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Dynamics of Retinal Waves Are Controlled by Cyclic AMP

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Summary

Waves of spontaneous activity sweep across the developing mammalian retina and influence the pattern of central connections made by ganglion cell axons. These waves are driven by synaptic input from amacrine cells. We show that cholinergic synaptic transmission during waves is not blocked by TTX, indicating that release from starburst amacrine cells is independent of sodium action potentials. The spatiotemporal properties of the waves are regulated by endogenous release of adenosine, which sets intracellular cAMP levels through activation of A2 receptors present on developing amacrine and ganglion cells. Increasing cAMP levels increase the size, speed, and frequency of the waves. Conversely, inhibiting adenylate cyclase or PKA prevents wave activity. Together, these results imply a novel mechanism in which levels of cAMP within an immature retinal circuit regulate the precise spatial and temporal patterns of spontaneous neural activity.

Introduction

Neural activity shapes developing circuits throughout the central nervous system, refining initial crudely targeted axonal projections to the precise connectivity seen in the adult (Goodman and Shatz, 1993; Katz and Shatz, 1996; Crair, 1999). Activity-dependent synaptic remodeling has been studied extensively in the vertebrate visual system (Constantine-Paton et al., 1990; Shatz, 1990, 1996; Cline, 1991; Katz and Shatz, 1996), where there is evidence that axonal refinement may be driven by the precise spatial and temporal pattern of retinal ganglion cell (RGC) activity (Stryker and Strickland, 1984; Schmidt and Buzzard, 1993; Weliky and Katz, 1997). In the visual system of binocular mammals, RGC projections to their thalamic target, the lateral geniculate nucleus (LGN), are segregated into eye-specific layers. Even before vision, the eye-specific layers have formed out of initially overlapping projections from the two eyes (Rakic, 1976; Linden et al., 1981; Shatz, 1983; Sretavan and Shatz, 1986), a process dependent upon spontaneous activity (Shatz and Stryker, 1988) of retinal origin (Penn et al., 1998).

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The retinal activity responsible for layer segregation takes the form of recurrent spontaneous bursts of action potentials (Galli and Maffei, 1988) that propagate across the ganglion cell layer of the developing retina in a wave-like manner (Meister et al., 1991; Wong et al., 1993). Waves have distinct spatiotemporal properties (Feller et al., 1996, 1997), with individual waves having a limited domain size and not propagating across the entire retina. Waves occur at any given retinal location approximately every 1–2 min. Domain boundaries are set in part by a refractory period, during which an area of retina that participated in a wave is incapable of supporting another wave for 40–50 s. While previous experiments indicated that activity is necessary for normal development, the role of the *pattern* of activity in the refinement to the precise adult architecture has yet to be determined (Crair, 1999).

The mechanisms of wave propagation and dynamics are only partially understood. Segregation of eye-specific layers occurs during the first 2 postnatal weeks in ferrets (Linden et al., 1981), well before eye opening (at postnatal day 30 [P30]) and the onset of vision. At this age, only amacrine and ganglion cells are fully differentiated (Greiner and Weidman, 1981), and both cell types participate in waves (Wong et al., 1995). Waves are known to be synaptically driven, with RGCs receiving both cholinergic and GABAergic inputs during waves (Feller et al., 1996). However, at the earliest ages studied, only cholinergic synaptic transmission is required for wave initiation and propagation (Feller et al., 1996; Penn et al., 1998), while GABAergic, glycinergic, and glutamatergic transmission can modulate wave generation at older ages (Fischer et al., 1998; Wong, 1999). The primary source of acetylcholine (ACh) in the mammalian retina is a class of bistratified amacrine cells, the starburst amacrine cells (Masland et al., 1984; Tauchi and Masland, 1984), which are also present in the ferret retina at birth (Feller et al., 1996). Although starburst cells are capable of firing action potentials in early development (Zhou and Fain, 1996), they are not thought to spike during spontaneously occurring retinal waves (Zhou, 1998), whereas the ganglion cells do (Meister et al., 1991; Wong et al., 1993). To test whether secretion from starburst and other amacrine cells during waves requires action potentials, we examined retinal activity in the presence of the sodium channel blocker tetrodotoxin (TTX), which is known to block the spontaneous bursts of action potentials recorded in ganglion cells during waves (Meister et al., 1991; Wong et al., 1995).

While ACh is essential for the retinal waves within the amacrine and ganglion cell network, surprisingly it does not appear to regulate the specific spatiotemporal patterns of activity in mammals (but see Sernagor and Grzywacz, 1999). Rather, blocking cholinergic receptors completely eliminates all the activity. Starburst amacrine cells are also known to release GABA (Vaney and Young, 1988; O'Malley et al., 1992). In older animals, GABA partially mediates the developmental change in firing patterns that distinguish ON from OFF ganglion cells (Wong and Oakley, 1996; Fischer et al., 1998). GABA,

traditionally an inhibitory transmitter, can act as an excitatory transmitter early in retinal development (Yamashita and Fukuda, 1993; Bahring et al., 1994; Huang and Redburn, 1996; Fischer et al., 1998), as well as in other parts of the central nervous system (reviewed by Cherubini et al., 1991), and could therefore facilitate the propagation of waves.

The population of starburst amacrines displaced to the ganglion cell layer contains adenosine in addition to GABA and ACh in the adult (Blazynski, 1989). Adenosine is also found in ganglion cells and other amacrine cells in the adult retina (Braas et al., 1987), and A1 and A2 adenosine receptors are found throughout the adult retina (Blazynski, 1990). Adenosine can regulate rhythmic network behavior in a mature neural circuit (Dale and Gilday, 1996), and adenosine acting through A2 receptors has been shown to increase release of ACh (reviewed by Sebastiao and Ribeiro, 1996). Thus, adenosine is also a good candidate for contributing to or modulating the dynamics of retinal waves.

Here, we examine the effects of various agents on the spatiotemporal properties of retinal waves during the first 2 postnatal weeks of development in ferrets, when it is known that retinal waves are required for ganglion cell axons to segregate into eye-specific layers within the LGN (Penn et al., 1998). We show adenosine profoundly regulates the spatiotemporal properties of waves. Endogenous adenosine transmission is required for normal frequency, velocity, and area of waves, and adenosine agonists can increase these wave parameters dramatically. By immunohistochemistry, we demonstrate that A2 adenosine receptors are localized to a subset of cells within the inner retina at the relevant postnatal ages. The spatiotemporal effects of changes in adenosine transmission can be fully explained by changes in levels of cAMP, which regulate protein kinase A (PKA) activity and thereby increase the activity in the amacrine cell network. Together, these results demonstrate that an early purinergic network, independent of sodium action potentials, governs the specific spatiotemporal patterns of neural activity present in the developing mammalian retina.

Results

Retinas were isolated from newborn ferrets (P0–P10) during the period when spontaneous waves are present (Wong et al., 1993; Feller et al., 1996) and required for the formation of eye-specific layers within the LGN (Penn et al., 1998). The frequency of wave occurrence was monitored using fluorescence imaging of calcium indicators. As cells become depolarized, they experience an increase in intracellular concentration of calcium that can be used as a marker of cell activity. The calcium increase leads to a decrease in Fura 2 fluorescence when illuminated with 380 nm excitation light. Fluorescence changes were averaged over a small region of retina, typically 3200 μm^2, and $\sim$20 cells. Monitoring these fluorescence changes over a large fraction of the retinal surface area allows us to investigate the overall spatiotemporal properties of retinal waves (Feller et al., 1996).

Retinal Waves Persist in TTX

Waves are characterized by spontaneous bursts of action potentials recorded from RGCs, concomitant with increases in intracellular calcium concentrations in both ganglion and amacrine cells (Wong et al., 1995; Penn et al., 1998). To determine whether these bursts of action potentials are required for wave propagation, the sodium channel blocker TTX was bath applied to the retina, while a large population of both amacrine and ganglion cells were monitored using calcium imaging. TTX (2 μM) does not eliminate the increases in intracellular calcium associated with waves ($n = 5$ retinas; Figure 1A), although both the frequency of occurrence and the magnitude of the fluorescence change are diminished (Figure 1B). However, the loss of sodium action potentials in RGCs would be expected to significantly reduce the calcium influx through voltage-gated calcium channels and therefore lower the magnitude of the fluorescence change. This could result in some waves occurring below our threshold of detection and would exaggerate any true alteration in wave frequency.

To detect waves independently of intracellular calcium changes, we recorded from RGCs by whole-cell voltage clamp before and during bath application of TTX. Previous experiments have demonstrated that compound postsynaptic currents (PSCs) occur simultaneously with the changes in fluorescence associated with propagating waves and reflect synaptic input from amacrine cells (Feller et al., 1996). The cells (11 cells from 11 retinas) were all ganglion cells, as classified by large soma size and in some cases the existence of an axon when filled with Lucifer yellow during the recording ($n = 2$). Compound PSCs persist during application of TTX (Figure 1C) and do not vary significantly in amplitude ($105\% \pm 8\%$ of control amplitude, $p > 0.4$, Student's t test) but occur slightly less frequently than normal ($79\% \pm 6\%$ of control frequency, $p < 0.01$; Figure 1D). The TTX was sufficient to block sodium action potentials, as all cells lost their inward sodium currents evoked by voltage steps (data not shown), and spikes could not be induced by depolarizing the cells in current clamp ($n = 4$). Monitoring RGC membrane potentials showed little change in the slow depolarizations associated with waves, though the action potentials were blocked (Figure 1C). The waves that persist in TTX require cholinergic transmission, since the cholinergic agonist epibatidine blocks the waves (Penn et al., 1998), as measured by either calcium imaging or whole-cell voltage clamp ($n = 2$ retinas [imaging] and 2 cells in 2 retinas [physiology]; data not shown). These results imply that waves can occur in the absence of RGC action potentials and that transmitter release from cholinergic amacrine cells during waves is independent of activation of voltage-gated sodium channels.

$GABA_A$ Receptors Do Not Contribute to Wave Dynamics

GABA is the primary transmitter of the majority of amacrine cells and has been shown recently to depolarize RGCs in the neonatal ferret retina (Fischer et al., 1998), implying that GABA can act as an excitatory transmitter. Consistent with this finding, we also observed that focal application of 1 mM GABA onto a P2 retina caused an

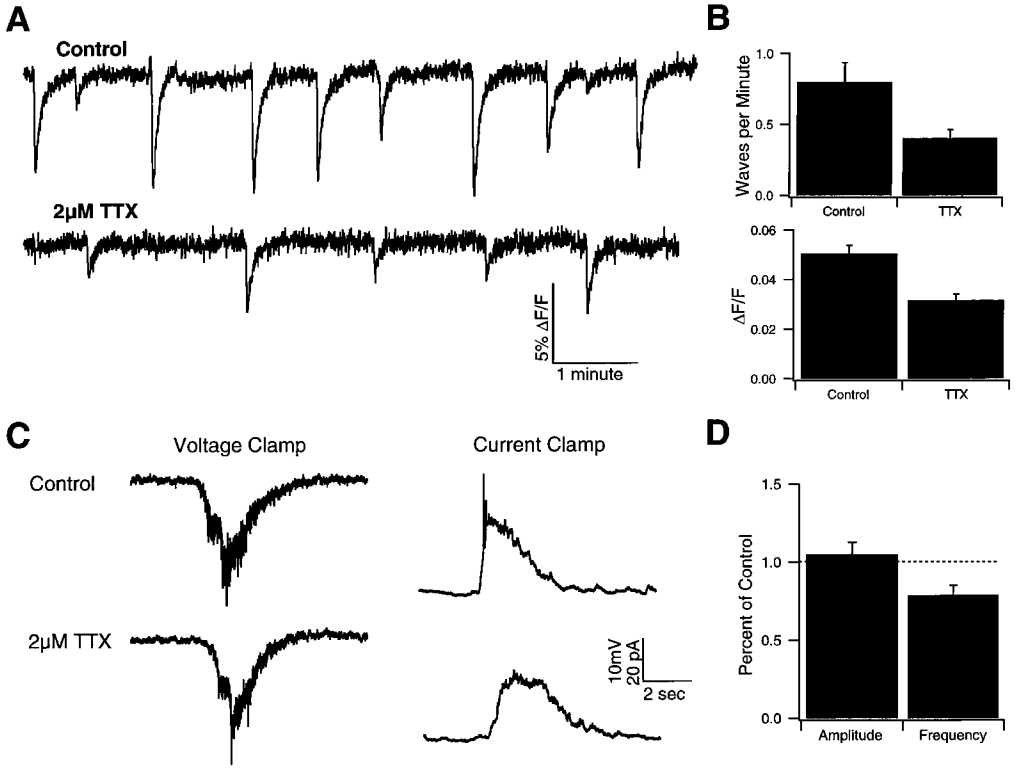

Figure 1. Retinal Waves Persist in TTX

(A) Fractional change in Fura 2 fluorescence averaged over a small region (3200 μm^2) of a P7 retina, in both control solution (top trace) and in 2 μM TTX (bottom trace). Downward deflections indicate increases in intracellular calcium, induced by propagating retinal waves.

(B) Pooled data from five retinas, showing the frequency of occurrence (top histogram) and the amplitude of fluorescence changes (bottom histogram) of waves in the presence of 2 μM TTX. (In this and all subsequent histograms, data is pooled from P0–P10 retinas.)

(C) Traces of whole-cell voltage clamp or current clamp recordings from a presumptive ganglion cell in a P10 retina, in control solution and in 2 μM TTX. Note that in TTX, there are no action potentials seen by current clamp.

(D) Pooled data from 11 retinas, showing integrated PSC amplitude and frequency of retinal waves measured with whole-cell voltage clamp in 2 μM TTX, as a percentage of values in control solution.

immediate decrease in Fura 2 fluorescence in the region surrounding the pipette (Figure 2A), indicating an increase in intracellular calcium, consistent with the idea that the cells are being depolarized and are opening voltage-sensitive calcium channels. Since GABA is depolarizing, it may contribute to the propagation of waves.

To examine whether activation of $GABA_A$ receptors modulates spatiotemporal properties of retinal waves in early neonates (P0–P10), we applied $GABA_A$ antagonists to the retina and monitored the wave parameters. RGCs are known to receive GABAergic inputs during waves (Feller et al., 1996). However, blocking $GABA_A$ receptors with 50 μM SR95531 ($n = 6$ retinas) did not alter wave propagation (Figure 2B; Fischer et al., 1998), even though 50 μM SR95531 was previously shown to be sufficient to block all GABAergic synaptic currents recorded from ganglion cells (Feller et al., 1996). Blockade of chloride channels with 100 μM picrotoxin (PTX) also does not modulate wave parameters ($n = 6$), indicating that $GABA_C$ receptors as well as $GABA_A$ receptors do not influence waves (Lukasiewicz and Shields, 1998). A detailed quantitative analysis of retinal activity showed little change in frequency of waves (0.63 $\pm$ 0.07 waves/min versus 0.59 $\pm$ 0.07 waves/min in SR95531 and 0.51 $\pm$ 0.04 waves/min in PTX), velocity of waves (165 $\pm$ 96 μm/s versus 156 $\pm$ 55 μm/s in SR95531), or amplitude of the fluorescence change associated with each wave (2.33% $\pm$ 0.52% $\Delta F/F$ versus 1.97% $\pm$ 0.35% $\Delta F/F$ in SR95531 and 2.10% $\pm$ 0.56% $\Delta F/F$ in PTX; Figure 2C). All of these conditions were not significantly different from each other. Wave areas also were unchanged in the presence of SR95531 (0.026 $\pm$ 0.022 mm^2 versus 0.026 $\pm$ 0.026 mm^2; Figure 2D). These observations confirm and extend previous conclusions that, although GABA contributes to the PSCs evoked in RGCs during waves, only ACh has been shown to be required for wave initiation and propagation at these particular ages. Moreover, GABA acting through $GABA_A$ receptors does not play a role in modulating retinal waves; it remains possible that $GABA_B$ receptors could modulate wave dynamics.

Adenosine Controls Parameters of Wave Dynamics

Since the classical neurotransmitters secreted by starburst cells are not involved in setting the spatiotemporal properties of the retinal waves, we next examined the neuromodulator adenosine, which is known to be colocalized with GABA and ACh in adult starburst amacrine cells (Blazynski, 1989). Bath application of 5′-N-ethylcarboxamidoadenosine (NECA), an adenosine agonist,

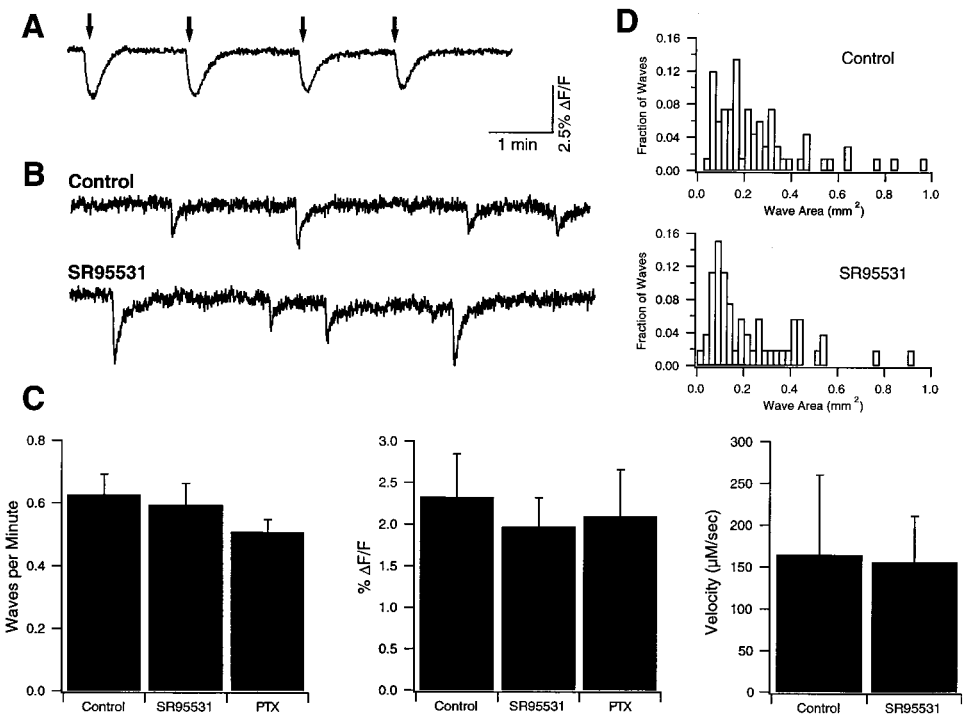

Figure 2. The Spatiotemporal Properties of Waves Are Independent of $GABA_A$ Receptor Activation

(A) Fractional change in Fura 2 fluorescence averaged over a small region of a P2 retina. Arrows indicate 50 ms focal applications of 1 mM GABA to the retina, delivered through a large-bore pipette.

(B) Representative fluorescence traces from a P10 retina exhibiting waves in normal ACSF (top trace) or 50 μM SR95531 (bottom trace).

(C) Group data of wave frequency, amplitude of calcium influx, and wave velocity in 50 μM SR95531 (n = 6 retinas) and 100 μM PTX (n = 6), compared to these measures in control ACSF. Velocity was calculated from 13 control waves and 19 waves in SR95531.

(D) Distribution of wave areas from two retinas during long recordings (60 min) in control ACSF (67 waves; top histogram) or 50 μM SR95531 (62 waves; bottom histogram).

led to a marked increase in wave frequency (Figure 3A). Waves, which normally occur at a rate of 0.84 ± 0.13 waves/min at any given location on the retina, occur at a rate of 2.52 ± 0.12 waves/min in NECA (n = 4 retinas).

To test whether endogenous adenosine release normally sets the frequency of waves, retinas were incubated in adenosine deaminase, an enzyme that degrades extracellular adenosine (Paes de Carvalho and de Mello, 1982). This manipulation produced a significant reduction in wave frequency (0.29 ± 0.05 waves/min versus 0.63 ± 0.06 waves/min for preincubation values, n = 5 retinas, $p \leq 0.001$; Figure 3B). Higher levels of enzyme reduced wave frequency to a barely detectable level (0.06 ± 0.02 waves/min, n = 4 retinas). Waves recover to a normal frequency of occurrence within 30 min of washing out the enzyme (0.52 ± 0.10 waves/min, $p > 0.1$; Figure 3B).

Adenosine acts through a number of classes of G protein–coupled receptors, the A1 and A3 receptors, which inhibit adenylate cyclase, and the A2 receptors, which stimulate the cyclase (van Calker et al., 1979). A number of neuromodulators act through similarly coupled receptors, such as dopamine, which has been extensively studied in the retina. Dopamine is known to mediate a number of functions including modulation of transmission through gap junctions (Baldridge et al., 1998), which are likely present at ages when waves occur (Penn et al., 1994). As with adenosine agonists, exogenously applied dopamine (100–400 μM) also increases wave frequency (from 0.78 ± 0.10 waves/min in control conditions to 5.06 ± 1.14 waves/min in dopamine, n = 5; Figure 4A). Higher doses of dopamine actually prevent all wave activity (1 mM dopamine, n = 3; data not shown). However, dopamine does not appear to be necessary in vivo for waves to have normal spatiotemporal properties. Mice deficient in dopamine (Zhou and Palmiter, 1995) have waves that occur at a frequency similar to their wild-type or heterozygous littermates (n = 23 controls, 0.51 ± 0.03 waves/min; n = 2 knockouts, 0.52 ± 0.02 waves/min; Figure 4B). Further, D1 receptor knockout mice have normal wave frequency (0.51 ± 0.07 waves/min, n = 7), and the D1 dopaminergic antagonist SCH-23390 (100 μM) has no effect on the frequency of waves in ferret retinas (n = 3; data not shown). The conclusion that dopamine does not contribute to retinal waves in vivo is consistent with observations that dopaminergic amacrine cells, as assayed immunohistochemically for tyrosine hydroxylase (TH), are not detectable at comparable ages in other mammals: TH staining is first seen at P6 in mouse retinas (Wulle and Schnitzer, 1989) and E59 in cat retinas (Mitrofanis et al., 1989), while waves are present at P0 in mice and as early as

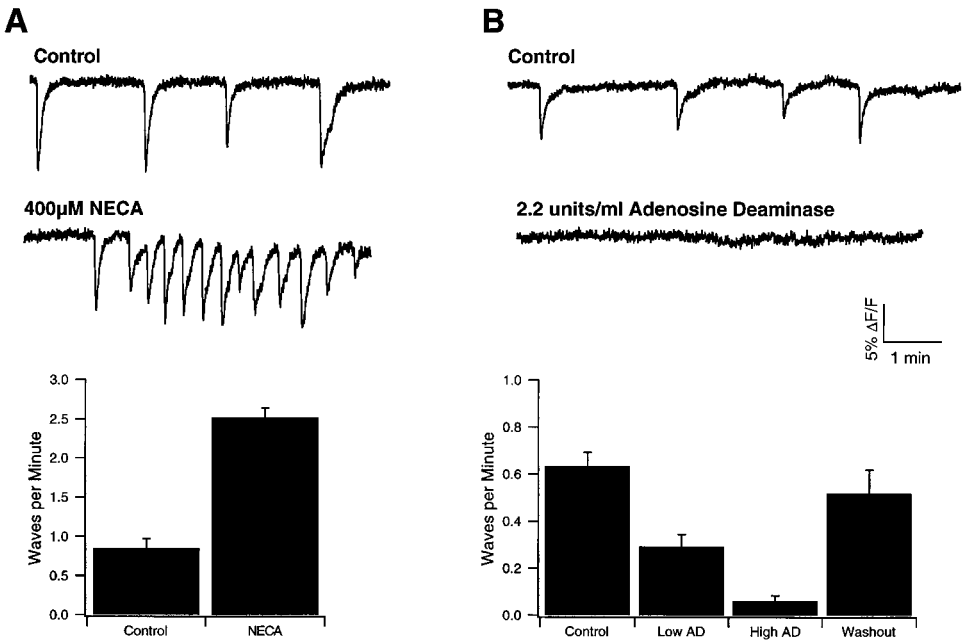

Figure 3. Adenosine Regulates Wave Frequency Endogenously

(A) Fluorescence traces from a P10 retina in control ACSF (top trace) and in 400 μM of the adenosine agonist NECA (bottom). Group data histograms show average frequency increase for four retinas.

(B) Fluorescence traces show retinal activity before (top) and after (bottom) a 15 min incubation in 2.2 U/ml of adenosine deaminase, to degrade extracellular adenosine. Group data histograms show average wave activity before (control) and immediately after incubation in low levels (0.5–1.25 U/ml, n = 5 retinas) or high levels (1.25–2.25 U/ml, n = 4 retinas) of adenosine deaminase, and after 30 min of washing out the drug.

E43 in cats (D. S. and C. J. S., unpublished data). These observations indicate that adenosine, but not dopamine, modulates wave parameters in vivo.

Adenosine Acts by Increasing cAMP through A2 Receptors

To determine if adenosine acts by increasing cAMP, through A2 receptors, or by decreasing cAMP, through A1 or A3 receptors, the levels of cAMP were manipulated with pharmacological agents. Bath application of forskolin, an activator of adenylate cyclase, increases wave activity in manner similar to adenosine agonists (0.69 ± 0.05 waves/min in control versus 3.20 ± 0.44 waves/min in 10 μM forskolin, n = 4; Figure 5A). This increase is dose dependent, indicating that wave frequency is tightly correlated with the level of cAMP (Figure 5B). Wave activity was blocked as usual by 100 μM curare, indicating that the waves are still propagated by cholinergic transmission (n = 2; data not shown). Conversely, cAMP levels can be decreased by blocking its production. Incubating retinas with the adenylate cyclase inhibitor SQ 22,536 reduces the frequency of waves to a barely detectable level (0.15 ± 0.07 waves/min, $p < 0.001$; Figure 5C).

The signaling cascade engaged by cAMP is known to involve protein kinase A. Thus, we examined whether inhibition of PKA with the specific inhibitor Rp-cAMPS could also decrease wave frequency in a manner similar to the effect of blocking cAMP production itself. Not only does incubating retinas in Rp-cAMPS significantly diminish wave frequency (0.21 ± 0.04 waves/min, $p < 0.002$; Figure 5D), but it entirely prevents the adenosine agonist NECA from increasing wave frequency (Figure 5D). The frequency of waves in retinas receiving a bath application of NECA after incubation in Rp-cAMPS (0.17 ± 0.11 waves/min, n = 5) was almost identical to that of retinas not treated with NECA (0.21 ± 0.04 waves/min). Thus, adenosine must act upstream of a pathway involving both cAMP and PKA. These observations are consistent with the finding that adenosine application to rabbit eye cup preparations (Blazynski et al., 1986) and to E17 chick retina (Paes de Carvalho and de Mello, 1982) increases levels of cAMP. Taken together, these results strongly suggest that adenosine modulates retinal wave dynamics by acting via the A2 receptors.

Are the appropriate adenosine receptors found in the developing retina? Both A2a and A2b receptor subtypes have been observed in the adult bovine retina (Blazynski and McIntosh, 1993), and A2 receptors are present in the inner plexiform layer (IPL) of the adult (Blazynski, 1990). Immunocytochemistry shows specific staining for A2a and A2b adenosine receptors in the developing ferret retina. Cross-sections of a P9 retina reveal an identical pattern of immunostaining for both receptor subtypes, with strong reactivity in a majority of cells in the ganglion cell layer, and weaker staining in a subpopulation of cells in the amacrine cell layer (Figure 6). Coexpression of both types of receptors by single retinal neurons has been reported previously in the adult (McIntosh and Blazynski, 1994). These results demonstrate that A2 receptors are indeed present in the inner retina at early stages of development, though we have not

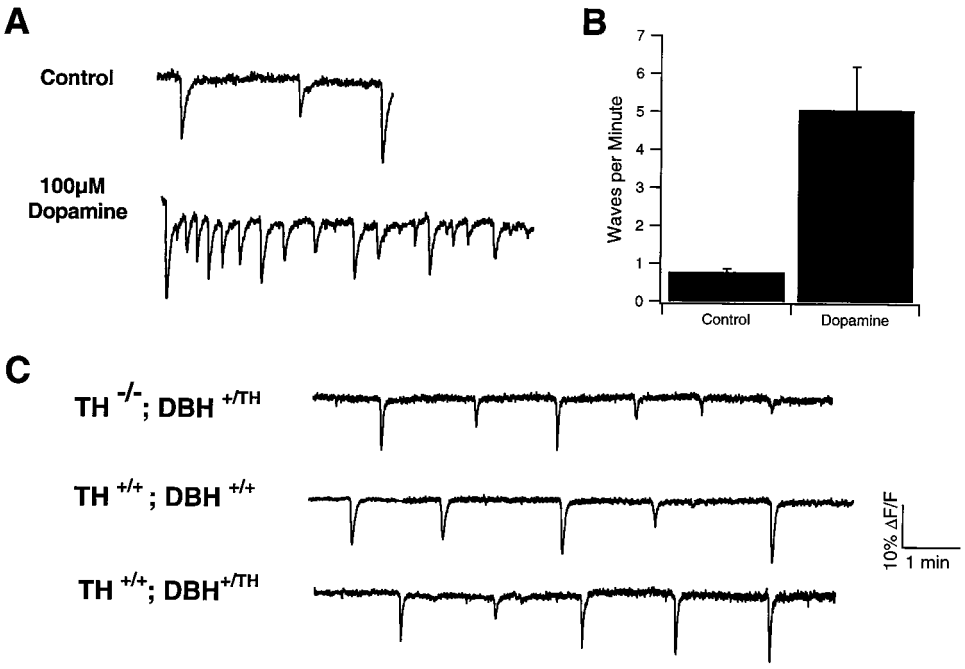

Figure 4. Waves Are Independent of Endogenous Dopamine Release

(A) Exogenously applied dopamine can increase wave frequency. Fluorescence traces show a P7 retina before (control; top trace) and during (bottom trace) bath application of 100 μM dopamine.

(B) Group data from five ferret retinas.

(C) Fluorescence traces from littermate mouse pups (P1) that are either dopamine deficient ($TH^{-/-}$; $DBH^{+/TH}$), wild type ($TH^{+/+}$; $DBH^{+/+}$), or wild type also carrying the knockin of tyrosine hydroxylase under the dopamine β-hydroxylase promoter ($TH^{+/+}$; $DBH^{+/TH}$).

determined whether the staining in the ganglion cell layer is on displaced amacrine cells, ganglion cells, or both. The presence of A2 receptors on the same neurons known to participate in retinal waves confirms the ability of adenosine transmission to set wave parameters in vivo.

cAMP Regulates Spatiotemporal Properties of Waves

How can a cAMP/PKA signaling pathway alter large-scale network activity in the developing retina? Detailed analysis of all waves occurring on retinas during long recording periods (25 control waves and 53 waves in 100 μM forskolin, n = 2 retinas) shows that forskolin not only causes an increase in wave frequency but also in wave area (Figure 7; control and forskolin-altered waves can be viewed in a movie available at http://www.neuron.org/cgi/content/full/24/3/673/DC1). The average area increased to 1.59 ± 0.10 mm^2 from the pre-forskolin value of 0.30 ± 0.06 mm^2. Velocities were then calculated from nine representative waves in each condition, and significant increases in forskolin were observed (659 ± 99 μm/s from 217 ± 26 μm/s in control). The increase in wave areas shown in Figure 7B, in fact, are underrepresented, as the forskolin waves often filled the viewing area (~2 mm^2) and propagated beyond the region of retina that could be monitored. The increase in wave area and velocity were not accompanied by significant increase in fluorescence change during a wave, with forskolin waves having 106.7% ± 9.1% of the fluorescence change seen in control waves (n = 12 control waves and 30 forskolin waves from 3 retinas; data not shown). Nor was there significant increase in the size of the PSCs recorded from RGCs during waves (111% ± 17% of control values, n = 9 cells from 9 retinas; data not shown). Similar increases in wave area and velocity are seen if the adenosine agonist NECA is applied instead of forskolin, with areas increasing to 1.57 ± 0.14 mm^2 (n = 18 waves) and velocity to 566 ± 130 μm/s (n = 6 waves). Wave areas also increased during application of 100 μM dopamine (1.04 ± 0.10 mm^2, n = 48 waves; data not shown), as might be expected since dopamine is known to elevate cAMP. Conversely, when cAMP levels are reduced by incubation in SQ 22,536, the few waves that do occur have smaller areas and slower velocities than normal (Figure 7). Wave areas (30 waves in SQ 22,536 and 23 waves after washout, n = 2 retinas) were reduced to 0.07 ± 0.01 mm^2 and shifted toward normal values after washout of the drug (0.21 ± 0.22 mm^2). Waves were also markedly slower (58 ± 12 μm/s, n = 9 waves) and sped up after washout (105 ± 12 μm/s, n = 9 waves). These results indicate that the spatiotemporal properties of the waves are correlated with the level of cAMP.

cAMP could control wave properties through a number of mechanisms. Modeling studies suggest that altering network excitability can affect wave propagation properties (Butts et al., 1999). One possibility is that adenosine-mediated increases in cAMP lead to an increase in neuronal sensitivity to released transmitter, by either insertion of new receptors in the membrane or by increasing the sensitivity of receptors already present, as has been seen in other systems (Gurantz et al., 1994).

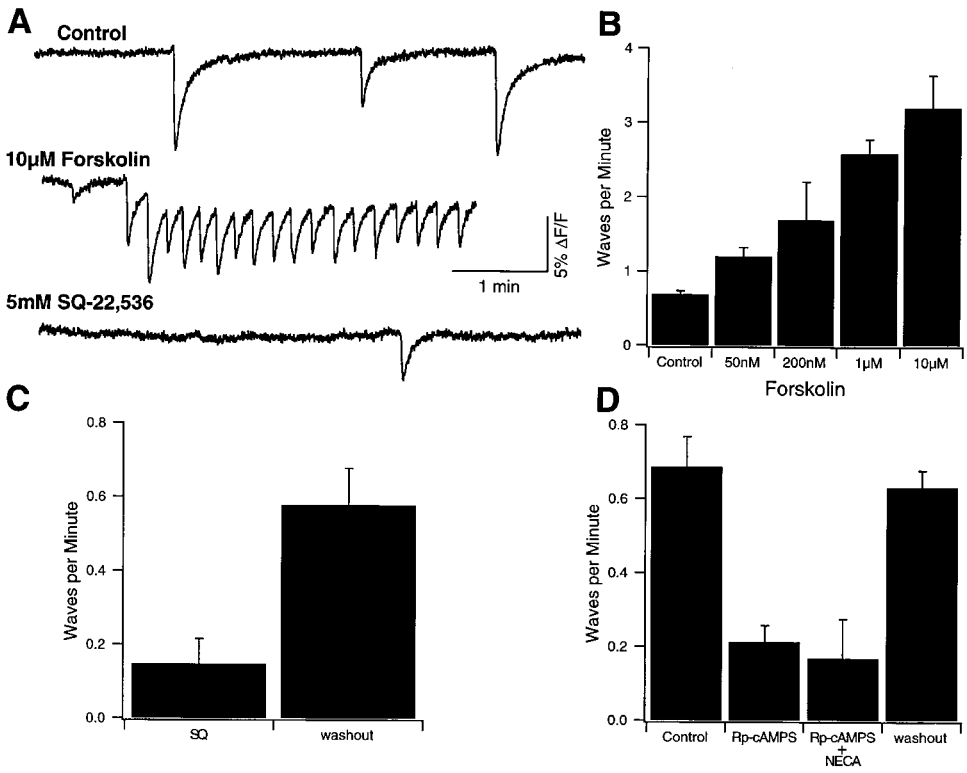

Figure 5. Changes in cAMP Can Alter Wave Dynamics

(A) Fluorescence traces from a P7 retina in control ACSF (top trace) and during treatment with 10 μM forskolin (middle trace), or after incubation in SQ 22,536 (bottom trace).

(B) Group data from all forskolin experiments (n = 3 retinas per forskolin dose; control is average of all 12 retinas in ACSF prior to forskolin treatment).

(C) Wave frequencies from retinas after incubation for at least 1 hr in the adenylate cyclase inhibitor SQ 22,536 (n = 7 retinas).

(D) Wave frequency measured from retinas before (control) and immediately following a 15 min incubation in the specific PKA inhibitor Rp-cAMPS (n = 10 retinas), or during bath application of the adenosine agonist NECA following incubation in Rp-cAMPS (n = 5 retinas). Recovery of wave activity was assessed after 30 min of washing out the drug.

We tested for alterations in transmitter sensitivity by measuring whole-cell currents in ganglion cells in response to focal applications of ACh. In the presence of 10 μM forskolin, ganglion cells showed no change in responsiveness to brief applications of 1 mM ACh, either in amplitude or decay of the induced current. The ACh response of forskolin-treated cells peaked at 94% ± 9% of the response of the same cells in normal artificial cerebrospinal fluid (ACSF) (n = 6 cells from 6 retinas). Thus, a change in postsynaptic AChR sensitivity is not likely to account for the observed effects of forskolin on wave dynamics.

Another possible explanation is that forskolin may elevate the resting membrane potential or otherwise increase membrane excitability, thereby lowering the threshold for a cell in the network to be recruited into a wave. We did observe a small tonic 3.7 ± 0.4 mV depolarization of RGCs induced by forskolin (n = 4 cells from 4 retinas) and a similar depolarization in the presence of dopamine (7 mV, n = 3). Nevertheless, the effects of forskolin, NECA, or dopamine on wave dynamics could not be reproduced simply by tonically depolarizing the cells. Increasing extracellular potassium (to 5 mM from the 2.5 mM in normal ACSF) to induce a similar change in resting membrane potential only induces a small increase in wave velocity (Feller et al., 1997) but does not significantly alter wave frequency (0.42 ± 0.11 waves/min versus 0.45 ± 0.13 waves/min in ACSF, n = 3 retinas) or the distribution of wave areas (0.176 ± 0.190 mm^2 versus 0.140 ± 0.136 mm^2 in ACSF, n = 258 waves [high K^+] and 328 waves [ACSF]). Therefore, it is unlikely that an increase in excitability can account for the effects of forskolin and other cAMP-dependent manipulations on wave dynamics. A remaining possibility is that cAMP increases the probability of transmitter release. Unfortunately, we cannot directly measure the probability of release in our preparation since TTX does not block the compound PSCs associated with waves and, therefore, miniature spontaneous synaptic events cannot be isolated. We consider this possibility more fully in the Discussion.

Discussion

The major finding of this study is that spatiotemporal properties of the spontaneous waves are determined by a cAMP–PKA cascade, which is regulated by endogenous adenosine within the developing mammalian retina. Increasing cAMP using a variety of pharmacological

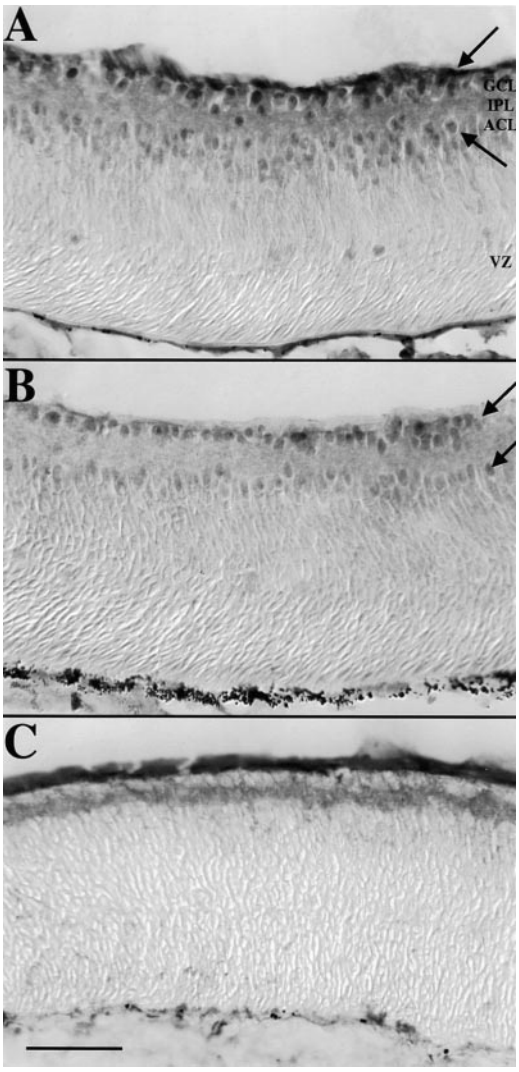

Figure 6. A2 Adenosine Receptor–like Immunoreactivity Is Found in the Developing Retina

Transverse sections through a P9 ferret retina immunostained with antibodies for the A2a subtype (A) or the A2b subtype (B), or with no primary antibody (C). Specific immunostaining is present on cell bodies located in both the amacrine cell layer (ACL) and ganglion cell layer (GCL) but not the ventricular zone (VZ) or other regions of the retina. Nonspecific staining (C) is present in the inner plexiform layer (IPL). The scale bar represents 50 μm for all micrographs.

manipulations generates larger, faster, and more frequent waves, while lowering levels of cAMP, either through direct inhibition or by using an enzyme that degrades extracellular adenosine, dramatically reduces wave activity. The changes in wave dynamics elicited by adenosine can be prevented by inhibiting PKA, implying the adenosine is acting through a cAMP–PKA pathway. In addition, we have shown that retinal waves can propagate via excitatory synaptic cholinergic transmission even in the presence of TTX. This observation has two implications. First, release of transmitter from amacrine cells during waves is independent of sodium action potentials. Second, action potentials in ganglion cells are not necessary for the initiation or propagation of waves. These results indicate that the dynamic properties of this developing synaptic network are regulated by a purinergic receptor-activated second-messenger cascade that is independent of sodium action potentials.

Release of Transmitter during Retinal Waves Is Independent of Action Potentials

Whole-cell voltage clamp recordings from RGCs during waves in the presence of TTX show no significant change in the amplitude of postsynaptic currents. Moreover, the currents that persist in TTX can still be blocked by blocking cholinergic synaptic transmission. These observations imply that starburst amacrine cells, the primary source of ACh in both the adult retina (Tauchi and Masland, 1984) and in development (Feller et al., 1996), can secrete transmitter in a subthreshold or graded response to membrane depolarizations. This conclusion is consistent with recent recordings from starburst cells demonstrating that, although they are capable of generating action potentials with sufficient depolarization, they do not spike during waves (Zhou, 1998). We showed previously that bath application of cadmium, a blocker of voltage-activated calcium channels, does block synaptic transmission during waves (Feller et al., 1996). Together, these findings indicate that release of ACh from starburst amacrines during waves is calcium dependent, though not action potential dependent. Calcium-dependent, graded release has been demonstrated for many cell types within the retina (reviewed by Dowling, 1987), though different classes of amacrine cells have different release properties (Borges et al., 1995; Bieda and Copenhagen, 1999). There is also growing evidence that calcium-induced calcium release can lead to action potential–independent forms of transmitter release via activation of intracellular calcium stores located near the plasma membrane (reviewed by Berridge, 1998), though no such mechanism has been reported as yet for retinal neurons. Blocking sodium channels, while not preventing waves, does lead to a small decrease in the frequency of compound PSCs. Though the cellular mechanisms underlying wave periodicity remain unknown, depolarization via sodium influxes may contribute slightly to the activity propagating through the network or modulate the time course of the refractory period (see below).

TTX also leads to a substantial reduction in the amplitude of periodic increases of intracellular calcium as measured by the average Fura 2 fluorescence change of a large number of amacrine and ganglion cells in the ganglion cell layer (see Experimental Procedures). Previous experiments demonstrated that the bursts of action potentials measured by extracellular multielectrode recordings of spontaneous ganglion cell activity can be blocked by TTX (Meister et al., 1991), suggesting that calcium influx through voltage-activated calcium channels is a major source of the spontaneous increases in calcium seen in normal conditions. This hypothesis is consistent with the observation that the falling phase of Fura 2 fluorescence traces (corresponding to an increase in intracellular calcium concentration) is simultaneous with the firing of action potentials in ganglion

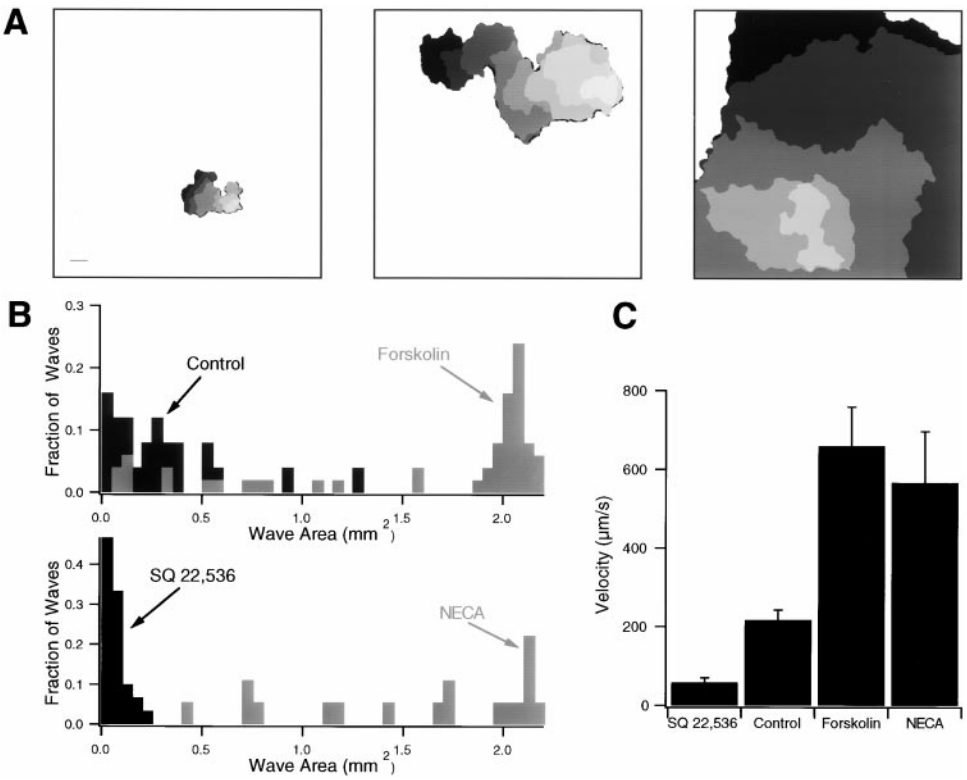

Figure 7. Spatiotemporal Properties of Waves Are Altered by Changing Levels of cAMP

(A) Examples of waves propagating in SQ 22,536 (left), ACSF (middle), or 100 μM forskolin (right). Each shade of gray represents the wave area for time steps of 0.5 s, with the wave propagating from light to dark grays. Scale bar represents 100 μm, and the border denotes the limits of the microscope viewing area.

(B) Distribution of wave areas before and following bath application of 100 μM forskolin (n = 25 control waves and 50 forskolin waves from 2 retinas; top histogram). Wave area distributions were also calculated for retinas following incubation in SQ 22,536 (n = 30 waves from 2 retinas; bottom histogram) and retinas treated with 400 μM NECA (n = 18 waves).

(C) Wave velocities computed from waves in each of the conditions (n = 9 waves each in control, forskolin, and SQ 22,536 and 6 waves in NECA).

cells (Penn et al., 1998). It should be noted that it was previously reported that calcium changes of individual retinal neurons as monitored by calcium imaging were not detected in the presence of TTX (Wong et al., 1995). However, these earlier imaging experiments may not have been sensitive enough to detect residual calcium changes occurring in amacrine and ganglion cells via voltage-activated calcium channels (Schmid and Guenther, 1999) or influx through neuronal nicotinic receptors (Rathouz et al., 1996). While it is clear that ganglion cell action potentials are not required to sustain retinal waves, they are of course necessary for the relay of retinal activity to the LGN and the consequent synaptic remodeling at the retinogeniculate synapse (Penn et al., 1998).

Implications for Mechanisms Underlying Spatiotemporal Properties of Waves

Adenosine, acting through cAMP and PKA, regulates the spatiotemporal properties of the waves. All known adenosine receptors either up- or downregulate cAMP production (van Calker et al., 1979), and increasing cAMP levels mimics the effect of adenosine agonists on all spatiotemporal properties of the waves (frequency, area, and velocity). Further, adenosine agonists cannot increase wave activity when the cAMP signaling cascade is blocked by inhibiting PKA. Finally, endogenous adenosine is known to increase cAMP levels in embryonic chick retina (de Mello et al., 1982; Paes de Carvalho and de Mello, 1982). These experiments do not address whether adenosine is released tonically, and therefore cAMP levels are relatively stable, or if adenosine is released during each wave event, which could result in oscillating levels of cAMP across the retina. In addition to adenosine, a number of other neuromodulators might influence cAMP production in vivo. Here, however, we have presented evidence that excludes endogenous dopamine from playing a role, at least at the early ages under study.

A wide variety of cellular mechanisms could be modulated by adenosine via a cAMP-mediated cascade. Note that these agents do not change the amplitude of periodic increases in intracellular calcium concentration or periodic compound PSC recorded from ganglion cells, indicating that the cellular processes responsible for governing the spatiotemporal properties are independent of the amount of excitatory synaptic input delivered to the ganglion cells. In the experiments presented here,

we detected no change in AChR sensitivity in the presence of elevated cAMP, thereby demonstrating that this enhancement of activity is unlikely to be due to an increase in receptor sensitization (Greengard et al., 1991; Gurantz et al., 1994). In addition, the dramatic change seen when cAMP levels are elevated cannot be reproduced by tonically depolarizing all cells with elevated levels of extracellular potassium, indicating that adenosine is not acting via a general increase in the membrane excitability of all cells in the network.

Adenosine is known to modulate presynaptic release of ACh in a number of systems (reviewed by Sebastiao and Ribeiro, 1996). Moreover, enhanced levels of cAMP modulate the release of transmitter at a variety of central synapses (Chavez-Noriega and Stevens, 1994; Heidelberger and Matthews, 1994; Weisskopf et al., 1994; Capogna et al., 1995; Trudeau et al., 1996; Chen and Regehr, 1997; Kondo and Marty, 1997), including depolarizing GABAergic synapses in the developing hypothalamus (Obrietan and van den Pol, 1997). Adenosine may also act to modulate the spatiotemporal properties of waves via modulation of transmission through gap junctions (Hampson et al., 1992; Mills and Massey, 1995), as has been seen in the developing chick retina (Catsicas et al., 1998; Wong et al., 1998). Tracer coupling of neurobiotin is known to exist between ganglion cells of the same classes, as well as between ganglion cells and amacrine cells, at the stage of development when waves are robust (Penn et al., 1994). These junctions may mediate either electrical or diffusional coupling between ganglion and amacrine cells. However, in the chick retina, dopamine, which has similar effects on wave frequency as reported here, does not affect dye coupling of ganglion cells (Catsicas et al., 1998). It remains to be seen whether agents such as adenosine or forskolin can modulate gap junctions in the developing mammalian retina.

A final possibility is that cAMP regulates the refractory period of retinal cells. After a wave passes through a region, that region enters a refractory period during which it cannot support another wave for 40–50 s (Feller et al., 1996). However, under conditions of elevated levels of cAMP, areas of retina experience waves as frequently as every 15 s. This observation suggests that the refractory period of retinal cells has been reduced. Though the cellular mechanisms underlying the refractory period are not yet known, some possibilities include altering the kinetics of nAChR desensitization (Gurantz et al., 1994) or modulating the rate of replenishment of the readily releasable pool of vesicles following depletion caused by a wave, as has been suggested to occur in the developing spinal cord (Fedirchuk et al., 1999). Experiments are in progress to address directly the cellular processes that determine the refractory period.

cAMP Levels, Wave Patterns, and Visual System Development

Are the specific spatiotemporal properties of waves critical for the detailed patterning of these connections throughout the visual system (Feller, 1999)? In most proposed models for the formation of eye-specific layers, neighboring ganglion cells within an eye should fire synchronously, while there should be no correlations between eyes (Katz and Shatz, 1996; Crair, 1999). Small, slow waves would guarantee nearest-neighbor correlations within an eye, and long intervals between events would make intereye correlations low. However, as the interwave interval is reduced intereye correlations should increase, and as the wave area and velocity increase intraeye correlations will broaden. Now that we have established a role for cAMP in setting the dynamic properties of retinal waves, in the future it should be possible to address experimentally the critical question of the importance of the pattern of wave activity.

The spatiotemporal properties of waves may be critical for development within the retina as well. Amacrine cell differentiation is regulated by the retinal environment (Holt et al., 1988), with the amacrine cells providing feedback that downregulates their production (Reh and Tully, 1986; Reh, 1987). Retinal differentiation can be regulated by intracellular levels of cAMP (Taylor and Reh, 1990). Similarly, in developing spinal cord neurons, the frequency of calcium oscillations can control neurotransmitter phenotype and growth cone motility (Gu and Spitzer, 1995, 1997; Gomez and Spitzer, 1999). In addition, intracellular levels of cAMP affect the responses of growth cones to guidance molecules (Ming et al., 1997). Together, these processes could control amacrine cell differentiation, as well as connectivity within the retina. Adenosine may also play a role in the emergence of different firing patterns for ON versus OFF RGCs (Wong and Oakley, 1996; Fischer et al., 1998) since adenosine is expressed only in ON cholinergic amacrine cells in the adult (Blazynski, 1989).

Regulation of cAMP levels via the spontaneous waves may also modulate cell survival in the retina, by selecting for RGCs that are synaptically integrated within the local retinal circuit. The waves occur during a period of massive programmed cell death in the retina, in which the number of RGCs decreases by half (Lam et al., 1982; Potts et al., 1982; Henderson et al., 1988). Cell survival in enriched cultures of embryonic RGCs, as well as developing spinal cord neurons, can be augmented by supplying appropriate trophic factors (Johnson et al., 1986; Rodriguez-Tébar et al., 1989), but responsiveness to these trophic factors is dependent upon either neural activity or elevated intracellular levels of cAMP (Meyer-Franke et al., 1995; Hanson et al., 1998). Our observations here suggest that the level of cAMP in the developing retina is set at least in part by adenosine transmission, and consequently adenosine may be an endogenous regulator of cell survival in the retina.

Experimental Procedures

Retinal Preparation

All procedures were performed in accordance with approved animal use protocols at the University of California, Berkeley. Retinas were isolated from newborn ferrets (P0–P10) or mice (P0–P5) that had been deeply anesthetized with halothane (ferrets) or ice (mice) and then decapitated. Retinas were dissected and placed ganglion cell layer up in a temperature-controlled chamber (30°C, Medical Systems) mounted on the stage of either an inverted microscope (Nikon, Diaphot 300) or an upright microscope (Technical Instruments) and were continuously perfused. All procedures were performed in ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 1.0 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 11 mM D-glucose). Solutions were buffered with $NaHCO_3$ and oxygenated with a mixture of 95% O_2/5% CO_2.

Optical Recording

Isolated retinas were incubated with 10 μM Fura 2-AM (Molecular Probes) in ACSF containing 1% DMSO and 0.02% pluronic acid for 2–6 hr in an oxygenated chamber at 28°C. All experiments were conducted with 380 nm illumination using either a 6.3× (Zeiss Neofluor) or 10× (Nikon) objective. Images were acquired with a SIT camera (Dage, MIT 300). Initially, a background frame was acquired that was then subtracted on a pixel-by-pixel basis from all subsequent frames to create a difference image. The difference image was averaged over four video frames, giving a time resolution of 120 ms/frame. Movies of fluorescence changes were acquired onto Hi-8 videotape (Sony) with Metamorph Software (Universal Imaging). Three locations, each ~3200 μm^2, were monitored on each retina to determine frequency and peak amplitude of fluorescence changes. Data are expressed as the average ± SEM. Fluorescence data are displayed as traces of ΔF/F versus time, where F is the amount of DC fluorescence corrected for bleaching, and ΔF is the deviation from this baseline. Some movies were then analyzed on a Macintosh computer using NIH Image software to determine area, frequency, and velocity of all recorded waves. The statistical significance of changes in wave parameters was evaluated by use of the Student's t test, with $p < 0.05$ being the measure of significance. More details of fluorescence imaging and wave data analysis are included in Feller et al. (1996, 1997).

Physiological Recording

Whole-cell patch clamp recordings were made, either in voltage clamp or current clamp mode, using an Axopatch 200A amplifier and pClamp6 software (Axon Instruments). The amplitude of PSCs was determined by integrating the total net current of the compound PSC for each event. Cells were held at −60 mV, with E_{Cl} set at −60 mV. Intracellular solutions consisted of 100 mM K-gluconate, 4 mM Na_2-ATP, 0.3 mM GTP, and 38 mM HEPES (pH 7.2). To identify ganglion cells, Lucifer fills were done by adding 0.1%–0.5% Lucifer yellow to the intracellular solution. Retinas were then fixed in 4% paraformaldehyde, mounted on slides, and viewed on an upright microscope (FX-A, Nikon). Ganglion cells were identified based upon dendrite morphology, soma size, and presence of an axon.

Pharmacology

Unless otherwise noted, drugs were purchased from Research Biochemicals and were dissolved in ACSF and bath applied to retinas. NECA and forskolin (Sigma) were predissolved in DMSO and then diluted into ACSF (final concentration <1% DMSO). Test solutions of ACSF with 1% DMSO had no effect on the frequency of retinal waves. Retinas were incubated in Adenosine Deaminase (Sigma), SQ 22,536, and Rp-cAMPS in an oxygenated, heated chamber. Ascorbic acid (0.3 μM, Sigma) or sodium bisulfate (0.05 mg/ml, Sigma) was added to all dopamine solutions to prevent oxidation of the dopamine. The antioxidants, at these concentrations, did not affect retinal activity. TTX (Calbiochem) was dissolved in the supplied citrate buffer and stored at −20°C. Forskolin was dissolved in DMSO and stored at −80°C until used. GABA and ACh were pressure-injected through a large-bore pipette directly onto patch-clamped cells or Fura 2–loaded retinas using a pneumatic picopump (WPI) with 50 ms pulses at 5 lb/in^2.

Adenosine Receptor Immunohistochemistry

Ferret eyes were removed, immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4°C, and then rinsed in PB for several days at 4°C. Retinas were then sectioned on a cryostat at 12 μm and stored at −20°C. Sections were incubated in a blocking solution of 5% fish gelatin (Sigma), 2.5% bovine serum albumin (Sigma), and 0.1% Tween in TBS (50 mM Tris, 150 mM NaCl) for 30 min at room temperature. Sections were then incubated in primary antibody (anti-A2a 1:200 and anti-A2b 1:100 [Chemicon] in blocking solution) for 1 hr at room temperature and rinsed four times for 10 min in TBS/Tween. Staining was amplified using a biotinylated secondary antibody and avadin–biotin conjugate (Vector) and visualized with True blue (Kirkegaard and Perry). Images were acquired with a Spot digital camera (Diagnostic Instruments) on an upright microscope (FX-A, Nikon) fitted with Nomarski optics and were montaged using Photoshop (Adobe).

Genotyping

Dopamine-deficient mice, generously provided by R. Palmiter, were genotyped by Southern blot, following the protocol of Zhou and Palmiter (1995). Note that in these mice TH, the rate limiting enzyme in dopamine production, was knocked in under the dopamine β-hydroxylase promoter to allow production of norepinephrine, in order to avoid embryonic lethality (see Zhou and Palmiter, 1995, for more details).

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