique and dynamic system which can adjust its signaling to signal over more than 10 log units of stimulus intensities. To accomplish this momentous feat, the retina adapts its processing over a wide range of ambient light levels. This results in the ability to specialize retinal signaling for different environmental light states. For example, at night, or under dark-adapted conditions, retinal signaling prioritizes sensitivity to light stimuli over visual acuity. However, during the day, or under bright-light conditions, the retina no longer needs to be highly sensitive since there is abundant background light, so it changes to favoring high visual acuity for detection of fine details in the environment. This is accomplished by modulating retinal receptive fields and inhibitory signaling at the level of the ganglion cell. However, we show in the current studies, in support of recent previous work, that modulation of inhibition and receptive fields at the bipolar cell level, specifically bipolar cells of the OFF pathway, plays an important role in mediating light-adapted retinal signaling changes.

In Chapter 2, we measured the magnitude and kinetics of light-evoked and spontaneous currents to OFF bipolar cells in dark- and light-adapted conditions. We found that there was a compensatory switch from glycine to GABA-mediated OFF bipolar cell inhibition in the light. Additionally, there was a light-induced reduction in the spontaneous inhibitory inputs to the OFF bipolar cells which ultimately increased the inhibitory signal-to-noise ratio. In Chapter 3 we examined a possible implication of the inhibitory switch to OFF bipolar cells with light adaptation. This was a potential change in the
spatial inhibitory input (inhibitory receptive field surround) to the OFF bipolar cells, which has been shown to be an important modulatory site for retinal acuity. We found that the spatial inhibitory input to OFF bipolar cells became significantly smaller and narrower with light adaptation with. The shift in center-surround balance to OFF bipolar cells was incorporated into a model of bipolar cell output strength onto a downstream ganglion cells. The model predicted this inhibitory change with light increased the strength of the bipolar cell signal. Lastly, in Chapter 4 we investigated a potential mechanism for mediating the inhibitory changes observed in Chapters 2 and 3. We focused on whether dopamine is directly involved as dopamine is released during the day and many inner retinal neurons express dopamine receptors. To address this question, light-evoked and spontaneous currents from OFF bipolar cells were recorded while mapping spatial inhibitory input under dark-adapted conditions with application of a D1 receptor agonist. We found that the inhibitory changes to OFF bipolar cells with light-adaptation are partially mediated by dopamine D1 receptor signaling, however, there are likely other factors involved in mediated inhibitory changes to OFF bipolar cells.

While the above experiments answered our initial questions, the results lead to further questions that would be important to investigate in the future. First, taking these protocols to the ganglion cells would be a set of key experiments to link the inner retina processing changes with the output neurons of the retina. This is especially true for the results from Chapter 3, in which the constructed model predicts a stronger signal output from the bipolar cells. This can be measured in the ganglion cells, utilizing a whole mount retinal preparation instead of a slice preparation, and the same dark- and light-adapted protocols
can be used. This would allow for the confirmation that the ganglion cells are indeed receiving a more distinct signal from smaller spatially close stimuli, thus connecting the bipolar cell changes directly to increases in retinal acuity. Along these lines, another logical step would be to measure the spatial inhibitory input to the ganglion cells. The above projects are focused on the bipolar cell inputs only but the ganglion cells themselves have inhibitory surrounds which are modulated by light. Understanding how the ganglion cell receptive fields are changing in our specific system would help to build a more complete model of retinal circuitry and signaling changes with light adaptation.

Additionally, there are other important factors to test as potential mechanisms for mediating the light-adapted inhibitory spatial changes. The first would be to investigate the role that gap junctions between inhibitory amacrine cells play by using specific connexin knockout mice or gap junction blockers. Since the coupling state of the retina is highly regulated and many cells are extensively coupled, it is likely that regulation of gap junctions contributes largely to changes in retinal signaling with light adaptation. Second, there is merit in investigating the contribution from the other subtypes of dopamine receptors as well, which can be accomplished via agonists and antagonists. Though these other receptors are not on the bipolar or amacrine cells directly, they may play an important role in network affects, which could lead to downstream changes in inhibition. It would also be interesting to measure the amount of dopamine that is present in the retina during different light-adapted states since this is currently unknown. This would be helpful in determining appropriate agonist concentrations for activation of the receptors to mimic certain light states. To further study the direct and specific effects of dopamine
receptors on inhibitory synaptic contacts to the OFF bipolar cells, a future experiment could take advantage of a transgenic mouse line in which all inhibitory amacrine cells express the light-sensitive cation channel Channelrhodopsin (ChR2). In this way, amacrine cell input can be directly stimulated while isolating the specific circuit which would help to decrease extra circuit affects onto the system. Finally, since our results suggest that dopamine may not be the only player, future studies should investigate the role of other neuromodulators, such as melatonin and NO, on inhibitory changes with light adaptation. All of the above experiments would be good future offshoots of this project to continue to elucidate how light adaptation causes inner retinal inhibitory changes and the role these changes play in contributing to retinal signaling and thus visual acuity.
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