MECHANISMS AND FUNCTIONAL CONSEQUENCES OF GLIAL SIGNALING IN THE RETINA

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ABSTRACT

Twenty years ago, glia were viewed as passive support cells for neurons. Since then, experiments have shown that glial cells have their own form of excitability with precise intracellular spatiotemporal dynamics, intercellular communication among themselves, a bidirectional dialog with neurons and synapses, and a key role in mediating blood flow changes in response to neuronal activity. Most of these experiments have been conducted in brain regions such as hippocampus, cortex, hypothalamus, and cerebellum. However, as work from our laboratory has shown, the mammalian retina is also an excellent preparation to study the active functions of glial cells.

Here, we describe two forms of active glial signaling in the retina. First, we tested the hypothesis that glial cells modulate synaptic activity in the retina. We measured synaptic strength by evoking excitatory postsynaptic currents (EPSCs) in ganglion cells with either light or an electrical stimulus. We then excited glial cells through several methods, including agonist ejection, photolysis of caged Ca^{2+}, and depolarization. The amplitude of the synaptic currents was altered by some, but not all, of these glial stimuli, leaving us unable to draw a definitive conclusion as to whether glial excitation alone is sufficient to modulate synaptic transmission in the retina.

Second, we characterized spontaneous intercellular glial Ca^{2+} waves in the retina. Glial cell excitability takes the form of transient intracellular Ca^{2+} elevations. One of the first recognized active properties of glia was their ability to propagate these Ca^{2+} elevations from cell to cell in a wave-like pattern. In most previous experiments, glial Ca^{2+} waves were initiated by an experimenter-driven stimulus, raising doubts about whether these waves occurred naturally in the organism. We demonstrate here that these

waves occur spontaneously both in intact tissue and in vivo, and that the rate of spontaneous wave generation increases as animals age. These spontaneous waves propagate by glial release of ATP and activation of ATP receptors on neighboring cells. Finally, spontaneous waves cause changes in blood vessel diameter. This is the first demonstration of a functional effect of spontaneous intercellular glial signaling.

These results suggest a functional role for glial cell signaling in the retina and raise the possibility that glial signaling may actively participate in the aging of the nervous system.

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Chapter 1

Background and Significance

Glial cells

Glial cells are non-neuronal cells in the nervous system. There are four times as many glial cells in the human cortex as neurons (Azevedo et al., 2009). Yet, glia have received only a small fraction of the study afforded to neurons, primarily because they lack the electrical excitability fundamental to the information processing of neurons.

Glia in the central nervous system are divided into three types: microglia, oligodendrocytes, and astrocytes. Microglia are the macrophages of the nervous system, and oligodendrocytes provide myelination for central axons. Cells of the third type, astrocytes, are now emerging as functional participants in the information processing of the nervous system. Astrocytes engage in a bidirectional dialog with neurons and also communicate between themselves, thereby forming an information processing circuit whose properties are just starting to be learned. Astrocytes are often identified immunohistologically by their expression of glial fibrillary acidic protein (GFAP), which is a structural element of the cytoskeleton.

This work described in this thesis investigates the functional roles of glial signaling in the mammalian retina. In the retina, the primary glial cell is the Müller cell (Newman and Reichenbach, 1996). These cells differ morphologically from forebrain astrocytes, but perform many of the same functions, and are therefore often categorized as astrocytes. In this chapter, we first discuss many of the functional signaling pathways discovered for astrocytes in the brain, and then describe the known functional roles of glia in the retina.

Calcium and glial excitability

Unlike neurons, glial cells are not electrically excitable. They do not express a high density of voltage-gated sodium channels to enable action potentials. Instead, glial cells respond to stimuli with transient elevations in cytosolic Ca^{2+} (Agulhon et al., 2008). Most of the Ca^{2+} is released from internal stores via activation of IP_3 receptors. In physiological signaling, this is often downstream of metabotropic G-protein coupled receptors such as metabotropic glutamate receptors (mGluRs), which activate phospholipase C to produce IP_3. Many functional consequences of glial activation, including modulation of synapses (Agulhon et al., 2008) and regulation of blood flow (Iadecola and Nedergaard, 2007), are dependent on cytosolic Ca^{2+} elevations in glia.

In culture (Cornell-Bell et al., 1990), as well as in thalamus (Parri et al., 2001), hippocampus (Nett et al., 2002), cortex (Hirase et al., 2004), retina (Newman, 2005), and cerebellum (Hoogland et al., 2009), glial cells exhibit spontaneous increases in intracellular Ca^{2+}, even when neuronal activity is blocked, implying that glial cells can independently generate Ca^{2+} signals. These spontaneous Ca^{2+} transients can induce neuronal N-methyl-D-aspartate receptor (NMDAR) currents (Parri et al., 2001), regulate neurite growth (Takano et al., 2006), and cause dilation of blood vessels (Kanemaru et al., 2007). Spontaneous transients remain confined to individual cells, although they may be spatially coordinated (Hirase et al., 2004).

Neuron-to-glia signaling

Astrocytes can respond to neuronal activity either electrically or with cytosolic Ca^{2+} increases. Astrocytes express a high density of K^+ channels and are directly

depolarized by K^+ efflux from nearby neurons, due to a change in the K^+ reversal potential (Orkand et al., 1966). Astrocytes also exhibit electrical responses to transmitters released from neurons. It was first observed in culture (Usowicz et al., 1989) that glial cells have functional glutamate receptors. In astrocyte-neuron co-cultures, spontaneous spiking in neurons (measured in patch clamp) coincides with inward currents in astrocytes (Murphy et al., 1993). Either AMPA receptors (Linden, 1997) or electrogenic glutamate transporters (Mennerick and Zorumski, 1994) can mediate electrical responses in glia to neuronal glutamate.

It is now believed that cytosolic Ca^{2+} elevation is a more important pathway in glial signaling than depolarization. The first discovery of glial cells responding to neuronal transmitters with Ca^{2+} elevations was in culture in response to glutamate (Cornell-Bell et al., 1990). In hippocampal slice, stimulation of glutamatergic afferent axons leads to Ca^{2+} elevations in astrocytes; this effect is blocked by tetrodotoxin (TTX) and by mGluR antagonists, implying that glutamate released from synapses acts on mGluRs on astrocytes (Dani et al., 1992; Porter and McCarthy, 1996; Pasti et al., 1997). Selective agonists for mGluRs evoke similar Ca^{2+} increases in astrocytes.

Functional receptors for many neurotransmitters have now been discovered on glial cells, including GABA (Kang et al., 1998), ATP (King et al., 1996), acetylcholine (Shelton and McCarthy, 2000), endocannabinoids (Navarrete and Araque, 2008), histamine (Inagaki et al., 1991), and norepinephrine (Kulik et al., 1999). The diversity of neurotransmitter receptors on glia has also been reviewed (Porter and McCarthy, 1997). For example, endocannabinoids released from neurons in the hippocampus act on CB1 cannabinoid receptors on astrocytes to produce Ca^{2+} increases. This in turn causes release

of glutamate from astrocytes, which produces slow inward currents in neurons (Navarrete and Araque, 2008).

Neuronal spiking also influences spontaneous glial signaling. In hippocampal slice (Aguado et al., 2002), astrocytes display spontaneous transient increases in intracellular Ca^{2+}, and groups of astrocytes exhibit transients that are synchronized within the group. TTX, an inhibitor of neuronal activity, reduces the degree to which these astrocytes are synchronized. Bicuculline, which causes bursts of neuronal activity, increases the number of glial transients and the degree of synchronization (Aguado et al., 2002). These results have been replicated in vivo in the cortex (Hirase et al., 2004), where astrocytes also display spontaneous Ca^{2+} transients. As in slice, bicuculline increases the frequency of these transients.

Glial responses to neuronal activity have a high degree of spatial selectivity, even within the processes of a single astrocyte. A single astrocyte in the hippocampus contacts tens of thousands of synapses (Bushong et al., 2002). The processes of an astrocyte form a highly ramified arbor, and astrocytes can experience local Ca^{2+} increases within sub-regions of this arbor. This was first discovered in culture (Inagaki et al., 1991), where astrocytes were grown at a low density so their processes could be individually observed. Bath application of a low concentration of histamine produced oscillations in cytosolic Ca^{2+} concentration that were localized to sub-regions of the astrocytic processes. These oscillations were not synchronized between the regions, indicating that the regions are functionally independent (Inagaki et al., 1991). One caveat is that the type-2 astrocytes used in these culture experiments may not exist in vivo (Butt et al., 1994). Fortunately, a similar phenomenon occurs in the cerebellar slice (Grosche et al., 1999). Ultrastructural

compartments of Bergmann glia, identified with serial-section electron microscopy, envelop parallel fiber-to-Purkinje cell synapses. These compartments, called "microdomains", exhibit local rises in intracellular Ca^{2+} when parallel fibers are stimulated (the point of stimulation was 200 µm away from the Bergmann glial cell) (Grosche et al., 1999). Astrocytes can also non-linearly integrate the information from their synaptic inputs (Perea and Araque, 2006).

An important question is whether glial cells are activated by neuronal signaling in the normal function of the brain. Three recent studies have addressed this question. In the isolated retina, Müller cells (the primary glial cell of the retina) normally exhibit spontaneous transient increases in cytosolic Ca^{2+}. Light stimulation increases the frequency of these transients, indicating a physiological signaling pathway from neurons to Müller cells (Newman, 2005). In vivo imaging in the barrel cortex of mice shows that astrocytes respond to whisker stimulation with Ca^{2+} increases (Wang et al., 2006). Finally, in the visual cortex of ferrets in vivo, astrocytes exhibit Ca^{2+} responses to visual stimuli, with receptive fields matching the nearby neurons (Schummers et al., 2008). These studies demonstrate that glial Ca^{2+} elevations are activated by natural sensory stimuli, and therefore neuron-glia communication likely plays a role in normal brain function.

There is another type of glial cell, the oligodendrocyte precursor cell (also known as an $NG2^{+}$ cell), that receives direct synaptic contacts from neurons (Paukert and Bergles, 2006). These cells are not closely related to astrocytes and will not be discussed in this thesis.

Glia-to-neuron signaling

Glial cells also release transmitters that act on neurons. This was first discovered in culture (Parpura et al., 1994). Stimulation of astrocyte cultures with bradykinin causes glutamate release, as measured by HPLC. The same stimulus given to astrocyte-neuron co-cultures leads to Ca^{2+} increases in neurons, which are blocked by NMDA receptor antagonists. Neurons cultured alone do not respond to bradykinin. Thus, stimulated astrocytes release glutamate that acts on neuronal NMDA receptors. Electrical stimulation of glia can also produce this effect (Nedergaard, 1994).

This phenomenon was confirmed in situ (Pasti et al., 1997; Bezzi et al., 1998), where a metabotropic glutamate receptor agonist evoked Ca^{2+} increases in astrocytes that were followed by Ca^{2+} increases in neurons. A combination of antagonists for NMDA and AMPA receptors abolished the neuronal, but not the glial, responses. On the other hand, tetanus toxin, which selectively blocks transmitter release from neurons, did not affect the responses. These results imply that mGluR stimulation causes astrocytes to release glutamate, which acts on neurons to produce Ca^{2+} elevations.

In hippocampal slice, glial stimulation also evokes slow inward currents in pyramidal neurons (Fellin et al., 2004; Angulo et al., 2004). These currents are mediated by NMDA receptors, and serve to synchronize the activity of neurons. Glutamate release from astrocytes can be directly imaged in culture and in slice (Bezzi et al., 1998; Innocenti et al., 2000), further supporting the theory that glutamate release mediates the effects of glial stimulation on neurons. Spontaneous Ca^{2+} increases in glia are sufficient to trigger NMDAR-mediated currents in neurons, arguing that glia-to-neuron signaling is physiologically realistic (Parri et al., 2001).

Many studies have shown that Ca^{2+} elevations in astrocytes are necessary for glutamate release (Montana et al., 2006). Specifically, astrocytes release glutamate through Ca^{2+}-dependent exocytosis. Electron microscopy shows vesicular compartments in astrocytes where vesicular glutamate transporters and vesicular SNARE proteins are co-localized (Bezzi et al., 2004). Acridine orange, a fluorescent dye that self-quenches at high concentrations inside vesicles, was incubated with astrocytes so they would take it up into vesicles. When the dye is released from a vesicle and is diluted, it becomes fluorescent. When astrocytes were stimulated with a mGluR agonist, small flashes of fluorescence were visible, indicating vesicular release of acridine orange. These flashes were prevented by incubating astrocytes with the membrane permeant Ca^{2+} chelator BAPTA-AM. Glutamate sniffing cells (which respond to glutamate with Ca^{2+} increases but alone do not respond to the mGluR agonist used in these experiments) were placed near astrocytes and monitored while stimulating astrocytes with the mGluR agonist. Transient Ca^{2+} elevations in the glutamate sniffing cells temporally matched the acridine orange fluorescence flashes seen in the astrocytes. Finally, incubation with tetanus toxin, which cleaves the vesicular fusion protein synaptobrevin, prevented both sniffer cell responses and acridine orange fluorescence flashes (Bezzi et al., 2004). Together these results indicate that astrocytes release glutamate by Ca^{2+}-dependent exocytosis.

In addition to glutamate, astrocytes release ATP (Coco et al., 2003) and D-serine (Mothet et al., 2005). Adenosine generated from ATP released by astrocytes in the brain regulates sleep pressure (Halassa et al., 2009) by acting on A1 receptors on neurons. Like glutamate release, ATP release from astrocytes is exocytotic: ATP release is reduced in transgenic mice in which vesicle fusion is selectively impaired in astrocytes (Pascual et

al., 2005). The role of D-serine in regulating synaptic plasticity is discussed in the next section.

Glial modulation of synaptic transmission

Glial cells extend processes that contact or envelop synapses (Spacek, 1985; Rothstein et al., 1994; Chaudhry et al., 1995; Ventura and Harris, 1999; Grosche et al., 1999) and are thus well-positioned to exert an influence on synaptic communication between neurons.

Indeed, it has been found in a variety of preparations that stimulation of glial cells causes a depression or facilitation of synaptic transmission. Following cytosolic Ca^{2+} elevations, which are often elicited by neuronal signals, glia release transmitters that act on synaptic receptors to influence transmission.

This phenomenon has been extensively reviewed (Newman, 2004; Volterra and Steinhauser, 2004; Allen and Barres, 2005; Fellin et al., 2006; Haydon and Carmignoto, 2006; Agulhon et al., 2008; Santello and Volterra, 2009). Here, we will briefly outline four distinct mechanisms that have been described for glial modulation of synapses.

First, glial cells may release transmitters that act on presynaptic receptors to control the probability of transmitter release. In astrocyte-neuron co-cultures, glial cells release glutamate when they are stimulated by photolysis of caged Ca^{2+}, and this glutamate acts on NMDARs to increase the frequency (but not the amplitude) of miniature post-synaptic currents (Araque et al., 1998). When stimulated by neuronal glutamate, glial cells in culture release ATP; this ATP causes synaptic depression through presynaptic P2Y receptors (Zhang et al., 2003). In hippocampal slice, ATP released from

glia is degraded to adenosine in the extracellular space, and this adenosine depresses glutamatergic synapses by acting on neuronal A1 receptors (Pascual et al., 2005). Glia can also modulate inhibitory neurotransmission: in hippocampal slice, glia are activated by GABA released from neurons and release glutamate to potentiate GABAergic neuron-to-neuron synaptic transmission (measured as an increase in miniature inhibitory post-synaptic current frequency) (Kang et al., 1998).

Second, transmitters released from glia can produce long-lasting changes in post-synaptic efficacy, likely by influencing AMPA receptor insertion. In hippocampal slice, norepinephrine triggers release of ATP from glial cells, and this ATP acts on postsynaptic P2X receptors to increase the strength of glutamatergic synapses through a phosphatidylinositol 3-kinase-dependent mechanism (Gordon et al., 2005).

Third, glial cells may release transmitters that function as co-agonists on postsynaptic receptors to increase the response to the primary neurotransmitter. D-serine, a co-agonist at the NMDA receptor, is synthesized and released by glia (Wolosker et al., 1999). D-serine released from glia in culture enables LTP (Yang et al., 2003). D-serine released from glia in the supraoptic nucleus controls the polarity of stimulus-induced synaptic plasticity (Panatier et al., 2006).

Fourth, glial cells may exhibit morphological plasticity and thereby alter the extracellular diffusion parameters or the proximity of neurotransmitter reuptake transporters to synapses. In the supraoptic nucleus, glia modulate synaptic transmission both by regulating synaptic glutamate levels through glutamate transporters and by physically advancing or withdrawing processes to change the diffusion rate of glutamate (Oliet et al., 2004).

Although astrocytes extend branching processes to span tens of microns, the processes of adjacent astrocytes do not interdigitate but instead form precise boundaries where the processes of one astrocyte end and those of another begin (Bushong et al., 2002; Ogata and Kosaka, 2002). Each astrocyte thus covers a spatial domain that does not overlap with the domains of other astrocytes despite its irregular shape. This raises the interesting possibility that each synapse is regulated by a unique astrocyte that is also integrating information from other synapses in its domain.

It is not yet known what computational purpose is served by glial modulation of synapses. The retina is an ideal system to study this, because a natural input (light) can be given, and because the circuitry that transforms patterns of light into output signals is well-studied. However, it is not yet known whether glia modulate synapses in the retina. In Chapter 3, we investigate this question.

Glia-to-glia signaling

Glial cells are coupled to one another by gap junctions. If one astrocyte is patched with a dye-filled pipette, the dye will spread out into many other astrocytes, revealing a glial syncytium. In addition to electrotonically coupling astrocytes, these gap junctions permit the spatial buffering of K^+ to prevent buildup in areas of high neuronal activity (Trachtenberg and Pollen, 1970).

Further, Ca^{2+} elevations can propagate between glial cells (Fig. 1). This was first illustrated by Stephen Smith's laboratory (Cornell-Bell et al., 1990), where cultured hippocampal astrocytes were stimulated with glutamate and responded with cytosolic Ca^{2+} increases that propagated from cell to cell in a wave-like pattern. Later studies

identified similar Ca^{2+} waves in intact tissue from retina (Newman and Zahs, 1997), corpus callosum (Schipke et al., 2002), cortex (Tian et al., 2006; Ding et al., 2007), and cerebellum (Hoogland et al., 2009). These waves can be evoked by mechanical, electrical, or chemical stimulation of glia; or indirectly by stimulation of neurons (Dani et al., 1992). Glial Ca^{2+} waves have the common feature of originating as a cytosolic Ca^{2+} elevation in a single glial cell, followed by propagation that spreads outward in a roughly circular pattern. Ca^{2+} elevations occur in all glial processes and somata that fall within the expanding radius, and the speeds of intra- and inter-cellular propagation are roughly equal. Waves can modulate neuronal activity (Nedergaard, 1994; Newman and Zahs, 1998) and blood flow (Mulligan and MacVicar, 2004; Metea and Newman, 2006). By coordinating glial activity over a wide area, Ca^{2+} waves may contribute to information processing in the nervous system.

The speed of wave propagation is remarkably consistent between preparations and typically falls in the range of 10-25 µm/sec. However, the peak diameter that waves reach is highly variable. Waves evoked by a strong external stimulus can spread for hundreds of microns and involve dozens of cells (Cornell-Bell et al., 1990; Newman and Zahs, 1997; Schipke et al., 2002), while spontaneously occurring waves are limited to a diameter of 50-100 µm (Hoogland et al., 2009) [also see Chapter 4 of this thesis].

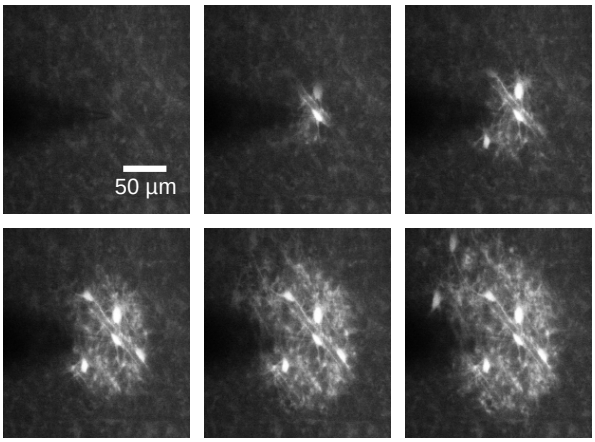

Fig. 1. Ca^{2+} elevations propagate between glial cells as a wave.

This time sequence of confocal fluorescence images shows the vitreal surface of a rat retina. Glial cells are loaded with a Ca^{2+} indicator dye. Between the first and second frames, the tip of a glass pipette, resting against the surface of an astrocyte, was advanced and retracted ~3 µm in 100 ms. This mechanical step produced a Ca^{2+} increase in the stimulated astrocyte which spread outward to nearby astrocytes and Müller cells. The pipette is visible on the left center of the images. Each image represents one second.

Two mechanisms have been identified for the propagation of intercellular Ca^{2+} waves in glia. The first mechanism is diffusion of a messenger molecule through gap junctions. In culture, blocking gap junctions prevents inter-, but not intracellular wave propagation; also, rapid flow of extracellular solution did not interfere with wave propagation in some experiments (Enkvist and McCarthy, 1992; Finkbeiner, 1992; Nedergaard, 1994). The molecule that travels through gap junctions is likely to be IP_3. Gap junctions are permeable to IP_3 (Saez et al., 1989), and blockade of IP_3 synthesis or IP_3-sensitive Ca^{2+} stores inhibits wave propagation (Venance et al., 1997). One caveat of these experiments is that gap junction blockers often have non-selective effects. Furthermore, although Ca^{2+} waves can be enabled in gap junction-deficient cells by artificial expression of connexins, the mechanism may be ATP release through hemichannels, rather than gap junction coupling (Cotrina et al., 1998).

The second mechanism of wave propagation is extracellular release of ATP and its action on the P2 receptors of nearby cells. It was discovered that some molecules that block gap junctions do not prevent the spread of Ca^{2+} waves (Guan et al., 1997). Furthermore, under some conditions, waves can propagate over cell-free spaces in culture (Hassinger et al., 1996), suggesting that an extracellular molecule is diffusing to carry the signal across this space. This molecule was identified as ATP. ATP can be detected in the extracellular solution when Ca^{2+} waves are evoked, and application of exogenous ATP can trigger a wave. P2 receptor antagonists block wave propagation (Guthrie et al., 1999). When ATP release is directly imaged using chemiluminescence, a wave of ATP can be seen in parallel with the Ca^{2+} wave (Wang et al., 2000). Mathematical modeling shows that waves mediated by ATP release are not qualitatively altered by the addition of gap

junction coupling between astrocytes (Iacobas et al., 2006). The mechanism of ATP release from glia is still debated, although there is evidence for release through hemichannels (Stout et al., 2002), exocytosis (Coco et al., 2003; Pascual et al., 2005), anion channels (Kimelberg et al., 2006), and pore-forming $P2X_7$ receptors (Duan and Neary, 2006).

The Ca^{2+} increase during waves is due to release from internal stores, rather than influx through the cell membrane, as Ca^{2+}-free external solution does not impair wave propagation (Charles et al., 1991). Instead, depletion of internal Ca^{2+} stores or blockade of IP_3 production reduces wave propagation.

An important question is whether the propagation of Ca^{2+} waves is regenerative. A non-regenerative model holds that all ATP and/or IP_3 is produced by the stimulated cell at the center of the wave, and simply diffuses outward to activate nearby cells. In culture, waves travel the same distance whether they are propagating through cells or through a cell-free space (Arcuino et al., 2004), supporting the non-regenerative model. This model is also supported in cerebellum, where wave speed decreases linearly with distance from the point of initiation, and waves spread farther along the axis of the parallel fibers, in which dimension diffusion is faster (Hoogland et al., 2009).

A regenerative model holds that the wave is amplified as it travels due to additional messenger molecules being release by cells that are recruited into the wave. Astrocyte ATP release can be visualized by incubating astrocyte cultures with the radioactive ^{14}C-ATP, which is taken up into the cells. Upon stimulation by normal ATP, ^{14}C-ATP is released (Anderson et al., 2004). This implies that the ATP released during Ca^{2+} waves would stimulate further ATP release. Regeneration of gap junction mediated

wave propagation may also be regenerative, through the Ca^{2+}-dependent production of IP_3 (via PLCδ), a process that has been mathematically modeled (Höfer et al., 2002), but for which there is no direct evidence. Mathematical modeling also suggests an explanation for how waves may be regenerative and yet not propagate indefinitely: the amplitude added by regeneration is less than the amplitude lost by propagation, so the wave diminishes in amplitude as it propagates (Höfer et al., 2002).

Until very recently, glial Ca^{2+} waves had only been observed in response to external stimuli (mechanical, chemical, or electrical) or under pathological conditions, and there was a growing consensus that waves were not physiologically relevant under normal conditions (Scemes and Giaume, 2006; Agulhon et al., 2008). In Chapter 4, we demonstrate the existence of spontaneous (i.e., unstimulated) waves in the retina, both in vivo and ex vivo, and we show that spontaneous waves exert control over blood vessel diameter. This establishes a role for Ca^{2+} waves that is both physiological and functional.

Glia in the retina

There are two types of macroglial cell in the adult retina: astrocytes and Müller cells (Fig. 2). Müller cells are in fact a type of specialized astrocyte, and functionally resemble the astrocytes of the brain (Newman and Reichenbach, 1996).

The astrocytes of the retina do not developmentally originate in the retina; instead, they migrate in along the optic nerve at around the time of birth (Stone and Dreher, 1987; Ling and Stone, 1988; Schnitzer, 1988; Huxlin et al., 1992). Upon reaching the retina, astrocytes remain in the nerve fiber layer, but spread out to cover most of the inner surface of the retina, reaching a density of approximately 200 astrocytes per mm^2 in

the adult primate (Ogden, 1978). This density is highest near the optic nerve and decreases toward the periphery of the retina. Like brain astrocytes, retinal astrocytes express glial fibrillary acidic protein (GFAP). Morphologically, the astrocytes of the retina resemble brain astrocytes, with round somata and processes ramifying outwards to a diameter of about 400 µm in the primate (Ogden, 1978). Many of these processes contact blood vessels (Zahs and Wu, 2001). Retinal astrocyte processes, unlike those of brain astrocytes, lie in a plane and, in general, do not penetrate into the depth of the retina.

Müller cells originate in the retina from a progenitor shared with the retinal neurons (Turner and Cepko, 1998). They are one of two radial glia in the central nervous system that persist into adulthood (the other is the Bergmann glial cell of the cerebellum). Their cell bodies lie in the inner nuclear layer of the retina, alongside the cell bodies of bipolar, amacrine, and horizontal cells; while their processes extend in both directions to span the entire thickness of the neural retina. Müller cell endfeet line the vitreal surface of the retina. Müller cell processes make contacts with blood vessels (Newman and Reichenbach, 1996) and also extend into the synaptic layers of the retina. Müller cells normally have a very low level of GFAP expression, but GFAP is up-regulated in injury conditions (see below). Müller cells can be readily identified immunohistochemically by their expression of the glutamate transporter GLAST (Derouiche and Rauen, 1995; Harada et al., 1998; Kugler and Beyer, 2003).

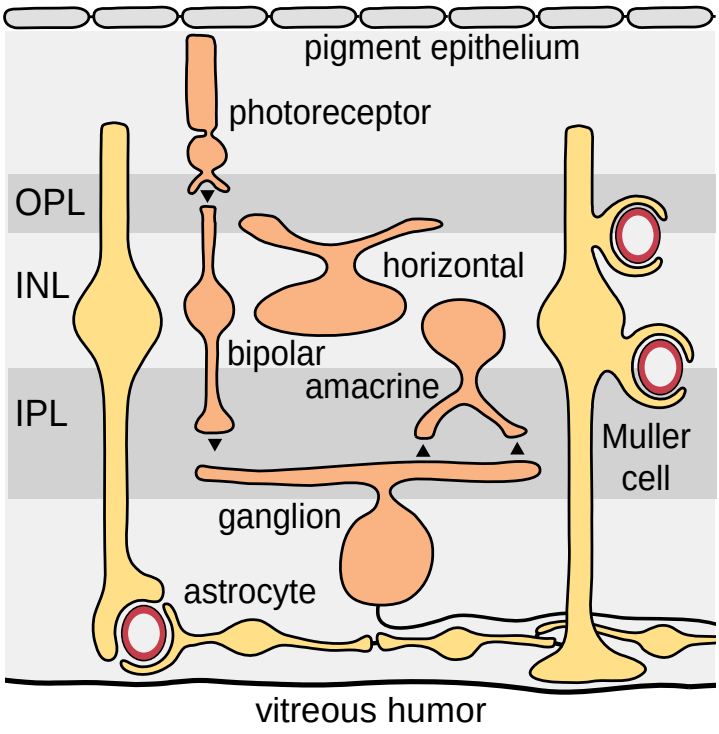

Fig. 2. The retina has two types of glial cell: Müller cells and astrocytes.

This diagram represents a cross-section of retina. The bottom of the diagram is toward the front of the eye. Astrocytes lie at the vitreal surface of the retina, in the nerve fiber layer, and make contacts with surface vessels. Müller cell bodies lie in the inner nuclear layer (INL) and extend processes to span the depth of the retina, as well as making contacts with blood vessels. Glia are shown in yellow, neurons in orange, and vessels in red.

Astrocytes are coupled to one another and to Müller cells by gap junctions (Zahs and Newman, 1997). When a small molecule dye such as neurobiotin is introduced into a single astrocyte, the dye spreads into many nearby astrocytes and Müller cells. On the other hand, if the dye is introduced into a Müller cell, the dye does not spread into any other cells. This implies an asymmetry of gap junction coupling in retinal glia (Zahs and Newman, 1997).

Retinal glia, like glial cells in the brain, express a high density of potassium channels. The predominant K^+ channel in Müller cells is Kir4.1, and this channel is concentrated in the endfeet (both those that line the vitreal surface and those that appose blood vessels) (Kofuji et al., 2000). Müller cells also express P2 purinergic receptors, which respond to extracellular ATP by triggering a rise in cytosolic Ca^{2+} via release from IP_3 receptor dependent internal stores. Recent evidence suggests that of the P2Y receptors, only $P2Y_1$ and $P2Y_4$ are expressed by Müller cells (Wurm et al., 2009).

Functions of retinal glial cells

Potassium siphoning (Newman et al., 1984) is a mechanism by which Müller cells shunt extracellular K^+ ions from the neural retina to the vitreous humor and blood vessels (which are effectively infinite sinks), preventing K^+ buildup in areas of high neuronal activity. When neurons release K^+ during action potentials, some of this K^+ enters Müller cells locally through K^+ channels. This depolarizes the Müller cell, driving K^+ out preferentially at Müller cell endfeet where the highest density of K^+ channels is expressed. The result is more efficient clearance of K^+ than diffusion alone could achieve

(Newman et al., 1984). Although K^+ siphoning was proposed (Paulson and Newman, 1987) to regulate blood vessels by delivering vasoactive K^+ to smooth muscle cells, this mechanism has been demonstrated not to operate in the retina (Metea et al., 2007).

Müller cells take up neurotransmitters released from neurons (including glutamate and GABA), preventing their extracellular accumulation (Bringmann et al., 2009). Müller cells also support neurons by converting used glutamate to glutamine, which is returned to neurons so that they can synthesize more glutamate (the glutamate-glutamine cycle) (Newman and Reichenbach, 1996). Regulation of Müller cell glutamate reuptake may influence synaptic transmission (Rauen et al., 1998).

A fascinating recently discovered function of the Müller cell is to act as a living optic fiber. As a tube of cytoplasm bridging the inner surface of the retina with the photoreceptors, Müller cells optically maximize the spatial resolution of the retina (Franze et al., 2007).

Retinal glia, like brain glia, signal to neurons. When glia in the retina are stimulated mechanically or chemically, they release ATP. This ATP is degraded to adenosine in the extracellular space, which acts on A1 adenosine receptors on ganglion cells (Newman, 2003). A1 receptor activation leads to opening of GIRK and SK channels, hyperpolarizing the ganglion cell (Clark et al., 2009). Glial cells are thus able to control the excitability of the output neurons of the retina.

In fact, it has been directly shown that glial cells regulate the light-evoked spiking of retinal ganglion cells. Ganglion cells fire action potentials in response to a flickering light stimulus. In these experiments (Newman and Zahs, 1998), a glial Ca^{2+} wave was initiated while recording spikes from a nearby ganglion cell. When the glial Ca^{2+} wave

reached the ganglion cell, the firing rate of the cell was altered. Some cells exhibited an increase in firing; others, a decrease. While a decreased firing rate could be accounted for by activation of A1 receptors due to glial ATP release, the mechanism for increased firing rate is not known.

Besides signaling to neurons, retinal glial cells also regulate blood flow. Stimulating the retina with light causes blood vessels to respond with constrictions or dilations (Metea and Newman, 2006). This makes sense, because light stimulates neuronal activity and thereby changes the energy demands of the retina. Glial cells have been recently shown to mediate this light-evoked vasomotion (Metea and Newman, 2006). Glial cell processes extensively contact blood vessels in the retina. When Ca^{2+} increases are artificially evoked in glial cells, nearby vessels show either constriction or dilation as a result. When neuron-to-glia signaling is blocked with a purinergic antagonist, light-evoked vasomotion is abolished. Together these results show that glial activation is sufficient and necessary for vessel responses. The polarity of the response (dilation vs. constriction) is influenced by both nitric oxide (NO) and oxygen (Mishra et al., 2009). At higher tissue oxygen concentrations, glial activation causes vessel constrictions, while at lower oxygen concentrations, glial activation causes dilations. This is consistent with the theory that glial cells mediate neurovascular coupling to signal changing energy demands. Glial stimulation leads to vessel diameters changes by the release of arachidonic acid metabolites. Cytosolic Ca^{2+} elevations in glia trigger arachidonic acid production through the Ca^{2+}-sensitive phospholipase A. Arachidonic acid is metabolized to vasoactive lipids including prostaglandin E2, 20-HETE, and 14,15-EET, via the enzymes COX, ω-hydroxylase, and CYP450 epoxygenase, respectively.

These lipids freely diffuse out of glial cells and modulate potassium channels on smooth muscle cells, thereby affecting muscle cell potential and causing constriction or relaxation.

Injury response

As in other areas of the brain, glial cells in the retina respond vigorously to tissue damage. Müller cell GFAP expression is up-regulated in response to elevated intraocular pressure (Woldemussie et al., 2004), light damage (Grosche et al., 1995), diabetic retinopathy (Rungger-Brandle et al., 2000), retinal detachment (Erickson et al., 1987), glaucoma (Savagian et al., 2008), or ischemia (Fitzgerald et al., 1990; Kalesnykas et al., 2008). In response to injury, Müller cells become more physiologically active, as well as experiencing proliferation and hypertrophy (Bringmann et al., 2006). Astrocytes also show higher GFAP expression with aging (Ramírez et al., 2001).

Glial release of ATP, and its conversion by ectonucliotidases to adenosine, may serve a neuroprotective role in the retina by hyperpolarizing neurons and thus reducing both energy demand and excitotoxicity (Newman, 2003; Clark et al., 2009).

Retinal glial Ca^{2+} waves

When retinal glia are stimulated focally by mechanical touch or agonist ejection, the resultant Ca^{2+} increases can propagate as a wave to the surrounding glial cells (Newman and Zahs, 1997).

Glial Ca^{2+} waves can modulate the information output of the retina (Newman and Zahs, 1998). In these experiments, ganglion cell spiking was recorded extracellularly, and

a Ca^{2+} wave triggered at some distance from the ganglion cell. When the wave propagated across the patched cell, the light-evoked spiking was either up- or down-regulated, depending on the cell (Newman and Zahs, 1998).

Wave propagation in the retina depends on both gap junction coupling and the diffusion of an extracellular messenger molecule (Newman, 2001). Mechanical stimulation of a retinal astrocyte triggered a Ca^{2+} wave that propagated through both astrocytes and Müller cells. This Ca^{2+} wave was accompanied by a wave of ATP, visualized by chemiluminescence. Waves could also be triggered by focal ejection of exogenous ATP. Either a P2 receptor antagonist or an ATP-degrading enzyme greatly reduced the extent of mechanically-evoked wave propagation in Müller cells, but spared propagation in astrocytes. When retinas were superfused with flowing Ringer's, the wave propagation was not circular but was elongated in the direction of superfusate flow. However, a P2 receptor antagonist eliminated this asymmetry. If waves were evoked by stimulating a Müller cell instead of an astrocyte, the waves spread only into other Müller cells, and this spread was largely blocked by a P2 receptor antagonist (Newman, 2001). These results imply that propagation of Ca^{2+} waves between astrocytes is mediated by gap junction coupling, while propagation into Müller cells is mediated by extracellular ATP.

Retinal Ca^{2+} waves are regenerative (Newman, 2001). When waves are stimulated mechanically, ATP release can be quantified by chemiluminescence. ATP receptor antagonists reduce the amount of ATP released, implying that ATP-stimulated ATP release occurs during the wave. Thus the wave extent is greater than it would be if ATP originated only from the stimulated cell.

Ca^{2+} waves in the retina may also modulate blood flow (Metea and Newman, 2006). A Ca^{2+} wave, evoked at a distance from an arteriole, constricts or dilates the arteriole upon reaching it. The constrictive effect is mediated by 20-HETE while the dilating effect is mediated by EETs; both are metabolic products of arachidonic acid. The balance between constriction and dilation is influenced by nitric oxide (NO). Although NO is typically thought of as a vasodilating agent (Ignarro, 2002), high NO levels favored constrictive responses to Ca^{2+} waves, while low NO levels favored dilating responses.

Chapter 2

Materials and Methods

Animals

Male Long-Evans rats were used in all experiments. Rats were 20-100 days old on arrival from the supplier (Harlan Laboratories). Some were maintained for as long as an additional 9 months in the University of Minnesota RAR facilities. Rats were sacrificed by cutting the diaphragm following deep isoflurane anesthesia. The animals used in this study were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Minnesota.

Isolated retina preparation

The isolated retina preparation has been described previously (Newman, 2001). Briefly, retinas were dissected from the hemisected eyeball and the vitreous removed. Retinas were cut into two pieces and laid flat in a superfusion chamber with the vitreal surface up. Isolated retinas were superfused with bicarbonate-buffered Ringer's at 2-3 mL/min at 22-23 °C. All experiments were performed in the isolated retina unless noted otherwise.

Whole cell recording from retinal ganglion cells

For all experiments in this chapter, excitatory postsynaptic currents (EPSCs) were evoked in ganglion cells of the retina. To record these currents, ganglion cells were patched with fire polished glass electrodes (tip resistance 6-12 MΩ). Recordings were made with a Multiclamp 700A (Axon Instruments). Cells were rejected if they did not generate action potentials with a peak voltage of at least +20 mV in response to a

depolarizing current pulse, or if their input resistance was outside the range of 50-500 MΩ. Cells were then voltage clamped at -60 mV to record synaptic currents.

Stimulation of bipolar cells

To evoke EPSCs in ganglion cells, bipolar cells were stimulated either indirectly with light or directly with an extracellular current injection. All experiments in Chapter 3 were conducted in 5 µM bicuculline and 1 µM strychnine to block GABAergic and glycinergic inputs from amacrine cells, as well as 200 nM TTX to block voltage-dependent sodium conductance.

Light stimuli were one second in duration and came from an incandescent bulb through a fiber optic bundle with a computer-controlled shutter (Uniblitz VMM-D1; Vincent Associates). The light illuminated the entire retina. The light produced an inward current in the ganglion cell, and the response amplitude was measured as the peak amplitude of this inward current. For each ganglion cell, the brightness was adjusted to produce an approximately 50 percent maximal synaptic response. Ganglion cells that did not exhibit light responses with a stable magnitude over time were discarded.

Current injection was produced by a NeuroLog NL800A constant current source. The negative terminal was connected to a silver chloride wire inserted into a monopolar glass electrode filled with Ringer's solution. The tip of this electrode was positioned in the outer plexiform layer to stimulate dendrites of bipolar cells. The light produced an inward current in the ganglion cell, and the response amplitude was measured as the peak amplitude of this inward current. Stimulus duration was always 20 ms, and intensity was adjusted for each ganglion cell to produce approximately 50 percent maximal synaptic

response. The stimulus ranged from 1 to 10 μA. The positive terminal of the current source was connected to a silver chloride wire placed in the recording chamber approximately 2 mm from the retina.

Calcium and ATP imaging

Our Ca^{2+} and ATP imaging methods have been described previously (Newman, 2001). Intracellular Ca^{2+} in glia was visualized with Fluo-4, a Ca^{2+}-sensitive dye. For bulk loading, retinas were incubated in Fluo-4 AM (25 μg/mL) and pluronic acid (1.75 mg/mL) for 25 minutes prior to imaging. To load individual Müller cells, the potassium salt of Fluo-4 (80 μg/mL) was added to the intracellular solution. Fluorescence images were captured with a confocal scanner (Odyssey, Noran), with images averaged for one second. Fluo-4 was excited with 488 nm laser light and emission was captured with a 515 nm longpass filter.

The luciferin-luciferase chemiluminescence assay was used to image ATP. Luciferase catalyzes the reaction of luciferin with ATP to emit light, allowing visualization of ATP release into the extracellular space. Superfusion was stopped and a solution of luciferin and luciferase gently pipetted into the chamber yielding final concentrations of 0.68 mg/mL luciferin and 0.61 mg/mL luciferase when imaging evoked ATP waves. Luciferase concentration was reduced by two thirds when imaging spontaneous waves because we found that luciferase itself acted as an inhibitor of spontaneous waves, presumably through its extracellular ATPase activity. The chemiluminescence was captured with a KS-1381 image intensifier (VideoScope) coupled to a CoolSnap ES camera (Photometrics) using a one second integration time

with 2x2 pixel binning. To maintain pH and oxygenation during ATP imaging, a collar was fitted around the objective and superfusion chamber and 95% O_2, 5% CO_2 gently blown over the surface of the chamber. All image acquisition was in MetaMorph (Molecular Devices). Luciferin/luciferase chemiluminescence images were calibrated as previously described (Newman, 2001) to calculate absolute ATP concentration at the retinal surface.

Calcium wave imaging and analysis

A 10X water immersion objective (Olympus) was used to count waves in the isolated retina, covering an area of approximately 0.5 mm^2. Waves were tallied by the number of waves starting in each frame. Waves were distinguished from other time-varying fluorescence signals by their pattern of concentric expansion and mesh-like appearance (ganglion cells, which show little to no fluorescence signal, form the dark spaces in the mesh). A custom image analysis program was written to automatically detect and tally waves.

In order to simultaneously monitor blood vessel diameter and glial Ca^{2+}, blood vessels were filled with dextran-conjugated fluorescein (2,000,000 MW; excites and emits at wavelengths similar to Fluo-4) by jugular vein injection prior to animal sacrifice to visualize the vessel lumen. In these experiments, retinas were incubated and superfused with 100 nM U-46619 (an analogue of thromboxane) to increase basal vessel tone (Metea and Newman, 2006).

In apyrase experiments (Fig. 11), after we recorded a baseline wave frequency for one minute, superfusion was stopped and apyrase gently pipetted into the recording

chamber. In control experiments for apyrase, superfusion was likewise stopped and an equal volume of Ringer's pipetted into the chamber. A gas collar was used as described for luciferase experiments. Apyrase controls showed a comparable wave frequency to other controls, where superfusion was not stopped.

Pressure ejection

To stimulate glial cells, ATP or ATPγS was ejected onto the surface of the retina at P_g = 5-10 psi using a glass pipette (tip resistance 1-4 MΩ). The pipette tip was positioned 20-30 μm from the patched ganglion cell and the pipette pointed directly at the ganglion cell soma.

Patching Müller cells

Müller cell endfeet line the vitreal surface of the retina. Endfeet were patched with fire polished glass electrodes (tip resistance 6-12 MΩ). Because Müller cells are not directly visible with DIC optics, we lowered the patch electrode until the tip was barely touching the surface of the retina and then applied gentle suction with a syringe. This would usually result in a patch on a Müller cell, which was confirmed by membrane potential (-80 to -85 mV) and input resistance (10 to 50 MΩ). The input resistance of Müller cells is low due to the high density of resting K^+ conductance.

Photolysis of caged Ca^{2+}

For bulk loading of caged Ca^{2+}, o-nitrophenyl EGTA AM (25 μg/mL) was added to the incubation solution. To load individual Müller cells, the potassium salt of NP-

EGTA was added to the intracellular solution used to patch on Müller cells. In both cases, a 5 second pulse of a 355 nm UV laser (DPSS), focused to a spot 50 μm in diameter, was used to uncage.

High light exposure

In the animal care facilities, rats were normally exposed to approximately 30 lux ambient light. (Light measurements were taken in the darkest part of cages where the rats huddled.) For high light exposure experiments, rats were housed in ambient lighting of 1,000 lux, beginning at 20 days of age. Rats were exposed to these high light conditions for one to four months. Both normal and high light exposure rats were on a 12 hour light / 12 hour dark cycle.

Mechanical stimulus

To artificially evoke Ca^{2+} waves, a glass pipette was positioned with its tip touching an astrocyte. The pipette, under control of a piezoelectric actuator (PZ-150 M, Burleigh), was advanced and retracted 3-4 μm in 100 ms. This mechanical step always triggered a large Ca^{2+} wave. Waves could be evoked repeatedly at a single location following a refractory period of 1-2 minutes, demonstrating that the mechanical stimulus did not injure the stimulated astrocyte.

Open-globe in vivo preparation

Rats were anesthetized by IP injection of urethane (1.3 g/kg initially, plus increments of 0.2 g/kg as needed to reach deep anesthesia). Core body temperature was

monitored and maintained at 37 degrees and hydration maintained by subcutaneous injection of saline. Animals were placed in a modified stereotaxic holder with a three-point head restraint. The right eye was secured to a metal ring by eight sutures passing through the connective tissue surrounding the globe. The cornea was removed by making an "X" cut with micro iris scissors and then cutting around the circumference of the cornea. The lens was removed by suction applied through a hypodermic needle after the anterior lens capsule was cut around its circumference with iris scissors. The posterior lens capsule was removed by tearing with two pairs of fine-tipped no. 5 forceps and cutting with iris scissors. The vitreous humor was removed completely by tearing with forceps and suction applied through the tip of a Pipetteman.

Glial cells were labeled with the Ca^{2+} indicator dye Oregon Green 488 BAPTA-1. The Oregon green solution (140 µg/mL Oregon Green, 7 mg/mL pluronic acid and 8 µM eserine in saline) was pipetted into the open globe following removal of the vitreous humor. After a 70 min incubation period, the open globe was rinsed with saline and then filled with a viscous saline solution. The stereotaxic holder and rat were fixed to a movable stage of an upright confocal microscope (Swept Field confocal, Prairie Technologies) with the open globe facing upwards. The retina was viewed through a 20X long working distance water immersion objective (Olympus).

Solutions and drugs

Bicarbonate-buffered Ringer's solution contained (in mM): 111 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 0.5 NaH_2PO_4, 15 dextrose, 32 $NaHCO_3$. The solution was bubbled with 95% O_2, 5% CO_2. Intracellular recording solution contained (in mM): 5 NaMeSO3, 128

KMeSO3, 5 K EGTA, 2 MgCl2, 1 Glutathione, 5 HEPES, 2 MgATP, 0.2 NaGTP. Intracellular solution pH was adjusted to 7.4 with KOH. The saline solution used for in vivo experiments contained (mM): 132.5 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 0.5 NaH_2PO_4, 10 HEPES; pH 7.4. Viscous saline was made by addition of hyaluronic acid, Na salt (15 mg/mL) to the saline solution. All reagents were purchased from Sigma except U-46619 (Cayman), TTX (Alomone Labs), NMDA, bicuculline, NBQX and DL-AP7 (Tocris), and Fluo-4, Oregon Green 488 BAPTA-1 and Pluronic acid (Invitrogen).

Statistics

Numerical values are given as mean ± SEM, and error bars in graphs represent SEM. The single-tailed Student's t test was used when comparing the means of two groups. ANOVA on a linear regression model was used when comparing the slope of the linear model to zero.

Chapter 3

Glial Modulation of Synaptic Transmission in the Retina

ABSTRACT

There is extensive evidence from hippocampus, cortex and culture that glial Ca^{2+} elevations lead to release of gliotransmitters (glutamate, ATP, D-serine) that facilitate or depress synaptic transmission between neurons. We investigated whether this phenomenon also occurs in the retina. We evoked EPSCs in ganglion cells by stimulating the outer retina with light or current. After establishing baseline EPSC amplitude, we stimulated glia by agonist ejection or UV photolysis of caged compounds. ATP ejection, ATPγS ejection, and uncaging of bulk-loaded caged Ca^{2+} all produced large Ca^{2+} increases in glia and also altered bipolar-to-ganglion cell synaptic transmission. Because all of these stimuli could potentially act directly on neurons, we also uncaged membrane-impermeant caged Ca^{2+} that was loaded directly into individual Müller cells. This stimulus also produced robust Ca^{2+} increases in glia, but it had no effect on synaptic transmission. We are unable to determine conclusively whether glia can modulate synaptic transmission in the retina.

INTRODUCTION

The retina presents an ideal setting to study how glial modulation of synapses affects neuronal information processing, because the transformation of natural light stimuli into ganglion cell spiking is well-characterized and can be measured as glia are manipulated. If glial activation modulates synaptic transmission in the retina, it would be possible to test experimentally how this modulation shapes receptive fields, light/dark adaptation, motion and color selectivity, and other parameters of retinal processing.

Müller cells extend processes into both synaptic layers of the retina and therefore are well positioned to have interactions with synapses. Light-evoked spiking in retinal ganglion cells is modulated by evoked glial Ca^{2+} elevation (Newman and Zahs, 1998), but it is not known whether this is due to modulation of synaptic transmission or a direct effect on intrinsic neuronal excitability. Stimulation of retinal glial cells triggers release of ATP, which is converted extracellularly to adenosine (Newman, 2003), raising the possibility that ATP released by glial cells could modulate synaptic transmission by acting on neuronal ATP or adenosine receptors. Also, D-serine is released from glial cells (Mothet et al., 2005), and endogenous D-serine in the retina modulates synaptic transmission (Stevens et al., 2003).

In the present study we directly test the hypothesis that stimulating glia changes the efficacy of synaptic transmission at the bipolar-to-ganglion cell synapse in the retina.

RESULTS

Photoreceptor cells in the retina make synaptic contacts on bipolar cells and horizontal cells. Bipolar cells in turn form glutamatergic synapses onto ganglion cells, which send their axons to the brain. We evoked synaptic responses in ganglion cells by stimulating bipolar cells either indirectly with light or directly with an electrical stimulus. In all the experiments in this chapter, we used whole-cell voltage clamp to record excitatory postsynaptic currents (EPSCs) from ganglion cells. The peak amplitude of the EPSC reported is measured from baseline.

The Müller cell, a radial glial cell, is the principle glial cell of the retina, playing a role similar to that of astrocytes in the brain (Newman and Reichenbach, 1996). We therefore hypothesized that activating Müller cells would modulate synaptic transmission. In the experiments in this chapter, we tested this hypothesis by first recording baseline synaptic current responses and then delivering a glial stimulus and looking for a change in synaptic strength.

ATP ejection facilitates synaptic transmission

Ejecting ATP (100 μM) onto the surface of the retina consistently produced large Ca^{2+} increases in astrocytes and Müller cells (Fig. 3A and B). We therefore tested whether ATP ejection would change the amplitude of EPSCs evoked in ganglion cells.

Baseline EPSC amplitude was first established by periodic stimulation. While holding a ganglion cell in voltage clamp, an electrical stimulus was delivered to bipolar cells through a monopolar electrode positioned in the OPL, evoking an inward current in the ganglion cell (Fig. 3C, black trace). Across ganglion cells, the peak amplitude of this

current ranged from 5-100 pA (n = 30 cells). The stimulus was repeated every 20 seconds. Between the fifth and sixth stimuli, ATP was ejected to trigger a Ca^{2+} increase in glial cells. This caused an increase of 160 ± 20 percent (n = 4 cells) in the amplitude of the EPSCs recorded in ganglion cells (Fig. 3C, red trace). The increase in EPSC amplitude lasted between 5 and 35 seconds (Fig. 3D, black squares), which matches the time course of the glial Ca^{2+} elevation (Fig. 3D, green trace).

We were also interested in whether glial stimulation potentiates EPSCs that are evoked with a physiological stimulus. Therefore, we repeated the previous experiment using a one-second global light flash instead of a current injection. To minimize photoadaptation, light stimuli were repeated every 40 seconds instead of every 20 seconds. Between the second and third light stimuli, ATP was ejected. ATP ejection caused a 35 ± 9 percent (n = 2 cells) increase in the light-evoked EPSC amplitude (Fig. 3E). This demonstrates that a stimulus producing a large increase in glial Ca^{2+} is sufficient to modulate the synaptic activity produced by a natural stimulus.

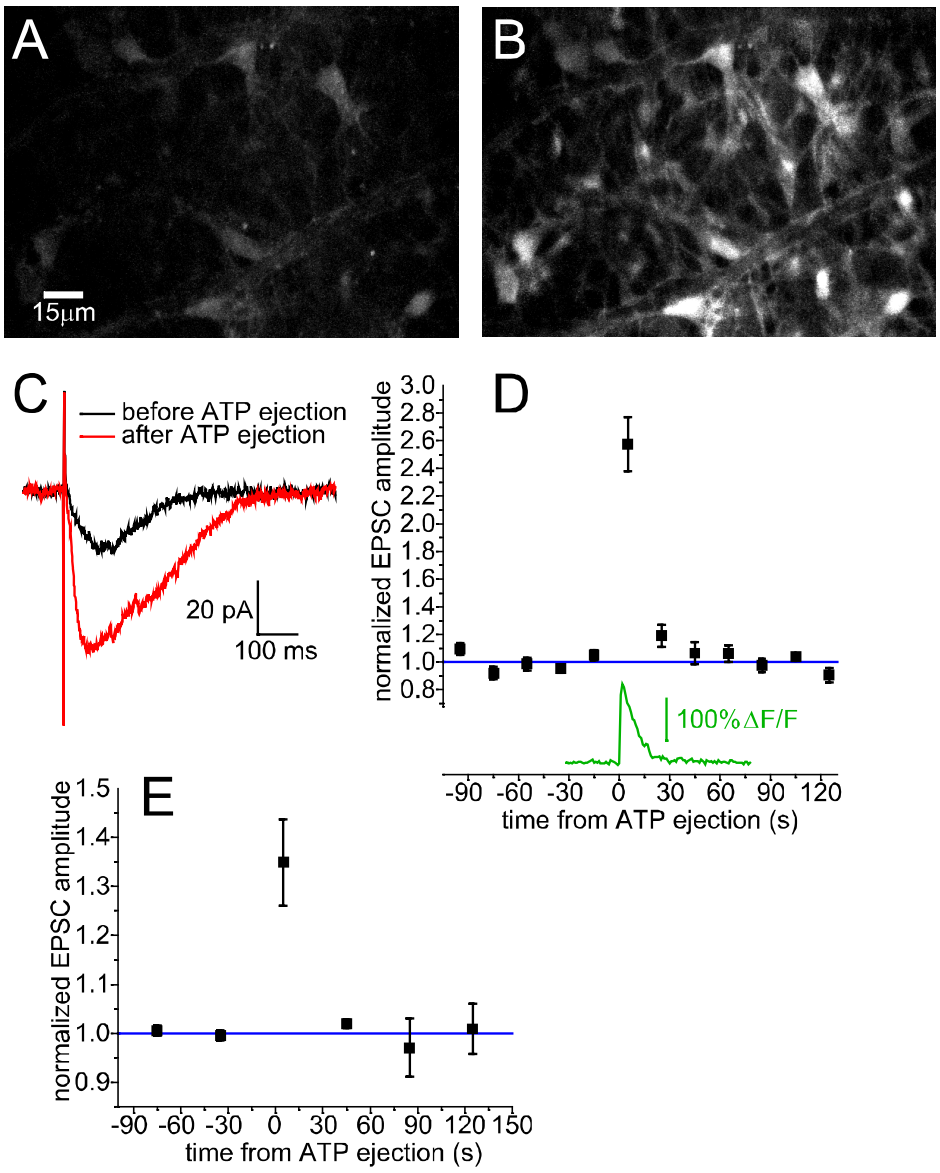

Fig. 3. ATP ejection stimulates glial Ca^{2+} elevation and increases EPSC amplitude.

A, Confocal fluorescence image of the vitreal surface of the retina prior to ATP ejection. Astrocytes and Müller cells are labeled with Fluo-4 AM, a Ca^{2+} indicator dye. **B**, The same field of view after ejection of 100 μM ATP. Both astrocytes and Müller cells responded with elevations in cytosolic Ca^{2+}. **C**, Example traces showing inward synaptic currents, recorded in ganglion cells, produced by light stimulation before (black) and

after (red) ejection of ATP. **D** and **E**, EPSCs were evoked repeatedly in ganglion cells by electrical or light stimulation. The amplitude of the inward current, normalized to pre-ejection baseline, is plotted on the vertical axis. Ejection of 100 μM ATP potentiated EPSCs evoked by either electrical (**D**) or light (**E**) stimulus. Horizontal blue lines represent the average baseline EPSC amplitude prior to ATP ejection. The green trace in **D** shows the increase in Ca^{2+} fluorescence signal averaged over the entire field, on the same time axis as the EPSC amplitudes.

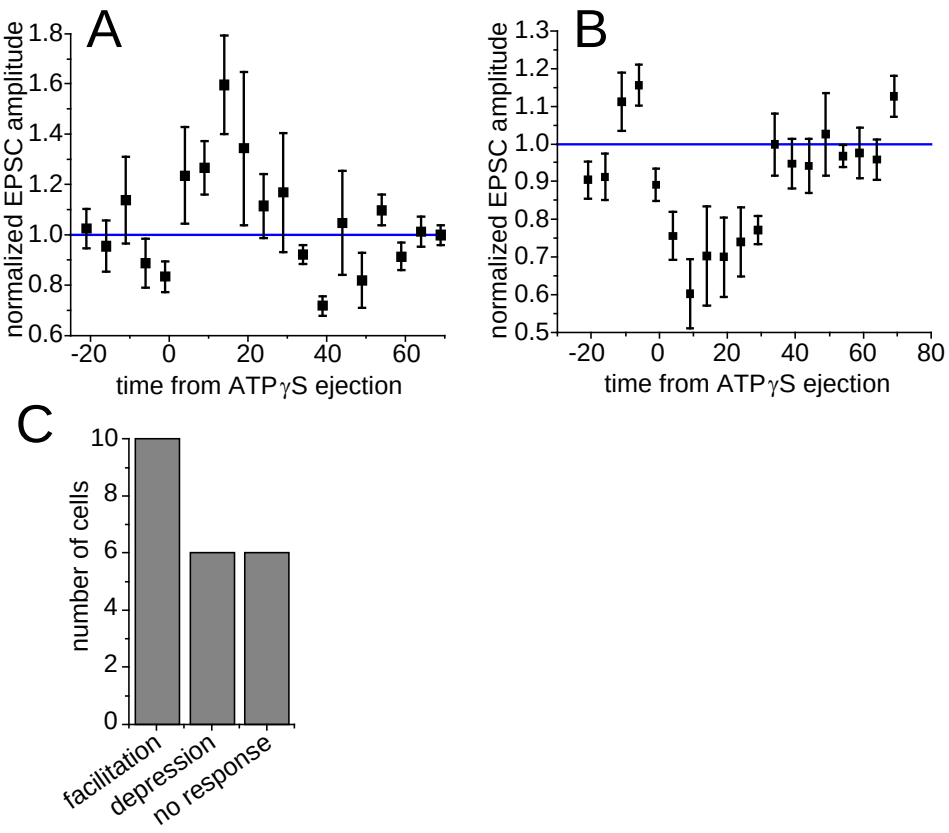

Fig. 4. ATPγS ejection facilitates or depresses EPSC amplitude.

A and **B**, EPSCs were evoked repeatedly in ganglion cells by electrical stimulation. The amplitude of the inward current, normalized to pre-ejection baseline, is plotted on the vertical axis. Ejection of 100 μM ATPγS either facilitated, depressed, or had no effect on EPSCs evoked by electrical stimulation. The trials with a facilitatory effect are averaged in **A**, and the trials with a depressive effect are averaged in **B**. **C**, Number of cells exhibiting facilitation, depression, or neither in response to ATPγS ejection.

ATPγS ejection facilitates or depresses synaptic transmission

Because ATP is rapidly degraded to ADP, AMP, and adenosine in the extracellular space (Newman, 2003), it is possible that the effect of ATP ejection is mediated by one or more of these other adenyl purines. We tested this by repeating the experiment described above, but ejecting ATPγS (100 μM), a non-hydrolyzable analogue of ATP, instead of ATP. In these experiments, EPSCs were always evoked by electrical stimulation in the OPL. Current pulses were delivered every five seconds to better resolve the timecourse of any modulation that resulted from glial stimulation. Like ATP ejection, ATPγS ejection produced a large Ca^{2+} increase in Müller cells and astrocytes (data not shown). However, we found that the effect of ATPγS on the amplitude of evoked EPSCs was variable. On some trials, EPSC amplitude increased following ejection, by an average of 60 ± 20 percent. These trials are averaged in Fig. 4A. On other trials, EPSC amplitude decreased following ejection, by an average of 40 ± 9 percent; these trials are averaged in Fig. 4B. In both cases, the timecourse of the change in EPSC amplitude matches the timecourse of the change in EPSC amplitude we saw with ATP ejection. Furthermore, in some trials ATPγS ejection had no effect on EPSC amplitude. The number of cells exhibiting each type of response to ATPγS ejection (increase, decrease, or no change in EPSCs) is summarized in Fig. 4C. By the proportion test, there was no significant difference in the number of cells exhibiting each of the three types of response. The fact that ATPγS ejection produces both depression and facilitation of synaptic transmission while ATP ejection produces only facilitation implies that ADP, AMP, or adenosine contributes to the effect of ATP ejection on synaptic transmission, perhaps by activating different receptors on glial cells or neurons. There was more

variability in the baseline responses to ATPγS ejection than to ATP ejection (e.g., compare Fig. 4A to Fig. 3D). In our experience it was common for the EPSC amplitude to be more variable when neuronal stimuli were given at a faster frequency (5 seconds for ATPγS versus 20 seconds for ATP). Thus we sacrificed stability for temporal resolution.

D-serine does not mediate the effect of ATP ejection on synapses

Because D-serine is released from glia (Mothet et al., 2005) and is an endogenous modulator of synaptic transmission in the retina (Stevens et al., 2003), we hypothesized that D-serine release from glia was the mechanism by which EPSCs are potentiated when glia are activated by ATP ejection. If this hypothesis is correct, then saturating the coagonist site on the NMDA receptor should block the potentiating effect of ATP ejection. To test this, we isolated the NMDAR-mediated component of light-evoked inward currents in ganglion cells by applying the AMPAR antagonist NBQX (10 μM). We confirmed that the remaining current was NMDAR-mediated by applying DL-AP7 (100 μM), an NMDAR antagonist, which fully blocked the remaining current (data not shown). An example of a light-evoked NMDAR-mediated inward current is shown in Fig. 5A, left panel, black trace. Ejecting ATP increased the amplitude of the EPSC (Fig. 5A, left panel, red trace), as we showed in Fig. 3E. After EPSC amplitude returned to baseline following ATP ejection, we waited five minutes and then bath-applied 100 μM D-serine (a saturating concentration by dose-response experiments; data not shown). D-serine alone increased the amplitude of light-evoked EPSCs (Fig. 5A, right panel, black trace). However, ejecting ATP in the presence of D-serine further increased the EPSC amplitude (Fig. 5A, right panel, red trace). In these experiments, EPSCs were evoked

every 20 seconds, and the timecourse of the response was 5-25 seconds (Fig. 5B). The average increase in EPSC amplitude produced by ATP alone was 94 ± 38 percent. D-serine alone produced an 89 ± 19 percent increase in EPSC amplitude, and ATP in the presence of D-serine generated an additional increase of 103 ± 49 percent of the pre-D-serine baseline. We observed this additive effect of ATP and D-serine on facilitation of synaptic transmission in three out of three ganglion cells tested. This additive effect was also evident whether current or light stimuli were used to evoke EPSCs. Therefore, we can reject our hypothesis that D-serine release mediates the effect of ATP ejection.

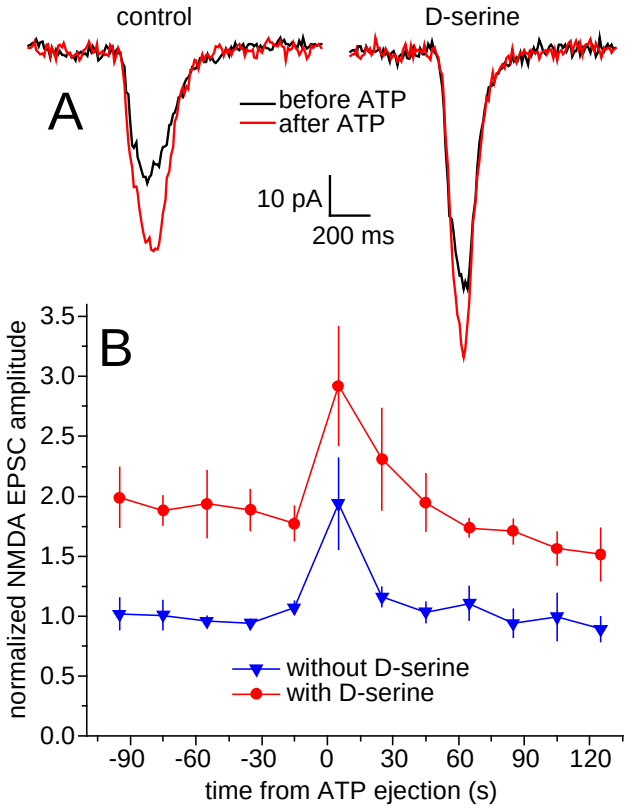

Fig. 5. D-serine does not mediate the potentiating effect of ATP ejection on EPSC amplitude.

A, Sample traces of EPSCs recorded from ganglion cells in response to light stimulation. The left panel shows EPSCs before and after 100 µM ATP ejection in the absence of exogenous D-serine; ATP ejection increases EPSC amplitude, as in Fig. 3. The right panel is from the same cell and also shows EPSCs before and after ATP ejection, but in the presence of 100 µM exogenous D-serine. The potentiating effect of ATP adds with the potentiating effect of D-serine. **B**, Same cell as **A**, showing peak EPSC amplitude on the vertical axis, plotted over time.

Uncaging bulk loaded Ca^{2+} from glial cells facilitates or depresses synaptic transmission

It is possible that ejection of ATP or ATPγS modulates synaptic transmission by acting directly on neurons. To selectively stimulate glial cells, we bulk loaded NP-EGTA AM, a photolabile Ca^{2+} cage. Uncaging with a focused UV laser caused an increase in the Fluo-4 fluorescence signal from glia but not from neurons (Fig 6A and B). This suggests that NP-EGTA AM loads preferentially into glial cells, which is consistent with the observation that AM esters of other molecules, such as Fluo-4 and Oregon Green BAPTA, load preferentially into glial cells in the intact retina. Of course, it is possible that NP-EGTA AM but not Fluo-4 AM loads into glial cells in the intact retina, in which case uncaging could cause a Ca^{2+} increase in neurons that we would be unable to detect.

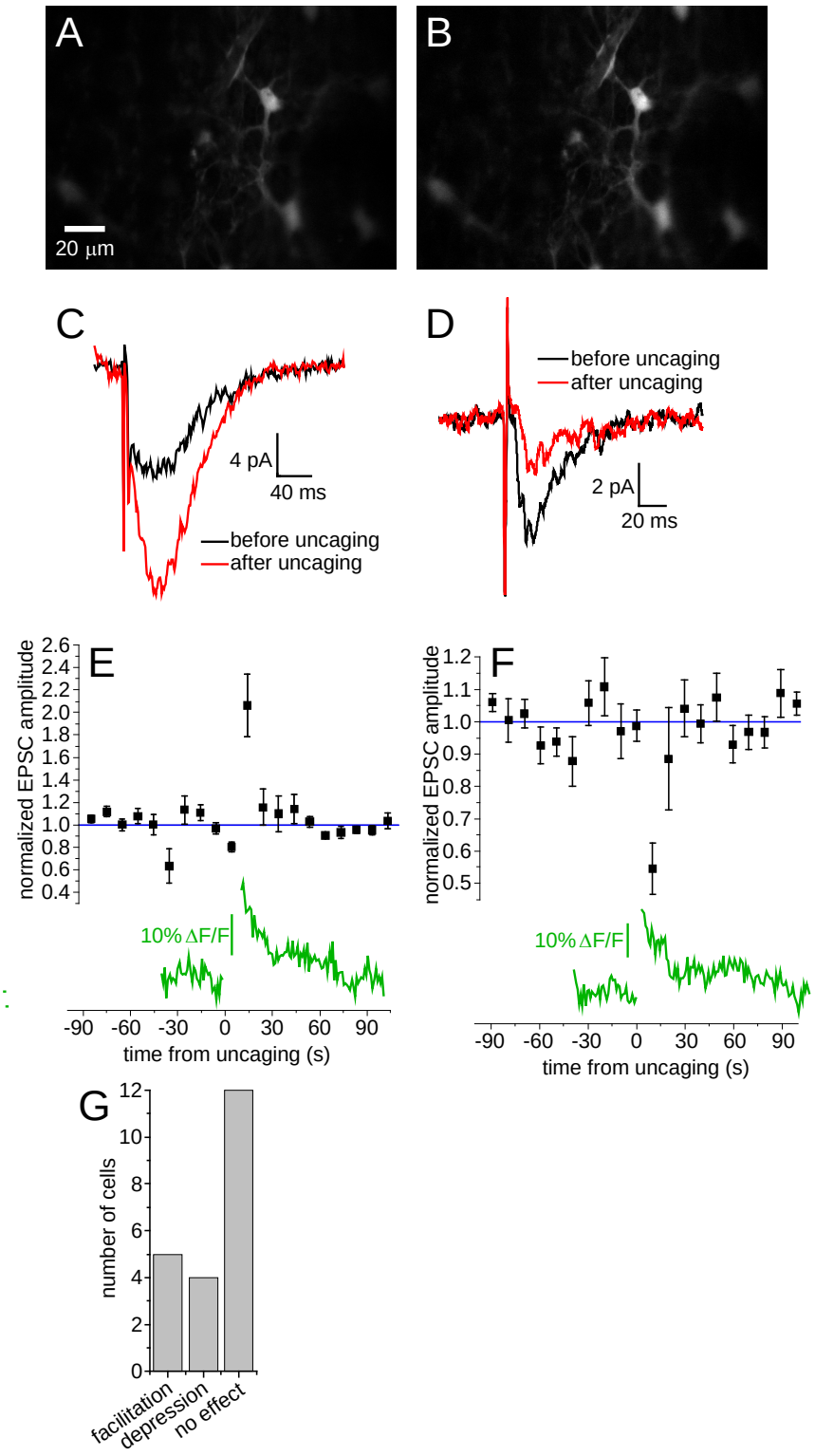

Fig. 6. Photolysis of bulk-loaded caged Ca^{2+} facilitates or depresses EPSC amplitude.

A, Confocal fluorescence image of the vitreal surface of the retina prior to uncaging Ca^{2+}. **B**, Same field of view after uncaging Ca^{2+}. Uncaging increases glial cytosolic Ca^{2+}. **C**, Sample traces of electrically-evoked EPSCs in ganglion cells, recorded before (black) and after (red) the uncaging UV laser flash. EPSC amplitude is increased following uncaging. **D**, Same as **C**, but from a cell that exhibited decreased EPSC amplitude following uncaging. **E**, Peak EPSC amplitude plotted over repeated stimulations. Averaged over cells in which uncaging potentiated EPSC amplitude. **F**, Same as **E**, but averaged over cells in which uncaging depressed EPSC amplitude. Green traces show examples of Ca^{2+} fluorescence signal from a Müller cell near the patched ganglion cell. **G**, Summary of the number of cells exhibiting facilitation, depression, or neither in response to photolysis of bulk-loaded caged Ca^{2+}.

Electrical stimuli in the OPL were used to evoke EPSCs in ganglion cells every 10 seconds. Example inward currents from before uncaging are shown in Fig. 6C and 6D, black traces. Like ATPγS ejection, uncaging Ca^{2+} produced a variable effect on the amplitude of evoked EPSCs. Some ganglion cells exhibited an increase in EPSC amplitude following uncaging (Fig. 6C, red trace). The trials exhibiting an increase in EPSC amplitude are averaged in Fig. 6E, showing an average increase of 110 ± 30 percent. On the other hand, some ganglion cells exhibited a decrease in EPSC amplitude following uncaging (Fig. 6D, red trace). The trials exhibiting a decrease in EPSC amplitude are averaged in Fig. 6F, showing an average decrease of 45 ± 8 percent. The number of cells exhibiting synaptic facilitation, depression, or no effect as a result of Ca^{2+} uncaging are summarized in Fig. 6G. We note that the Ca^{2+} elevation in glial cells produced by uncaging (Fig. 6E and 6F, green traces) is shorter lasting than the Ca^{2+} elevation produced by ATP ejection (cf. Fig. 3D, green trace), and this matches the shorter timecourse of the change in EPSC amplitude (Fig. 6E and 6F).

Uncaging Ca^{2+} from single Müller cells has no effect on synaptic transmission

We cannot rule out that when bulk loading glia with caged Ca^{2+}, some caged Ca^{2+} enters neurons, causing the uncaging UV laser to produce synaptic modulation without the involvement of glia. Therefore, we loaded caged Ca^{2+} into individual Müller cells by patching a Müller cell with a pipette containing the potassium salt of NP-EGTA, which is membrane-impermeant. The pipette also contained the potassium salt of Fluo-4 to visualize Ca^{2+} levels in the Müller cell. Because we were monitoring synaptic

transmission at the bipolar-to-ganglion cell synapse, we visualized Fluo-4 in the inner plexiform layer of the retina where these synapses are made (Fig. 7A).

In these experiments, we first patched onto a ganglion cell, and then with a second pipette patched onto a Müller cell ~50 µm away from the ganglion cell soma. This distance was chosen to maximize the likelihood that the patched Müller cell was within the dendritic arbor of the ganglion cell where synaptic contacts are made (Jakobs et al., 2008). Electrical stimuli were used to evoke EPSCs in ganglion cells every 10 seconds (example inward current shown in Fig. 7C, black trace). The tip of the glass pipette used to deliver the electrical stimulus was placed in the OPL, directly beneath the patched Müller cell. Between the 9th and 10th electrical stimuli, a focused UV laser pulse was delivered to the patched Müller cell, resulting in a large Ca^{2+} increase in Müller cell processes in the IPL (Fig. 7B). However, the amplitude of evoked EPSCs was unaltered following uncaging (n = 3 ganglion cells) (Fig. 7C, red trace; Fig. 7D), despite the large magnitude of the Ca^{2+} increase in the Müller cell (Fig. 7D, green trace).

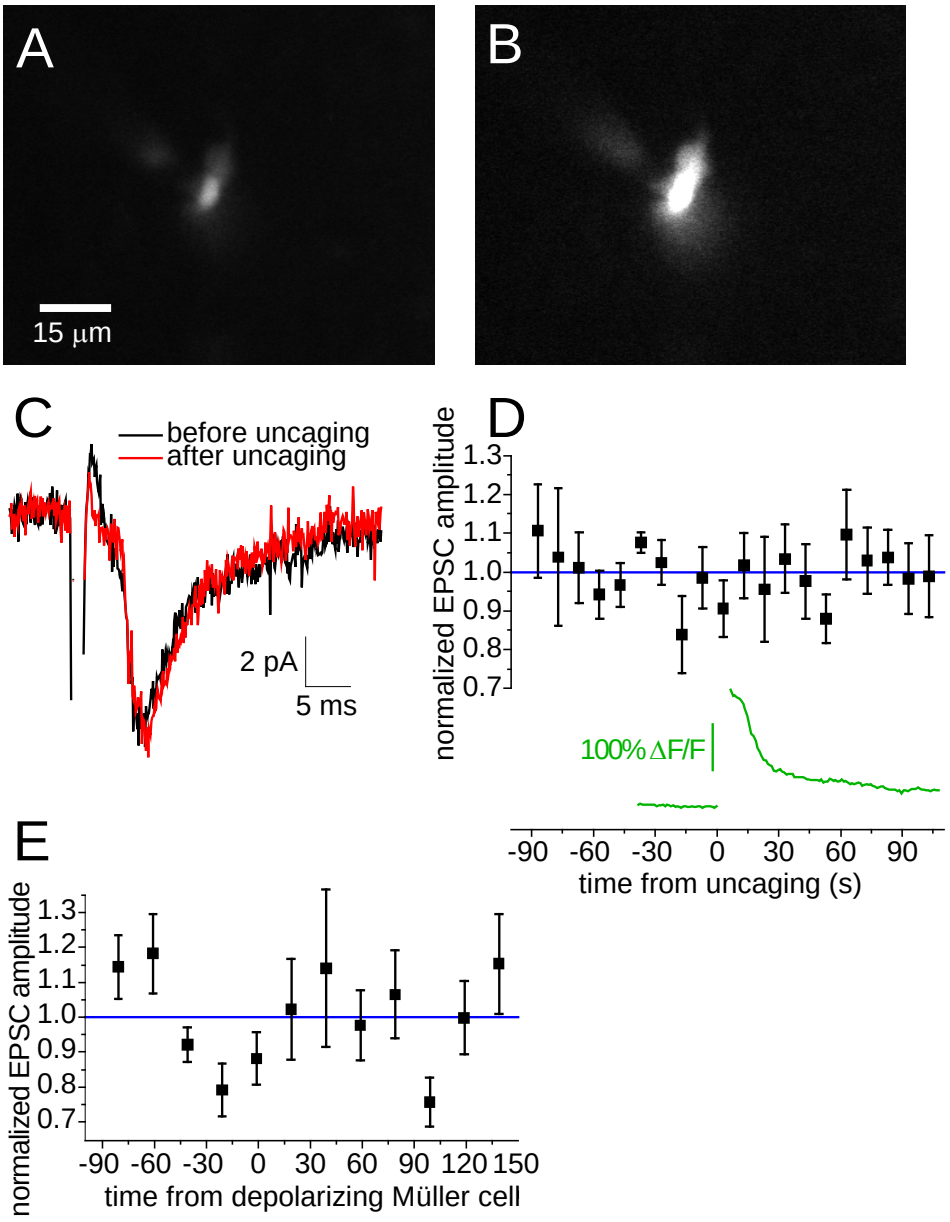

Fig. 7. Selective stimulation of individual Müller cells has no effect on EPSC amplitude.

A and **B**, Confocal fluorescence images before (**A**) and after (**B**) uncaging of cell-impermeant caged Ca^{2+} from a single Müller cell. The plane of focus is in the inner plexiform layer, and Ca^{2+} increases are visible both in the stalk (central bright area) and

processes (surrounding halo) of the Müller cell. **C**, Sample traces of electrically-evoked EPSCs in ganglion cells, recorded before (black) and after (red) the uncaging UV laser flash. Uncaging had no effect on the amplitude of EPSCs. **D**, Peak EPSC amplitude plotted over repeated stimulations. **E**, Depolarization of a single Müller cell from -80 to +60 mV had no effect on EPSC amplitude.

Depolarizing single Müller cells has no effect on synaptic transmission

If glia are indeed responsible for modulating synaptic transmission at the bipolar to ganglion cell synapse, it is possible that uncaging Ca^{2+} in Müller cells does not activate the requisite intracellular signaling pathways in Müller cells to produce this modulation. As another method of selectively stimulating Müller cells, we directly depolarized individual Müller cells. Glial cells have previously been shown to respond to electrical stimuli with Ca^{2+} increases (Nedergaard, 1994), and a train of depolarizing current pulses delivered to astrocytes in the hippocampus causes modulation of synaptic transmission (Kang et al., 1998). First, we patched onto a ganglion cell, and then with a second pipette patched onto a Müller cell ~50 µm away from the ganglion cell soma. The Müller cell was held in voltage clamp at -80 mV. Electrical stimuli in the OPL were used to evoke EPSCs in ganglion cells every 20 seconds. Between the fifth and sixth electrical stimuli, we applied a train of four depolarizing pulses (to +60 mV) to the Müller cell. Each pulse lasted 500 ms and pulses were separated by 1500 ms. This depolarizing pulse train had no effect on EPSC amplitude (n = 5) (Fig. 7E).

DISCUSSION

We set out to test the hypothesis that activating glial cells alters the efficacy of synaptic transmission in the retina. In other brain areas, selective stimulation of glia causes release of transmitters that act on neurons to alter synaptic strength. Glial cells in the retina release ATP when stimulated (Newman, 2001; Newman, 2003). Retinal glia also have processes in the inner plexiform layer where bipolar cells synapse onto ganglion cells. We therefore speculated that stimulation of retinal glia could lead to modulation of the bipolar-to-ganglion cell synapse.

To measure synaptic strength at the bipolar-to-ganglion cell synapse, we evoked EPSCs in ganglion cells using either a diffuse light flash (which activates photoreceptors, leading to activation of bipolar cells through the normal circuitry of the retina) or an extracellular current pulse near the bipolar cell dendrites. After recording baseline EPSC amplitude, we stimulated glia using one of five different methods. Although three types of stimuli (ATP ejection, ATPγS ejection and photolysis of bulk loaded caged Ca^{2+}) that produced glial Ca^{2+} elevations also modulated synaptic transmission, the two most selective glial stimuli tested (uncaging from single Müller cells and Müller cell depolarization) had no effect on the amplitude of evoked synaptic currents. There are two possible interpretations of these data.

First, it may be that the modulation of synaptic transmission produced by the less selective stimuli (ATP ejection, ATPγS ejection and photolysis of bulk loaded caged Ca^{2+}) was a direct effect of those stimuli on neurons, rather than being mediated by glial cells. For example, ejected ATP or ATPγS could act directly on purinergic receptors at the synapse to modulate transmission (Cunha and Ribeiro, 2000). Likewise, if some

caged Ca^{2+} enters bipolar cells or ganglion cells during bulk loading, photolysis of this caged Ca^{2+} could act pre- or post-synaptically to modulate synaptic currents.

Second, it may be that the modulation of synaptic transmission we observed in Figs. 3-5 was indeed mediated by glial cells, but the more selective stimuli we used may not have been sufficient to engage the glial intracellular signaling pathways that lead to altered synaptic transmission. For example, activation of more than one Müller cell may be required. It is possible that when loading membrane-impermeant caged Ca^{2+} into single Müller cells, the caged Ca^{2+} molecule does not diffuse efficiently into the fine processes that abut synapses. It is also possible that patching onto a Müller cell, and consequently dialyzing it, could interfere with the intracellular signaling pathways that lead to transmitter release. It has been recently observed that different methods of triggering seemingly identical Ca^{2+} elevations in glia produce different physiological responses (Shigetomi et al., 2008). The mechanism of ATP release from Müller cells is not known (Newman, 2003), although in other tissues, ATP is released from glial cells through hemichannels (Stout et al., 2002), exocytosis (Coco et al., 2003; Pascual et al., 2005), anion channels (Kimelberg et al., 2006), and pore-forming $P2X_7$ receptors (Duan and Neary, 2006).

We cannot reach a conclusion from these data as to whether glial activation is sufficient to modulate synaptic transmission in the retina. Future experiments should take advantage of genetic techniques to compare synaptic transmission in the retina between wild-type mice and mice lacking vesicular release from glia (Pascual et al., 2005), and to express foreign receptors (for which no endogenous ligand exists) selectively on glia to allow stimulation of glia but not neurons (Fiacco et al., 2007).

If modulating synaptic transmission is a physiological function of glial cells, what consequences might this modulation have? Glial cells may provide a direct link to carry information between synapses. Astrocytes bridge synapses to allow heterosynaptic plasticity (Kang et al., 1998). Glia can synchronize neuronal activity through extrasynaptic NMDA receptors (Fellin et al., 2004); it is possible that synaptic modulation also contributes to coordinating neuronal activity. Glial cells are sensitive to synaptic neurotransmitter and integrate information from multiple synapses (Perea and Araque, 2006). Modulating synaptic transmission could be a mechanism to return this integrated information to neurons. Müller cells span the thickness of the retina and are therefore well positioned to carry information between layers. A neuroprotective role has been suggested for glial adenosinergic signaling in the retina (Clark et al., 2009); Müller cells could potentially also suppress synaptic inputs onto ganglion cells under conditions of hypoxia to reduce metabolic demand.

Another question raised by our experiments is why ATP consistently potentiates synaptic transmission while ATPγS can have either a potentiating or depressive effect. To our knowledge, the only difference between these molecules is that ATP is rapidly degraded to ADP, AMP, and adenosine, while ATPγS (in which one oxygen on the third phosphate group is replaced with a sulfur atom) is not degraded. Thus, the action of these other adenyl purines, either on neurons or on glia, may bias synaptic modulation toward potentiation. Yet another possibility is that ATPγS, by acting as a non-hydrolyzable substrate for ATPases, interferes with ATP-dependent molecular processes involved in synaptic transmission or in glial modulation of synaptic transmission. This seems less

likely because the effect of ATPγS pressure ejection is rapid and may not allow enough time for ATPγS to be internalized into the cell where it could have an effect.

Glia modulate synaptic transmission in the brain; if this does not occur in the retina, we must ask why. The brain undergoes synaptic plasticity throughout life to encode new information, whereas long-term synaptic plasticity has never been observed in the retina. The retina is thought to serve a largely time-invariant computational function (with the notable exceptions of light/dark adaptation, for which short-term plasticity occurs in the retina (Demb, 2008)). Therefore, there may not be as much need in the retina for glial regulation of synaptic plasticity. However, it is still an open question whether glia participate in the normal information processing of the retina by dynamically modulating synapses over short time scales. In the supraoptic nucleus, the slow time scale of glial morphological plasticity is well suited to modulate endocrine function (Oliet et al., 2004).

In the brain, glia signal to neurons by releasing ATP (Pascual et al., 2005), glutamate (Bezzi et al., 1998), and D-serine (Yang et al., 2003; Panatier et al., 2006). In the retina, ATP is the primary gliotransmitter (Newman, 2003), and there is no evidence for functional glial release of glutamate. The ability to release glutamate could be tied to a more active functional role of glia in the brain versus the retina. Endogenous D-serine regulates synaptic transmission in the retina (Stevens et al., 2003), but it is not clear whether the D-serine originates from neurons or glia.

Recent work by McCarthy and colleagues (Fiacco et al., 2007; Petravicz et al., 2008) casts some doubt on whether glial Ca^{2+} elevation leads to synaptic modulation under physiologically realistic conditions in the brain. Transgenically expressing a

foreign G-protein coupled receptor on a glial cell specific promoter allows selective stimulation of the glial intracellular signaling pathways believed to be involved in the transmitter exocytosis that causes synaptic modulation. Activation of this receptor leads to glial Ca^{2+} increases but not changes in synaptic transmission (Fiacco et al., 2007). Conversely, selectively knocking out the astrocytic IP_3 receptor abolishes astrocyte Ca^{2+} increases but has no effect on synaptic transmission (Petravicz et al., 2008). The authors argue that in previous studies, the stimuli (such as photolysis of caged Ca^{2+}) used to activate glial cells were not realistic.

In live, intact animals, sensory stimulation is sufficient to cause Ca^{2+} elevations in glial cells (Wang et al., 2006; Schummers et al., 2008), but it is not known if these naturally-evoked Ca^{2+} elevations cause synaptic modulation the way artificially evoked Ca^{2+} increases do. The artificial stimuli used to generate Ca^{2+} elevations may engage processes that are not engaged by natural stimuli. However, spontaneous transients in astrocytes are sufficient to activate NMDA receptors on neurons (Parri et al, 2001). Any possible endogenous role for glial Ca^{2+} elevations to alter synaptic transmission remains unexplored.

Chapter 4

Spontaneous Intercellular Glial Ca^{2+} Waves in the Retina Increase with Age and Regulate Blood Flow

ABSTRACT

Intercellular glial Ca^{2+} waves constitute a signaling pathway between glial cells. Artificial stimuli have previously been used to evoke these waves, and their physiological significance has been questioned. We report here that Ca^{2+} waves occur spontaneously in rat retinal glial cells, both in the isolated retina and in vivo. These spontaneous waves are propagated by ATP release. In the isolated retina, suramin (P2 receptor antagonist) reduces the frequency of spontaneous wave generation by 53 percent, and apyrase (ATP-hydrolyzing enzyme) reduces frequency by 95 percent. Luciferin/luciferase chemiluminescence reveals waves of ATP matching the spontaneous Ca^{2+} waves, indicating that ATP release occurs as spontaneous Ca^{2+} waves are generated. Wave generation also depends on age. Spontaneous wave frequency rises from 0.27 to 1.0 per minute per mm^2, as rats age from 20 to 120 days. The sensitivity of glia to ATP does not increase with age, but the ATP released by evoked waves is 31 percent greater in 120 day old than in 20 day old rats, suggesting that increased ATP release in older animals could account for the higher frequency of wave generation. Simultaneous imaging of glial Ca^{2+} and arterioles in the isolated retina demonstrates that spontaneous waves alter vessel diameter, implying that spontaneous waves may have a significant impact on retinal physiology. Spontaneous intercellular glial Ca^{2+} waves also occur in the retina in vivo, with frequency, speed and diameter similar to the isolated retina. Increased spontaneous wave occurrence with age suggests that wave generation may be related to retinal pathology.

INTRODUCTION

It has been recently demonstrated that intercellular glial Ca^{2+} waves occur spontaneously in vivo, answering the question of whether waves occur in the absence of artificial stimulation. In healthy animals, spontaneous waves have been observed in cerebellum (Hoogland et al., 2009). Spontaneous waves also occur in neocortex near amyloid plaques in transgenic Alzheimer's disease mice (Kuchibhotla et al., 2009), suggesting a possible role of waves in pathology. However, these studies do not establish a functional role for spontaneous glial Ca^{2+} waves.

We report here that spontaneous glial Ca^{2+} waves occur in the retina, both in the isolated retina and in vivo. Like evoked waves, spontaneous waves in the retina are propagated by the release of ATP and can modulate blood vessel diameter. We also find that spontaneous waves increase in frequency as animals age. The results demonstrate that glial Ca^{2+} waves are a physiological signaling mechanism in the retina and that glia can independently regulate blood flow. The results also suggest that glial Ca^{2+} waves may be involved in the aging process.

RESULTS

Two types of macroglial cells are present in the mammalian retina: astrocytes and Müller cells. Astrocyte somata lie on the vitreal surface of the retina and extend processes that contact blood vessels in the innermost vascular layer. Müller cells are radial glia whose somata lie in the inner nuclear layer. Müller cell processes span the entire thickness of the retina and make extensive contacts with blood vessels and synapses (Newman and Reichenbach, 1996). Müller cell endfeet line the vitreal surface of the retina. Astrocytes are coupled to one another and to Müller cells by gap junctions (Zahs and Newman, 1997). Mechanical, electrical, or chemical stimulation of the inner retina evokes waves of Ca^{2+} elevation that propagate between both types of glial cells (Newman and Zahs, 1997; Newman, 2001).

Spontaneous glial Ca^{2+} waves occur in the retina

Glial Ca^{2+} was monitored with confocal microscopy at the inner surface of the isolated retina, where Ca^{2+} signals were imaged in Müller cell endfeet and astrocyte somata and processes. Glia exhibited spontaneous (i.e., unstimulated) waves of Ca^{2+} elevation that propagated between cells (Fig. 8A). The waves followed a pattern of concentric expansion similar to evoked waves, expanding for 4-8 seconds from a central point of initiation and then contracting over the next 5-10 seconds.

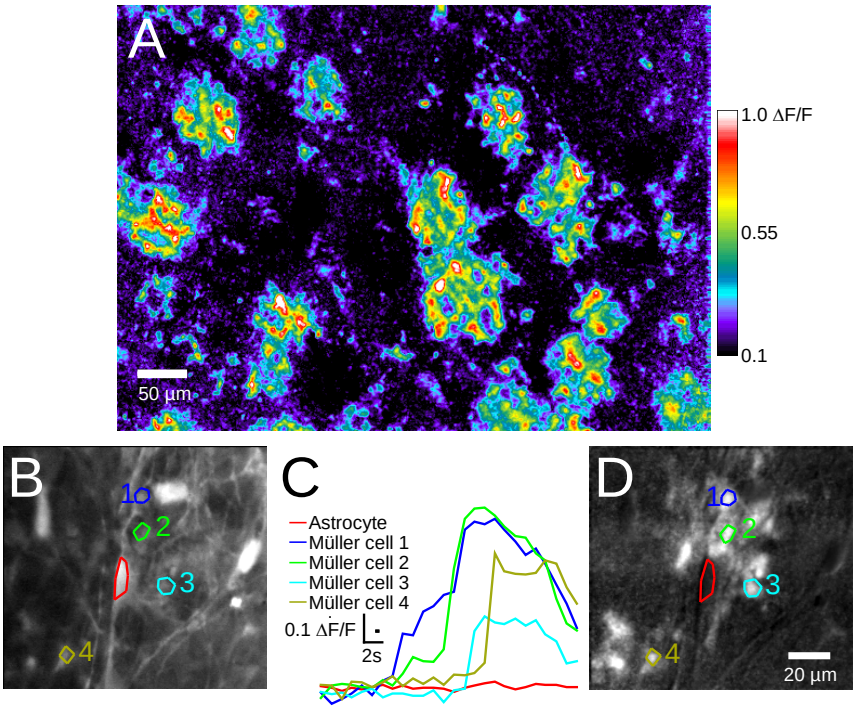

Fig. 8. Spontaneous intercellular glial Ca^{2+} waves in the isolated retina begin in and propagate through Müller cells.

A, Spontaneous waves are initiated from multiple points on the retina. This pseudocolor ratio image collapses 60 seconds of video by a maximum ΔF/F projection. 15 spontaneous waves are generated during the 60 second period. **B-D**, A single Ca^{2+} wave shown at higher magnification. Like all waves we observed, this wave was initiated in and propagated through Müller cells but not astrocytes. **B**, Calcium fluorescence image before the wave was initiated (average of 5 frames). Regions 1-4 are Müller cells participating in the wave. The red region is an astrocyte. **C**, The brightness increase (ΔF/F) in each region plotted over time. Although the wave propagates across the soma of the astrocyte, $[Ca^{2+}]_i$ did not rise in the cell. **D**, A maximum ΔF/F projection of the frames during the wave (same field of view as B), revealing cells in which there is a $[Ca^{2+}]_i$ increase.

Spontaneous Ca^{2+} waves propagated exclusively through Müller cells and were not observed to propagate into astrocytes. In 15 waves recorded at high magnification, the waves were always initiated in Müller cells and propagated past astrocytes without a Ca^{2+} elevation occurring in the astrocyte (n = 8 animals; Fig. 8B-D). However, we cannot rule out the possibility that astrocytes occasionally also experience Ca^{2+} increases. Waves propagated at 24.3 ± 1.7 μm/s and reached a maximal diameter of 68.4 ± 2.2 μm (n = 18 waves from 4 animals).

Spontaneous waves increase with age

The frequency of spontaneous wave generation was dependent on the age of the animal (Fig. 9A). The frequency equaled 1.0 per minute per mm^2 in 120 day old animals, 3.8 times higher than the 0.27 per minute per mm^2 value in 20 day old animals. These measurements of wave frequency were made after the retina had been in the superfusion chamber for 20-70 minutes.

Some retinal areas from older animals exhibited a very high frequency of spontaneous wave generation immediately after the retinas were placed in the superfusion chamber. This high frequency of wave generation decayed within approximately 15 minutes (see Fig. 11A). A stable baseline rate of wave generation was reached after 15 minutes and was similar to the rate seen in areas of the retina where a high frequency of wave generation did not occur. The probability of retinas displaying an initial high rate of wave generation increased with the age of the animal (Fig. 9B). The maximal wave rate, measured in the first minute of recording, also increased as a function of the animal's age

(Fig. 9C). In retinas from 12 month old animals, the rate was 56 ± 24 waves per minute per mm^2.

Because the rate of glial Ca^{2+} wave generation increased with age, we speculated that wave occurrence could be a consequence of cumulative damage, perhaps caused by exposure to light. To test this, we exposed rats to 1,000 lux ambient light (~30 times normal light levels) for a period of 1 to 4 months, hypothesizing that this would hasten the development of waves. We found no effect of light treatment on the incidence of waves, however, indicating that the generation of spontaneous Ca^{2+} waves was not due to retinal damage caused by light exposure (Fig. 9D). In fact, there was a trend toward reduced wave generation in light-exposed animals, but the interaction between light exposure and age was not significant by two-way ANOVA ($p = 0.07$).

Spontaneous waves are propagated by release of ATP

Artificially evoked glial Ca^{2+} waves in the retina are propagated from cell to cell through two mechanisms. Astrocyte-to-astrocyte propagation is mediated by diffusion of a small molecule (probably IP_3 (Venance et al., 1997)) through gap junctions, while astrocyte-to-Müller cell and Müller cell-to-Müller cell propagation are mediated by release of ATP and its action on P2 purinergic receptors (Newman, 2001).

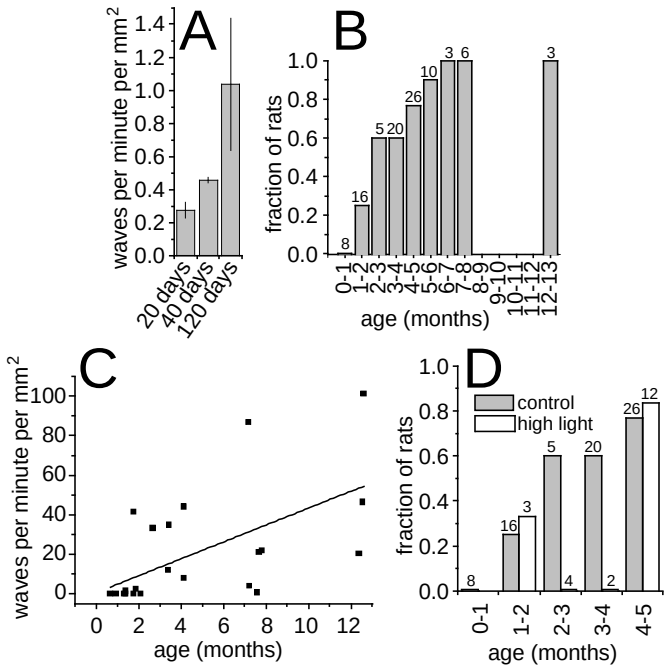

Fig. 9. The frequency of spontaneous Ca^{2+} wave generation increases with age.

A, The average frequency of spontaneous waves (counted from 20 to 70 minutes after the start of imaging) in animals of different ages. The frequency increases with age ($p < 0.05$). **B**, The fraction of animals at each age that exhibited a high initial frequency of waves (defined as three or more waves occurring within the first six minutes of imaging). Numbers above the bars indicate the total number of animals in each bin. **C**, The highest wave rate observed in a rat increased with the rat's age ($p < 0.005$). Each point represents one animal. **D**, Exposure to high light levels for 1 to 4 months did not increase the fraction of rats displaying a high initial wave frequency.

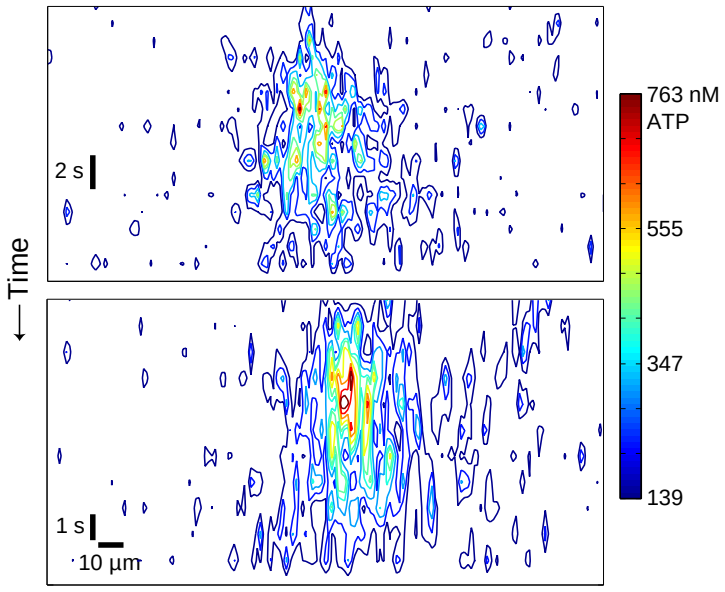

Fig. 10. Spontaneous waves of ATP release in the isolated retina.

ATP release was visualized with luciferin-luciferase chemiluminescence in areas of the retina with a high frequency of spontaneous Ca^{2+} waves. The graphs are linescan images of chemiluminescence and are represented as contour plots of brightness with time shown on the vertical axis. In these two examples, the linescans are centered at the middle of each wave. Like spontaneous Ca^{2+} waves, the ATP waves expand from a central point (at the center/top of each graph) and then shrink, over a total period of 8-15 seconds.

We asked whether the propagation of spontaneous waves through Müller cells was also mediated by ATP release. The luciferin-luciferase ATP chemiluminescence assay was used to monitor ATP release into the extracellular space during spontaneous Ca^{2+} wave generation. Calcium imaging was first used to locate a wave-rich region of an isolated retina from an older rat (175-325 days). ATP chemiluminescence was then imaged from the same wave-rich retinal region for several minutes. We observed multiple spontaneous chemiluminescence signals that spread outwards, indicating spontaneous waves of ATP release (Fig. 10). The propagation velocity of the spontaneous ATP waves (22.0 ± 2.1 μm/s; n = 4) closely matched the propagation velocity of spontaneous Ca^{2+} waves. The maximal diameter of the ATP waves (85.3 ± 3.1 μm) was somewhat larger than that of the Ca^{2+} waves. The luciferin-luciferase chemiluminescence images were calibrated to determine the absolute concentration of ATP at the retinal surface. Peak extracellular ATP concentration equaled 2 μM at the center of spontaneous waves of ATP release. This is an order of magnitude smaller than the peak ATP levels generated by mechanically-evoked waves (Newman, 2001). This is reasonable, as the mechanically evoked waves were substantially greater in magnitude, having significantly larger diameters.

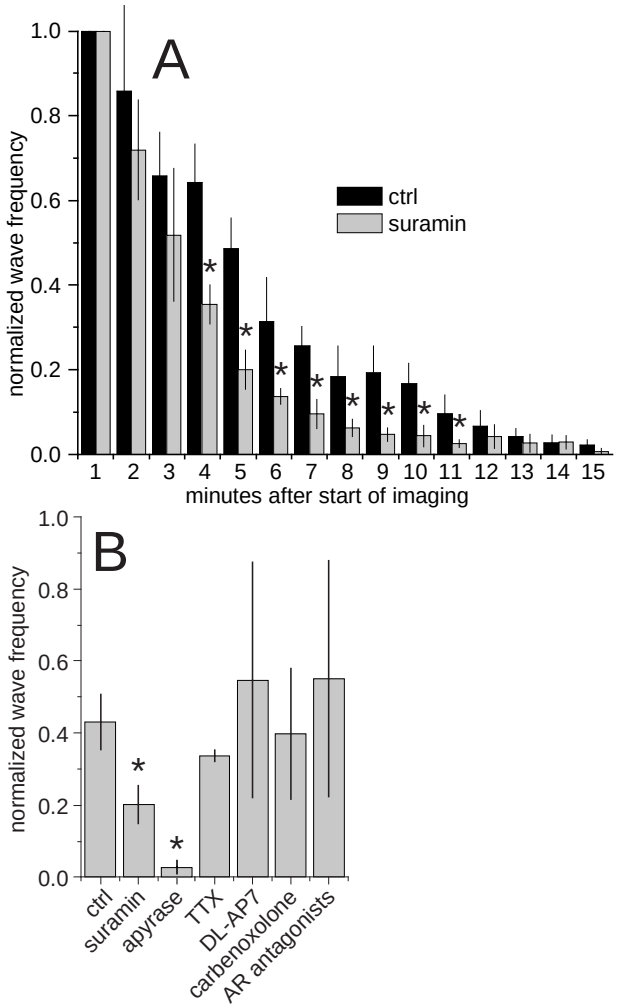

Fig. 11. Spontaneous Ca^{2+} wave generation is dependent on P2 receptors and extracellular ATP. **A**, Suramin (100 µM; P2R antagonist) decreases the frequency of spontaneous waves. The difference between control and suramin values is superimposed on the decay of the high initial rate of wave generation. Spontaneous Ca^{2+} wave counts are binned in 1 minute intervals, and each trial is normalized to the wave count during the first minute of the trial. **B**, Suramin (100 µM) and apyrase (100 U/mL; ATP-degrading enzyme), but not TTX (200 nM), DL-AP7 (100 µM; NMDAR antagonist), carbenoxolone (100 µM; gap junction blocker), or a cocktail of adenosine receptor antagonists (100 nM DPCPX, 1 µM MRS 1191, 1 µM MRS 1706, 1 µM ZM 241385) reduced the frequency of spontaneous wave generation. Spontaneous wave counts were averaged from 3-6 minutes after the start of imaging and were normalized to wave counts during the first minute. Drugs were added 1 minute after the start of imaging.

* indicates $p < 0.05$.

The luciferin-luciferase chemiluminescence results demonstrate that ATP is released in a spatial pattern matching the spontaneous Ca^{2+} waves. To test whether ATP plays a causal role in wave propagation, we blocked the action of ATP on glial purinergic receptors. Pharmacology experiments were performed during the period of high initial wave frequency in older rats (116-327 days). Suramin (a P2 receptor antagonist; 100 µM) reduced the frequency of spontaneous wave generation by 52 ± 7 percent (n = 7) (Fig. 11A,B). Suramin has non-selective effects and might be blocking Ca^{2+} waves by a mechanism other than antagonizing P2 receptors. We therefore blocked the action of ATP with apyrase, an enzyme that selectively hydrolyzes ATP in the extracellular space. Apyrase (100 units/mL) reduced the frequency of spontaneous waves by 95 ± 4 percent (n = 4) (Fig. 11B). The greater effect of apyrase compared to suramin may reflect an incomplete block of P2 receptors by suramin.

We also tested whether gap junctions participate in spontaneous wave propagation by applying carbenoxolone, a gap junction blocker. Carbenoxolone (100 µM) did not affect the number of spontaneous waves generated (n = 3) (Fig. 11B) or the average speed or diameter of waves (data not shown), indicating that gap junctions do not play a significant role in the propagation of spontaneous waves. This is consistent with the lack of astrocyte involvement in spontaneous waves, as wave propagation between retinal astrocytes is mediated by gap junctions but propagation between Müller cells is not (Newman, 2001). We also applied TTX to determine if neuronal spiking contributes to the generation of spontaneous waves. TTX (200 nM) did not affect the number of spontaneous waves generated (n = 4) (Fig. 11B). In addition, neither DL-AP7 (n = 5) (100 µM; an NMDA receptor antagonist) nor a cocktail of adenosine receptor antagonists

(n = 4) (100 nM DPCPX for A1, 1 μM ZM 241385 for $A2_A$, 1 μM MRS 1706 for $A2_B$, and 1 μM MRS 1191 for A3) had any effect on the number of spontaneous waves generated (Fig. 11B).

ATP sensitivity does not increase with age

We speculated that an increase in glial ATP sensitivity with age could account for the increase in wave generation frequency observed in older animals. To test this hypothesis, we bath-applied 3 μM ATP to retinas from animals of different ages. The application of ATP always produced a Ca^{2+} increase in Müller cells and astrocytes, but we found no correlation between the magnitude of this increase and the age of the animal (n = 24) (Fig. 12A). To ensure that the ATP response was not saturated, we also tested ATP at 1 μM and 0.3 μM. While the Ca^{2+} responses were smaller, we saw no effect of age on the magnitude of responses at these concentrations (data not shown). The results demonstrate that a change in glial cell ATP sensitivity does not underlie the increased rate of spontaneous wave generation in older animals.

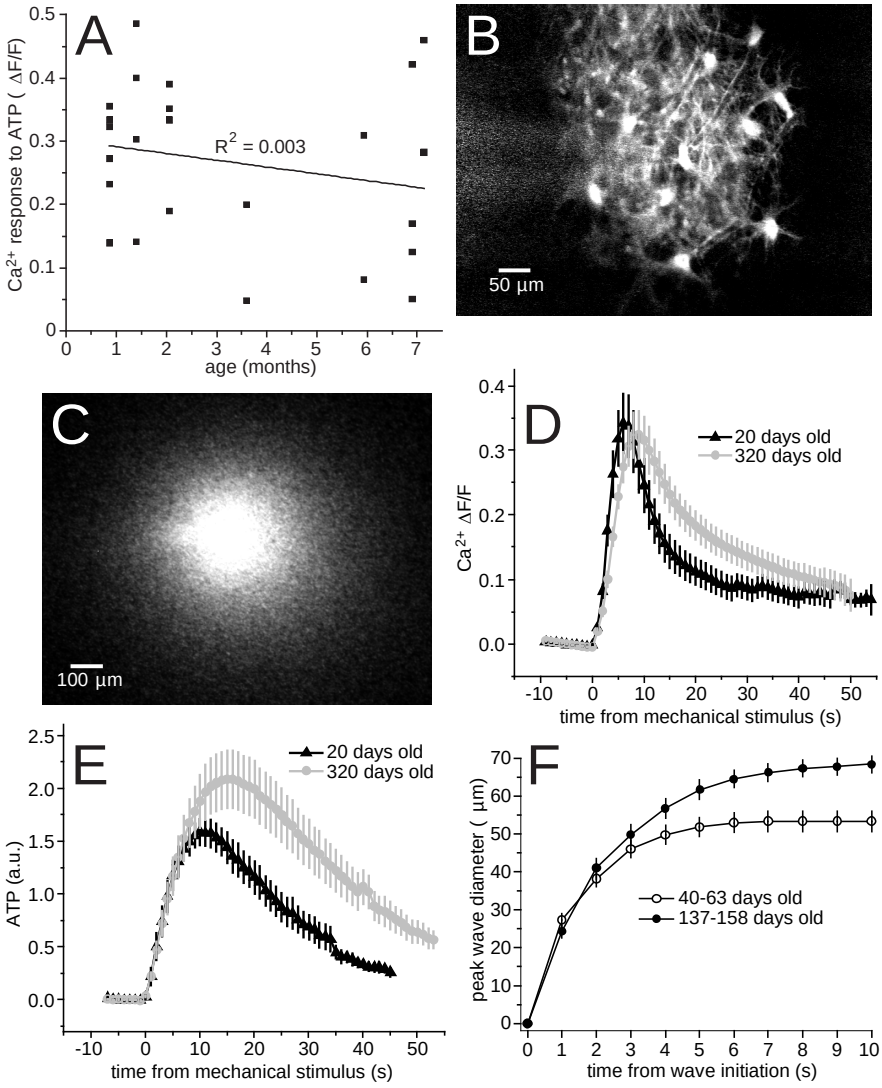

Fig. 12. ATP release, but not ATP sensitivity, increases with age.

A, ATP sensitivity of glial cells is age-independent. Bath-application of 3 µM ATP produced a Ca^{2+} increase in Müller cells and astrocytes that was not correlated to the age of the animal. **B**-**E**, Mechanical stimulation of astrocyte somata triggered waves of Ca^{2+} and ATP. The amount of ATP released during evoked waves was greater in older animals. **B**, An evoked Ca^{2+} wave (maximum brightness projection). **C**, An evoked ATP wave (single frame at peak diameter). **D**, The peak Ca^{2+} increase during evoked waves in

old rats is not different from young rats. **E**, The peak ATP concentration during evoked waves in old rats is higher than in young rats. Differences are significant ($p < 0.05$) from 23 to 34 seconds. In **D** and **E**, brightness measurements for Ca^{2+} fluorescence and ATP chemiluminescence are averages over the entire microscopic field. **F**, Spontaneous Ca^{2+} waves reach a larger maximal diameter in older animals. The diameter shown at each time point is the maximum diameter reached by that time. Differences between younger and older rats are significant when analyzed per wave ($p < 0.001$) or per animal ($p < 0.005$).

More ATP is released by glial cells in older animals

Calcium wave propagation involves both ATP release and a Ca^{2+} elevation in response to the released ATP. Since the Ca^{2+} elevation observed in response to ATP does not increase with age (Fig. 12A), we speculated that the amount of ATP released during Ca^{2+} wave propagation might increase with age. We tested this by evoking Ca^{2+} waves with a mechanical stimulus in retinas from either 20 day old or 320 day old animals. We alternated trials between imaging glial Ca^{2+} with Fluo-4 (Fig. 12B) and imaging ATP release with luciferin/luciferase (Fig. 12C). Although the peak Ca^{2+} increase of evoked waves was unchanged from 20 to 320 days (Fig. 12D), the peak ATP concentration generated by evoked waves was significantly higher in older animals than in younger animals (Fig. 12E). In intercellular glial Ca^{2+} waves, ATP is released in a regenerative fashion (Newman, 2001). It is likely that the difference in ATP signal between older and younger animals in Fig. 12E represents an increase in regenerative ATP release, because the difference grows from 5 to 20 seconds after stimulation (Fig. 12E). Consistent with this greater ATP release in older animals, we also found that spontaneous waves reach a maximal diameter in older animals (137-158 days; n = 18 waves analyzed) that is 28 ± 4 percent larger than in young animals (40-63 days; n = 21 waves analyzed) (Fig. 12F).

Spontaneous waves cause blood vessels to constrict

Artificially evoked glial Ca^{2+} waves, as well as Ca^{2+} increases evoked in single glial cells by photolysis of caged compounds, can dilate and constrict blood vessels in the retina (Metea and Newman, 2006). We investigated whether spontaneously generated Ca^{2+} waves have a similar effect on retinal vessels. Blood vessels and glial Ca^{2+} were

monitored simultaneously in retinas from older animals (139-224 days) exhibiting a high frequency of Ca^{2+} wave generation. In three out of three trials (each from a different animal) where a spontaneous wave propagated from one side of an arteriole to the other, the vessel exhibited a constriction that was synchronized with the wave (Fig. 13). In each case, the Ca^{2+} wave started at least 20 µm from the vessel and at least two seconds before the constriction began. The constriction always began on the same video frame in which the Ca^{2+} wave crossed the vessel. The constrictions were 24%, 11%, and 9% of baseline diameter. These constrictions occurred over a small length of the vessel within the area of the spontaneous wave and lasted only as long as the glial Ca^{2+} elevation. Calcium waves which approached an arteriole but did not propagate past the vessel did not trigger vasoconstriction (n = 41 waves from 7 animals). Vasodilations were not observed, as expected since our Ringer's was bubbled with 95% oxygen, which favors vessel constriction over dilation (Gordon et al., 2008; Mishra et al., 2009).

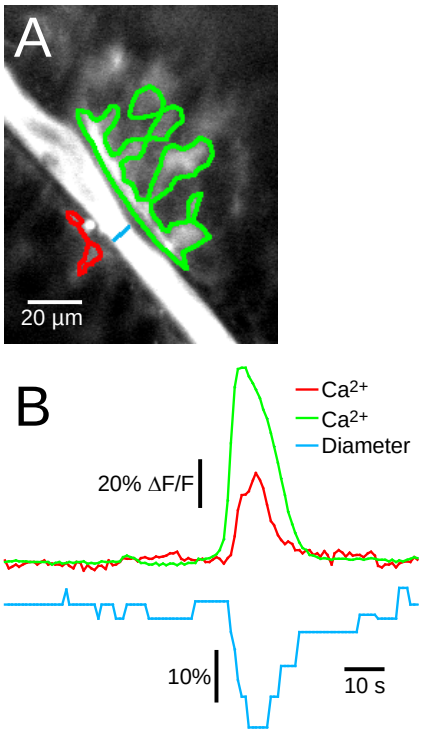

Fig. 13. Spontaneous Ca^{2+} waves cause constrictions in arterioles.

A, A confocal fluorescence image showing a spontaneous Ca^{2+} wave (ΔF/F ratio image). Glial cells are labeled with Fluo-4 and the arteriole filled with dextran-fluorescein to visualize the lumen. **B**, The arteriole constricts in synchrony with the spontaneous Ca^{2+} wave. The large Ca^{2+} rise in both red and green regions represents a spontaneous wave propagating across the vessel. The Ca^{2+} signals were measured in the two regions outlined in **A** and the vessel diameter measured at the blue line in **A**.

Spontaneous waves occur in vivo

Spontaneous Ca^{2+} waves could be an artifact of the isolated retina preparation and might not occur in vivo. We tested this by looking for spontaneous waves in retinas in vivo. We used an open-globe in vivo preparation where glial Ca^{2+} could be monitored in Müller cells and astrocytes with confocal microscopy. In the absence of stimulation, we observed waves of Ca^{2+} elevation in glia propagating outwards from a central point of initiation (Fig. 14A). Waves in vivo displayed more variability in size and rate of expansion than they did in the isolated retina. However, on average, the waves expanded at 22.1 ± 5.0 μm/s and achieved a maximal diameter of 67.7 ± 9.3 μm (n = 3 waves), very similar to the values measured in the isolated retina.

In rats 24-53 days old (n = 3), we observed an average of 0.6 spontaneous waves per minute per mm^2. In older rats, 84-270 days old (n = 4), we observed an average of 6.5 spontaneous waves per minute per mm^2. Thus, there is a trend toward an increase in the frequency of spontaneous Ca^{2+} wave generation with age in the in vivo retina (Fig. 14B), although this effect does not reach significance. Interpolating between these values, the rate of wave generation in 120 day old rats would be approximately 1.8 waves per minute per mm^2, similar to the baseline wave generation rate of 1.0 per minute per mm^2 measured in the isolated retina of 120 day old rats.

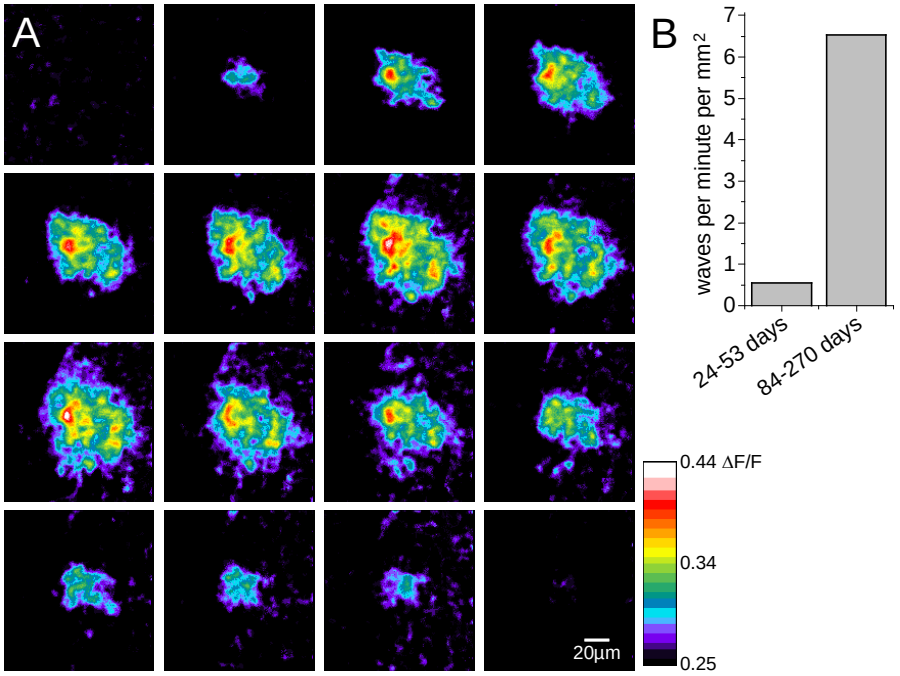

Fig. 14. Spontaneous Ca^{2+} waves occur in the retina in vivo.

A, A time series (left to right, top to bottom) of a single spontaneous Ca^{2+} wave in vivo, showing pseudocolor ΔF/F ratio images. Each frame represents one second. The focal plane is a few microns beneath surface of the retina, in the ganglion cell layer, where the brightest Müller cell Ca^{2+} signals are observed. **B**, Spontaneous waves in vivo occur more frequently in older animals than in younger animals. In each rat, the best-labeled areas of the retina were imaged for 25-110 minutes. Fluorescence signals were counted as waves only if 1) their shape was roughly circular, 2) they expanded concentrically over at least two frames, and 3) their peak diameter was between 30 and 100 µm.

DISCUSSION

Intercellular glial Ca^{2+} waves constitute a coordinated signaling mechanism between glial cells and can, potentially, modulate neuronal excitability and blood flow. Recently, spontaneous waves have been observed in vivo in the cerebellum (Hoogland et al., 2009) and the cortex (Kuchibhotla et al., 2009), demonstrating that glial Ca^{2+} waves are a physiologically relevant phenomenon and not just a product of artificial stimulation. We report here that spontaneous waves, propagated by the release of ATP, occur in both the isolated retina and the retina in vivo.

The spontaneous retinal Ca^{2+} waves resemble the spontaneous waves reported in vivo in the cerebellum and cortex (Hoogland et al., 2009; Kuchibhotla et al., 2009) in some respects but differ in others. Whereas the waves in cortex were seen only near amyloid plaques in Alzheimer's mice (and did not occur in wild type animals), waves in retina and cerebellum occurred in the absence of apparent pathology. The waves in cortex, although expanding at a rate similar to waves in retina, reached a maximum diameter of ~200 μm, similar to waves seen in culture, but much larger than the spontaneous waves observed in cerebellum and in the retina. The rate of spontaneous wave generation was not reported in cerebellum or cortex.

Like the artificially evoked Ca^{2+} waves reported in the isolated retina (Newman and Zahs, 1997) and in brain slices (Dani et al., 1992), spontaneous waves in the retina are mediated by release of ATP and activation of glial purinergic receptors. ATP imaging reveals spontaneous waves of ATP release matching the spontaneous Ca^{2+} waves (Fig. 10). Both suramin, a purinergic antagonist, and apyrase, an ATP-degrading enzyme, reduce the frequency of spontaneous waves in the retina. Neither gap junctional coupling

between glial cells nor neuronal activity play a significant role in the generation and propagation of spontaneous waves. Spontaneous Ca^{2+} waves observed in vivo in the cerebellum are also mediated by activation of purinergic receptors (Hoogland et al., 2009).

Spontaneous Ca^{2+} waves in the retina are initiated in and propagate through Müller cells and do not appear to involve astrocytes. Although evoked glial Ca^{2+} waves in the retina propagate through astrocytes if they are initiated in astrocytes, evoked waves that are initiated in Müller cells rarely propagate into astrocytes (Newman, 2001). The difference could be attributable to astrocytes having a higher threshold for Ca^{2+} activation by purinergic receptors than Müller cells.

Some retinas, especially from older animals, exhibited a high initial frequency of spontaneous waves that decayed to a stable baseline within 15 minutes. We speculate that the high initial wave rate may be a consequence of mechanical trauma suffered during dissection. However, it is likely that the waves that occur during the initial high frequency period are generated by the same mechanism as those observed during the stable period of low frequency wave generation as they have similar properties, including propagation velocity and size.

The frequency of spontaneous Ca^{2+} wave generation increases significantly with the age of the animal. In the isolated retina, the stable, basal rate of wave generation increases from 0.27 to 1.0 waves per minute per mm^2 as animals age from 20 to 120 days. A similar increase in the rate of spontaneous wave generation is seen in the retina in vivo. The increase could be related to pathology associated with aging. It is not due to damage produced by light exposure, however, as animals exposed to high ambient light for 1-4

months did not exhibit increased wave generation (Fig. 9D). ATP-evoked Ca^{2+} increases in retinal glial cells did not increase with age, suggesting that increased wave frequency is not due to an upregulation of P2 receptors or downstream signaling molecules in glial cells. However, the ATP released during evoked Ca^{2+} waves did increase with age, suggesting that an increase in glial ATP release could account for the age-related increase in spontaneous wave generation. In the isolated retina, individual Müller cells, but not astrocytes, frequently display transient, non-propagating Ca^{2+} increases (Newman, 2005). If spontaneous waves begin as single-cell Ca^{2+} transients, it is possible that greater ATP release in older animals in response to Ca^{2+} increases permits more of these transients to propagate as waves. This could account for the higher frequency of wave generation observed in older animals. Consistent with an age-dependent increase in glial ATP release, spontaneous Ca^{2+} waves reached a larger peak diameter in older animals (Fig. 12F).

Spontaneous glial Ca^{2+} waves could contribute to pathology in the CNS. Under ischemic conditions in the cortex in vivo, not only are spontaneous glial Ca^{2+} transients increased compared to healthy brain, but they are also spatially coordinated and contribute to neuronal death (Ding et al., 2009). In Alzheimer's mice, spontaneous Ca^{2+} waves occur near amyloid plaques (Kuchibhotla et al., 2009), suggesting that the generation of waves could be triggered by pathology.

Conversely, spontaneous Ca^{2+} waves could act to protect retinal cells. Activation of P2Y receptors on astrocytes boosts the resistance of both the astrocytes and nearby neurons to oxidative stress (Wu et al., 2007). Spontaneous waves could serve as a source of extracellular ATP to stimulate this protective effect, which would become increasingly

important in older animals as oxidative stress increases (Sohal and Weindruch, 1996; Kregel and Zhang, 2007).

Besides their possible role in pathology, spontaneous glial Ca^{2+} waves comprise a functional signaling pathway that may have important downstream effects on blood vessels and neurons. Artificially evoked Ca^{2+} waves regulate the diameter of retinal arterioles by the release of arachidonic acid metabolites (Mulligan and MacVicar, 2004; Metea and Newman, 2006). We demonstrate here that spontaneously generated Ca^{2+} waves can also regulate arteriole diameter, implying that spontaneous waves contribute to the regulation of blood flow. Arteriole constrictions were observed, as expected because the retina was superfused with Ringer's bubbled with 95% oxygen. When the oxygen concentration is lowered to a physiological level, it is likely that spontaneous Ca^{2+} waves would evoke vasodilations instead of vasoconstrictions (Gordon et al., 2008; Mishra et al., 2009).

Spontaneous waves may also influence neuronal activity. Evoked increases in glial Ca^{2+} lead to gliotransmitter release that can modulate neuronal excitability and synaptic transmission (Newman, 2004; Agulhon et al., 2008). ATP release from glia causes depression of synaptic transmission in cultured hippocampal neurons (Koizumi et al., 2003) and brain slices (Pascual et al., 2005). Spontaneous Ca^{2+} transients in individual glial cells cause NMDA receptor-mediated currents in neurons (Parri et al., 2001; Fellin et al., 2004; Angulo et al., 2004). In the retina, stimulated Ca^{2+} increases in glia cause neuronal hyperpolarization mediated by ATP release (Newman, 2003) and evoked waves can facilitate or depress light-evoked neuronal spiking (Newman and Zahs, 1997). In light of these functional consequences of glial Ca^{2+} increases, changes in wave

generation over the course of early adulthood could represent age-dependent changes in information processing in the retina.

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