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GLIAL CELL FUNCTION

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Calcium signaling in retinal glial cells and its effect on neuronal activity

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Introduction

Intercellular glial calcium waves have been studied extensively in culture (Finkbeiner, 1993; Verkhratsky et al., 1998). Stimulation of a single astrocyte in a confluent culture evokes an increase in intracellular Ca^{2+} levels which propagates outwards through the network of astrocytes.

These glial Ca^{2+} increases have been shown to modulate neuronal activity. In co-cultures of astrocytes and neurons, Ca^{2+} increases in glial cells evoke Ca^{2+} increases in neighboring neurons (Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995). In addition, glial Ca^{2+} increases modulate the electrical activity of neighboring neurons, evoking inward currents and modulating neurotransmitter release at neuronal synapses (Hassinger et al., 1995; Araque et al., 1998a,b; Sanzgiri et al., 1999).

Characterization of glial Ca^{2+} signaling and glial-neuronal interactions has been largely restricted to work on cultured cells, leaving critical questions unanswered: Do intercellular glial Ca^{2+} waves occur in intact CNS tissue? If they do, are these glial Ca^{2+} waves capable of modulating the electrical activity of neurons?

This chapter will review work we have done on glial Ca^{2+} signaling and glial-neuronal interactions

in the freshly isolated mammalian retina. Although our experiments were conducted on excised tissue, the conditions in our preparation are presumably similar to those in the intact animal.

Calcium waves in the mammalian retina

The mammalian retina offers many advantages for studying glial Ca^{2+} signaling and glial-neuronal interactions in situ. Two principal macro glial cells are present in mammals with vascularized retinas: astrocytes and Müller cells (Fig. 1). See Newman (1999, 2000) for reviews. Astrocytes are largely restricted to the nerve fiber layer at the inner border of the retina. Müller cells, a type of radial glial cell, span the entire neural retina, from the vitreal surface to the photoreceptor layer. Retinal neurons lie directly beneath astrocytes and the endfeet of Müller cells in the ganglion cell layer. The somata and dendrites of these neurons are surrounded by Müller cell processes.

If one looks down on the inner surface of the retina, astrocyte somata and processes and the endfeet of Müller cells are visible (see Figs. 2 and 7). Tracer studies have shown that these glial cells are coupled together by gap junctions (Robinson et al., 1993; Zahs and Newman, 1997). When a single astrocyte is filled with a tracer, the tracer spreads to many other astrocytes as well as to Müller cells. Coupling between astrocytes and Müller cells is weaker than is coupling between astrocytes. A small tracer (neurobiotin) spreads into both types of glial cells while a larger tracer (Lucifer yellow) spreads

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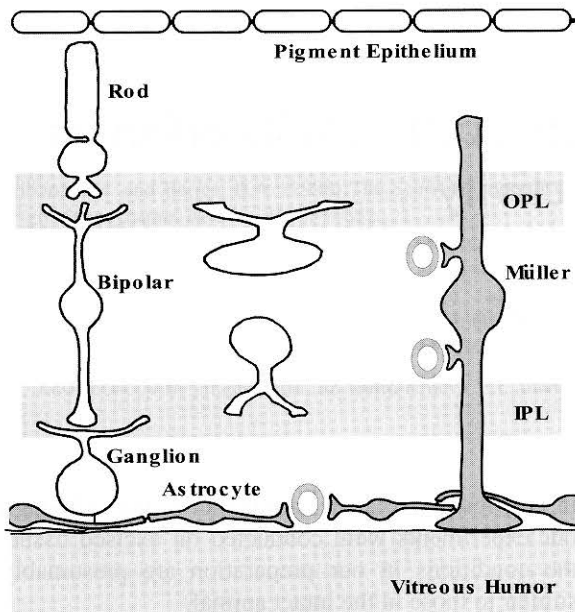


Fig. 1. Schematic of vascularized mammalian retina. Retinal glial cells (Müller cells and astrocytes) are shaded. Endfoot processes of both types of glial cells are shown terminating on retinal blood vessels (shaded ovals). OPL, outer plexiform layer; IPL, inner plexiform layer.

only into astrocytes. In addition, coupling between astrocytes and Müller cells is asymmetric. Neurobiotin passes from astrocytes to Müller cells, but not from Müller cells to astrocytes.

Calcium waves were monitored in our experiments on isolated whole-mount rat retinas and rat eyecups using Ca^{2+} indicator dyes (Calcium Green-1 or Fluo-4). The vitreal surface of the retina was imaged with a video-rate confocal microscope (Noran Odyssey) and images stored and analyzed using image processing software (MetaMorph, Universal Imaging).

Stimulation of astrocyte somata on the retinal surface evoke robust intercellular Ca^{2+} waves (Newman and Zahs, 1997; Fig. 2). Chemical (ATP, carbachol, phenylephrine, but not glutamate), mechanical and electrical stimulation are all effective in evoking waves. Regardless of the method of stimulation, the waves propagate outwards at a velocity of approximately $23 \mu\text{m/s}$. Similar propagation velocities have been noted in astrocytes in culture (Finkbeiner, 1993).

The increases in intracellular Ca^{2+} concentra-

tion that occur during Ca^{2+} wave propagation in the rat retina are generated by the release of Ca^{2+} from internal stores rather than from the influx of Ca^{2+} from extracellular space (Newman and Zahs, 1997). Normal Ca^{2+} waves are observed when the retina is superfused with nominally Ca^{2+} -free solution (Fig. 3A,B). Calcium waves are substantially reduced, however, when retinas are treated with thapsigargin, an endoplasmic reticulum Ca^{2+} pump poison that depletes internal Ca^{2+} stores (Fig. 3C,D).

These results demonstrate that glial cells in freshly excised central nervous system (CNS) tissue are capable of supporting the propagation of intercellular Ca^{2+} waves. The Ca^{2+} waves behave similarly to those observed in cultured astrocytes. One notable difference, however, is that Ca^{2+} increases in retinal glial cells are not evoked by application of glutamate (Newman and Zahs, 1997), while cultured astrocytes isolated from the brain are quite sensitive to this transmitter (Finkbeiner, 1993). This difference may arise because glial cells at the inner surface of the retina are, *in vivo*, continually exposed to $10 \mu\text{M}$ glutamate, the concentration normally present in the vitreous humor (Dreyer et al., 1996; Ambati et al., 1997).

The remainder of this chapter will address three key questions concerning Ca^{2+} waves in retinal glial cells:

- (1) What is the mechanism by which Ca^{2+} waves are propagated between retinal glial cells?
- (2) Do Ca^{2+} increases in retinal glial cells lead to changes in the activity of neighboring retinal neurons?
- (3) Does Ca^{2+} signaling in glial cells occur normally in intact retinal tissue?

Mechanism of propagation of intercellular calcium waves

Propagation of Ca^{2+} waves through networks of astrocytes was originally believed to occur by the diffusion of an internal messenger between cells (Sanderson et al., 1994; Sanderson, 1996). Astrocytes are coupled together, both *in vivo* and *in culture*, by an extensive network of gap junctions. When blockers of gap junctional coupling are added to cultured astrocytes, Ca^{2+} wave propagation is diminished (Finkbeiner, 1992; Enkvist and McCarthy,

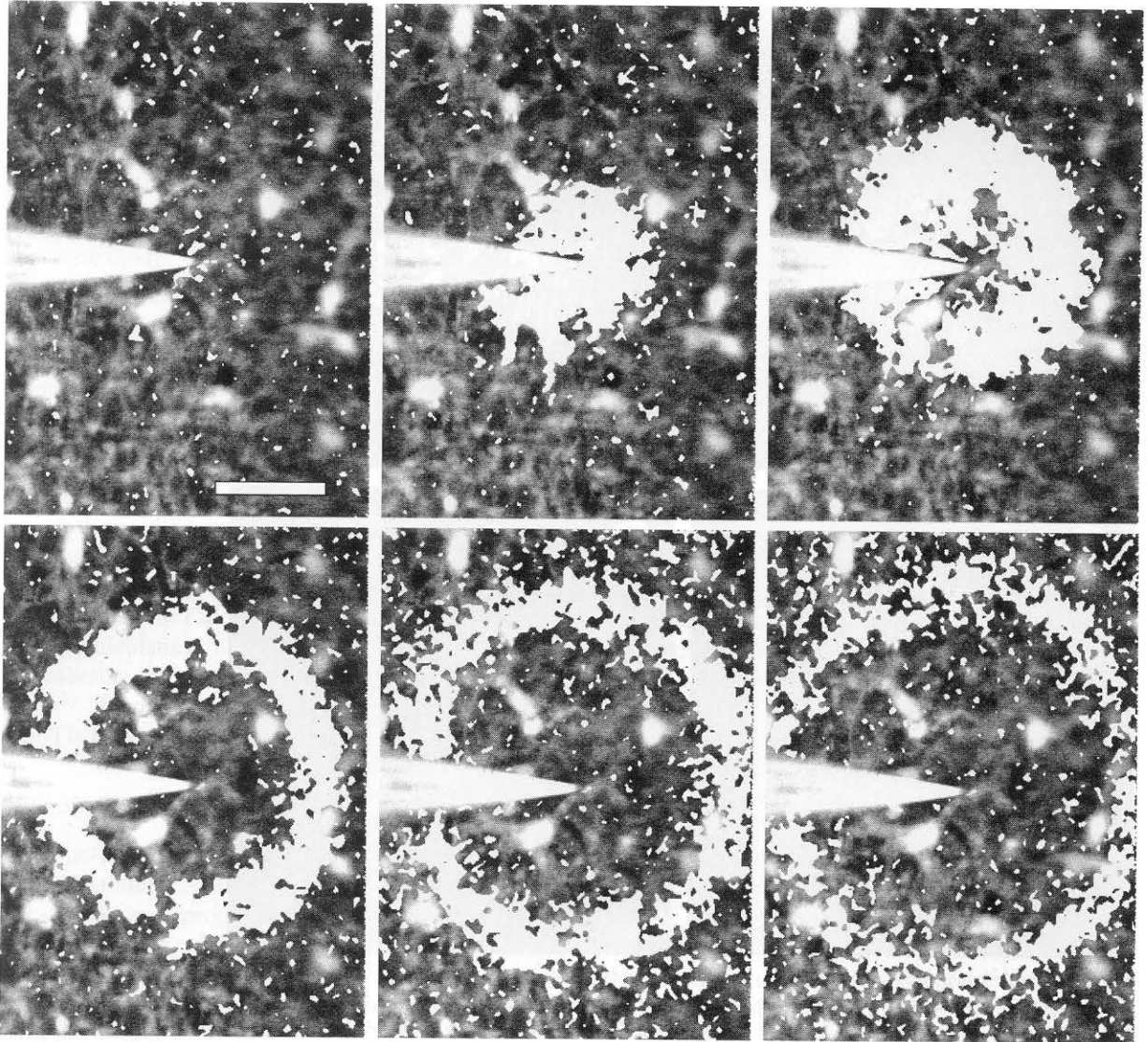


Fig. 2. Propagation of an intercellular glial Ca^{2+} wave in the retina. Fluorescence image of the retinal surface is shown in black and white. Superimposed white rings mark the leading edge of the Ca^{2+} wave (where the change in fluorescence between successive images exceeded a threshold value). Isolated whole-mount rat retina. Scale bar: 50 μm . From Newman and Zahs, 1997.

1992). In addition, blockers of IP_3 signaling within cells are effective in reducing the spread of astrocyte Ca^{2+} waves (Boitano and Dirksen, 1992; Charles et al., 1993; Newman and Zahs, 1997). Taken together, these results indicate that Ca^{2+} wave propagation occurs by the diffusion of IP_3 (and perhaps Ca^{2+}) between glial cells through gap junctions.

Recent evidence has challenged this model of Ca^{2+} wave propagation. In culture, Ca^{2+} waves are

able to propagate through a network of astrocytes, even when direct contact between cells is interrupted by gaps in the confluent culture (Hassinger et al., 1996; Guthrie et al., 1999). This suggests that an extracellular messenger is acting as a signaling molecule to bridge gaps between cells when they are not directly coupled. In addition, Ca^{2+} wave propagation is diminished by addition of purinergic receptor antagonists (Guan et al., 1997; Cotrina et

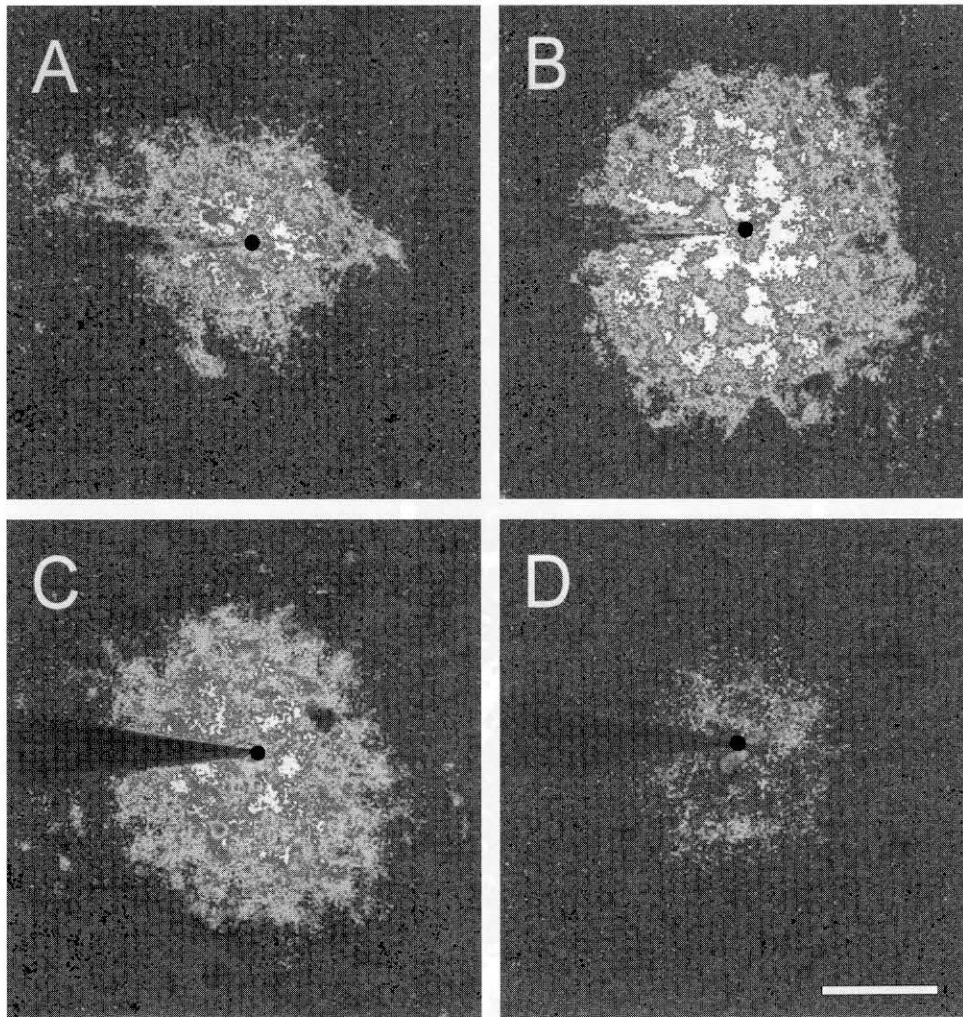


Fig. 3. Glial Ca^{2+} increases are generated by release from internal stores. (A) Control Ca^{2+} wave evoked by an electrical stimulus. (B) Calcium wave evoked in the same retina after it was bathed in 0 mM Ca^{2+} , 0.5 mM EGTA for 31 min. The Ca^{2+} wave is not diminished. (C) Control Ca^{2+} wave. (D) Calcium wave evoked in the same retina after it was bathed in 1.5 μM thapsigargin for 16 min. The Ca^{2+} wave is reduced substantially in intensity and size. The images in all frames represent the ratio of images acquired after stimulation to images acquired before stimulation. Isolated whole-mount rat retina. Scale bar: 50 μm . From Newman and Zahs, 1997.

al., 1998a,b, 2000; Guthrie et al., 1999; Fam et al., 2000) and by apyrase (Cotrina et al., 1998a,b, 2000; Guthrie et al., 1999), which hydrolyzes ATP. Release of ATP into the culture medium has also been demonstrated during Ca^{2+} wave propagation (Cotrina et al., 1998b; Guthrie et al., 1999). Together, these results support a mechanism of Ca^{2+} wave propagation involving the release of ATP, which functions as an extracellular messenger.

We have studied Ca^{2+} wave propagation in glial

cells of the retina to determine which of these competing models of wave propagation operates in intact CNS tissue (Newman, 2001). Calcium waves were imaged at high magnification (using a 40 $\times$ objective lens) and with high temporal resolution (30 Hz) in the rat eyecup in order to visualize the spread of waves within single glial cells and between adjacent cells. Waves were stimulated mechanically by advancing the tip of a stimulating probe 15 to 25 μm for 10 ms with a piezoelectric actuator. The

TABLE 1

Effect of agents on Ca^{2+} wave propagation in the rat eyecup

Condition	Astrocyte wave radius (μm)	Müller cell wave radius (μm)	Astrocyte $\rightarrow$ Müller cell delay (s)
<i>Astrocyte stimulation</i>			
Control	84.7 ± 3.2 (24)	82.2 ± 3.4 (24)	0.85 ± 0.09 (18)
Octanol (0.5 mM)	100.6 ± 3 (20) ^a	100.6 ± 3 (20) ^a	0.99 ± 0.06 (12)
Suramin (100 μM)	54.5 ± 6.2 (21) ^a	12.9 ± 5.2 (21) ^a	2.38 ± 0.13 (4) ^a
PPADS (20 and 50 μM)	69.0 ± 3.7 (33) ^a	49.8 ± 6.0 (33) ^a	2.14 ± 0.14 (22) ^a
Apyrase (80 U/ml)	63.3 ± 9.6 (9) ^a	20.7 ± 6.8 (9) ^a	
<i>Müller cell stimulation</i>			
Control (eyecup)		64.4 ± 2.2 (18)	
Control (slice)		66.0 ± 3.0 (28)	
Suramin (slice, 100 μM)		32.6 ± 3.5 (22) ^a	

Calcium wave radii were measured 10 s after waves were evoked. In the last column, the time between the onset of a Ca^{2+} increase in an astrocyte process and the onset in an adjacent Müller cell endfoot is given. In all tables, mean $\pm$ SEM (*n*) are given. From Newman (2001).

^a $P < 0.01$, control vs. test.

magnitude of calcium wave propagation was quantified by measuring the extent of wave spread 10 s after stimulation. Calcium wave spread was evaluated by calculating 'ratio images'; images of Ca^{2+} fluorescence during wave propagation were divided by Ca^{2+} fluorescence images obtained in a control period before stimulation.

Astrocyte stimulation

When an astrocyte soma is stimulated with a mechanical stimulus, a Ca^{2+} wave is initiated in the soma. The wave propagates outwards through the processes of the stimulated cell and spreads into neighboring astrocytes with little additional delay. The Ca^{2+} wave also spreads into adjacent Müller cells, but with a delay of ~ 0.85 s (Table 1). Under control conditions, the wave spreads to a radius of $84.7 \mu\text{m}$ in astrocytes and $82.2 \mu\text{m}$ in Müller cells at 10 s (Table 1, Fig. 4A).

Wave propagation was not diminished by the addition of 0.5 mM octanol, which blocks astrocyte gap junctional coupling in cultured cells (Finkbeiner, 1992) and astrocyte–Müller cell coupling, but not astrocyte–astrocyte coupling (Zahs and Newman, 1997), in the rat retina. Stimulation of an astrocyte evoked a Ca^{2+} wave which propagated through both astrocytes and Müller cells as it did under control conditions (Fig. 4B). The spread of the wave in both astrocytes and Müller cells in the presence of

octanol was $100.6 \mu\text{m}$, somewhat greater than the spread under control conditions.

Addition of purinergic receptor antagonists, in contrast, substantially reduced Ca^{2+} wave propagation in the rat retina. The antagonists suramin (100 μM) and PPADS (20–50 μM) produced similar changes. In the presence of these purinergic blockers, Ca^{2+} wave propagation through Müller cells was compromised. Stimulation of an astrocyte sometimes evoked a Ca^{2+} wave that traveled through adjacent astrocytes, but not into Müller cells (Fig. 4C). In other trials, Ca^{2+} waves propagated into Müller cells, but only after a longer delay (2.1–2.4 s) than in control trials. In these trials, the spread of the wave through Müller cells was substantially less than under control conditions (Table 1). In addition, the spread of the wave within astrocytes was somewhat decreased in the presence of the purinergic blockers (Table 1).

Addition of apyrase, which hydrolyzes ATP, produced results similar to those seen with purinergic receptor blockers. Astrocyte stimulation evoked a Ca^{2+} wave that traveled through adjacent astrocytes while propagation into Müller cells was either reduced or blocked completely (Fig. 4D, Table 1).

ATP release

These results suggest that Ca^{2+} wave propagation in retinal glial cells involves the release of ATP. This

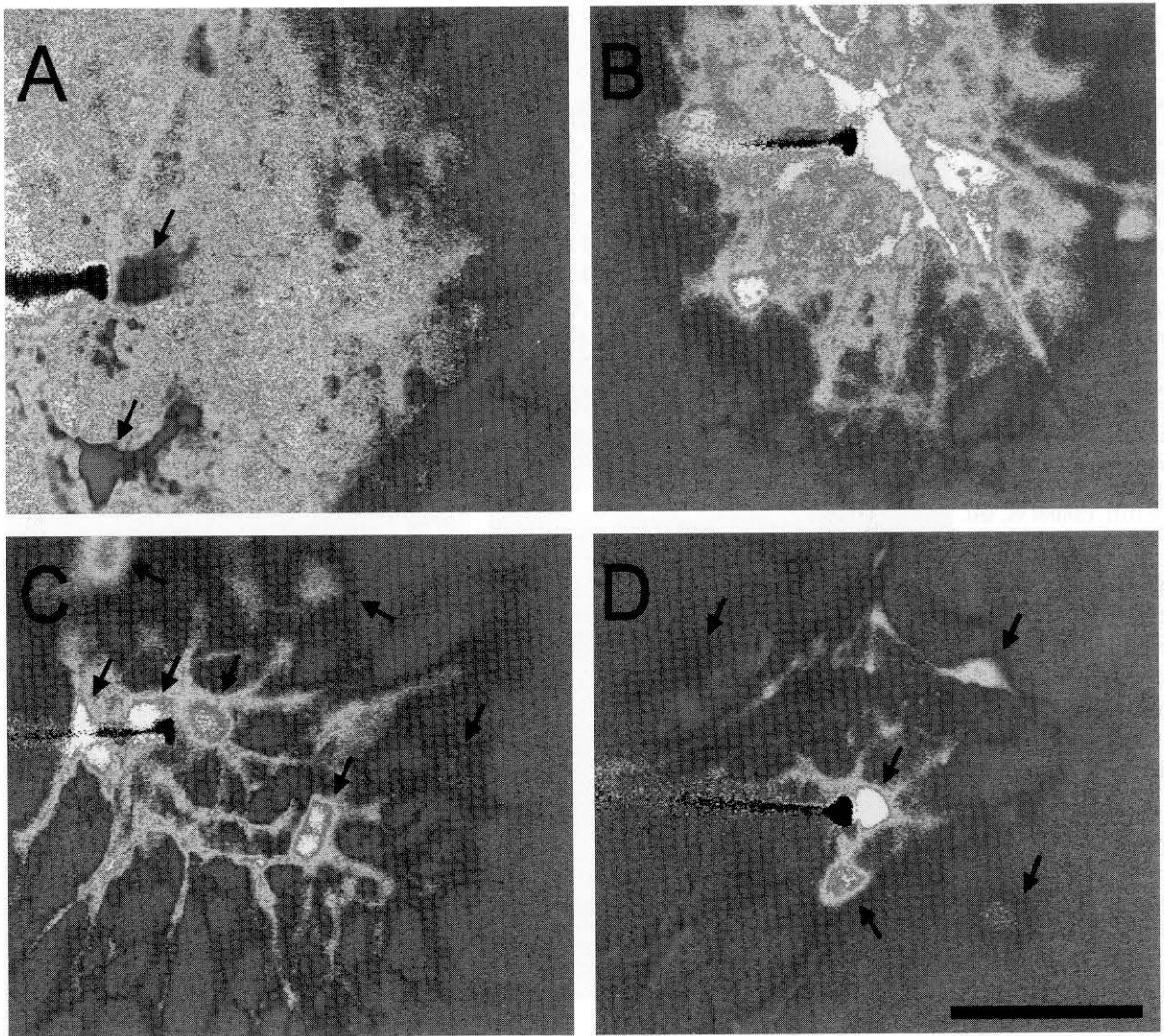


Fig. 4. Effect of agents on intercellular glial Ca^{2+} wave propagation in the retina. Waves were evoked by mechanical stimulation of astrocyte somata. Ratio images show the spread of waves 10 s after stimulation. (A) Control Ca^{2+} wave propagates through both astrocytes and Müller cells. Note that Ca^{2+} levels increase in all regions within the wave. (Calcium fluorescence in some astrocytes (arrows) was saturated; the fluorescence ratio does not show an increase.) (B) Octanol (0.5 mM), has little effect on Ca^{2+} wave propagation. Calcium levels increase in both astrocytes and Müller cells. (C) In the presence of suramin (100 μM), the Ca^{2+} wave propagates through several astrocytes (arrows), but not into Müller cells (darker regions between astrocytes). (D) Similarly, in the presence of apyrase (80 U/ml) the Ca^{2+} wave propagates through astrocytes (arrows), but not into Müller cells. Rat eyecup. Scale bar: 50 μm .

prediction was tested by employing the luciferin-luciferase assay to detect ATP release during Ca^{2+} wave propagation. Luciferin and luciferase were added directly to the superfusate and the bioluminescence produced by ATP hydrolysis monitored with a sensitive intensified cooled CCD camera. For technical reasons, experiments were performed on

isolated whole-mount rat retinas rather than eyecups.

Mechanical stimulation of the retinal surface evoked release of ATP as indicated by a substantial increase in bioluminescence (Newman, 2001). ATP release began at the site of stimulation and spread outwards as a wave (Fig. 5). The radius of the

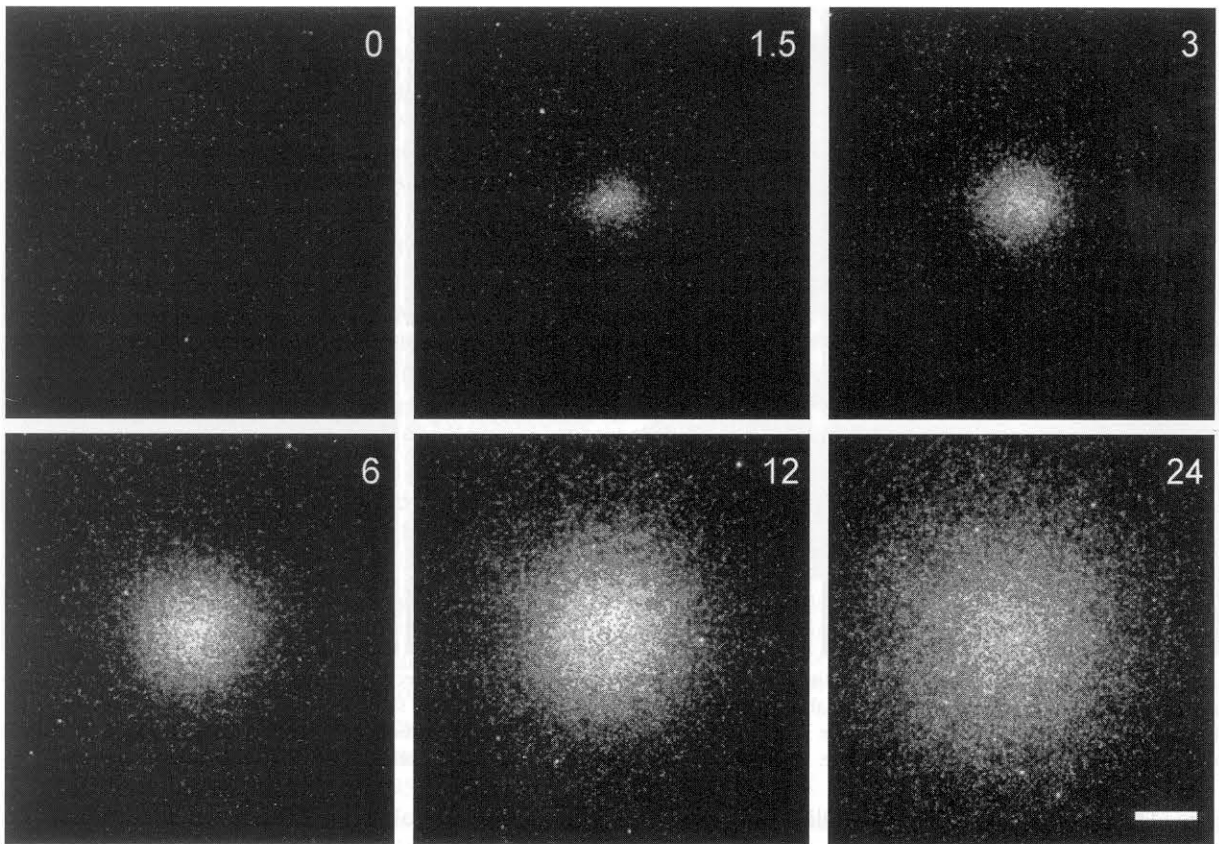


Fig. 5. ATP release during Ca^{2+} wave propagation. A mechanical stimulus that reliably evokes a Ca^{2+} wave was applied to the retina. ATP release was monitored using the luciferin–luciferase assay. Stimulation evokes a wave of ATP release which propagates outwards from the stimulation site. The elapsed time (in s) following stimulation is indicated in each frame of the sequence. Isolated whole-mount rat retina. Scale bar: 50 μm .

ATP wave 10 s after stimulation was $162 \pm 9 \mu\text{m}$ ($n = 8$), somewhat greater than the radius of Ca^{2+} waves at the same time.

The luciferin–luciferase assay was calibrated by adding a series of ATP standards to the superfusate solution. Maximal ATP release at the site of wave stimulation, based on this calibration, was $78 \pm 3 \mu\text{M}$ ($n = 8$).

It could be argued that the ATP released following mechanical stimulation originates from damaged cells at the stimulation site rather than from the physiological release accompanying Ca^{2+} wave propagation. This possibility was tested by monitoring ATP release in the presence of suramin. ATP release evoked by mechanical stimulation was substantially reduced by suramin, demonstrating that, under control conditions, the ATP release imaged

using the luciferin–luciferase assay was not due to liberation from damaged cells.

Müller cell stimulation

Propagation of Ca^{2+} waves in retinal glial cells was also examined for waves evoked by stimulation of Müller cells, rather than astrocytes (Newman, 2001). Müller cells were mechanically stimulated with a probe positioned within the inner plexiform layer, at a depth of $\sim 15\text{--}30 \mu\text{m}$ below the retinal surface. Stimulation within the inner plexiform layer evoked Ca^{2+} increases (imaged at the retinal surface) which propagated outwards radially from the stimulation site. The waves initially traveled through Müller cells and propagated from Müller cells into adjacent astrocytes only after a delay of $3.0 \pm 0.3 \text{ s}$ ($n = 12$).

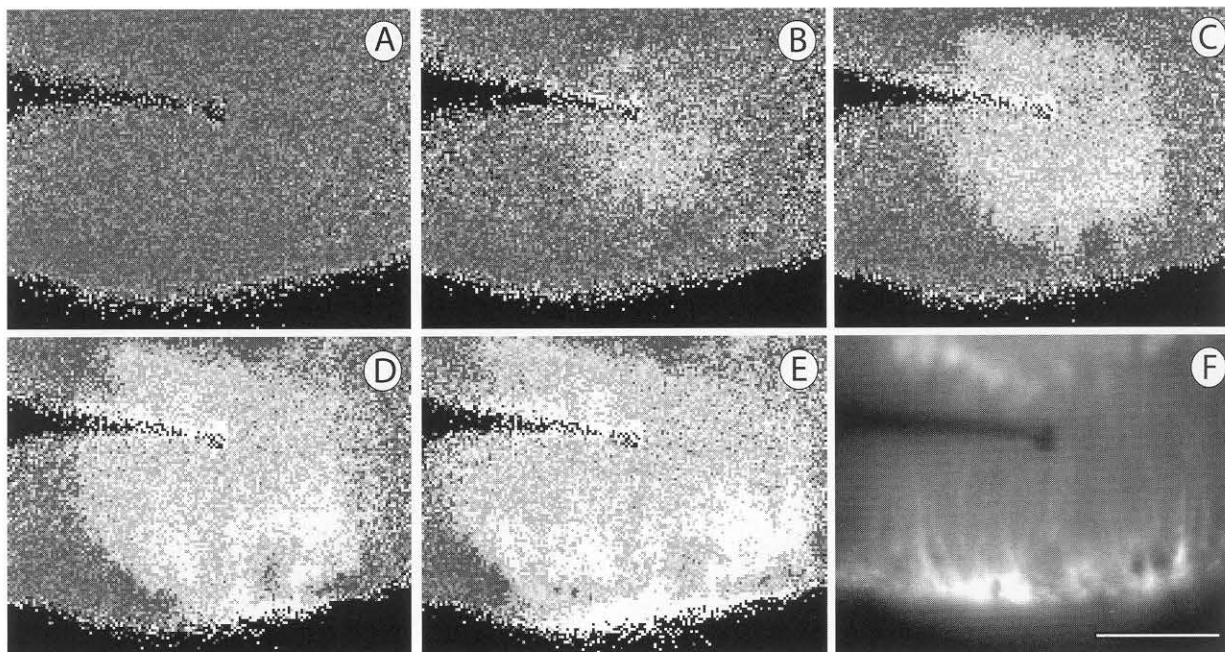


Fig. 6. Calcium wave propagation in the retinal slice. A Ca^{2+} wave was evoked by a mechanical stimulus within the inner plexiform layer, $\sim 50 \mu\text{m}$ below the cut surface of the slice. A time sequence of ratio images (A–E) show the concentric spread of the wave from the point of stimulation. A Ca^{2+} fluorescence image (F) shows the Müller cells through which the wave traveled. In frame F, Müller cell somata (top) and endfeet (bottom) are visible. The vitreal border of the retina is at the bottom. Rat retinal slice. Scale bar: $50 \mu\text{m}$.

Spread of the Ca^{2+} waves within Müller cells was $64 \mu\text{m}$ (Table 1).

These Ca^{2+} waves were imaged at the retinal surface. From this vantage point, it is not possible to ascertain how the Ca^{2+} waves are propagated through Müller cells. Following Müller cell stimulation in the inner plexiform layer, Ca^{2+} waves could be propagated from Müller cell to Müller cell within the inner plexiform layer. Alternately, the waves could travel initially through the processes of the stimulated cells to the retinal surface where they would then propagate into neighboring Müller cells and astrocytes.

Calcium waves were imaged in retinal slices in order to distinguish between these two possibilities. The cut surface of retinal slices ($\sim 500 \mu\text{m}$ thick) were imaged. A stimulating probe was positioned $25\text{--}75 \mu\text{m}$ below the cut surface of the slice within the inner plexiform layer. Stimulation of Müller cells in the inner plexiform layer evoked a Ca^{2+} wave that traveled outwards concentrically from the stimulation site (Fig. 6). Spread of the wave parallel to the retinal surface was $66 \mu\text{m}$, similar to the spread observed

in the eyecup following Müller cell stimulation. Addition of suramin substantially reduced Ca^{2+} wave propagation through Müller cells in the retinal slice. Spread in the presence of $100 \mu\text{M}$ suramin was only $33 \mu\text{m}$.

Calcium wave propagation: conclusion

Our results indicate that Ca^{2+} waves are propagated through retinal glial cells by a combination of two mechanisms: the diffusion of an internal messenger through gap junctions and the release of an external messenger. Calcium waves can be propagated from astrocyte to astrocyte via direct gap junctional coupling. This form of propagation occurs even in the presence of purinergic receptor antagonists or apyrase, when propagation by ATP release is blocked. Propagation of Ca^{2+} waves between astrocytes is probably enhanced by the release of ATP, however, as agents which antagonize ATP propagation also reduce, to a degree, the spread of the waves within astrocytes.

Calcium wave propagation from astrocytes to

Müller cells does not occur effectively via gap junctional coupling, even though the two types of glial cells are coupled together. Following astrocyte stimulation, wave propagation from astrocytes to Müller cells is greatly reduced or is blocked completely by purinergic antagonists and by apyrase. Both suramin and PPADS reduce propagation into Müller cells, indicating that P2X/Y receptors are involved in propagation by ATP release.

The concentric spread of Ca^{2+} waves within the inner plexiform layer, viewed in the retinal slice preparation (Fig. 6), demonstrates that wave propagation from Müller cell to Müller cell can occur along the entire length of these cells, not just at the retinal surface. Müller cell processes within the inner plexiform layer are presumably able to release ATP and respond to ATP released from other Müller cells.

Modulation of neuronal activity by glial cells

Astrocytes have been shown to modulate the electrical activity of neurons in glial–neuronal co-cultures. Increases in Ca^{2+} within astrocytes evoke inward currents and modulate neurotransmitter release in adjacent neurons (Hassinger et al., 1995; Araque et al., 1998a,b; Sanzgiri et al., 1999). This glial–neuronal signaling is mediated by the release of glutamate from astrocytes (Parpura et al., 1994; Hassinger et al., 1995; Araque et al., 1998a,b; Sanzgiri et al., 1999). Increases in glial Ca^{2+} in culture have been shown to evoke the release of glutamate into the culture medium (Parpura et al., 1994; Bezzi et al., 1998). In addition, stimuli which evoke Ca^{2+} waves in networks of astrocytes in culture also evoke waves of glutamate release from astrocytes (Innocenti et al., 2000).

Similar glial–neuronal signaling could occur in intact CNS tissue. We have examined whether such signaling occurs in the mammalian retina (Newman and Zahs, 1998). Extracellular single-unit activity was recorded from neurons in the ganglion cell layer of the rat eyecup while the retina was stimulated with flashes of light (Fig. 7). Changes in neuronal response to these light flashes was assessed as mechanically evoked glial Ca^{2+} waves passed by the neuron being monitored. Neurons were classified as ON, ON–OFF, or OFF cells, depending on their response to light flashes. Both ganglion cells and dis-

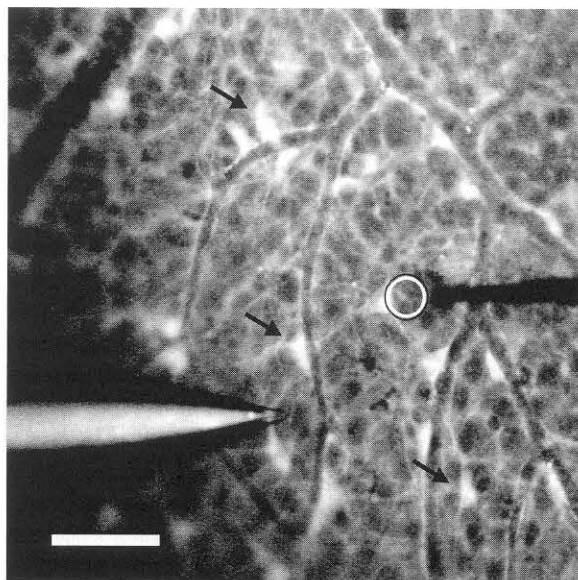


Fig. 7. Calcium fluorescence image of the retinal surface. Labeled astrocytes (arrows) and Müller cell endfeet (diffuse background fluorescence) are seen. The pipette used to initiate Ca^{2+} waves is to the left and the microelectrode used to record ganglion cell activity to the right. Rat eyecup. From Newman and Zahs, 1998.

placed amacrine cells, which function as inhibitory interneurons in the retina, may have been monitored, as both are present in the ganglion cell layer of the rat retina.

The activity of a majority of the retinal neurons we monitored were modulated by increases in Ca^{2+} in adjacent glial cells (Newman and Zahs, 1998). In recordings from single neurons, modulation was observed in trials in which a Ca^{2+} wave propagated past the cell but not in trials in which the Ca^{2+} wave was initiated but died out before reaching the cell (Fig. 8). Of the 53 neurons fully characterized, five displayed excitatory modulation (Table 2). Many more cells, 25 in all, were inhibited by glial cells. ON, ON–OFF and OFF cells displayed inhibitory modulation while only ON cells showed excitatory modulation (Table 2).

Inhibitory modulation of neuronal activity was characterized in greater detail. The magnitude of the inhibitory modulation was correlated with the amplitude of Ca^{2+} increases in neighboring glial cells. In individual trials, greater inhibitory modulation was associated with larger glial Ca^{2+} increases (Fig. 9).

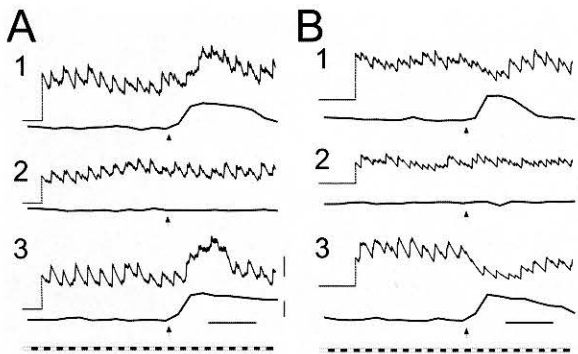


Fig. 8. Glial cell modulation of neuronal activity in the rat retina. (A) Excitatory modulation in an ON neuron. (B) Inhibitory modulation in an ON-OFF neuron. For each trial (1–3), in A and B, the spike activity of the neuron (top trace, plotted as a running average of spike frequency) and glial cell Ca^{2+} adjacent to the neuron (bottom trace, circle in Fig. 7) are shown. For each trial, a Ca^{2+} wave is initiated by mechanical stimulation of a distant astrocyte (arrow). When the Ca^{2+} wave reaches the neuron (trials 1 and 3 in A and B) the spike activity of the neuron is modulated. When the Ca^{2+} wave is initiated, but fails to reach the neuron (trial 2 in A and B), there is no change in spike activity. Rat eyecup. From Newman and Zahs, 1998.

Inhibitory glial modulation of neuronal activity was also characterized in pharmacological experiments. Inhibitory modulation was abolished by bicuculline and strychnine, antagonists of GABA and glycine, the two major inhibitory neurotransmitters in the retina (Table 3). Similarly, NBQX and D-AP7,

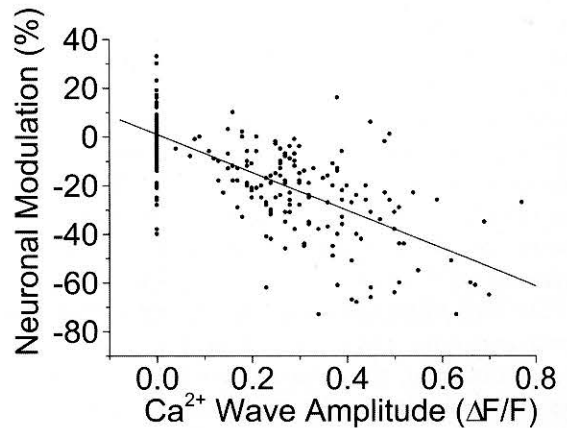


Fig. 9. Correlation between modulation of neuronal activity and glial Ca^{2+} increases. The magnitude of inhibition of neuronal activity is plotted vs. the amplitude of glial Ca^{2+} increases. Points represent single trials from all cells showing significant inhibitory modulation. The magnitude of inhibitory modulation increases with larger Ca^{2+} wave increases. Points to the left ($\Delta\text{F}/\text{F} = 0$) represent trials in which the Ca^{2+} wave failed to reach the neuron. From Newman and Zahs, 1998.

antagonists of AMPA and NMDA glutamate receptors, blocked inhibitory modulation of neuronal activity (Table 3).

Together, these results provide convincing evidence that glial cells are capable of modulating the electrical activity of neighboring neurons in intact

TABLE 2

Modulation of neuronal activity by glial cell Ca^{2+} increases

	Neuronal type			
	ON		ON-OFF	OFF
<i>Number of cells with significant modulation</i>				
Inhibition	7 of 24		9 of 15	9 of 14
Excitation	5 of 24		0 of 15	0 of 14
<i>Magnitude of cell modulation</i>				
	Inhibition	Excitation	Inhibition	Inhibition
No Ca^{2+} wave ^a	+3 ± 5% (30)	−1 ± 3% (20)	+1 ± 2% (39)	−1 ± 1% (36)
Large Ca^{2+} wave ^b	−35 ± 3% (35)	+27 ± 5% (27)	−25 ± 3% (35)	−25 ± 2% (44)

Number of cells: for each cell tested, modulation was judged significant if the modulation in those trials when a Ca^{2+} wave reached the cell ($\Delta\text{F}/\text{F}$ Ca^{2+} signal >22%) was significantly different than when a Ca^{2+} wave failed to reach the cell ($\Delta\text{F}/\text{F}$ <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 Ca^{2+} wave reached the cell and did not reach the cell. For ON neurons, separate means are given for cells with excitatory and inhibitory modulation. From Newman and Zahs, 1998.

^a Trials when the $\Delta\text{F}/\text{F}$ Ca^{2+} signal was <1%; ^b trials when $\Delta\text{F}/\text{F}$ was >22%.

TABLE 3

Effect of neurotransmitter antagonists on the inhibitory glial modulation of neuronal activity

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

Modulation magnitude for trials when a Ca^{2+} wave reached the neuron are shown. Only neurons with significant inhibitory modulation were tested. Antagonists were applied for 15 min prior to testing. Recovery followed a 60-min washout. From Newman and Zahs, 1998.

^a $P < 0.01$; ^b $P < 0.05$, antagonist vs. control, recovery vs. antagonist.

CNS tissue. Increases in Ca^{2+} in glial cells are associated with either excitatory or inhibitory modulation of neuronal activity.

The mechanism by which glial cells modulate neuronal activity in the retina remains to be characterized. Our pharmacological results suggest, however, that the mechanism may be similar to that operating in glial-neuronal co-cultures. Increases in glial Ca^{2+} associated with Ca^{2+} waves may result in the release of glutamate from astrocytes, Müller cells, or both types of glial cells, leading to the ac-

tivation of retinal neurons (Fig. 10). Both amacrine cells and ganglion cells could be directly excited by glutamate release and display excitatory modulation. Only ganglion cells are likely to show inhibitory modulation, however, as they would be inhibited by amacrine cell activity triggered by glutamate release from glia. This inhibitory modulation would be blocked by both glutamate antagonists (blocking glial excitation of amacrine cells) and GABA and glycine antagonists (blocking amacrine inhibition of ganglion cells), precisely the pharmacological actions we observed.

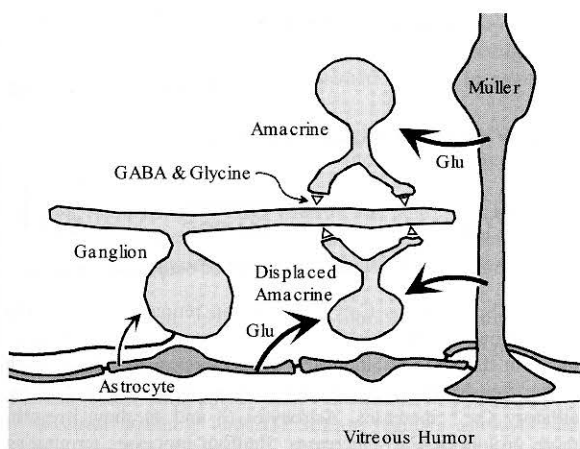


Fig. 10. Proposed mechanism of inhibitory glial modulation of neuronal activity. Increases in Ca^{2+} within astrocytes and/or Müller cells results in the release of glutamate (Glu) which excites amacrine cells. The subsequent release of GABA and glycine from amacrine cells inhibit ganglion cells. Glutamate released from glial cells may also excite ganglion cells directly.

Spontaneous calcium signaling in retinal glial cells

We have demonstrated that Ca^{2+} waves can be propagated through networks of retinal glial cells (Newman and Zahs, 1997) and that these Ca^{2+} waves can modulate neuronal activity (Newman and Zahs, 1998). It remains to be determined whether such glial modulation of neuronal activity occurs in vivo. Key to this question is whether Ca^{2+} waves, or other types of glial Ca^{2+} signaling, occurs under normal physiological conditions.

Evidence from a number of experimental preparations suggests that Ca^{2+} signaling occurs in vivo. Application of neurotransmitters, including glutamate (Kriegler and Chiu, 1993; Porter and McCarthy, 1995a,b; Pasti et al., 1997; Shao and McCarthy, 1997), norepinephrine (Shao and McCarthy, 1997), and ATP (Porter and McCarthy, 1995a) evoke

Ca^{2+} increases in glial cells in acutely isolated brain tissue. Furthermore, electrically evoked neuronal activity results in glial Ca^{2+} increases in hippocampal slices (Dani et al., 1992; Porter and McCarthy, 1996; Pasti et al., 1997), cerebellar slices (Grosche et al., 2000) and acutely isolated optic nerve (Kriegler and Chiu, 1993). This neuronal–glial signaling can be mediated by the release of glutamate from activated neurons (Porter and McCarthy, 1996; Pasti et al., 1997). Transmitter-evoked Ca^{2+} signaling in glial cells also occurs in the intact retina. As described above, neurotransmitter agonists, including ATP, carbachol and phenylephrine, evoke Ca^{2+} increases in astrocytes and Müller cells in the freshly excised retina (Newman and Zahs, 1997).

Spontaneous Ca^{2+} increases also occur in retinal glial cells. In the first 2 h after isolation, spontaneous Ca^{2+} transients and oscillations are observed in both astrocytes and Müller cells in the rat eyecup (Fig. 11). Calcium oscillations occur in Müller cells, while irregular Ca^{2+} increases are observed, less frequently, in astrocytes. These spontaneous Ca^{2+} increases are smaller in magnitude than those observed during evoked Ca^{2+} waves. We are currently conducting experiments to determine whether these spontaneous Ca^{2+} signals are capable of modulating the activity of neighboring neurons.

Conclusion

Our results, together with those from other laboratories, indicate that glial Ca^{2+} signaling and communication between glial cells and neurons is likely to occur *in vivo*. We have demonstrated that Ca^{2+} waves can be propagated through networks of glial cells in the freshly excised retina. Propagation occurs both by spread of internal messengers through gap junctions and by the release of ATP and the stimulation of purinergic receptors. Furthermore, Ca^{2+} increases in glial cells result in the modulation of the activity of neighboring neurons, perhaps by the release of glutamate from glial cells. Calcium signaling in retinal glial cells is likely to occur *in vivo* as spontaneous Ca^{2+} oscillations are observed in freshly excised eyecups.

Calcium increases in glia, whether they occur spontaneously or in response to neuronal activity, may exert a modulatory influence on neighboring

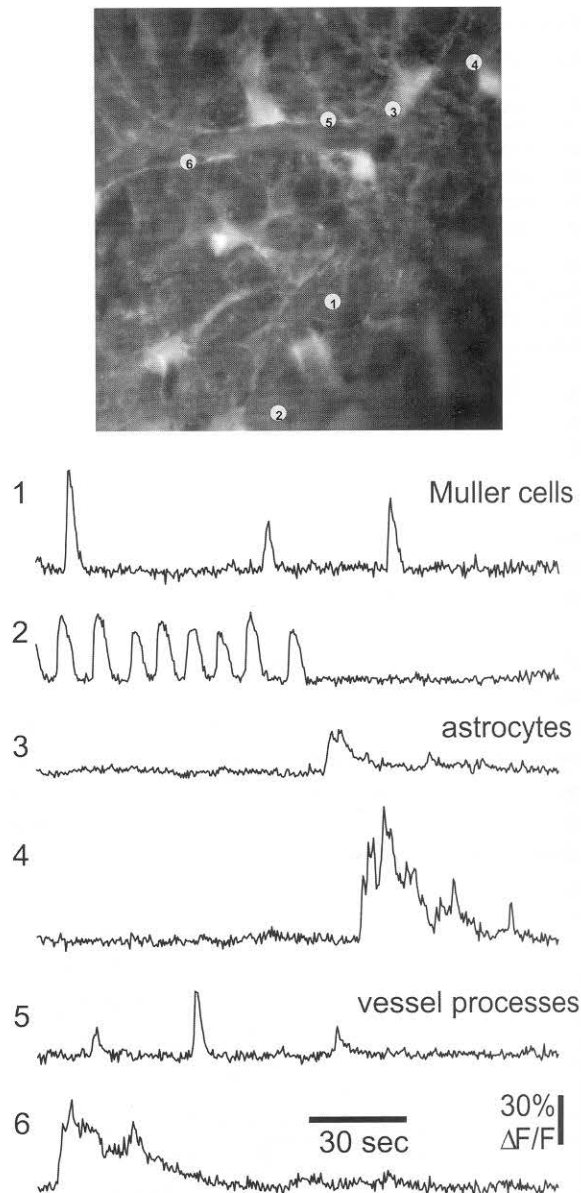


Fig. 11. Spontaneous Ca^{2+} increases in retinal glial cells. The traces show variations in Ca^{2+} fluorescence recorded simultaneously in the six cells indicated in the fluorescence micrograph at the top. Müller cells (1 and 2) show brief transient or oscillatory Ca^{2+} increases. Astrocytes (3 and 4) show irregular, more prolonged Ca^{2+} increases. Endfoot processes terminating on blood vessels (5, Müller cell? and 6 astrocyte?) also show spontaneous Ca^{2+} increases. Recorded from a rat eyecup 30 min after isolation.

neurons. This modulation could be either excitatory or inhibitory, depending on the prominence of inhibitory interneurons in the local circuitry. The magnitude of this modulation and its effect on information processing in the CNS must be determined in future experiments.

Abbreviations

ATP	adenosine triphosphate
CNS	central nervous system
GABA	γ -aminobutyric acid
NMDA	N-methyl-D-aspartate

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