

Modulation of Neuronal Activity by Glial Cells in the Retina

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Glial–neuronal communication was studied by monitoring the effect of intercellular glial Ca^{2+} waves on the electrical activity of neighboring neurons in the eyecup preparation of the rat. Calcium waves in astrocytes and Müller cells were initiated with a mechanical stimulus applied to the retinal surface. Changes in the light-evoked spike activity of neurons within the ganglion cell layer occurred when, and only when, these Ca^{2+} waves reached the neurons. Inhibition of activity was observed in 25 of 53 neurons (mean decrease in spike frequency, $28 \pm 2\%$). Excitation occurred in another five neurons (mean increase, $27 \pm 5\%$). Larger amplitude Ca^{2+} waves were associated with greater modulation of neuronal activity. Thapsigargin, which reduced the amplitude of the glial Ca^{2+} increases, also reduced the magnitude of neuronal modulation. Bicuculline and strychnine,

inhibitory neurotransmitter antagonists, as well as 6-Nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) and $\text{D}(-)$ -2-amino-7-phosphonoheptanoic acid (D-AP7), glutamate antagonists, reduced the inhibition of neuronal activity associated with glial Ca^{2+} waves, suggesting that inhibition is mediated by inhibitory interneurons stimulated by glutamate release from glial cells. The results suggest that glial cells are capable of modulating the electrical activity of neurons within the retina and thus, may directly participate in information processing in the CNS.

Key words: calcium waves; glial cells; astrocytes; Müller cells; neurons; ganglion cells; retina; modulation; glial–neuronal interaction

Intercellular Ca^{2+} waves have been observed in various cell types, including glial cells of the CNS (Finkbeiner, 1993). These Ca^{2+} waves are transient increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) that propagate through networks of cells coupled together by gap junctions. They are initiated by various types of focal stimuli.

Intercellular Ca^{2+} waves in glial cells have been observed in a number of *in vitro* preparations (Finkbeiner, 1993). They have been recorded in syncytia of cultured astrocytes (Cornell-Bell et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Enkvist and McCarthy, 1992) and in glial cells of organotypic hippocampal slices (Dani et al., 1992). The waves can be initiated by mechanical stimuli or application of neurotransmitters (Finkbeiner, 1993).

Recently, we demonstrated that intercellular Ca^{2+} waves can also be propagated through networks of glial cells *in situ* in the freshly isolated mammalian retina (Newman and Zahs, 1997). These waves are initiated by electrical or mechanical stimuli as well as focal application of neurotransmitters. The waves travel out concentrically across the retinal surface and travel synchronously in astrocytes and Müller cells, the two principal glial cells of the mammalian retina.

These glial Ca^{2+} waves could have several possible functions. The waves could serve as a signaling mechanism permitting glial cells to communicate with each other over long distances. Such communication could serve to coordinate glial cell activity. Calcium waves might also represent a mechanism by which glial cells

communicate with and modulate the activity of neighboring neurons.

Support for such communication between glial cells and neurons has come from work in cell culture. When glial Ca^{2+} waves are initiated in co-cultures of astrocytes and neurons, increases in neuronal $[\text{Ca}^{2+}]_i$ are seen as Ca^{2+} waves are propagated through the underlying glial cells (Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995). A fascinating record showing the electrical excitation of a neuron, initiated by a glial Ca^{2+} wave, has also been reported (Hassinger et al., 1995). In these cell culture experiments, glial modulation of neuronal activity is thought to be mediated by the release of glutamate from astrocytes, because glutamate antagonists block the increases in neuronal $[\text{Ca}^{2+}]_i$ associated with the glial Ca^{2+} waves (Parpura et al., 1994; Hassinger et al., 1995).

The glial–neuronal signaling observed in cell culture is intriguing and suggests an important modulatory role for glial cells in the CNS. The results must be interpreted with caution, however, because cultured glial cells are known to differ from cells *in situ* in important respects (Barres et al., 1990; Duffy and MacVicar, 1995; Porter and McCarthy, 1995a,b). It remains to be shown that similar glial–neuronal signaling occurs in intact CNS tissue. We have examined this issue in the present work by studying glial–neuronal signaling in the freshly excised eyecup preparation of the rat. We have investigated whether the propagation of intercellular Ca^{2+} waves in retinal glial cells results in the modulation of the firing rate of neighboring retinal neurons. Our results suggest that glial cells can indeed modulate neuronal activity.

MATERIALS AND METHODS

Eyecup preparation. Male Long–Evans rats (250–400 gm) were deeply anesthetized with sodium pentobarbital administered intraperitoneally, and the eyes were removed. The posterior one-third of the eyes was everted over a Plexiglas dome and held in place with a stainless steel retaining ring. Most of the vitreous humor was removed by suction, and the eyecups were incubated in Calcium Green-1 AM (70 $\mu\text{g}/\text{ml}$; Molec-

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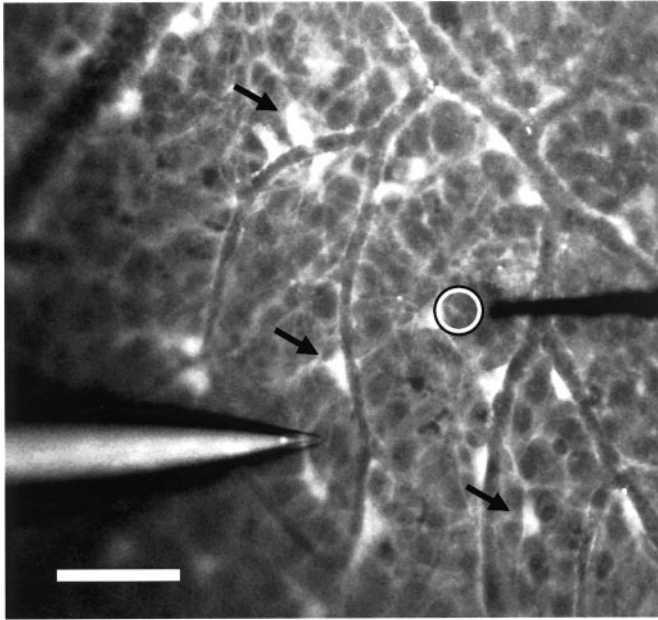


Figure 1. Confocal fluorescence image of the retinal surface of the eyecup labeled with Calcium Green-1. Astrocytes (arrows) and Müller cell endfeet (dim, diffuse fluorescence) were labeled. Calcium waves were initiated by advance of a micropipette (left). Extracellular activity of single neurons within the ganglion cell layer was recorded with a metal microelectrode (right). Calcium Green-1 fluorescence was measured within a 15 μm diameter region near the microelectrode tip (open circle). Scale bar, 50 μm .

ular Probes, Eugene, OR) and pluronic acid (4.7 mg/ml) in Ringer's solution for 35 to 60 min at 22°C. After incubation, eyecups were perfused with HCO_3^- -buffered Ringer's at 24°C. The Ringer's solution, containing 117 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 0.5 mM NaH_2PO_4 , 15 mM dextrose, and 26 mM NaHCO_3 , was equilibrated with 5% CO_2 in O_2 , pH 7.4. In contrast to earlier experiments (Newman and Zahs, 1997), neither ATP nor glutamate was added to the Ringer's solution to potentiate Ca^{2+} waves.

Imaging of intracellular Ca^{2+} . Incubation of rat eyecups with Calcium Green-1 AM selectively labeled astrocytes and Müller cells but left retinal neurons essentially unlabeled (Fig. 1). Calcium Green-1-labeled cells were viewed with a video-rate Noran Odyssey Confocal scanner (Middleton, WI) and a BX60 Olympus microscope with a 20 \times , 0.5 numerical aperture water immersion objective. Calcium Green-1 fluorescence was monitored with 488 nm argon excitation and a 515 nm long-pass barrier filter. Averaged images (average of 16 frames) were acquired every 2.55 sec, with the laser excitation source turned on for 1.10 sec during each acquisition cycle. MetaMorph software (Universal Imaging, West Chester, PA) was used to acquire, store, and analyze images.

Calcium waves were monitored to determine whether, in a particular trial, a wave reached the neuron being recorded. Calcium Green-1 fluorescence from glial cells within a circular region 15 μm in diameter, positioned just past the tip of the recording microelectrode (Fig. 1, open circle), was measured off-line. Normalized fluorescence values, the change in fluorescence divided by the baseline fluorescence ($\Delta F/F$), are reported.

Neuronal recording. Extracellular recordings from neurons were made with metal-in-glass microelectrodes plated with gold and platinum (Dowben and Rose, 1953). The recordings were from cell somata, as indicated by the recorded action potential waveforms, which often displayed both axonal and somatal components. Recordings were made in the ganglion cell layer within a few micrometers of the retinal surface.

Stimulation of the retina. Glial Ca^{2+} waves were initiated by a mechanical prodding of single glial cells at the retinal surface with the tip of a micropipette. The micropipette was advanced 15 μm for 10 msec, under the control of a piezoelectric actuator (Burleigh Instruments, Fishers, NY). The heat-sealed pipette was filled with a fluorescent dye to make it visible under confocal optics.

Neurons within the retina were stimulated by repetitive light flashes of the confocal laser excitation source. During an experimental trial, the laser was turned on for 1.10 sec every 2.55 sec. The excitation source illuminated a square region $\sim 300 \mu\text{m}$ on a side.

Analysis. Results are given in the form mean \pm SEM (number of samples). Statistical significance between samples was assessed using the Student's *t* test (unpaired samples).

RESULTS

The two principal types of macroglial cells within the mammalian retina are astrocytes and Müller cells (Ramon y Cajal, 1995). Astrocytes are confined largely to the nerve fiber layer at the inner retinal boundary, whereas radial Müller cells extend from the inner retinal surface to the photoreceptors in the outer retina. When Ca^{2+} waves are initiated at the retinal surface, increases in intracellular Ca^{2+} are observed in astrocyte somata and processes as well as in Müller cell endfeet and processes within the ganglion cell and inner plexiform layers (Newman and Zahs, 1997).

In the present study, Ca^{2+} waves in retinal eyecups were initiated by mechanically stimulating astrocyte somata or Müller cell endfeet. The resulting Ca^{2+} waves, traveling through astrocytes and Müller cells, were similar to those observed previously in the isolated retina (Newman and Zahs, 1997), although the propagation velocities were somewhat slower: $13.8 \pm 0.4 \mu\text{m}/\text{sec}$ (57) compared with 23.1 $\mu\text{m}/\text{sec}$ in the isolated retina (where the bathing solution was supplemented with glutamate and ATP). In the eyecup, the largest Ca^{2+} waves attained a diameter of $\sim 400 \mu\text{m}$. We studied the effect of these Ca^{2+} waves on neuronal activity by recording the spike activity of single neurons in the ganglion cell layer while simultaneously monitoring the propagation of glial Ca^{2+} waves at the retinal surface.

Light-evoked neuronal activity

More than 90% of all of the neurons recorded within the ganglion cell layer responded to flashes of light. Cells were classified into three groups according to their responses to the on and off of the light stimulus: ON, ON-OFF, and OFF cells. Peristimulus time histograms of three representative cells are illustrated in Figure 2.

Modulation of neuronal activity

The light-evoked activity of a neuron was frequently altered when a glial Ca^{2+} wave traveled past the cell. One example of this change in activity is illustrated in Figure 3. The activity of the neuron, an ON cell, is indicated in traces 1 and 2, and the Calcium Green-1 fluorescence within glial cells adjacent to the neuron is shown in trace 3. After a 30 sec control period, a Ca^{2+} wave was initiated by a mechanical stimulus (arrow). After a delay of ~ 3 sec, the Ca^{2+} wave arrived at the neuron. An increase in the firing rate of the neuron occurred after a similar delay. A movie illustrating this experimental trial can be viewed at <http://enlil.med.umn.edu/www/phsl/work/caw.htm#neuron>.

The majority of the neurons we monitored showed changes in their firing rate that were correlated with the arrival of glial Ca^{2+} waves. Modulation of the light-evoked responses of neurons, as well as neuronal spontaneous activity, was observed. The modulation was either excitatory or inhibitory, depending on the neuron being recorded.

An example of excitatory modulation is illustrated in Figure 4A, where records from three sequential trials in an ON neuron are shown. In all three trials, a mechanical stimulus initiated a glial Ca^{2+} wave. The Ca^{2+} wave reached the neuron in trials 1 and 3. In trial 2, the wave died out before reaching the cell. As shown in Figure 4A, the firing rate of the neuron increased in

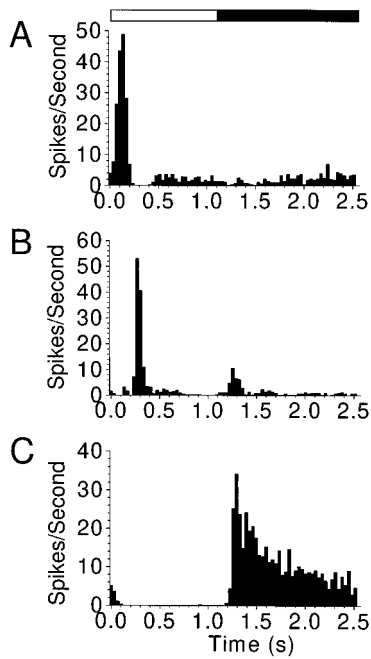


Figure 2. Peristimulus time histograms of the light-driven activity of an ON (*A*), an ON–OFF (*B*), and an OFF (*C*) neuron, each representative of one of the three response classes observed. Open and closed bars above the histograms indicate periods of light ON and OFF, respectively. Activity was recorded during control periods, before initiation of Ca^{2+} waves.

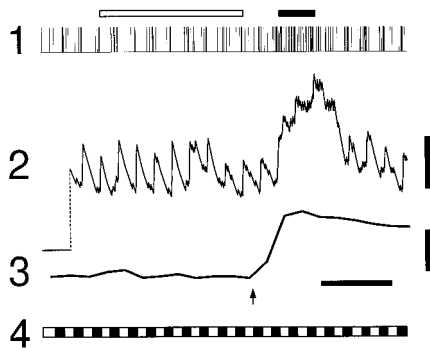


Figure 3. Modulation of neuronal activity in an ON neuron. Cell activity is plotted as a spike display in trace 1 (vertical lines represent single action potentials) and as a frequency plot in trace 2 (abscissa represents the running average of spike frequency, i.e., a leaky integrator with a linear decay to 0% at 10 sec; 0 frequency is indicated at left end of the trace). Trace 3, Calcium Green-1 fluorescence in a circular region near the recording microelectrode tip. Trace 4, light stimulus, indicating periods of light ON (open segments) and OFF (closed segments). Arrow indicates initiation of the Ca^{2+} wave. Open and closed bars above trace 1 indicate control and test periods, respectively, during which average spike frequency was measured. Neuron spike frequency and glial cell fluorescence increase concurrently several seconds after initiation of the glial Ca^{2+} wave. Calibration bars: trace 2 (spike frequency), three spikes/sec; trace 3 (Calcium Green-1 fluorescence), 20% $\Delta F/F$; time, 10 sec.

those trials when the Ca^{2+} wave reached the cell but remained unchanged when the wave failed to reach the cell.

An example of inhibitory modulation in an ON–OFF neuron is illustrated in Figure 4*B*. A decrease in the firing rate of the neuron occurred when the glial Ca^{2+} wave reached the cell (trials 1 and 3). When a Ca^{2+} wave was initiated but failed to reach the cell (trial 2), the firing rate of the neuron remained unaltered.

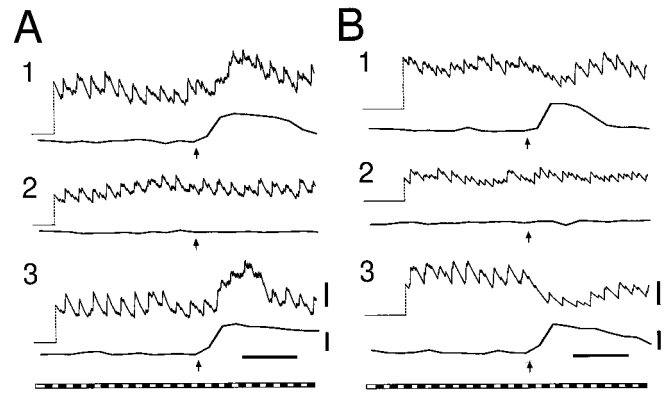


Figure 4. Excitatory and inhibitory modulation of neuronal activity. A frequency plot of cell activity (top trace) and glial cell Calcium Green-1 fluorescence (bottom trace) are shown for each trial. *A*, Excitatory modulation in an ON neuron. Spike frequency increases in those trials (1 and 3) in which $[\text{Ca}^{2+}]_i$ in neighboring glial cells also increases. *B*, Inhibitory modulation in an ON–OFF neuron. Spike frequency decreases when glial $[\text{Ca}^{2+}]_i$ increases. The peristimulus time histograms of cells in *A* and *B* are shown in Figure 2, *A* and *B*, respectively. Calibration bars are the same as in Figure 3.

Although changes in neuronal firing rate were associated with increases in glial Ca^{2+} , the onset and decline of neuronal modulation did not necessarily follow the time course of changes in glial Ca^{2+} precisely. The neuronal firing rate sometimes recovered toward control levels even as glial Ca^{2+} levels remained elevated (e.g., Fig. 3).

Magnitude of neuronal modulation

Changes in the firing rate of neurons were quantified by comparing the average frequency of spike activity in a control period, before initiation of a Ca^{2+} wave, to activity in a test period during propagation of the wave. The control period spanned the eight on–off light cycles immediately preceding initiation of the Ca^{2+} wave (Fig. 3, open bar above trace 1). The test period consisted of the two light cycles centered at the peak of the Ca^{2+} wave (Fig. 3, closed bar). For trials in which the Ca^{2+} wave did not reach the neuron being monitored, the test period was determined by estimating when the wave would have arrived if it had propagated past the neuron (based on its propagation velocity). Modulation of the neuronal firing rate was expressed as the percentage change in average spike frequency:

$$\text{Modulation} = \left(\frac{\text{Freq}_{\text{test period}} - \text{Freq}_{\text{control period}}}{\text{Freq}_{\text{control period}}} \right) \cdot 100$$

The relation between the change in neuronal firing rate and Ca^{2+} levels in adjacent glial cells was analyzed for a total of 53 neurons. For each neuron tested, 9–14 trials were conducted. In some of these trials, the glial Ca^{2+} wave propagated past the neuron (defined as a $\Delta F/F$ fluorescence change of $>22\%$ in the glial cells adjacent to the neuron). In other trials, a Ca^{2+} wave was initiated but failed to reach the neuron ($\Delta F/F < 1\%$). For each neuron, the average value of neuronal modulation was calculated for those trials in which the Ca^{2+} wave reached the cell and for those trials in which the wave failed to reach the cell.

In 30 of the 53 neurons analyzed, there was a significant difference in the modulation of spike activity when a Ca^{2+} wave reached the cell, compared with the modulation observed when the Ca^{2+} wave did not reach the cell (Table 1). Twenty-five of the

Table 1. Modulation of neuronal activity

Neuronal type	ON	ON-OFF	OFF
Number of cells with significant modulation			
Inhibition	7 of 24	9 of 15	9 of 14
Excitation	5 of 24	0 of 15	0 of 14
Magnitude of cell modulation			
No Ca ²⁺ wave ^a	+3 ± 5% (30)	-1 ± 3% (20)	+1 ± 2% (39)
Large Ca ²⁺ wave ^b	-35 ± 3% (35)	+27 ± 5% (27)	-25 ± 3% (35)

Number of cells. For each cell tested, modulation was judged significant if the modulation in those trials when a Ca²⁺ wave reached the cell ($\Delta F/F$ Ca²⁺ signal > 22%) was significantly different than when a Ca²⁺ wave failed to reach the cell ($\Delta F/F < 1%$) ($p < 0.05$).

Magnitude of cell modulation. Shown are the average modulation values for trials in cells with statistically significant modulation. Separate means are given for trials when a Ca²⁺ wave reached the cell and did not reach the cell. For ON neurons, separate means are given for cells with excitatory and inhibitory modulation.

^a Trials when the $\Delta F/F$ Ca²⁺ signal was <1%.

^b Trials when $\Delta F/F$ was >22%.

53 cells showed a significant decrease in firing rate associated with the arrival of a Ca²⁺ wave. Modulation averaged $-28 \pm 2\%$ (114 trials) in these cells. Another five cells showed a significant increase in firing rate associated with a Ca²⁺ wave. Modulation averaged $28 \pm 5\%$ (27 trials) in these neurons. There was no modulation of neuronal activity when a Ca²⁺ wave failed to reach a neuron. In these trials, modulation equaled $+1 \pm 2\%$ (125 trials) for the 30 cells with Ca²⁺ wave-associated modulation and $-3 \pm 1\%$ (235 trials) for all 53 neurons analyzed.

Examples of inhibition of spike activity were seen in all three classes of neurons, ON, ON-OFF, and OFF cells. Interestingly, enhancement of spike activity was seen only in ON neurons (Table 1). In trials when significant modulation of neuronal activity was observed, the distance between the neurons and the initiation site of the Ca²⁺ wave averaged $60 \pm 1 \mu\text{m}$ (74). The distance exceeded $75 \mu\text{m}$ in eight trials.

Relation between Ca²⁺ increases and neuronal modulation

The results summarized above demonstrate that a change in neuronal firing rate is associated with the arrival of a glial Ca²⁺ wave at that neuron. In addition, the *magnitude* of the change in neuronal activity was correlated with the *amplitude* of the Ca²⁺ increase. This relation was analyzed for all cells displaying significant inhibitory modulation (Fig. 5A). In trials in which the amplitude of the glial [Ca²⁺]_i increase was 0, i.e., when the wave failed to reach the neuron, neuronal modulation was near 0. As the amplitude of the [Ca²⁺]_i increase grew larger, the magnitude of inhibitory modulation also increased (greater negative modulation).

The timing of the modulation of neuronal activity was correlated with the timing of the Ca²⁺ wave as well. When the time to the peak of inhibitory modulation was plotted as a function of the time to the peak of the Ca²⁺ wave, a clear relation was evident (Fig. 5B). Such a relation would not exist if the mechanical stimulus used to initiate the Ca²⁺ wave was directly modulating the activity of the neurons.

Thapsigargin and neuronal modulation

If increases in glial [Ca²⁺]_i result in the modulation of neuronal firing, then pharmacological manipulation of the Ca²⁺ increase should alter the modulation. This was tested by application of

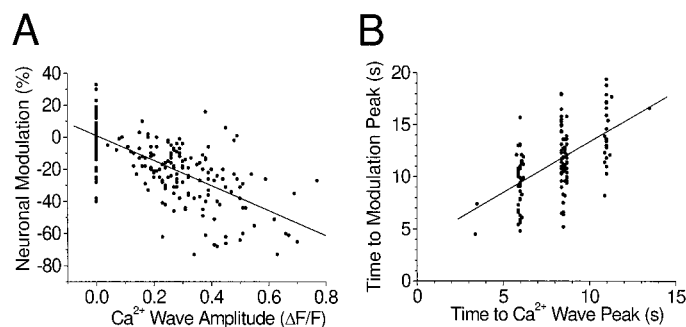


Figure 5. Correlation between neuronal modulation and Ca²⁺ increases. *A*, Relation between the magnitude of inhibition of neuronal activity and the amplitude of the Ca²⁺ wave. Points represent single trials from all cells showing significant inhibitory modulation. Points at the left of the plot ($\Delta F/F = 0$) represent trials in which the Ca²⁺ wave failed to reach the neuron. Least-squares fit, $R^2 = 0.42$. *B*, Relation between the time to the peak of neuron modulation and the time to the peak of the Ca²⁺ wave. Trials in which the inhibition of activity was 25% or greater are included. The discrete Ca²⁺ wave times reflect the 2.55 sec acquisition period of fluorescence images. Least-squares fit, slope = 0.97, intercept = 3.6 sec, $R^2 = 0.30$.

thapsigargin, a compound that depletes intracellular Ca²⁺ stores (Thastrup et al., 1990) and substantially reduces the magnitude of glial Ca²⁺ waves (Newman and Zahs, 1997). Experiments were conducted on neurons that displayed prominent inhibitory modulation associated with increases in glial [Ca²⁺]_i. Under control conditions, the average inhibitory modulation observed in these neurons equaled $-23 \pm 3\%$ (16). Modulation was reduced to $-12 \pm 3\%$ (22) after 30 min exposure to $3.0 \mu\text{M}$ thapsigargin ($p < 0.02$) (Fig. 6). In the same trials, the increase in [Ca²⁺]_i measured in the glial cells was reduced from $28 \pm 4\%$ ($\Delta F/F$) to $4 \pm 1\%$ by thapsigargin. In all trials, Ca²⁺ waves were initiated sufficiently close to the neuron being monitored, so that under control conditions the waves would have reached the neuron (48 ± 1 and $44 \pm 1 \mu\text{m}$ from the neurons in control and test trials, respectively).

Transmitter antagonists and neuronal modulation

If glial cells cause the observed modulation of neuronal activity, it is possible that the modulation is mediated by the release of

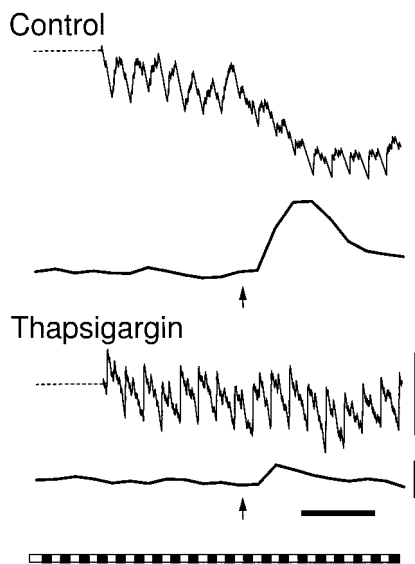


Figure 6. Effect of thapsigargin on the modulation of neuronal activity. Thapsigargin ($3.0 \mu\text{M}$) reduces the glial Ca^{2+} increase and the inhibition of activity in an OFF neuron. Calibration bars for trials in Figures 6 and 7: spike frequency, two spikes/sec; Calcium Green-1 fluorescence, 20% $\Delta F/F$; time, 10 sec. A frequency plot of neuronal activity and Calcium Green-1 fluorescence are shown in each trial.

neurotransmitters from the glial cells. This appears to be the case in cell culture where Ca^{2+} -dependent release of glutamate from glial cells has been demonstrated (Parpura et al., 1994, 1995) and where neuronal $[\text{Ca}^{2+}]_i$ increases associated with glial Ca^{2+} waves are blocked by glutamate antagonists (Parpura et al., 1994; Hassinger et al., 1995). We performed a number of pharmacological experiments to investigate whether glutamate or other neurotransmitters were involved in the modulation of neuronal activity associated with glial Ca^{2+} waves. In these studies, only trials in which the Ca^{2+} wave reached the neuron being monitored were analyzed. Experiments were restricted to the characterization of inhibitory modulation, because neurons showing excitatory modulation were rarely encountered.

Antagonists to the inhibitory neurotransmitters GABA and glycine were both effective in reducing the inhibition of spike activity associated with glial Ca^{2+} waves (Table 2; Fig. 7A). The GABA_A antagonist bicuculline ($5 \mu\text{M}$) and the glycine antagonist strychnine ($1 \mu\text{M}$) each reduced inhibitory modulation by more than half, whereas both antagonists applied together completely abolished the inhibitory modulation. The effects of both bicuculline and strychnine were largely reversible (Table 2). Neither antagonist reduced the amplitude of the Ca^{2+} waves.

Glutamate antagonists were also effective in reducing the inhibition of spike activity associated with glial Ca^{2+} waves (Table 2; Fig. 7B). The AMPA antagonist NBQX ($10 \mu\text{M}$) and the NMDA antagonist D-AP7 ($200 \mu\text{M}$) both reduced the magnitude of inhibitory modulation by one-half or more, and the two antagonists applied together nearly abolished the inhibition of spike activity. The effects of the antagonists were partially reversible, and neither antagonist reduced the amplitude of the Ca^{2+} waves.

DISCUSSION

Glial modulation of neuronal activity

The results presented in this study suggest that the propagation of intercellular Ca^{2+} waves through glial cells in the retina results in

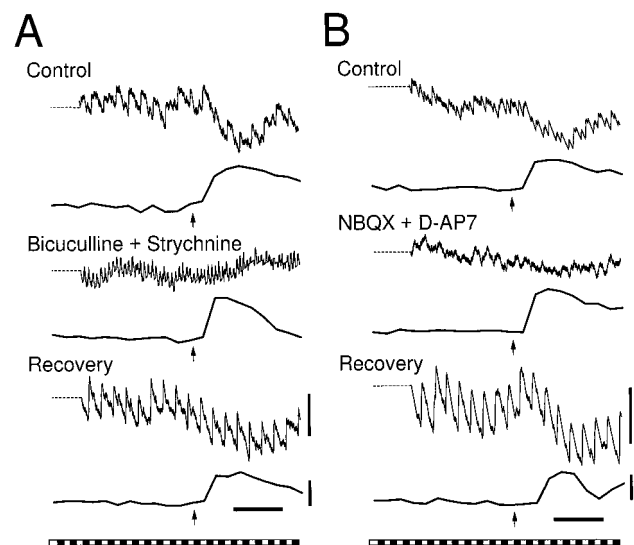


Figure 7. Effect of neurotransmitter antagonists on neuronal modulation. *A*, Bicuculline ($5 \mu\text{M}$) and strychnine ($1 \mu\text{M}$), applied together, abolish the inhibition of activity in an ON–OFF neuron without attenuating the glial Ca^{2+} increase. *B*, NBQX ($10 \mu\text{M}$) and D-AP7 ($200 \mu\text{M}$) together reduce the inhibition of activity in an ON–OFF neuron without reducing the glial Ca^{2+} increase. As illustrated, light responses of neurons often changed during drug application and recovery.

the modulation of the firing rate of retinal neurons. Modulation of neuronal activity was not dependent on the initiation of a glial Ca^{2+} wave, per se, but rather on whether the Ca^{2+} wave reached the neuron. In addition, the magnitude of the modulation of neuronal activity was correlated with the amplitude of the Ca^{2+} increase in the glial cells adjacent to the neuron. The timing between the modulation of neuronal activity and the glial Ca^{2+} increase was similarly related. Finally, attenuation of glial Ca^{2+} increases by thapsigargin resulted in a significant decrease in the magnitude of neuronal modulation. All of these findings support the hypothesis that increases in glial $[\text{Ca}^{2+}]_i$, or some other response associated with the propagation of Ca^{2+} waves through glial cells, results in the modulation of neuronal firing.

Alternative explanations of neuronal modulation

It is conceivable that a mechanism other than direct glial–neuronal communication was responsible for the observed modulation. The mechanical stimulus used to initiate glial Ca^{2+} waves could stimulate, in theory, the neurons being monitored and alter their firing rate. This is highly unlikely, however. Identical mechanical stimuli applied to the same retinal location had different effects on neuronal firing, depending on whether the stimulated Ca^{2+} wave reached the cell. In addition, direct mechanical stimulation of a neuron would have a latency of milliseconds rather than the several seconds observed.

It is possible that neuronal modulation was mediated by a signal propagated within a network of neurons. The mechanical stimulus used to initiate glial Ca^{2+} waves might also initiate intercellular Ca^{2+} waves within neurons, for instance. Such Ca^{2+} waves, propagated through ganglion cells and amacrine cells, have been observed in the neonatal ferret retina (Wong et al., 1995; Feller et al., 1996). This mechanism seems unlikely, however. First, it is not known whether these waves can be initiated mechanically; they occur spontaneously in the ferret retina (Wong et al., 1995). Furthermore, the mechanical stimulus used to initiate glial Ca^{2+} waves in our study was small in amplitude ($15 \mu\text{m}$) and was

Table 2. Effect of neurotransmitter antagonists on the inhibitory modulation of neuronal activity

Condition	Control	Antagonist	Recovery
Inhibitory neurotransmitter antagonists			
Bicuculline (5 μ M)	$-31 \pm 5\%$ (15)	$-13 \pm 3\%$ (17)*	$-26 \pm 3\%$ (16)*
Strychnine (1 μ M)	$-28 \pm 5\%$ (20)	$-8 \pm 3\%$ (17)*	$-25 \pm 4\%$ (19)*
Bicuculline + strychnine	$-28 \pm 4\%$ (28)	$0 \pm 1\%$ (23)*	$-20 \pm 2\%$ (32)*
Glutamate antagonists			
NBQX (10 μ M)	$-25 \pm 5\%$ (22)	$-6 \pm 3\%$ (22)*	$-15 \pm 2\%$ (23)*
D-AP7 (200 μ M)	$-24 \pm 5\%$ (21)	$-12 \pm 4\%$ (18)**	$-22 \pm 5\%$ (14)
NBQX + D-AP7	$-18 \pm 2\%$ (19)	$-2 \pm 2\%$ (19)*	$-12 \pm 3\%$ (18)*

Shown are the average modulation values for trials when a Ca^{2+} wave reached the neuron. Only neurons with significant inhibitory modulation were tested. Antagonists were applied for 15 min before testing. Recovery followed a 60 min washout.

* $p < 0.01$, antagonist versus control, recovery versus antagonist.

** $p < 0.05$.

directed at individual glial cells. The stimulus would presumably be less effective in initiating Ca^{2+} waves in neurons lying beneath the surface. In addition, neuronal Ca^{2+} waves, if they were initiated, would have to mimic the glial Ca^{2+} waves in many respects, including their extent of travel, magnitude, and timing, to account for the observed results. The chance of this occurring in many of the trials is highly unlikely.

Identity of neurons that are modulated

The neurons recorded in this study lay just beneath the surface of the retina in the ganglion cell layer and thus were presumably ganglion cells. Their responses to light resembled those reported previously for ganglion cells in the rat (Brown and Rojas, 1965). It is possible, however, that some of these cells were displaced amacrine cells, which are prevalent in the ganglion cell layer of the rat (Perry and Walker, 1980) and generate spike responses similar to those of ganglion cells (Bloomfield, 1996).

Mechanism of modulation of neuronal activity

The following model of the *inhibition* of neuronal activity by glial cells is suggested by our experiments. The arrival of a Ca^{2+} wave in a glial cell leads to the release of glutamate from the cell and to the excitation of inhibitory interneurons. The interneurons, in turn, release GABA and glycine onto ganglion cells, leading to a decrease in their firing rate. Amacrine cells, which are stimulated by glutamate and release both GABA and glycine (Miller, 1994), are likely to be the interneurons that mediate the inhibition of ganglion cell activity.

This model is supported by our transmitter antagonist experiments as well as by the known pharmacology of retinal neurons. GABA and glycine antagonists each reduced the inhibition of neuronal activity, as they would if they interrupted the inhibitory link between amacrine cells and ganglion cells. Glutamate antagonists also reduced inhibition of ganglion cell activity, as they should if the major effect of glutamate release from glial cells is to excite amacrine cells rather than ganglion cells. (If glutamate released by glia primarily excited ganglion cells, then glutamate antagonists would *increase* rather than *decrease* the inhibition of ganglion cell activity.) It has been shown previously that amacrine cells possess both AMPA and NMDA receptors and that ganglion cells possess both GABA_A and glycine receptors (Miller, 1994).

In cell culture, the release of glutamate from glial cells is thought to be mediated by an increase in $[Ca^{2+}]_i$ (Parpura et al., 1994, 1995). Such a Ca^{2+} -dependent release of glutamate may

occur in the retina as well. Not only is modulation of neuronal activity associated with $[Ca^{2+}]_i$ increases within glial cells in our experiments, but neuronal modulation is reduced when these Ca^{2+} increases are diminished by thapsigargin. [The thapsigargin-induced decrease in neuronal modulation (48%) was less than the observed decrease in Ca^{2+} wave magnitude (86%), suggesting that a Ca^{2+} -dependent mechanism may not fully account for the results.]

The *excitation* of neuronal activity observed in a few of the ON cells in our experiments could arise in several ways. These neurons may be ganglion cells which receive an unusually large glutamatergic input from glial cells. Alternately, the neurons may be amacrine cells, which by our model receive excitatory glutamatergic input from glial cells.

An important feature of our model that must be explained is why glutamate released from glial cells primarily stimulates amacrine cells rather than ganglion cells. Glutamate may be released preferentially from those regions of astrocytes or Müller cells that directly contact amacrine cells, but a morphological correlate to account for this specialization is not known.

Glial Ca^{2+} waves in the retina travel through both astrocytes and Müller cells (Newman and Zahs, 1997). It remains to be determined which of these two types of glial cells (or both) mediated the modulation of neuronal firing.

Glial modulation of neuronal activity *in vivo*

Our work indicates that glial cells, when stimulated mechanically, modulate the firing rate of neurons in the retina. But do glial cells modulate neuronal activity *in vivo*? This remains an open question, because it is not known whether glial Ca^{2+} waves, or other Ca^{2+} signals, are generated *in vivo*. Glial Ca^{2+} increases are stimulated by neuronal activity in a number of *in situ* preparations, however. In organotypic hippocampal slices, electrical stimulation of neuronal fiber tracts results in the initiation of intercellular Ca^{2+} waves in astrocytes (Dani et al., 1992). In acutely isolated hippocampal slices, electrical stimulation (Porter and McCarthy, 1996), as well as the addition of glutamatergic (Porter and McCarthy, 1995b) and purinergic (Porter and McCarthy, 1995a) agonists, leads to increases in astrocytic $[Ca^{2+}]_i$. Finally, in preliminary experiments on the eyecup preparation in our own laboratory, light flashes trigger transient Ca^{2+} increases within Müller cells. These increases are as large as those observed during propagated Ca^{2+} waves (E. Newman, unpublished observations).

Significance of glial modulation of neuronal activity

There is growing evidence that glial cells can influence neuronal activity in a number of ways. Glial cells control neuronal excitability by regulating K^+ levels (Newman, 1995) and pH (Chesler, 1990) in the extracellular space. Glial cells regulate synaptic efficacy by their active uptake of neurotransmitters (Martin, 1995) and may promote the development of functional synaptic connections between neurons (Pfrieger and Barres, 1997).

Our work provides evidence for an additional mechanism of glial control of neuronal excitability. As the studies cited above indicate, neuronal activity may stimulate Ca^{2+} increases within glial cells, leading to the glial release of glutamate. The resulting neuronal–glial–neuronal feedback loop could provide either a positive or negative input onto neurons and serve to reinforce or dampen neuronal activity, depending on the prominence of inhibitory interneurons in the feedback circuit. Our results, along with those of others, provide evidence that glial cells modulate neuronal activity and, as such, may directly participate in information processing in the brain.

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