Synaptic regulation of the light-dependent oscillatory currents in starburst amacrine cells of the mouse retina.

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Running Title: Light-dependent response of starburst amacrine cells

Pages: 41

Figures: 9

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Abstract

Responses of on-center starburst amacrine cells to steady light stimuli were recorded in the dark adapted mouse retina. The response to spots of dim white light appear to show two components, an initial peak that correspond to the onset of the light stimulus, and a series of oscillations that ride on top of the initial peak relaxation. The frequency of oscillations during light stimulation was three time higher than the frequency of spontaneous oscillations recorded in the dark. The light-evoked responses in starburst cells were exclusively dependent on the release of glutamate likely from presynaptic bipolar axon terminals and the binding of glutamate to AMPA/kainate receptors because they were blocked by CNQX. The synaptic pathway responsible for the light responses was blocked by AP4, an agonist of metabotropic glutamate receptors that hyperpolarize on-center bipolar cells upon activation. Light responses were inhibited by the calcium channel blockers cadmium ions and nifedipine suggesting that the release of glutamate was calcium dependent. The oscillatory component of the response was specifically inhibited by blocking the glutamate transporter with TBOA, suggesting that glutamate reuptake is necessary for the oscillatory release. GABAergic antagonists bicuculline, SR 95531 and picrotoxin increased the amplitude of the initial peak while they inhibit the frequency of oscillations. TTX had a similar effect. Strychnine, the blocker of glycine receptors did not affect the initial peak but strongly decreased the oscillations frequency. These inhibitory inputs onto the bipolar axon terminals shape and synchronize the oscillatory component.

Keywords: amacrine cells, oscillations, retina, glutamate
Introduction

Oscillatory activity amongst neuronal ensembles has been reported throughout the central nervous system (Leznick et al., 2002; Llinas et al., 1994). In the retina, rhythmic discharges, in the form of spontaneous propagating waves, first appear prenatally and are crucial to the proper development of synaptic circuitry within both the retina and lateral geniculate nucleus (Meister et al., 1991; Wong, 1993, 1999). In the adult, the oscillatory potentials (OPs) are a prominent component of the electroretinogram, indicating that widespread rhythmic activity exists across the retina. There is now strong evidence that the OPs reflect postsynaptic activity of amacrine and ganglion cells (Zhou et al., 2007). Indeed, adult ganglion cells display oscillatory activity that is both light dependent and independent (Neuenschwander et al., 1999). The light dependent rhythms show a wide range of frequencies that can be altered by changes in stimulus size and contrast (Stephens et al., 2006). Synaptically-driven oscillatory activity has been described for amacrine cells in the fish retina (Sakai and Naka, 1992). Consistent with these findings, the presynaptic bipolar cells show calcium-dependent oscillations of their membrane potential that leads to pulsatile release of transmitter and oscillatory activity of postsynaptic targets (Burrone and Lagnado, 1997; Ma and Pan, 2003). Interestingly, oscillations have also been reported in other amacrine cell subtypes in fish and mouse that survive cell isolation and are thus independent of synaptic drive (Feigenspan et al., 1998; Solessio et al., 2002). Thus, both synaptically-mediated and intrinsically-driven oscillatory activity occurs in the retina.
Recently, we described spontaneous, subthreshold oscillatory activity in starburst amacrine cells, a unique subtype that releases both acetylcholine and GABA and thereby subserves both excitatory and inhibitory circuits within the proximal retina (Petit-Jacques et al., 2005). Our results indicated that this spontaneous rhythmic activity is synaptically driven, derived from pulsatile, calcium-dependent glutamate release from presynaptic bipolar cells. This mechanism resides in the proximal retina and is independent of light as evidenced by its experimental induction in the absence of photoreceptor signaling.

Here, we report that starburst amacrine cells also show prominent light-dependent oscillatory activity. The light-evoked responses of starburst cells consist of two components: an initial transient peak inward current that relaxes during the presentation of a light stimulus, and oscillatory potentials that ride atop this relaxation phase. Our results indicate that both components result from glutamate release from presynaptic bipolar cell axon terminals. However, they are affected differentially by a number of pharmacological agents that act upon inhibitory synaptic innervation of bipolar cell terminals or glutamate reuptake transporters. Taken together, these results suggest that the two response components result from the sequential release of glutamate from a single pool or discrete pools within presynaptic bipolar cell endings.
Material and Methods

Mouse retina-eyecup preparation

All animal procedures complied with National Institutes of Health guidelines for the ethical use of animals. C57BL6 wild-type (25-60 days old) were deeply anesthetized with an intraperitoneal injection of pentobarbital (0.08g/kg body weight). Lidocaine hydrochloride (20 mg/ml) was applied locally to the eyelids and surrounding tissue. A flattened retinal-scleral eyecup preparation developed for rabbit by Hu et al. (2000) was adopted and modified for the mouse. Briefly, the eye was removed under dim red illumination and hemisected anterior to the ora serrata. Animals were sacrificed immediately after enucleation by cervical dislocation. The lens and vitreous humor were removed and the resultant eyecup preparation was placed on the base of a submersion-type recording chamber. Several radial incisions were made peripherally and the retina was flattened in the chamber vitreal side up. The chamber was mounted on a microscope stage within a Faraday cage and superfused (1-2 ml/min) with an oxygenated mammalian Ringer solution composed of (in mM): NaCl 120, KCl 5, NaHCO₃ 25, Na₂HPO₄ 0.8, NaH₂PO₄ 0.1 MgSO₄ 1, CaCl₂ 2, D-Glucose 10. A pH of 7.4 was maintained by bubbling with 95% O₂ - 5% CO₂ at room temperature of 20-22 °C.

Electrophysiological recordings

Recordings were made in the whole cell patch mode with an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA). Cells were visualized with near infrared light (>775 nm) at 80X magnification with a Nuvicon tube camera (Dage-MTI, Michigan City,
IN) and differential interference optics (DIC) on a fixed-stage microscope (BX51WI; Olympus, Tokyo, Japan). Currents were recorded under voltage clamp, filtered at 1 kHz, sampled at 20 kHz, and stored directly on the computer’s hard drive using a Digidata 1322A A/D interface (Axon Instruments). For the characterization of voltage responses, neurons were recorded in the fast current clamp mode of the amplifier. The resting potential of neurons was adjusted to –70 mV with small injections of direct current.

pCLAMP (v. 9.0; Axon Instruments) software was used for data acquisition with subsequent data analysis performed off-line using Minianalysis (v. 6.0.1; Synaptosoft, Decatur, GA) and Origin (v. 6.1; OriginLab, Northampton, MA) software packages. Patch electrodes (3-5 MΩ) were pulled from standard wall borosilicate glass tubing (World Precision Instruments, Sarasota, FL) with a Flaming/Brown type micropipette puller (Sutter Instruments, Novato, CA). Pipettes were filled with a K-gluconate internal solution composed of (in mM): K-gluconate 144, MgCl2 3, EGTA 0.2, HEPES 10, ATP-Mg 4, GTP-Tris 0.5, pH 7.3 with KOH, and biocytin (0.2% w/v, Sigma, St. Louis, MO). All recordings were made in dark-adapted retinas.

**Light stimulation**

The light stimuli were generated by the Vision Works software Neurophysiology, outputted through a video projector onto a coherent fiber optic and delivered to the retina through the microscope objective. The stimulus intensity was maintained in the mesopic illuminance range; for example, a 200 μm diameter spot of white light stimulus had an intensity of 0.7 μW/cm² as measured with a radiometer/photometer (Ealing Electro-
Optics). Spot stimuli of various diameters were used and were always visually centered over the starburst cell soma under study.

**Biocytin labeling**

Neurons were labeled by allowing biocytin to diffuse from the micropipette during patch recordings. After electrophysiological experiments were completed, retinas were fixed in a cold (4°C) solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.3) overnight. Retinas were then washed in phosphate buffer and soaked in a solution of 0.18% hydrogen peroxide in methyl alcohol for one hour. This treatment completely abolished the endogenous peroxidase activity. Retinas were then washed in phosphate buffer and reacted with the Elite ABC kit (Vector Laboratories, Burlingame, CA) and 1% Triton X-100 in sodium phosphate-buffered saline (9% saline, pH=7.5). Retinas were subsequently processed for peroxidase histochemistry using 3,3’-diaminobenzidine (DAB) as the chromagen, dehydrated and flat-mounted for light microscopy.

**Statistical analyses**

Data were analyzed using Student’s *t*-test statistic. Presentation of data is in the form mean ± standard error (SEM) throughout.
Results

Basic electrophysiological properties of starburst amacrine cells in the mouse retina.

Recordings were made from on-center starburst amacrine cells, which have somata displaced to the ganglion cell layer (GCL) and dendritic arbors stratifying within sublamina-\(b\) of the inner plexiform layer (IPL). By specifically targeting small, round somata in the GCL, we achieved a success rate of more than 60% in identifying starburst cells. After electrophysiological experiments, each recorded cell was injected with biocytin to confirm their identity by post hoc histology. Starburst amacrine cells in the mouse showed the typical morphology described previously in this (Ozaita et al., 2004; Petit-Jacques et al., 2005) and other species (Bloomfield and Miller, 1986; Famiglietti, 1983; Tauchi and Masland, 1984). This consisted of 5 -7 main dendritic branches that formed a proximal zone, which then divided into thinner intermediate segments that divided further into a plexus of distal branches showing numerous varicosities (Fig. 1A).

As we have described previously, mouse starburst cells displayed a number of basic and stereotypic electrophysiological properties (Petit-Jacques et al, 2005). One characteristic feature of starburst cell was their membrane voltage response to the injection of current steps. Membrane depolarization triggered by pulses larger than \(+ 50\) pA tended to saturate, and it was therefore not possible to depolarize the membrane to potentials more positive than \(-20\) mV, even with injections of large current steps of \(+ 450\) pA (Fig. 1B). Under our recording conditions, starburst cells never showed any spontaneous or evoked spiking, consistent with our previous data from mouse (Petit-Jacques et al., 2005) and studies of the rabbit retina using the whole cell recording
technique (Peters and Masland, 1996; Taylor and Wässle, 1995). Large depolarizing current pulses did evoke a small transient component, but it never reached potentials above 0 mV and it was insensitive to TTX. The absence of spiking activity was consistent with voltage clamp recordings that showed a total absence of inward currents for a full range of membrane depolarization of -70 to +50 mV (Fig. 1C). In contrast to the absence of inward currents, membrane depolarization above -20 mV did trigger very large outward currents comprised of two components: a transient current immediately followed by a delayed rectifier component that did not inactivate. Upon repolarization, the delayed rectifier component displayed very fast deactivation tail currents. These properties are consistent with those of voltage-gated Kv3 channels, which have been shown to carry most of the outward current in starburst cells of the mouse retina (Ozaita et al., 2004). Whereas all starburst cells showed the delayed rectifier current, only a subset of these cells showed the transient outward component upon depolarization (Fig. 1C). These results are consistent with our previous finding that some starburst cells lack the transient outward current (Ozaita et al., 2004) and support the recent finding of two types of murine starburst cells with different physiological properties (Kaneda et al., 2007). However, we saw no differences in the light-evoked responses of these two subsets of starburst cells and so we do not differentiate them in the results described below.

Light-evoked responses of starburst amacrine cells.

In a previous study, we showed that starburst amacrine cells in the mouse retina display spontaneous current oscillations (Petit-Jacques et al., 2005). In the dark-adapted
retina, starburst cells held at -70 mV, exhibit random, spontaneous inward currents of varying shape and amplitude (Fig. 1D, Petit-Jacques et al., 2005). To further investigate the characteristics of these oscillatory currents, we examined how they are affected by presentation of light stimuli. At the resting potential (~ -70 mV) in current clamp mode, the presentation of a small spot (70 µm diameter) of light centered on the starburst cell soma triggered a postsynaptic potential (PSP) consisting of an initial peak followed by smaller-amplitude oscillations of the membrane potential that lasted for the duration of the light stimulus. At light offset, the final oscillatory wave gradually returned to the resting membrane potential (Fig. 1E). Likewise, light stimulation of cells voltage clamped at -70 mV resulted in a large, initial inward current peak followed by a series of current oscillations that lasted for the duration of the stimulus (Fig. 1F). Similar light-evoked responses have been observed for on-center starburst amacrine cells in the rabbit retina (Bloomfield, 1992; Peters and Masland, 1996). These results indicate that current oscillations not only appear spontaneously, but are also an active component of the light response of starburst cells in the mouse retina. Interestingly, the frequency of the light-evoked oscillatory currents was more than three times that of the spontaneous oscillations for individual cells (3.79 +/- 0.26 Hz for spontaneous and 11.97 +/- 0.98 Hz for light-evoked oscillations, n=10 cells, Fig. 2B).

An analysis of the light-evoked current response indicated that it consisted of two discrete components: an initial transient peak at light onset followed by a burst of oscillations riding atop the relaxation phase of the initial peak. Figure 2A illustrates a typical light response of a starburst amacrine cell to a 70-µm diameter spot of light. The maximal point of relaxation for each current oscillation was measured and is represented
by the square data points. The relationship between these points could be described by a first-order exponential decay that tightly matched the relaxation kinetics of the initial peak.

On average, starburst cells in the mouse retina responded to light stimulation with a synaptic delay of 108.5 +/- 2.4 ms. Interestingly, the delay between the light offset and the disappearance of the light response was twice as long as the synaptic delay at light onset (average of 233.9 +/- 2.9 ms; n=88 tests in 10 cells, not shown). The synaptic delay for excitatory responses appears to be shorter in the rabbit retina in which values near 60 ms have been reported (Lee and Zhou, 2006; Peters and Masland, 1996).

Light-evoked oscillations in starburst amacrine cell responses are mediated by glutamate.

The spontaneous oscillations in starburst cells are synaptically mediated and are dependent on the excitatory drive from presynaptic bipolar cells (Petit-Jacques et al., 2005). Bipolar cells form glutamatergic synapses onto starburst cells that involve AMPA/kainate ionotropic receptors (Brandstätter et al., 1998; Firth et al., 2003; Thoreson and Witkovsky, 1999). To determine whether glutamate release was responsible for both of the light-evoked response components of starburst cells, cells were stimulated in the presence of CNQX, a specific blocker of AMPA/kainate receptors. Application of CNQX (10 µM) almost totally blocked all response components to a small spot of light (Fig. 3A, B). Upon washout, the actions of CNQX were totally reversed and both phases of the light response were recovered (Fig. 3C). On average, CNQX reduced the maximal amplitude of the initial peak by 87% (Fig. 3D; n=27 stimulations in 3 cells, p<0.0001).
Our results thus indicate that both the peak and oscillatory response components of starburst cells are dependent on the glutamate release from presynaptic bipolar cells.

Light responses of starburst cells are derived from the ON pathway.

On-center bipolar cells receive glutamate input from photoreceptors via the metabotropic glutamate receptors mGluR6 (Nomura et al., 1994; Ueda et al., 1997). The activation of these receptors leads to a hyperpolarization of on-center bipolar cells and a reduction of their excitatory inputs to more proximal neurons (Nakajima et al., 1993; Tian and Slaughter, 2003). Application of AP4, an agonist of these receptors, totally blocked the light response of starburst cells to small spots of light. Only the spontaneous, light-independent random activity was visible in the presence of the drug (Fig. 3E, F). At its steady state effect, AP4 reduced the maximal amplitude of the initial peak by 95% and eliminated all light-dependent current oscillations (Fig. 3G; p<0.0001).

Glutamate release from bipolar cell terminals is dependent on calcium channels.

The glutamate release from bipolar cells is modulated by the activity of different types of calcium channels (Berntson et al., 2003; Pan 2000, 2001), which co-localize with the vesicle docking sites at ribbon synapses (Sterling and Matthews, 2005). We showed previously that the spontaneous oscillatory currents of starburst amacrine cells in mouse retina are completely inhibited by calcium channel blockers (Petit-Jacques et al., 2005). Here, we examined the effect of these blockers on the light-evoked responses of starburst cells. In the presence of cadmium ions, a nonspecific blocker of calcium channels, the light response to a small spot of light was totally blocked, with loss of both the initial
peak and the oscillatory components (Fig. 4A, B). For the example illustrated in Figure 4, the average maximal amplitude of the initial peak was 5.47 +/- 0.44 pA/pF (n=9) and the average frequency of oscillations was 11.25 +/- 0.29 Hz (n=8) in control, both of which were eliminated by cadmium (n=8). The only events detectable under cadmium superfusion were light-independent spontaneous miniature events. The effect of cadmium was extremely fast with a maximal inhibition apparent after only 2 minutes, and, although the full light response returned upon wash out, the recovery was slow. Nifedipine, a specific blocker of L-type calcium channels, also had a strong inhibitory effect on the light response of starburst cells, although less effective than that of cadmium. Nifedipine blocked only 54% of the initial peak and decreased the current oscillations frequency by 74% (n=9 tests) for the cell illustrated in Figure 4C.D. Similar results were found for 5 additional starburst cells. These data suggest that both components of the light response of starburst cell are dependent on the calcium-dependent release of glutamate from the bipolar cell synaptic terminals, although cadmium and nifedipine could also affect more distally-located calcium channels.

**Blockade of glutamate transporters differentially affects the light-evoked response components of starburst amacrine cells.**

Different types of glutamate transporters are present in the mammalian retina, localized to presynaptic terminals of photoreceptors and bipolar cells (Hasegawa et al., 2006; Palmer et al., 2003). The glutamate transporters in the axon terminals of rod bipolar cells are effectively blocked by DL-threo-β-benzylxoxaspartic acid (TBOA), a non-selective non-transported blocker (Veruki et al., 2006). Interestingly, application of
TBOA (20 µM) differentially affected the peak and oscillatory response components of mouse starburst cells (Fig. 5A, B). TBOA had no significant effect on the amplitude of the initial peak component (Fig. 5C; 7.10 +/- 0.64 pA/pF in control, 6.97 +/- 0.70 pA/pF in TBOA, n=26 and 27 tests in 3 cells), but it reduced the average frequency of the current oscillations by 83% (Fig. 5D; 10.99 +/- 0.18 Hz in control compared to 1.85 +/- 0.16 in TBOA, p<0.0001). The different pharmacology of these two components suggests that the peak and oscillatory components are generated by distinct mechanisms related to the release of glutamate from bipolar cell axon terminals (see Discussion).

*Effects of GABA blockers on the light-evoked responses of starburst cells.*

The excitatory release of glutamate from bipolar cell synaptic terminals can be modulated by GABAergic feedback inhibition from postsynaptic amacrine cells (Freed et al., 2003; Matsui et al., 2001; Shen and Slaughter, 2001; Wässle et al., 1998). We have reported previously that GABA receptors antagonists have a strong stimulatory effect on the spontaneous current oscillations of mouse starburst cells (Petit-Jacques et al., 2005). Here, we extended the study of the different GABA receptors blockers by examining their effects on the light-evoked responses of starburst cells. The GABA<sub>A</sub> receptor blockers bicuculline (BMI, 10 µM) and SR 95531 (10 µM) strongly enhanced the amplitude of the initial peak response, yet slightly decreased the frequency of current oscillations (Fig. 6A-D). Picrotoxin (PTX, 50 µM), an antagonist of A- and C-type GABA receptors, had an effect similar to that of the GABA<sub>A</sub> blockers, triggering a comparable increase in the amplitude of the initial peak response. However, PTX had a stronger inhibitory effect than the GABA<sub>A</sub> antagonists on the frequency of the
oscillations (Fig. 6E, F). On average, the amplitude of the initial peak showed a 35% increase following BMI (p<0.05, n=17 and 18 tests in 2 cells), a 22% increase following SR 95531 (p<0.01, n=18 and 16 tests in 2 cells), and 26% increase following PTX application (p< 0.001, n=18 tests in 2 cells) when compared to control conditions (Fig. 5G). In contrast, the average frequency of oscillations showed an 11% decrease following BMI (p<0.05), a 20% decrease following SR 95531 (p<0.001) and a 43% decrease following PTX application (p<0.001) from control levels (Fig. 5H).

Interestingly, studies of murine rho subunits have suggested that the native GABA<sub>C</sub> receptors in mouse to be insensitive to PTX (Greka et al., 1998, 2000). However, our finding that PTX produce a significantly larger decrease in the oscillatory frequency than either BMI or SR 95531 argues against this. Taken together, these data suggest that the GABAergic feedback inhibition of bipolar cell axon terminals plays an important role in the modulation of the glutamate release.

In the inner retina, spike-dependent feedforward and feedback inhibition from amacrine cells are thought to play an important role in the spatial tuning of ganglion cells (Cooks and McReynolds, 1998; Cooks et al., 1998; Shields and Lukasiewicz, 2003; Taylor, 1999). To test the possible involvement of spike-dependent inhibition in the regulation of starburst cell responses, we applied tetrodotoxin (TTX), a specific inhibitor of voltage dependent sodium channels, after application of PTX. In the presence of PTX, TTX further increased the amplitude of the initial peak due to a presumed blockade of non-GABAergic spiking amacrine cells. Application of TTX also strongly inhibited the current oscillations (Fig. 7A, B). Upon wash out, the amplitude of the initial peak was slightly increased and the current oscillations fully recovered (Fig 7C). On average, TTX
slightly increased the amplitude of the initial peak by 7%, although the difference was not significant when compared to PTX alone (Fig. 7D). The major effect of TTX was a 40% inhibition of the oscillations maximal amplitude when compared to PTX (Fig. 7E, p<0.0005) and a 34% inhibition of the current oscillations frequency (Fig. 7F, p<0.05). Application of TTX alone showed similar effects on the two response components. These results suggest that spike-dependent inhibition, at least partly non-GABAergic, modulates the oscillatory release of glutamate from bipolar cells.

Effect of strychnine of starburst cell light responses.

The results with TTX suggest a role for glycinergic inhibition in modulating glutamate release from bipolar cells and thereby affecting starburst cell responses (Cui et al., 2003; Du and Yang, 2002; Eggers and Lukasiewicz, 2006; Ivanova et al., 2006). We therefore examined the effect of the glycine receptor blocker, strychnine, on the light-evoked response of starburst cells. Application of strychnine (10 µM) almost totally abolished the oscillatory currents, whereas the initial peak component was largely unaffected (Fig. 8A, B). Strychnine did produce a slight decrease in the amplitude of the initial peak response, but this was not significant (Fig. 8C, from 8.9 +/- 0.56 pA/pF to 7.49 +/- 0.43 pA/pF, n=18 tests in 2 cells). In contrast, the oscillatory component frequency was reduced by 69% (Fig. 8D, from 10.1 +/- 0.22 Hz to 3.1 +/- 0.28 Hz, p<0.0001). These data suggest that glycinergic inhibition of bipolar cell axon terminals specifically controls the releasable pool of glutamate that underlies the starburst cell oscillatory responses.
Discussion

Taken together with our previous report (Petit-Jacques et al., 2005), the present results indicate that starburst amacrine cells in mouse retina show spontaneous and light-evoked oscillatory activity. Both apparently arise from a common mechanism, a pulsatile release of glutamate from presynaptic bipolar cell axon terminals, which can be induced in darkness or by light. The light-evoked responses of dark-adapted starburst cells were comprised of two distinct components: a prominent peak of inward current at stimulus onset and oscillations that rode along the relaxation phase of the initial peak. Our results indicate that these response components both result from the temporal properties of glutamate release from presynaptic bipolar cells. However, our finding that the transient and oscillatory response components were differentially affected by a number of pharmacological agents suggests that they result from two distinct mechanisms related to the release of glutamate from presynaptic bipolar cell axon terminals (Fig.9).

Basic electrophysiological characteristics of starburst cells.

There is a controversy as to whether adult starburst amacrine cells support Na+-dependent spike activity. In the rabbit, some studies reported light-evoked spiking of starburst cells (Bloomfield, 1992; Cohen, 2001; Gavrikov et al., 2003), whereas others found them to be totally absent (Peters and Masland, 1996; Taylor and Wässle, 1995; Zhou and Fain, 1996). Under our experimental conditions, we never recorded spiking behavior in starburst amacrine cells of the mouse retina (Ozaita et al., 2004; Petit-Jacques
et al., 2005 and see Fig. 1). Likewise, we did not record any inward currents in response to the depolarization of the cell membrane (Ozaita et al., 2004 and see Fig. 1). Consistent with our findings, Kaneda et al. (2007) recently reported the absence of voltage-gated Na⁺ currents in murine starburst cells. However, they did report two types of voltage-gated Ca²⁺ currents in starburst cells. This discrepancy between their data and ours is difficult to explain, but it may relate to differences in experimental conditions. Kaneda et al. (2007) recorded from starburst cells in retinal slices and dissociated in culture, whereas our experiments were performed on intact retinas. Nevertheless, we are confident in our observation that starburst cells lacked voltage-gated Na⁺ and Ca²⁺ currents under our experimental conditions because we could record robust voltage-gated inward currents and associated spiking activity from ganglion cells in the same retinas. The absence of voltage-gated inward currents in our starburst cells is consistent with our conclusion that the effects of TTX, cadmium, and nifedipine on light-evoked responses do not reflect direct actions on starburst amacrine cells, but rather the glutamate release from presynaptic bipolar cells. Consistent with our previous report (Ozaita et al., 2004), we found that starburst cells displayed prominent K⁺-mediated outward currents. Kaneda et al. (2007) reported recently that a starburst cell in the mouse can show one of two types of outward current activity. Some cells showed both transient and delayed outward currents, whereas others showed only the delayed current. Here, we illustrated starburst amacrine cell responses with both the transient and delayed outward currents (Fig. 1C) and we described previously starburst cells in the mouse retina with only delayed outward currents (Ozaita et al., 2004). Therefore, we agree with the findings of Kaneda et al. (2007) that two types of starburst cell responses exist in the mouse retina with
regard to their voltage-gated outward currents. The delayed outward current in starburst cells is mediated mainly by Kv3.1/3.2 channels (Ozaita et al., 2004).

*Light-evoked responses in starburst cells are carried by glutamate.*

Both the transient and oscillatory light-evoked response components were blocked by CNQX, indicating that they are dependent on the binding of glutamate to AMPA/kainate receptors located on starburst cell postsynaptic membranes. Similar results have been reported previously for starburst amacrine cells in the rabbit retina (Peters and Masland, 1996). The light-evoked response components were also blocked by the mGluR6 agonist, AP4, indicating that they are derived from the ON pathway. In addition, our results indicate that the glutamate release from bipolar cell axon terminals that gives rise to the two starburst cell response components is calcium-activated and largely dependent on the activity of L-type calcium channels sensitive to nifedipine. In bipolar cells, calcium channels organized in clusters around the ribbon synapse control the exocytosis of glutamate vesicles through a calcium-induced calcium release process, which is triggered by a light-dependent depolarization (Burrone et al., 2002; Llobet et al., 2003; Sterling and Matthews, 2005).

Although both light-evoked response components are dependent on glutamate release, we found that they were differentially affected by pharmacological agents. The agents TBOA, bicuculline, SR95531, TTX, and strychnine all affected either one of the response components or affected the two in opposite directions. These observations indicate the later oscillatory component is not the result of the initial peak component
activating a mechanism intrinsic to the starburst cell such as voltage-gated ionic channels. Rather, they support our conclusion that these components both result synaptically, from the glutamate release from bipolar cell terminals.

The differential effects of TBOA, a blocker of the glutamate transporter, were most striking. Application of TBOA had an insignificant effect on the amplitude of the initial peak component, but it suppressed almost all of the current oscillations. The inability of TBOA to affect the initial peak amplitude, even after lengthy application, indicates that this component is not readily dependent on the recycling of glutamate from the synaptic cleft. In contrast, the light-evoked oscillations were largely inhibited during the first minutes of the TBOA application, indicating that they arise from glutamate stores dependent on the transporter for their recycling process. The dependence of the oscillatory component on glutamate recycling may simply reflect the fact that it occurred sequentially after the transient peak component and a possible corresponding depletion of transmitter. However, these data also raise the possibility that the two starburst cell response components reflect the existence of two distinct releasable pools of glutamate from bipolar axon terminals with different depletion kinetics. Consistent with this notion, the vesicular release at bipolar cell synaptic terminals exhibits two distinct components, a fast pool released within a few milliseconds and a sustained pool that is released over the next several hundred milliseconds (Mennerick and Matthews, 1996; Singer and Diamond, 2003; Von Gersdorff et al., 1998). The fast pool corresponds to the vesicles docked at the base of the ribbon synapse, whereas the sustained pool is dependent on vesicles tethered to the ribbon in higher rows more distant from the plasma membrane (Sterling and Matthews, 2005). It has been suggested that filaments connecting vesicles to the
ribbon could constitute a molecular motor that transports primed vesicles in higher rows of the ribbon in successive waves to the base, where they fuse with the plasma membrane. Such a structural organization of the ribbon may underlie successive waves of release that could give rise to the oscillations we observed.

The glutamate transporter located at the bipolar cell axon terminal is a co-transporter of chloride ions inside the presynaptic terminal (Kugler and Beyer, 2003; Palmer et al., 2003; Rauen et al., 1996). This coupled anion current can counteract the stimulus-evoked depolarization of the bipolar axon terminal and thereby suppress transmitter release (Veruki et al., 2006). Such a mechanism may also be involved in the development of an oscillatory release of glutamate. In this scheme, each vesicle or group of vesicles that fuse into the plasma membrane to release a pool of glutamate is followed by the co-transport of chloride ions that will repolarize the synaptic terminal membrane and suppress further release. Thus, the cascade of calcium channel-induced depolarization, glutamate release, activation of transporter/chloride current, hyperpolarization and suppression of release, could trigger successive oscillatory waves of glutamate release (Fig. 9). Furthermore, the oscillatory release would stimulate the release of GABA and glycine from amacrine cells, including starburst cells. The timing of these inhibitory inputs arriving on the bipolar cell terminal would follow each wave of the oscillatory release with a slight delay, participating in the suppression of the release and the return to the current baseline between each wave. Further, the interventions of these different inhibitory inputs would likely be synchronized with the system of glutamate release-activity of the glutamate transporter.
Regulation of glutamate release by presynaptic receptors.

Bipolar cell axon terminals receive a variety of synaptic inputs that can modulate the release of glutamate. GABAergic inhibition derived from amacrine cell processes feedback onto bipolar cell terminals to limit and synchronize the release (Euler and Masland, 2000; Freed et al., 2003; Shields et al., 2000). Activation of GABA$_A$, GABA$_C$ and glycine receptors all differentially affect light-evoked signaling in mouse retinal bipolar cells (Cui et al., 2003; Eggers and Lukasiewicz, 2006; Eggers et al., 2007; Frech and Backus, 2004). For example, some GABAergic and glycinergic inputs onto bipolar cell terminals participate in the lateral inhibition of ganglion cells and are spike-dependent (Cook et al., 1998; Shields and Lukasiewicz, 2003). Our results suggest that inhibitory circuits are involved in the regulation of glutamate release from bipolar cells to starburst cells during light stimulation (Fig. 9). GABAergic and glycinergic inhibitory inputs, the latter which is likely spike-dependent in part, influence the response of starburst cells to light. Activation of both GABA$_A$ and GABA$_C$ receptors is involved in the regulation of the light responses, which likely includes both feedforward and feedback circuitry. Since glycinergic synapses appear not to occur onto cone bipolar cells, but do occur on rod bipolar cells in the murine retina (Cui et al., 2003; Ivanova et al., 2006), the strychnine effects likely reflect suppression of glycinergic receptors on rod bipolar cell axon terminals.

The increased amplitude of the initial peak component following application of the GABA blockers and TTX appears to be the logical result of reducing feedback inhibition to bipolar cell terminals, thereby increasing glutamate release. In contrast, the mechanism of the reduced frequency of the oscillatory component by these agents is less
clear. However, our results are consistent with the idea that feedback inhibition plays a crucial role in the synchronization of the oscillatory release of glutamate from bipolar cell terminals (Euler and Masland, 2000; Freed et al., 2003). Our TTX data suggest that, under control conditions, repetitive spike-dependent inhibition repolarizes the bipolar cell terminal membrane at regular intervals, reinforcing an oscillatory release of glutamate. Overall, our results indicate that inhibition derived from amacrine cells, some of which is spike dependent, act to shape the oscillatory release of glutamate from bipolar cell terminals.

Physiological role of light-evoked oscillations of starburst cells.

We showed previously that oscillations form an important mechanism of spontaneous transmitter release from bipolar cell axon terminals (Petit-Jacques et al., 2005). Our present results indicate that oscillatory release from bipolar cells is also triggered by light and thus likely plays a role in propagating visual signals. While our pharmacological data indicate that the mechanisms that modulate the transient peak and oscillatory components of starburst cell response are different, those for the latter are similar to those we reported previously for spontaneous oscillatory activity (Petit-Jacques et al., 2005). These results suggest that the oscillatory glutamate release from bipolar cells is modulated both dependently and independently of light. One idea is that spontaneous oscillatory release corresponds to a basal synaptic noise. Thus, changes in the frequency of oscillatory events during stimulation by light could provide a mechanism for postsynaptic starburst cells to distinguish light-evoked signals from synaptic noise (Singer et al., 2004).
In the visual cortex, oscillatory potentials may play a role in the binding of separate neuronal aggregates into sensory units. Oscillatory responses form a time/frequency coding mechanism for neurons to detect the physical properties of a stimulus and are thereby involved in sensory information processing relevant for perceptual grouping (Neuenschwander and Singer, 1996; Sannita, 2000). We propose that the oscillatory component of light-evoked responses in the retina constitute an important early step in the coding of visual clues transmitted to the brain. The specificity of the pharmacology of the oscillatory component provides compelling evidence of its importance for encoding visual signals. Considering the major role played by starburst amacrine cells in the computation of direction selectivity in the retina (Fried et al., 2005; Hausselt et al., 2007; Taylor and Vaney, 2003) it will be important in future studies to determine how the oscillatory component is modulated in response to moving light stimuli.
GRANTS

This work was supported by NIH Grants EY07360 (SAB).
References


Zhou ZJ, Fain GL. Starburst amacrine cells change from spiking to nonspiking neurons during retinal development. *Proc Natl Acad Sci USA* 93: 8057-8062, 1996.
Figures Legend

Figure 1. Characteristics of ON starburst amacrine cells in the mouse retina. A: a photomicrograph of a starburst amacrine cell in the mouse retina labeled with biocytin shows the characteristic dendritic arborization. The bar in the upper right corner is 50 μm long. B: representative current clamp recording from a starburst amacrine cell. Steps of currents were injected in the cell for 600 ms and the membrane voltage responses were recorded under whole cell patch clamp. Between pulses, the cell was maintained at a voltage of -75 mV by constant injection of a small amount of current (indicated by the arrow at the left of the traces). The voltage traces are in response to injection of 50 pA current steps from -100 to +450 pA. Note the saturation of the membrane depolarization at -20 mV for current pulses greater than +50 pA.

C: representative voltage clamp recording from the same starburst cell. The cell was maintained at a holding potential of -70 mV, and the membrane was depolarized by 10 mV steps from -70 to +50 mV during 150 ms. Upon return from depolarization, the cell was maintained at -40 mV for 70 ms to visualize the deactivation tail currents. Note the total absence of inward currents, but the presence of large outward currents with a fast transient outward component and a slower delayed rectifier component. The holding current was +25 pA.

D: spontaneous oscillatory currents were recorded in voltage clamp at -70 mV in another starburst cell. In absence of light, oscillations of variable amplitude and shape emerged from the baseline current and the miniature events. Holding current was -10 pA.

E: in current clamp mode, voltage membrane variations were recorded at -70 mV in the same cell as in D. During the application of a 70 μm diameter spot of light
(represented by the horizontal line below the voltage trace) the cell membrane potential displayed a series of outward oscillations with a large initial peak followed by events with decreasing amplitude. After the light stimulation was cutoff, the oscillations disappeared and the membrane voltage returned to the baseline. Holding voltage was -72 mV. F: in voltage clamp at a holding potential of -70 mV, the light stimulation triggered a series of oscillatory inward currents with a large initial peak followed by events of decreasing amplitude. After the light cutoff, the oscillatory currents gradually disappeared into the baseline current. Same cell as in D and E. Holding current was -18 pA.

Figure 2. Characteristics of light-evoked responses in starburst amacrine cells. A: a representative light-evoked response recorded in voltage clamp at -70 mV. The light stimulation triggered oscillatory inward currents with the initial “On” peak followed by a group of oscillations. Points of maximal relaxation for each oscillation are represented by the square symbols. The relationship between these points could be described by a first order exponential decay (represented by the dashed line joining the points) and totally superposed with the fitting of the relaxation of the initial “On” peak. The duration of light stimulation is represented by the horizontal line below the current trace. Holding current was -10 pA. B: comparison between average frequency of spontaneous and light-evoked current oscillations recorded at -70 mV for 10 different cells. For the light-evoked responses, the initial “On” peak was not counted as an oscillation and the light stimulation was a 70 μm diameter spot of light. Note that the frequency of light-evoked oscillations is more than 3 times higher than the frequency of spontaneous oscillations.
Figure 3. Glutamate binding onto AMPA/kainate receptors and the “On” pathway define the light-evoked responses in starburst amacrine cells. A, B, C: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (A), in the presence of 10 μM CNQX (B) and after return in control condition (C). Note the almost total disappearance of light response in the presence of CNQX, a blocker of AMPA/kainate receptors. The horizontal lines below the current traces represent the light stimulation durations. Holding current was -10 pA, -5 pA and 0 pA. D: the maximum amplitude of current is shown as current density in control condition and in the presence of CNQX. The blocker inhibited 87% of the maximum amplitude. E, F: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (E), and in the presence of 50 μM AP4 (F). The light response totally disappeared in the presence of AP4. Note that the peak appearing after the light cutoff is not light dependent but just a random spontaneous activity. The horizontal lines below the current traces represent the light stimulation durations. Holding current was -30 pA and -40 pA. G: the maximum amplitude of current expressed as current density is shown in control condition and in the presence of AP4. The amplitude was inhibited 95% by AP4.

Figure 4. The presynaptic release of glutamate occurring during the light response is calcium dependent. A, B: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (A) and in the presence of 200 μM CdCl₂, a non specific blocker of voltage-dependent calcium channels (B). Note the total disappearance of the light response in the presence of cadmium ions. Holding current was – 4 pA and 0 pA. C, D: the light response to a 200 μm diameter spot was recorded at -70 mV in control
condition (C) and in the presence of 30 μM nifedipine, a specific blocker of L-type calcium channels (D). With nifedipine, the initial “On” peak amplitude was largely reduced and its kinetics slowed, and the current oscillations disappeared. The horizontal lines below the current traces represent the light stimulation durations. Holding current was – 4 pA and -10 pA.

Figure 5. Presynaptic glutamate reuptake through a glutamate transporter is necessary for the oscillatory release of glutamate during light stimulation. A, B: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (A) and in the presence of 20 μM TBOA (B). Note the disappearance of current oscillations in the presence of TBOA. The horizontal lines below the current traces represent the light stimulation durations. Holding current was -50 pA and -57 pA. C: the maximum amplitude of current expressed as current density is shown in control condition and in the presence of TBOA. The amplitude of the initial “On” peak was not modified by TBOA. D: the average frequency of current oscillations is shown for control condition and for the steady state effect of TBOA. The frequency was decreased by 83% in the presence of the glutamate transporter blocker.

Figure 6. Effect of GABA_A and GABA_C receptors blockers on the light-evoked responses in starburst amacrine cells. A, B: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (A) and in the presence of 10 μM bicuculline (BMI), a GABA_A receptor antagonist (B). BMI largely increased the amplitude of the initial “On” peak without significantly affecting the current oscillations. Holding current
was +9 pA and -4 pA. C, D: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (C) and in the presence of 10 μM SR 95531, another GABA<sub>A</sub> receptor antagonist (D). SR 95531 increased the amplitude of the initial peak and slightly reduced the current oscillations frequency. Holding current was -10 pA and -18 pA. The horizontal lines below the current traces represent the light stimulation durations. E, F: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (E) and in the presence of 50 μM picrotoxin (PTX), a GABA<sub>A/C</sub> receptors antagonist (F). PTX increased the amplitude of the initial peak and strongly reduced the number of current oscillations. The horizontal lines below the current traces represent the light stimulation durations. Holding current was +16 pA and +20 pA. G: the maximum amplitude of the initial peak is shown as current density for control condition and for BMI (left panel), SR 95531 (center panel) and for PTX (right panel). The three drugs significantly increased the amplitude of the initial peak compared to control condition. H: the average frequency of current oscillations is shown for control condition and for BMI (left panel), SR 95531 (center panel) and for PTX (right panel). The three GABA receptors antagonists reduced the frequency of oscillations, with PTX showing the most potent effect.

Figure 7. The presynaptic oscillatory release of glutamate is regulated by a spiking inhibitory input. A, B, C: the response to a 70 μm diameter spot of light was recorded at -70 mV in the presence of PTX (A), in the presence of PTX and 0.3 μM tetrodotoxin (TTX) a specific blocker of voltage-dependent sodium channels (B), and after return to PTX (C). Note that in the presence of TTX, most of the current oscillations disappeared.
The horizontal lines below the current traces represent the light stimulation durations. Holding current was +20 pA, +28 pA and +40 pA. D, E, F: the average effect of TTX is shown for the maximum amplitude of the initial “On” peak (D), for the maximum amplitude of current oscillations (E), and for the oscillations frequency (F). When compared to PTX, TTX did not significantly increase the amplitude of the initial peak, but it strongly reduced the oscillations maximum amplitude and frequency.

Figure 8. The presynaptic oscillatory release of glutamate is regulated by a glycine/nergic input. A, B: the response to a 70 μm diameter spot of light was recorded at -70 mV in control condition (A) and in the presence of 10 μM strychnine, a glycine receptors antagonist (B). Almost all current oscillations disappeared during the application of strychnine. The horizontal lines below the current traces represent the light stimulation durations. Holding current was -30 pA and -50 pA. C: the maximum amplitude of the initial peak expressed as current density is shown in control condition and in the presence of strychnine. The antagonist did not significantly affect the initial peak amplitude. D: the average frequency of current oscillations is shown for control condition and for the steady state effect of strychnine. Strychnine strongly reduced the current oscillations frequency.

Figure 9: Schematic of the synapse between bipolar cell and starburst amacrine cell showing the different mechanisms regulating the glutamate release from the bipolar cell presynaptic terminal. In response to a light stimulus, the ribbon synapse of the bipolar cell terminal release glutamate into the synaptic cleft. The light response in starburst cells
shows two components, a large initial peak in response to the onset of light followed by a series of oscillations that last the entire duration of the light stimulus. The oscillations depend on an oscillatory release of glutamate that is dependent on the reuptake of glutamate from the synaptic cleft by the glutamate transporter (labeled T, it cotransport glutamate and Cl⁻ ions, GLUT/Cl⁻). The initial peak release and the oscillatory release (symbolized by the double arrow labeled GLUT for glutamate) are both dependent on the activity of presynaptic calcium channels that localize with the ribbon synapse. Three types of presynaptic inputs onto the bipolar cell terminal participate in the regulation of the glutamate release. A GABAergic input that could be a feedback from starburst amacrine cell or a direct input from a non-spiking amacrine cell affects both components of the release, the initial peak and the oscillations. Three different GABA receptors blockers, bicuculline, SR 95531 and picrotoxin increase the amplitude of the initial peak and decrease the frequency of the oscillations. The two other inputs seem to affect predominantly the oscillatory component of the glutamate release. A spiking-dependent inhibitory input is blocked by a specific blocker of voltage-dependent sodium channels, tetrodotoxin (TTX), it might be glycineric in nature. A glycineric input is blocked by the glycine receptors specific antagonist, strychnine. Both TTX and strychnine significantly decrease the frequency of oscillations.
Figure 1
Figure 2

A

B

Average Frequency (Hz)

n=10

Spontaneous Light-Evoked
Figure 3

A

Control

B

CNQX 10μM

C

Wash

D

Max Amplitude (pA/pF)

Control

n=27

E

Control

F

AP4 50μM

G

Max Amplitude (pA/pF)

Control

AP4

n=18
Figure 4

A
Control

B
CdCl₂ 200μM

C
Control

D
Nifedipine 30μM
Figure 5

A

Control

B

TBOA 20μM

C

Max Amplitude (pA/pF)

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D

Average Frequency (Hz)

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Figure 6
Figure 7

A. PTX 50 µM

B. PTX + TTX 0.3 µM

C. return to PTX

D. Initial Peak

E. Oscillations

F. Oscillations

Max Amplitude (pA/pF)

n=18

PTX PTX + TTX

Max Amplitude (pA/pF)

n=18

PTX PTX + TTX

Average Frequency (Hz)

n=18

PTX PTX + TTX

return to PTX
Figure 8
Figure 9