**Chao Sun, Colenso M. Speer, Guo-Yong Wang, Barbara Chapman and Leo M. Chalupa**
*J Neurophysiol* 100:3253-3263, 2008. First published Oct 15, 2008; doi:10.1152/jn.90303.2008

**You might find this additional information useful...**

This article cites 46 articles, 20 of which you can access free at:
http://jn.physiology.org/cgi/content/full/100/6/3253#BIBL

Updated information and services including high-resolution figures, can be found at:
http://jn.physiology.org/cgi/content/full/100/6/3253

Additional material and information about ***Journal of Neurophysiology*** can be found at:
http://www.the-aps.org/publications/jn

This information is current as of October 18, 2009 .

***Journal of Neurophysiology*** publishes original articles on the function of the nervous system. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2005 by the American Physiological Society. ISSN: 0022-3077, ESSN: 1522-1598. Visit our website at http://www.the-aps.org/.

Downloaded from jn.physiology.org on October 18, 2009

*J Neurophysiol* 100: 3253–3263, 2008.
First published October 15, 2008; doi:10.1152/jn.90303.2008.

# Epibatidine Application In Vitro Blocks Retinal Waves Without Silencing All Retinal Ganglion Cell Action Potentials in Developing Retina of the Mouse and Ferret

**Chao Sun,[1,*] Colenso M. Speer,[2,*] Guo-Yong Wang,[4] Barbara Chapman,[1,2] and Leo M. Chalupa[1,2,3]**

[1]*Department of Neurobiology, Physiology, and Behavior,* [2]*Center for Neuroscience, and* [3]*Department of Ophthalmology and Vision Science, School of Medicine, University of California, Davis, Davis, California; and* [4]*Department of Structural and Cellular Biology, Tulane University, New Orleans, Louisiana*

Submitted 25 February 2008; accepted in final form 7 October 2008

**Sun C, Speer CM, Wang G-Y, Chapman B, Chalupa LM.** Epibatidine application in vitro blocks retinal waves without silencing all retinal ganglion cell action potentials in developing retina of the mouse and ferret. *J Neurophysiol* 100: 3253–3263, 2008. First published October 15, 2008; doi:10.1152/jn.90303.2008. Epibatidine (EPI), a potent cholinergic agonist, disrupts acetylcholine-dependent spontaneous retinal activity. Early patch-clamp recordings in juvenile ferrets suggested that EPI blocks all retinal ganglion cell (RGC) action potentials when applied to the retina. In contrast, recent experiments on the developing mouse that relied on multielectrode array (MEA) recordings reported that EPI application decorrelates the activity of neighboring RGCs and eliminates retinal waves while preserving the spiking activity of many neurons. The different techniques used in previous studies raise the question of whether EPI has different effects on RGC activity in mouse compared with that in ferret. A resolution of this issue is essential for interpreting the results of developmental studies that relied on EPI to manipulate retinal activity. Our goal was to compare the effects of EPI on the spontaneous discharges of RGCs in mouse and ferret using 60-electrode MEA as well as patch-clamp recordings during the developmental stage when retinal waves are driven by acetylcholine in both species. We found that in both mouse and ferret EPI decorrelates RGC activity and eliminates retinal waves. However, EPI does not block all spontaneous activity in either species. Instead, our whole cell recordings reveal that EPI silences more than half of all RGCs while significantly increasing the activity of the remainder. These results have important implications for interpreting the results of previous studies that relied on this cholinergic agonist to perturb retinal activity.

## INTRODUCTION

It is widely accepted that spontaneous retinal activity during early stages of development contributes to the emergence of visual circuits (Firth et al. 2005; Huberman 2007; Huberman et al. 2008a; Katz and Shatz 1996). Early in development neighboring retinal ganglion cells (RGCs) discharge in a temporally correlated manner, resulting in propagating waves of depolarization that spread across the retinal surface (Maffei and Galli-Resta 1990; Meister et al. 1991; Wong et al. 1993). These retinal waves are mediated by different cellular mechanisms and neurotransmitter systems at different developmental stages (Firth et al. 2005; Wong 1999).

There is good agreement in the literature that epibatidine (EPI) abolishes cholinergic retinal waves assessed either by $Ca^{2+}$ imaging (Huberman et al. 2002; Penn et al. 1998) or by multielectrode array (MEA) recording (Cang et al. 2005; Pfeiffenberger et al. 2005). Initial experiments investigating the role of retinal waves in the development of the visual system in the ferret reported that EPI completely blocked cholinergic-driven spontaneous retinal activity, silencing all spiking activity in RGCs (Penn et al. 1998). This was demonstrated by making patch-clamp recordings from RGCs treated with EPI in vitro. The EPI treatment in vivo caused retinal afferents from the two eyes to remain commingled within the dorsal lateral geniculate nucleus (dLGN) (Huberman et al. 2002; Penn et al. 1998) and ocular dominance columns failed to emerge in primary visual cortex (Huberman et al. 2006). These results were interpreted to indicate that silencing all retinal activity has a profound effect on eye-specific segregation during development.

More recently, several studies of spontaneous activity have been performed in the mouse using EPI to block retinal waves. Unlike in the ferret, EPI application to the mouse retina was reported not to abolish spontaneous activity. Instead, the discharges of neighboring cells were not correlated, indicating a loss of retinal waves (Cang et al. 2005; Pfeiffenberger et al. 2005). The mouse studies relied on MEA recordings in vitro to assess the effects of EPI on retinal activity. Similar to what has been reported in ferret, intraocular injections of this drug in the developing mouse resulted in abnormal retinogeniculate, retinocollicular, and geniculocortical projections (Cang et al. 2005; Chandrasekaran et al. 2005; Huberman et al. 2008b; Pfeiffenberger et al. 2005). These results were interpreted to indicate that altering patterns of retinal activity during development has a profound effect on eye-specific segregation and retinotopic map formation.

The different results reported in mouse and ferret could signify a genuine species difference or they could be spurious, reflecting the different techniques used to record retinal activity in the two species. Clarification of this issue is required for interpreting the results of studies examining retinogeniculate and geniculocortical development and determining whether RGC activity is playing an instructive or a permissive role in setting up precise connections in the visual system.

* These authors contributed equally to this work.

Address for reprint requests and other correspondence: L. M. Chalupa, Department of Neurobiology, Physiology, and Behavior, UC Davis, Davis, CA 95616 (E-mail: lmchalupa@ucdavis.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.


0022-3077/08 $8.00 Copyright © 2008 The American Physiological Society


Downloaded from jn.physiology.org on October 18, 2009

## METHODS

### *Animals*

Timed-pregnant ferrets were obtained from Marshall Farms (New Rose, NY); C57BL/6 black mice were bred at University of California, Davis. Postnatal day zero (P0) represents the day of pup birth. All experimental procedures were performed in accordance with National Institutes of Health guidelines using protocols approved by the Animal Care and Use Committee of UC Davis.

### *Surgery and tissue preparation*

The surgical and tissue preparation procedures for the MEA recordings have been described previously (Warland et al. 2006). Briefly, neonatal mice and ferrets were administered a lethal intraperitoneal dose of Euthana-6 (0.1–0.2 ml, pentobarbital sodium; Western Medical Supply, Arcadia, CA). The eyes were enucleated, and the retinae were removed and stored in buffered and oxygenated media (Eagle's minimum essential medium [MEME], M7278; Sigma–Aldrich, St. Louis, MO) at room temperature. For both patch-clamp and multielectrode recording, the retinae were cut into 5- to 8-$mm^2$ rectangles.

### *Patch-clamp recordings and analysis*

We recorded the whole cell activity of 10 RGCs from retinae of seven mice and 14 RGCs from retinae of seven ferrets. The patch-clamp recording procedures have been described previously (Liets et al. 2003; Wang et al. 2001). The bath solution within the recording chamber was maintained at 37°C during each experiment. Patch pipettes with a tip resistance between 3 and 7 MΩ were pulled from thick-walled 1.5-mm-OD borosilicate glass on a Sutter Instruments (Novato, CA) puller (model P-97). Current-clamp recordings were made with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The data were low-pass filtered at 1 kHz and digitized at 4 kHz before storage on an IBM computer for subsequent off-line analysis. Patch recordings were made from cells with clear, nongranular cytoplasm. High-resistance seals were obtained by moving the patch electrode onto the cell membrane and applying gentle suction. After formation of a high-resistance seal between the electrode and the cell membrane, transient currents caused by pipette capacitance were electronically compensated by the circuit of the Axopatch 200B amplifier. The series resistance was 7–16 MΩ. After attaining whole cell configuration, the resting membrane potential was read off the amplifier and monitored regularly throughout the recording. For our recordings, the input resistance varied between 310 and 620 MΩ. Following whole cell patch of each RGC, spontaneous activity was recorded for 7–15 min in control bath solution prior to introduction of epibatidine and for an additional 7–15 min following drug application.

### *Multielectrode array recordings*

A piece of retina was placed ganglion cell layer down onto a 60-channel MEA (Multi-Channel Systems, Tubingen, Germany), held in place with a piece of dialysis membrane (Spectrapore 132130; Spectrum, Los Angeles, CA), and superfused with buffered medium (MEME, M7278; Sigma–Aldrich) at 1–2 ml/min at 37°C. The array electrodes were 30 $\mu$m in diameter, arranged on an 8 × 8 rectilinear grid with 200-$\mu$m interelectrode spacing. For a 5-mm-diameter eye, the array covered 50% of the area between the optic disc and the peripheral edge, so the MEA recordings sampled from a large fraction of the available retina. At this interelectrode spacing, the signal of a given cell appeared on only one electrode, so each cell was assigned the coordinates of the electrode that recorded its signal. Analog data were acquired at 20 kHz per channel simultaneously from each of the 60 electrodes. Following experimental setup, retinae were allowed to acclimate for 5–20 min. On emergence of retinal wave epochs, recordings were performed for a period of 15–20 min, during which time overall firing rates of the ensemble appeared stable. Following control recordings, 10 nM EPI was applied to the perfusion medium and spontaneous activity was recorded for an additional 15–20 min.

### *Spike identification*

Before sorting spike events, the data were digitally filtered with a 125-Hz high-pass filter (four-pole Butterworth). A threshold of 6SD was set for each channel and 1 ms of data before a threshold-crossing event and 4 ms after the threshold event were stored for each negative-slope event. These candidate spike waveforms were then sorted with the OfflineSorter (Plexon, Denton, TX) using the first three principal components of the spike waveforms. Coincident events within 0.5 ms of one another that occurred on all electrodes were attributed to perfusion noise and removed. Clusters were first identified using an EM cluster algorithm by Shoham et al. (2003) and then manually edited for clustering errors. Typically, the activity of one to three cells was recorded by each electrode.

### *Statistical analysis*

BURST ANALYSIS. The burst duration was measured using the burst analysis algorithm provided by Neuroexplorer (Nex Technologies, Littleton, MA). The spike train was scanned until an interspike interval (ISI) of ≤0.1 s was found. This defined the beginning of the burst. Subsequent spikes with ISIs of <1 s were included in the burst, whereas an ISI of >1 s denoted the end of the burst. If an interval between two bursts was <5 s, the two bursts were merged and considered to be one burst. Bursts with a duration <0.5 s or with fewer than four spikes were discarded. Isolated cells exhibiting fewer than five bursts during the recording were excluded from the burst analysis. The burst analysis algorithm provided the burst frequency, burst duration, interburst interval, ISI of spikes within bursts, number of spikes per burst, and percentage of spikes in bursts relative to total spike number for the recording. Two-sample Kolmogorov–Smirnov (KS) tests were applied to the data for group comparison.

CORRELATION ANALYSIS. To quantify the degree of correlated firing between recorded pairs of cells, all cross-correlation functions were calculated and assigned a correlation index (CI). The CI measures the likelihood relative to chance that a pair of cells fired together within a particular time window. The CI was computed as described by Wong et al. (1993) using the following formula: $N_{ab}(-w, +w) \times T/[N_a(0, T) \times N_b(0, T) \times (2 \times w)]$, where $N_{ab}(-w, +w)$ is the number of spike pairs from cells a and b for which cell b fires within w seconds of cell a, T is the duration of the recording in seconds, $N_a(0, T)$ and $N_b(0, T)$ are the total number of spikes from cell a and b during the recording, and $2 \times w$ is the width of the correlation window. $N_{ab}$ was computed using $w = 0.1$ s and the cross-correlation function was binned at 0.05 s. The particular values of the CI depend on the choice of the correlation window w. A value of 0.1 s was chosen based on what has been commonly used by other investigators as a reasonable timescale for activity-dependent modification of synaptic strength (Meister et al. 1991).

WAVE FREQUENCY ANALYSIS. Naïve experimenters visually inspected the raw multielectrode analog data and counted wave events directly. A wave was defined as coincident bursting on four or more electrode sites.

## RESULTS

To evaluate the effect of EPI on RGC physiology, we performed whole cell patch and MEA recordings of isolated mouse and ferret retinae during acute bath application of control or 10 nM EPI [(±)-epibatidine dihydrochloride;



Downloaded from jn.physiology.org on October 18, 2009

Sigma–Aldrich] solutions. This drug dosage was previously reported to abolish all spontaneous retinal activity in neonatal ferret retinae (Penn et al. 1998) and to disrupt correlated wave activity in mouse retinae (Cang et al. 2005; Pfeiffenberger et al. 2005).

### *Patch-clamp recordings*

For retinal ganglion cell recordings, the resting membrane potentials varied between −50 and −70 mV for all cells at the onset of recording. In control whole cell patch-clamp recordings, RGCs of both mouse ($n = 10$ cells) and ferret ($n = 14$ cells) exhibited depolarizing events with an average frequency of 0.0289 ± 0.0324 Hz. For some cells this resulted in the generation of bursts of action potentials (Fig. 1, *A*, *B*, and *D*). For other cells, depolarizations were insufficient to generate individual spikes or bursts of spikes and only excitatory postsynaptic potentials (EPSPs) of varying amplitudes were recorded (Fig. 1*C*). RGCs usually exhibited periods of quiescence ranging from 1 to 3 min between depolarizations, a time course paralleling retinal wave frequency. Acute application of 10 nM EPI (Fig. 1, *A–D*, black arrows) to the perfusion bath resulted in the abolishment of all depolarizing events in some RGCs of both mouse (Fig. 1*A*; P4–P8, $n = 6$ of 10 recorded cells) and ferret (Fig. 1*C*; P4–P8, $n = 8$ of 14 recorded cells). In other recordings, however, EPI application elicited tonic activation at enhanced levels relative to normal (Fig. 1*B*, mouse P4–P8, $n = 4$ of 10 recorded cells; Fig. 1*D*, ferret P4–P8, $n = 6$ of 14 recorded cells). Acute application of EPI to the bath solution often elicited an immediate depolarization in recorded RGCs. This effect was seen both in cells that were silenced following EPI application (Fig. 1, *A* and *C*) and in cells whose activity was increased following drug treatment (Fig. 1*D*). This is likely due to the initial transient activity of EPI as a partial agonist of nicotinic acetylcholine receptors (nAChRs) (see DISCUSSION).

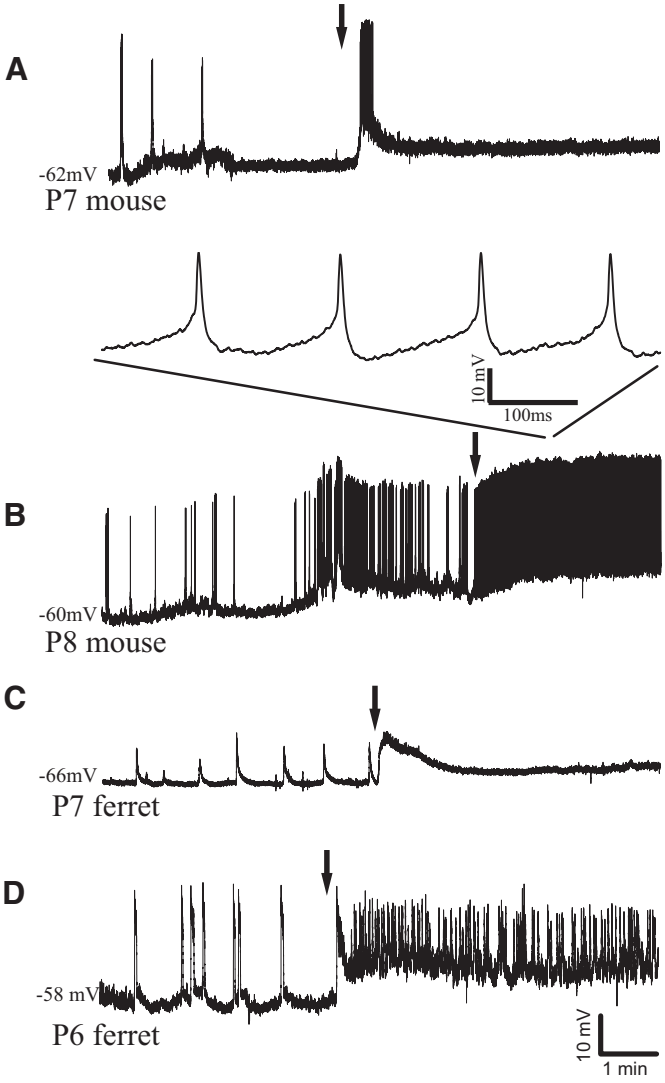


FIG. 1. Whole cell current-clamp recordings from retinal ganglion cells (RGCs) in mouse and ferret. Following (±)-epibatidine dihydrochloride (EPI) application (black arrows) some RGCs of mouse (*A*) ($n = 4$ of 8 total cells recorded) and ferret (*C*) ($n = 8$ of 16 total cells recorded) are silenced. Other RGCs exhibit increased tonic activation following EPI application (*B* and *D*) ($n = 4$ of 8 cells recorded in mouse; $n = 6$ of 14 cells recorded in ferret). Resting membrane potentials are shown adjacent to recording traces. Expanded trace in *B* shows tonic spiking elicited by EPI application.

### *MEA recordings*

Recordings of retinal activity on a 60-channel MEA allowed us to gather physiological data from 40–120 RGCs during typical recording sessions. As expected, retinal activity of neighboring cells in normal retinae was highly correlated, occurring at periodic intervals (Fig. 2, *A* and *C*, mouse and ferret, P4). At finer timescales, spontaneous retinal activity could be seen to sweep across neighboring electrodes, a phenomenon characteristic of retinal waves (Fig. 2*A*, *bottom*). Acute application of 10 nM EPI disrupted these correlated discharges in both species (Fig. 2, *B* and *D*, mouse and ferret, P4). Many RGCs whose activity could be isolated from the raw MEA recordings showed enhanced tonic firing following acute EPI application (Fig. 2, *B* and *D*, mouse and ferret, P4), confirming the observations made in the patch-clamp recordings described earlier.

### *EPI increases firing rate*

Mean firing frequency increased in both control and EPI-treated retinae from postnatal day 1 (P1) to P12 in mouse (Fig. 3*A*) and P0–P10 in ferret (Fig. 3*D*) ($n = 2$ or 3 retinae per time point for each species). Additionally, statistically significant differences between EPI-treated retinae and controls were found at many developmental time points ($^*P < 0.05$, two-sample KS test). We analyzed EPI-dependent changes in mean firing frequency during the developmental period of retinogeniculate segregation, when cholinergic signaling within the starburst amacrine cell network is the key factor for wave propagation (Feller et al. 1996; Zheng et al. 2006). Retinogeniculate segregation occurs in mouse from P0 to P8 and in ferret from P0 to P10. Group data collected from each species during these ages were pooled. As may be seen in Fig. 3, in both species acute EPI application significantly increased the overall level of recorded RGC activity (Fig. 3, *B* and *E*, mouse and ferret; $^*P < 0.05$, two-sample KS test). Mean firing frequency (spikes/s) during these time points was about 14% higher in EPI-treated mouse retinae and about 31% higher in EPI-treated ferret retinae compared with control RGC firing



Downloaded from jn.physiology.org on October 18, 2009

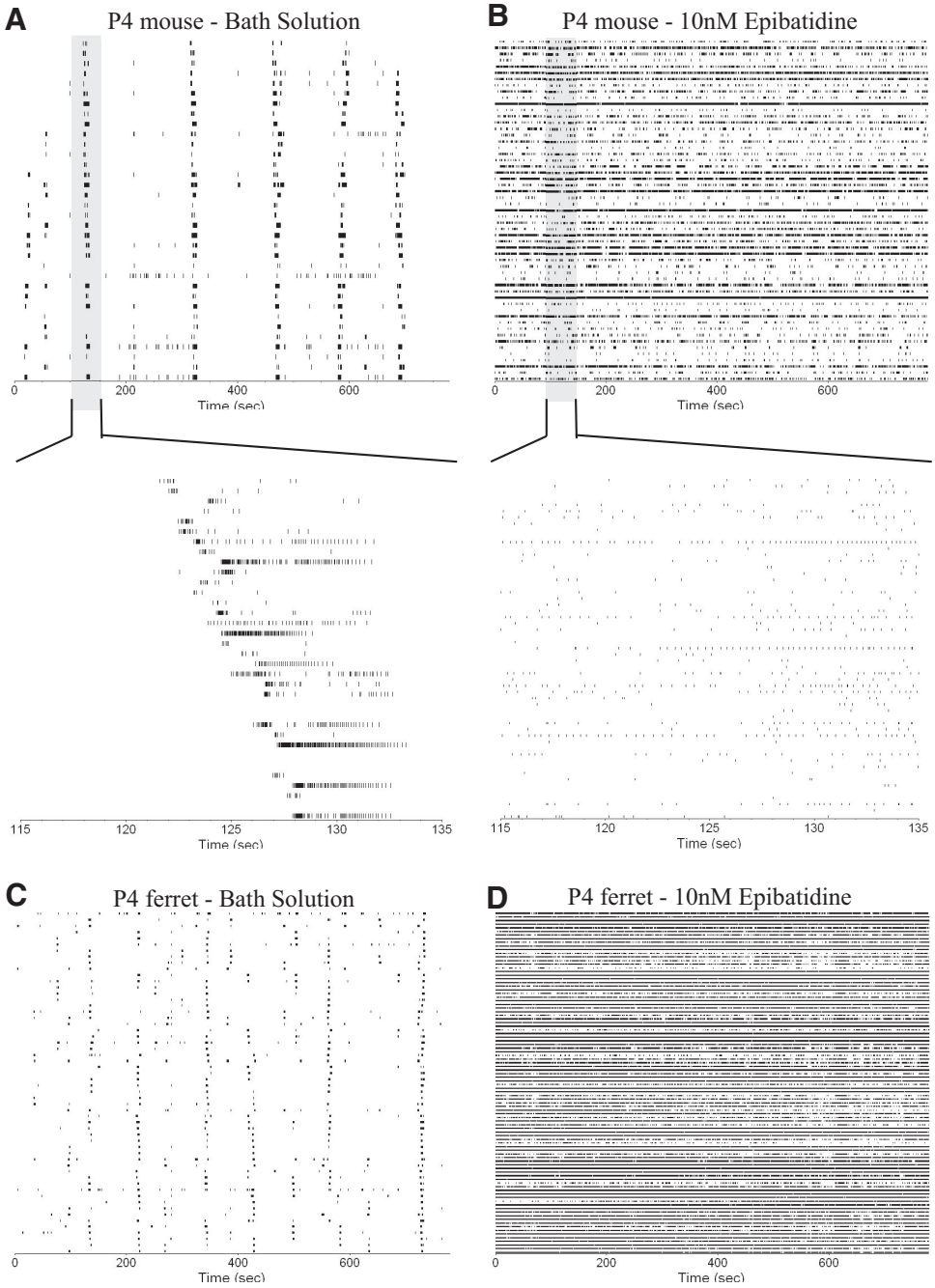


FIG. 2. Raster plots of individual RGC spike activity obtained via multielectrode array (MEA) recording. Each row is the spike train from a single RGC. Within each row, vertical lines represent spikes from the corresponding cell. The interval of time within the shaded region is expanded at a finer scale below each raster. Propagating waves of spontaneous activity were recorded under control conditions in both mouse (*A*) and ferret (*C*) at all developmental ages (postnatal day 4 [P4] shown). Application of 10 nM EPI resulted in the abolishment of normal wave activity in both species, yet RGC spiking persisted in all cases (*B*, mouse; *D*, ferret).

frequency. It should be noted, however, that this analysis does not take into account those cells that were silenced by EPI treatment.

### *EPI abolishes correlated activity*

Using a standardized algorithm for quantifying correlations among recorded RGC spike trains, we plotted CI values for control and EPI-treated retinal recordings in both mouse and ferret (see METHODS). Analyses of control recordings (Fig. 4*A*, mouse P4; Fig. 4*B*, ferret P4) revealed strong correlations between neighboring RGCs, which decreased as a function of distance between cell pairs (see regression line fit to individual data points). In contrast, acute EPI application to both mouse (Fig. 4*C*) and ferret (Fig. 4*D*) retinae eliminated distance-dependent correlations so that nearest-neighbor cells no longer showed temporal alignment in spike firing. Analysis of the maximum CI values measured at each developmental age confirmed this finding in mouse (Fig. 4*E*) and ferret (Fig. 4*F*) at all ages studied. Under control conditions, retinae in both species showed high correlation coefficients at early time points when waves are known to be acetylcholine dependent. As reported previously in control retinal recordings in ferret (Wong et al. 1993), we recorded a reduction in the maximum CI that occurs as a function of age in both mouse and ferret. This can be explained by increases in wave area and frequency that increase chance firing between cell pairs at greater distances and, additionally, by an increase in spike activity outside



Downloaded from jn.physiology.org on October 18, 2009

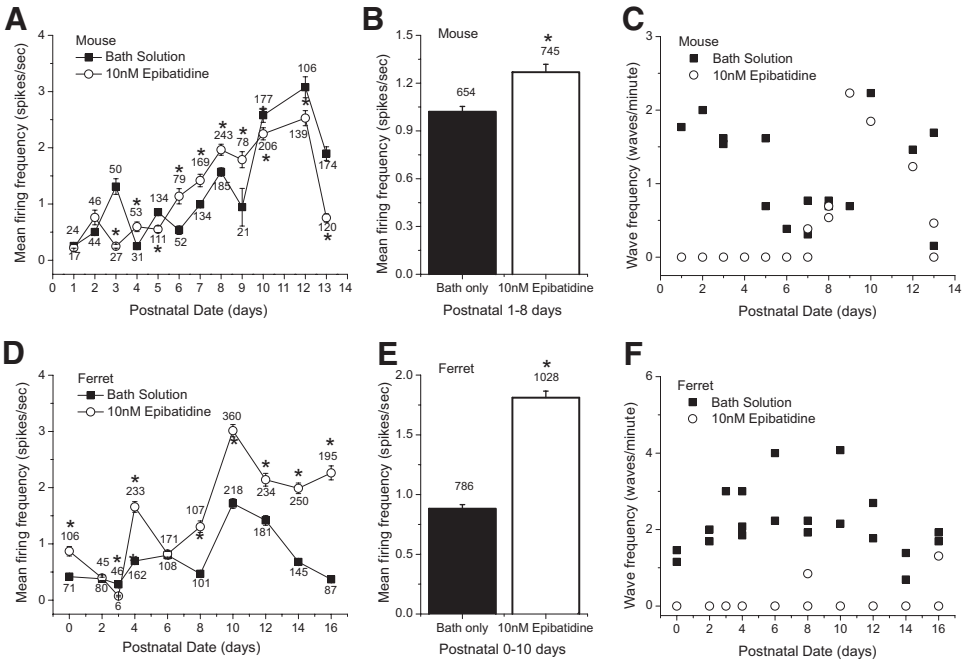


FIG. 3. Analysis of mean firing frequency and wave frequency as a function of developmental age. The mean firing frequency (spikes/s) of RGCs increased in both mouse (*A*) and ferret (*D*) as a function of developmental age. During the period of eye-specific segregation in the dorsal lateral geniculate nucleus (dLGN) occurring from P0 to P8 in mouse (*B*) and P0 to P10 in ferret (*E*), EPI increased overall mean firing frequency. EPI eliminated retinal waves from P0 to P7 in mouse (*C*) and P0 to P16 in ferret (*F*). Numbers in *A*, *B*, *D*, and *E* reflect RGCs isolated during MEA recording. Data points in *A* and *D* reflect the mean for all recorded retinae. Error bars reflect ±SE. Data points in *C* and *F* reflect separate retinae. $*P < 0.05$; 2-sample Kolmogorov–Smirnov (KS) test.

of wave events. In combination, these changes lower the maximum correlation values for each cell pair examined. Acute EPI application reduced the maximum CI values for both species to near zero for the entire developmental period examined, consistent with the elimination of correlations inherent to retinal waves and an increase in uncorrelated spike activity. The increase in overall activity of some RGCs following EPI treatment could itself lower the calculated correlation coefficients, potentially masking underlying correlated wave activity. However, for each data set acquired, independent observers evaluated the MEA output by eye to verify the absence of wave activity in EPI-treated retinae. Quantification of individual wave events in both control and EPI-treated retinae confirmed that acute EPI treatment eliminated retinal waves in mouse (Fig. 3*C*) and ferret (Fig. 3*F*) ($n = 2$ or 3 retinae per time point for each species) during the aforementioned period of ACh-dependent spontaneous retinal activity. In mouse, retinal waves persisted with EPI treatment at ages >P7 (Fig. 3*C*) ($n = 2$ or 3 retinae per time point). In ferret, EPI treatment eliminated retinal waves at all ages studied, up to P16 (Fig. 3*F*) ($n = 2$ or 3 retinae per time point). At the oldest age examined, however, retinal waves were not eliminated but were markedly reduced in frequency, which may reflect the maturation of glutamate-dependent spontaneous activity (Fig. 3*F*) (P16, $n = 1$).

### *Properties of EPI-resistant activity*

To further identify the properties of RGC discharges following EPI treatment we analyzed the structure of the remaining spontaneous activity in EPI-treated retinae. Notably, although many units continued to exhibit burst activity following EPI treatment, the pattern of RGC spike output appeared more tonic in the presence of the drug (Fig. 2, *B* and *D*). This was evidenced by an increase in the number of single spikes occurring between bursts and thus a statistically significant decrease in the percentage of total recorded spikes occurring within bursts during the period of retinogeniculate development in both mouse (Fig. 5*A*) and ferret (Fig. 5*B*) ($n = 2$ or 3 retinae per time point for each species). In addition, the frequency of bursts is increased by EPI application and the average interval between bursts is smaller in the presence of the drug (Fig. 5, *C–F*). Individual bursts were longer in duration and contained more spikes per burst in the presence of EPI (Fig. 6, *A–D*). However, the ISI within bursts was increased by EPI application (Fig. 6, *E* and *F*). All of these changes in burst properties are consistent with an overall change in spontaneous retinal activity from regular episodic bursts characteristic of normal waves to more tonic spike trains with increased spike output following EPI treatment (Fig. 3, *A*, *B*, *D*, and *E*).

Following acute EPI treatment, the percentage of total recorded cells engaged in burst activity was reduced in a concentration-dependent manner and the magnitude of this effect was age dependent (Fig. 7*A*, mouse). Additionally, the percentage of time RGCs fired at rates >10 Hz was significantly reduced in mouse (Fig. 7*B*) and ferret (Fig. 7*C*) ($n = 2$ or 3 retinae per time point for each species) following EPI treatment. The effect on high-frequency output following EPI treatment persisted in the ferret until P16 (Fig. 7*C*).

## DISCUSSION

Epibatidine, a cholinergic agonist, has been used to assess the role of retinal activity in the development of retinogeniculate (Huberman et al. 2002; Penn et al. 1998; Pfeiffenberger et al. 2005), retinocollicular (Chandrasekaran et al. 2005; Huberman et al. 2008b), and geniculocortical (Cang et al. 2005; Huberman et al. 2006) projections. This drug was introduced to the field of visual system development by Penn et al. (1998) who reported, on the basis of patch-clamp recordings from RGCs in the isolated ferret retina, that EPI silenced all retinal activity. More recently, MEA recordings from the isolated mouse retina have revealed that EPI application per-



Downloaded from jn.physiology.org on October 18, 2009

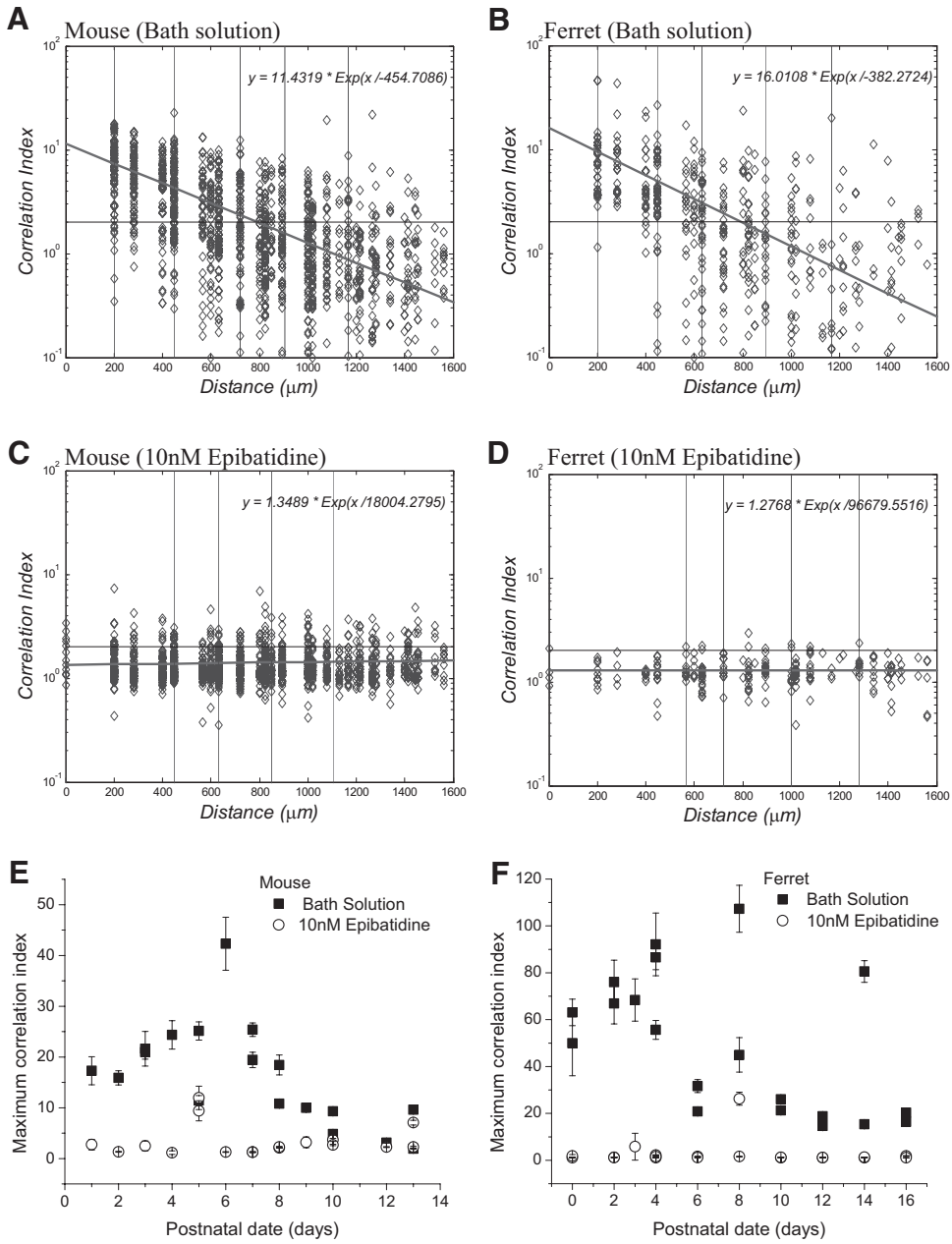


FIG. 4. Correlation index (CI) as a function of intercell distance for pairs of recorded cells. The location of each cell isolated on the array was assigned the position of the electrode on which it was recorded. For each pair of recorded cells, the CI was plotted logarithmically against the intercell distance. The regression line represents an exponential fit to the data. The *y*-intercepts (maximum CI) from the scatterplots are a measure of the strength of the overall correlations recorded from cell pairs. In both mouse (*A*) and ferret (*B*) RGCs exhibit highly correlated spontaneous activity as a function of intercell distance. Application of EPI to retinae of both species (*C* and *D*) eliminates correlations between recorded pairs. At all ages examined in both mouse (*E*) and ferret (*F*), the maximum CI values are reduced by EPI treatment. The maximum CI obtained from recordings of mouse and ferret, respectively, is plotted for each age together with the 95% confidence limits (error bars) obtained from the exponential fit. Vertical lines denote quintile boundaries and the horizontal thin line denotes the 99% limit of the shuffle analysis.

turbs retinal waves while preserving ganglion cell activity (Cang et al. 2005; Pfeiffenberger et al. 2005). We have sought to reconcile these seemingly conflicting reports in ferret and mouse retina by performing whole cell patch-clamp and MEA recordings in these two species at a comparable stage of development.

### *EPI disrupts spontaneous retinal activity in mouse and ferret*

In line with previous reports, we found that EPI treatment eliminated retinal waves in ferret and mouse retinae. However, our results demonstrate that this drug silences the activity of some but not all ganglion cells in both the ferret and the mouse. Additionally, in both species EPI significantly increased the frequency of action potential firing for those cells that remained active after drug application.

The differences between our results in ferret retinae and the previously reported silencing of all RGC action potentials in the presence of EPI (Penn et al. 1998) may be attributed to a number of factors. First, it is possible that the reported lack of active cells in the previous study could reflect a small sample size (five RGCs recorded in the presence of EPI). That study relied primarily on $Ca^{2+}$ imaging results to show the disruption of waves. Alternatively, it is possible that other methodological differences could account for the different results. The earlier experiments may have been performed under different recording temperature (unreported in Penn et al. 1998 vs. 37°C in our experiments) and in a different medium (artificial cerebrospinal fluid in Penn et al. 1998 vs. MEME in our experiments). Both temperature and medium affect excitability and wave characteristics in retinae from mice lacking normal cholinergic transmission due to knock out of the gene coding for the beta-2



Downloaded from jn.physiology.org on October 18, 2009

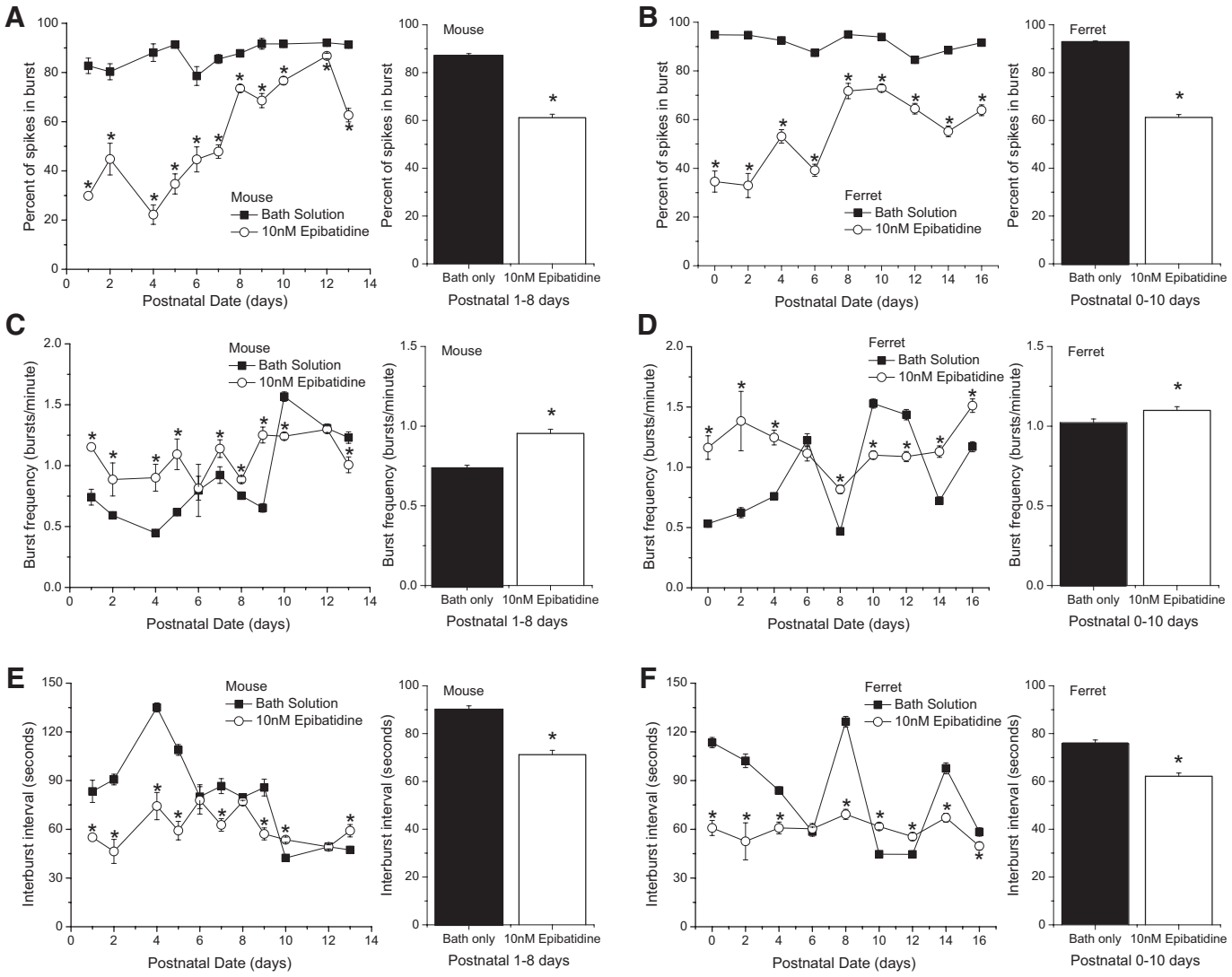


FIG. 5. Spontaneous activity of individual RGCs recorded by MEA. The percentage of total spikes recorded that occurred within bursts was significantly reduced at all recorded ages following EPI treatment in both mouse (*A*) and ferret (*B*) (line graphs). Data pooled from time points reflecting eye-specific segregation (P0–P8 in mouse and P0–P10 in ferret) are shown in bar graphs. Overall burst frequency (*C* and *D*) was increased following EPI treatment and the mean interburst interval was reduced (*E* and *F*) during time points of eye-specific segregation in both mouse and ferret. Individual data points reflect mean values and error bars are ±SE; $*P < 0.05$; 2-sample KS test.

subunit of the ACh receptor (Sun et al. unpublished data; D. Feldheim and B. Stafford, unpublished results). Although the basis of the differences between our results and those of Penn et al. are unknown, our findings clearly demonstrate that in vitro application of EPI has equivalent effects on the developing ferret and mouse retina when recordings are made under the same conditions.

In addition to silencing over half of all RGCs, EPI application severely perturbs the activity of those RGCs still spiking after drug treatment. Under normal conditions, retinal waves drive RGCs to fire action potentials, which are temporally correlated among neighboring cells. In contrast, EPI abolished all correlations among spiking RGC cell pairs at all distances, concomitant with a complete breakdown of observable retinal waves.

The residual activity of RGCs in the presence of EPI consisted of both bursts and single spikes, although many RGCs failed to engage in burst activity following EPI treatment. The loss of burst activity in some, but not all, RGCs exhibiting residual spike activity could be taken as additional evidence for differences in EPI sensitivity among varying RGC types. We found that, for those cells exhibiting bursts in the presence of EPI, many properties of the bursts were affected in a manner that reflects an overall shift in spontaneous activity from regular intervals of consistent burst behavior to more tonic firing patterns. This increase in tonic spiking contributes to the flat correlation index observed in our analysis of post-EPI MEA data. The abolishing of bursting in a fraction of active RGCs combined with increased spiking not confined to bursts caused significant decreases in the measurement of the percentage of total spikes occurring in bursts as well as the percentage of time RGCs were observed to fire at rates >10 Hz.

### *Mechanisms of EPI action on spontaneous retinal activity*

Why was the activity of some retinal ganglion cells eliminated by EPI, whereas the firing frequency of other ganglion cells was increased by this drug? In the mamma-



Downloaded from jn.physiology.org on October 18, 2009

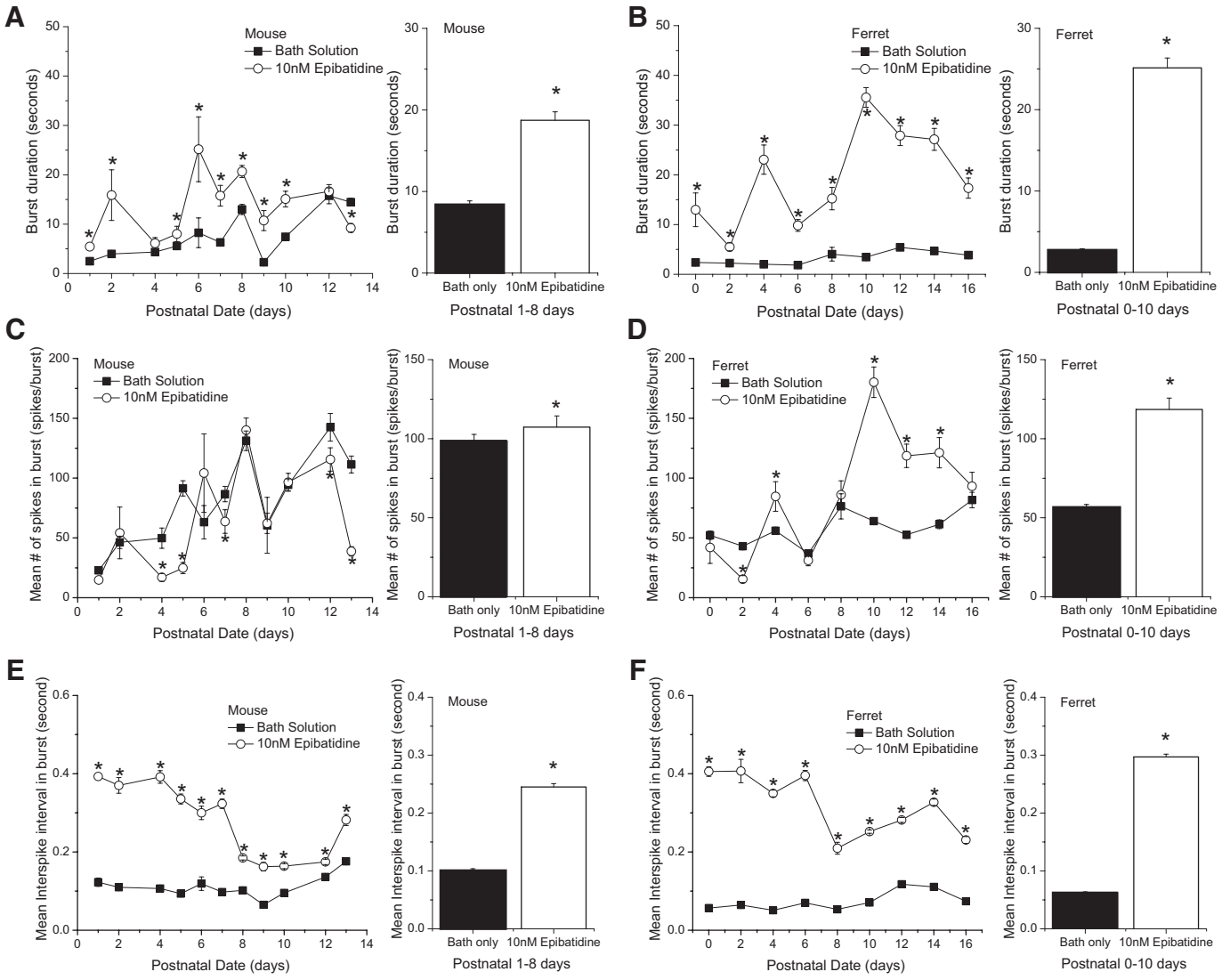


FIG. 6. Burst structure exhibited by individual RGCs recorded by MEA. Following EPI application, the average burst duration was significantly increased for RGCs of both mouse (*A*) and ferret (*B*) over the time course of eye-specific segregation (bar graphs). The average number of spikes/burst was also increased in both species in the presence of EPI during time points of eye-specific segregation (*C* and *D*, bar graphs). The mean interspike interval occurring within individual bursts was significantly increased at all time points evaluated in each species (*E* and *F*, line graphs), which resulted in an overall increase in this metric during the period of eye-specific segregation (*E* and *F*, bar graphs). Individual data points reflect mean values and error bars are ±SE; $*P < 0.05$; 2-sample KS test.

lian retina at least seven different nAChR subtypes are expressed and it is likely that different ganglion cells are characterized by different variants of this receptor (Feller 2002; Gotti et al. 2007; Lecchi et al. 2005; Marritt et al. 2005; Moretti et al. 2004). Since the activation kinetics of different nAChR subtypes are known to be different (Dani and Bertrand 2007), it seems reasonable to think that the differential effects of EPI on individual ganglion cells depend on specific subtypes of nAChRs found in the membrane of a given neuron (Lecchi et al. 2005). At the concentrations used in our study, EPI likely acts to elicit a prolonged desensitization of certain nAChRs, thereby reducing normal excitatory conductance following endogenous ACh release, which would be sufficient to prevent action potential generation in some ganglion cells. Those expressing the common $\alpha4\beta2$ receptor subunit combination may be particularly sensitive to EPI binding to inactivated receptors, locking them with high binding affinity in this inactive conformation (Buisson et al. 2000).

In ganglion cells lacking the nAChR subtypes by which EPI elicits a prolonged desensitization, the action of the drug might be expected to increase spiking activity. This seems unlikely, however, since studies performed on the binding of EPI to nAChRs have shown that at low concentrations, similar to that used in the present study, this drug did not elicit receptor activation, but rather diminished the responses of nAChRs to simultaneously applied ACh (Busisson et al. 2000). A more nuanced hypothesis for explaining EPI-dependent increased activation of some RGCs merits attention. It is known that during ACh-dependent waves, the activity of RGCs and cholinergic amacrine cells can be modulated by GABAergic and glycinergic signaling (Feller et al. 1996; Fischer et al. 1998; Johnson et al. 2003; Stellwagen et al. 1999; Syed et al. 2004; Zheng et al. 2004, 2006). Although γ-aminobutyric acid



Downloaded from jn.physiology.org on October 18, 2009

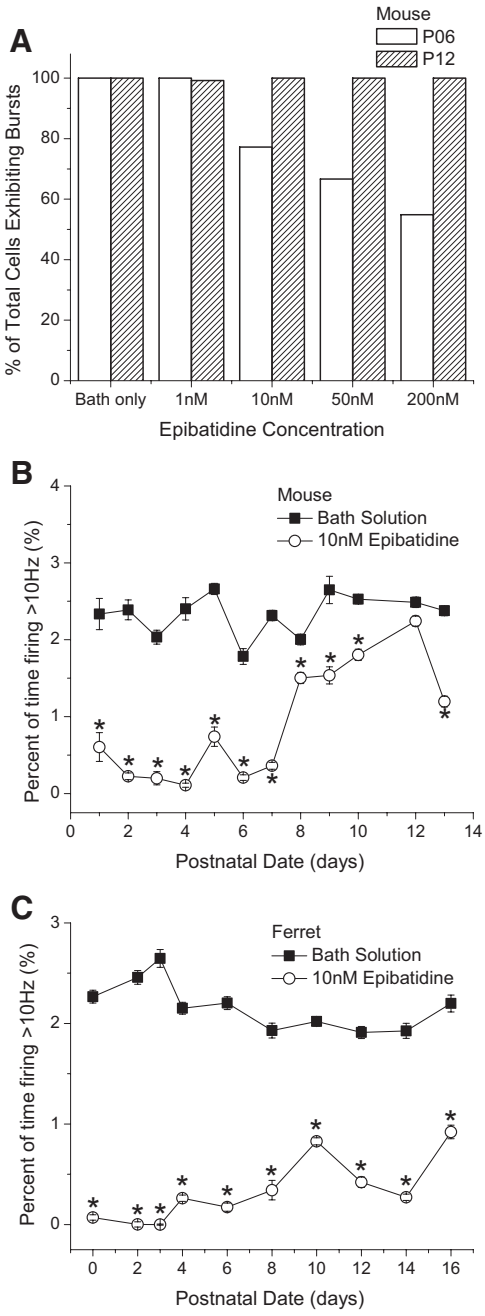


FIG. 7. EPI application to mouse retina resulted in a dosage- and age-dependent decrease in the percentage of total recorded RGCs engaged in burst activity (*A*). EPI application decreased the percentage of time-recorded RGCs spent firing at rates >10 Hz in both mouse (*B*) and ferret (*C*). Individual data points reflect mean values and error bars are ±SE; $*P < 0.05$; 2-sample KS test.

(GABA) is depolarizing for RGCs at young ages (Fischer et al. 1998; Stellwagen et al. 1999), tonic activation of $GABA_A$ receptors in the retina reduces RGC and amacrine cell excitability, perhaps eliciting a resting conductance that may serve to shunt excitatory inputs (Wang et al. 2007). It has also been demonstrated that many GABAergic amacrine cells express nAChRs (Dmitrieva et al. 2001). Additionally, starburst amacrine cells corelease and respond to both GABA and ACh, raising the possibility that EPI may reduce overall GABA release in the retina, thereby modulating RGC and amacrine cell outputs in a way that would increase spike activity for cells normally constrained by tonic $GABA_A$ activation (Wang et al. 2007; Zheng et al. 2004). Further pharmacological studies will be needed to clarify this issue.

The mechanism by which EPI abolishes retinal waves could reflect the effects of this drug on RGCs as well as cholinergic amacrine cells. The direct action of EPI on RGCs, discussed earlier, could be sufficient to abolish retinal waves, with some cells being silenced by EPI, whereas others fire at a higher-than-normal rate. This would act to decorrelate the discharges of neighboring ganglion cells, thereby abolishing retinal waves and decreasing the maximum correlation index. It is also likely that the activity of cholinergic amacrine cells is perturbed by EPI application. During ACh-dependent retinal waves, rhythmic oscillations in the membrane conductances of these retinal interneurons result in the release of ACh onto the dendrites of RGCs and other cholinergic amacrine cells (Zheng et al. 2006). Cholinergic amacrine cells are known to express multiple ACh receptors (Dmitrieva et al. 2001; Keyser et al. 2000; Yamada et al. 2003; Zheng et al. 2004; Zhou and Zhao 2000), so EPI binding to nAChRs expressed by cholinergic amacrine cells would be expected to disrupt the efficacy of the reciprocal excitatory signaling within this amacrine cell network. This altered pattern of spontaneous activity in the cholinergic amacrine cell network would in turn block the generation of RGC wave activity. Most likely, both the direct binding of EPI to nAChRs expressed by RGCs and the indirect action of this drug via the cholinergic amacrine cells contribute to the elimination of retinal wave activity, although the relative impact of these two factors remains to be established.

Although the precise mechanism by which EPI blocks retinal waves is unknown, our results demonstrate that in the mouse this occurs from P0 through P7; at later ages the drug had no obvious effects on retinal physiology. Previous work on the mouse retina showed that blocking nACh transmission using curare abolished retinal waves as late as P11, but by P12 the waves are driven by glutamate (Bansal et al. 2000). In the ferret, we found that EPI eliminated or significantly perturbed retinal waves from P0 until P16. In the ferret retina, little is known about the timing of the shift from cholinergic to glutamatergic waves. It was previously shown that at P7/P8 in the ferret, ACh is required for waves to occur, but that blocking glutamatergic transmission even at this early age caused a significant reduction in wave frequency (Wong et al. 2000). Blocking cholinergic transmission no longer affected waves at P20, but time points between P8 and P20 were not studied (Wong et al. 2000). Taken together, these results suggest that there is likely a gradual shift from waves driven solely by ACh to waves driven solely by glutamate and that different pharmacological agents used to block transmission and different methods of assessing waves may lead to different assessments of the relative importance of the two transmitters during the transition phase. Additionally, the duration during which both ACh and glutamate are involved in wave generation appears to be prolonged in the ferret compared with the mouse.

### *EPI as a tool for investigating activity-dependent development*

We have focused on the effects of EPI on retinal activity because this drug has been used by a number of previous



Downloaded from jn.physiology.org on October 18, 2009

studies to assess the role of retinal activity in the development of retinogeniculate (Huberman et al. 2002; Penn et al. 1998; Pfeiffenberger et al. 2005), retinocollicular (Chandrasekaran et al. 2005; Huberman et al. 2008b), and geniculocortical (Cang et al. 2005; Huberman et al. 2006) projections. Although it is widely believed that retinal activity is required for normal development of the visual pathways (Firth et al. 2005; Huberman et al. 2008a), it is not clear which parameters of retinal activity are required for a particular feature of the visual system to be normally formed.

With respect to the role of retinal activity in the formation of eye-specific retinogeniculate projections several conflicting reports remain to be reconciled (Chalupa 2007). An initial study reported that infusion of the voltage-gated $Na^+$ channel blocker tetrodotoxin (TTX) into the region of the optic chiasm prevented the segregation of eye specific inputs in the dLGN of the fetal cat (Shatz and Stryker 1988). In contrast, intraocular injections of TTX delayed, rather than prevented, eye-specific layers from forming in the postnatal ferret (Cook et al. 1999). In this context, the study by Penn et al. (1998) that intraocular injections of EPI, which they reported silenced all RGC activity, prevented eye-specific segregation in the ferret seemed to refute the results of Cook et al. (1999). Our results clearly show, however, that EPI does not block retinal activity; as discussed earlier: some RGCs are silenced by this drug, whereas others spike at a higher-than-normal frequency. Previous experimental work suggested that correlated spontaneous activity is critical for the normal emergence of eye-specific circuits (Cang et al. 2005; Grubb et al. 2003; Torborg et al. 2005). Modeling studies propose that the structure of burst activity in individual ganglion cells establishes limits on the refinement of connectivity (Butts et al. 2007). Together, these studies argue that the structure of RGC activity is important for the formation of eye-specific projections. However, testing this hypothesis requires an agent that changes RGC output in a defined manner without changing the overall level of activity. Although we find that EPI decorrelates spontaneous retinal activity and decreases overall bursting, we also find that it changes levels of activity in the retina, increasing firing of some cells while silencing others. Therefore EPI experiments cannot answer the question of whether retinal waves are instructive in visual system development or whether normal levels of activity are merely permissive for normal connections to form.

Our results illustrate the utility of applying multiple techniques in the examination of spontaneous retinal activity. Although MEA recording allows for large-scale assessment of spontaneous RGC output, this technique is not without limitations. Analysis of data obtained using MEAs relies on the experimenter's ability to identify individual output neurons based on physiological signatures that are unique for each cell. In the case of pharmacological manipulations that affect the spike waveform of RGC output, as in the present study using EPI, individual cells isolated prior to drug application cannot be subsequently identified based on physiological parameters. Therefore it was not possible to assess the number of cells silenced by drug application when comparing pre- and post-drug MEA recordings. Patch-clamp recording mitigates this difficulty by allowing direct evaluation of the effects of drug treatment on the spontaneous output of individual RGCs. However, patch-clamp recordings preclude one's ability to examine patterns of spontaneous activity across the retina. Additionally, it is difficult to obtain large samples for statistical analysis using this method. Therefore an approach that blends the strengths of multiple techniques is perhaps the optimal means of evaluating the constellation of effects a given manipulation may induce.

Finally, it is important to note that a common assumption in the field of visual system development is that acute effects of pharmacological agents observed in vitro are representative of their chronic effects in vivo. Further experiments are needed to determine the chronic effects of EPI on retinal physiology in vivo.

## ACKNOWLEDGMENTS

We thank C. Tan and K. Zhou for assistance in visually inspecting raw multielectrode array recordings to assess retinal wave frequency and X. Shan for technical support.

## GRANTS

This work was supported by National Institutes of Health Grants EY-11369 to B. Chapman, EY-13301 to G. Wang, and EY-03991 and P20MH-60973 (Research to Prevent Blindness) to L. M. Chalupa.

## REFERENCES

**Bansal A, Singer JH, Hwang BJ, Xu W, Beaudet A, Feller MB.** Mice lacking specific nicotinic acetylcholine receptor subunits exhibit dramatically altered spontaneous activity patterns and reveal a limited role for retinal waves in forming ON and OFF circuits in the inner retina. *J Neurosci* 20: 7672–7681, 2000.

**Buisson B, Vallejo YF, Green WN, Bertrand D.** The unusual nature of epibatidine responses at the alpha4beta2 nicotinic acetylcholine receptor. *Neuropharmacology* 39: 2561–2569, 2000.

**Butts DA, Kanold PO, Shatz CJ.** A burst-based "Hebbian" learning rule at retinogeniculate synapses links retinal waves to activity-dependent refinement. *PLoS Biol* 5: e61, 2007.

**Cang J, Renteria RC, Kaneko M, Liu X, Copenhagen DR, Stryker MP.** Development of precise maps in visual cortex requires patterned spontaneous activity in the retina. *Neuron* 48: 797–809, 2005.

**Chalupa LM.** A reassessment of the role of activity in the formation of eye-specific retinogeniculate projections. *Brain Res Rev* 55: 228–236, 2007.

**Chandrasekaran AR, Plas DT, Gonzalez E, Crair MC.** Evidence for an instructive role of retinal activity in retinotopic map refinement in the superior colliculus of the mouse. *J Neurosci* 25: 6929–6938, 2005.

**Cook PM, Prusky G, Ramoa AS.** The role of spontaneous retinal activity before eye opening in the maturation of form and function in the retinogeniculate pathway of the ferret. *Vis Neurosci* 16: 491–501, 1999.

**Dani JA, Bertrand D.** Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol* 47: 699–729, 2007.

**Dmitrieva NA, Lindstrom JM, Keyser KT.** The relationship between GABA-containing cells and the cholinergic circuitry in the rabbit retina. *Vis Neurosci* 18: 93–100, 2001.

**Feller MB.** The role of nAChR-mediated spontaneous retinal activity in visual system development. *J Neurobiol* 53: 556–567, 2002.

**Feller MB, Wellis DP, Stellwagen D, Werblin FS, Shatz CJ.** Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. *Science* 272: 1182–1187, 1996.

**Firth SI, Wang CT, Feller MB.** Retinal waves: mechanisms and function in visual system development. *Cell Calcium* 37: 425–432, 2005.

**Fischer KF, Lukasiewicz PD, Wong RO.** Age-dependent and cell class-specific modulation of retinal ganglion cell bursting activity by GABA. *J Neurosci* 18: 3767–3778, 1998.

**Gotti C, Moretti M, Gaimarri A, Zanardi A, Clementi F, Zoli M.** Heterogeneity and complexity of native brain nicotinic receptors. *Biochem Pharmacol* 74: 1102–1111, 2007.

**Grubb MS, Rossi FM, Changeux JP, Thompson ID.** Abnormal functional organization in the dorsal lateral geniculate nucleus of mice lacking the beta 2 subunit of the nicotinic acetylcholine receptor. *Neuron* 40: 1161–1172, 2003.



Downloaded from jn.physiology.org on October 18, 2009

**Huberman AD.** Mechanisms of eye-specific visual circuit development. *Curr Opin Neurobiol* 17: 73–80, 2007.

**Huberman AD, Feller MB, Chapman B.** Mechanisms underlying development of visual maps and receptive fields. *Annu Rev Neurosci* 31: 479–509, 2008a.

**Huberman AD, Manu M, Koch SM, Susman MW, Lutz AB, Ullian EM, Baccus SA, Barres BA.** Architecture and activity-mediated refinement of axonal projections from a mosaic of genetically identified retinal ganglion cells. *Neuron* 59: 425–438, 2008b.

**Huberman AD, Speer CM, Chapman B.** Spontaneous retinal activity mediates development of ocular dominance columns and binocular receptive fields in V1. *Neuron* 52: 247–254, 2006.

**Huberman AD, Stellwagen D, Chapman B.** Decoupling eye-specific segregation from lamination in the lateral geniculate nucleus. *J Neurosci* 22: 9419–9429, 2002.

**Johnson J, Tian N, Caywood MS, Reimer RJ, Edwards RH, Copenhagen DR.** Vesicular neurotransmitter transporter expression in developing postnatal rodent retina: GABA and glycine precede glutamate. *J Neurosci* 23: 518–529, 2003.

**Katz LC, Shatz CJ.** Synaptic activity and the construction of cortical circuits. *Science* 274: 1133–1138, 1996.

**Keyser KT, MacNeil MA, Dmitrieva N, Wang F, Masland RH, Lindstrom JM.** Amacrine, ganglion, and displaced amacrine cells in the rabbit retina express nicotinic acetylcholine receptors. *Vis Neurosci* 17: 743–752, 2000.

**Lecchi M, McIntosh JM, Bertrand S, Safran AB, Bertrand D.** Functional properties of neuronal nicotinic acetylcholine receptors in the chick retina during development. *Eur J Neurosci* 21: 3182–3188, 2005.

**Liets LC, Olshausen BA, Wang GY, Chalupa LM.** Spontaneous activity of morphologically identified ganglion cells in the developing ferret retina. *J Neurosci* 23: 7343–7350, 2003.

**Maffei L, Galli-Resta L.** Correlation in the discharges of neighboring rat retinal ganglion cells during prenatal life. *Proc Natl Acad Sci USA* 87: 2861–2864, 1990.

**Marritt AM, Cox BC, Yasuda RP, McIntosh JM, Xiao Y, Wolfe BB, Kellar KJ.** Nicotinic cholinergic receptors in the rat retina: simple and mixed heteromeric subtypes. *Mol Pharmacol* 68: 1656–1668, 2005.

**Meister M, Wong RO, Baylor DA, Shatz CJ.** Synchronous bursts of action potentials in ganglion cells of the developing mammalian retina. *Science* 252: 939–943, 1991.

**Moretti M, Vailati S, Zoli M, Lippi G, Riganti L, Longhi R, Viegi A, Clementi F, Gotti C.** Nicotinic acetylcholine receptor subtypes expression during rat retina development and their regulation by visual experience. *Mol Pharmacol* 66: 85–96, 2004.

**Penn AA, Riquelme PA, Feller MB, Shatz CJ.** Competition in retinogeniculate patterning driven by spontaneous activity. *Science* 279: 2108–2112, 1998.

**Pfeiffenberger C, Cutforth T, Woods G, Yamada J, Renteria RC, Copenhagen DR, Flanagan JG, Feldheim DA.** Ephrin-As and neural activity are required for eye-specific patterning during retinogeniculate mapping. *Nat Neurosci* 8: 1022–1027, 2005.

**Shatz CJ, Stryker MP.** Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. *Science* 242: 87–89, 1988.

**Shoham S, Fellows MR, Normann RA.** Robust, automatic spike sorting using mixtures of multivariate t-distributions. *J Neurosci Methods* 127: 111–122, 2003.

**Stellwagen D, Shatz CJ, Feller MB.** Dynamics of retinal waves are controlled by cyclic AMP. *Neuron* 24: 673–685, 1999.

**Syed MM, Lee S, Zheng J, Zhou ZJ.** Stage-dependent dynamics and modulation of spontaneous waves in the developing rabbit retina. *J Physiol* 560: 533–549, 2004.

**Torborg CL, Hansen KA, Feller MB.** High frequency, synchronized bursting drives eye-specific segregation of retinogeniculate projections. *Nat Neurosci* 8: 72–78, 2005.

**Wang CT, Blankenship AG, Anishchenko A, Elstrott J, Fikhman M, Nakanishi S, Feller MB.** GABA(A) receptor-mediated signaling alters the structure of spontaneous activity in the developing retina. *J Neurosci* 27: 9130–9140, 2007.

**Wang GY, Liets LC, Chalupa LM.** Unique functional properties of on and off pathways in the developing mammalian retina. *J Neurosci* 21: 4310–4317, 2001.

**Warland DK, Huberman AD, Chalupa LM.** Dynamics of spontaneous activity in the fetal macaque retina during development of retinogeniculate pathways. *J Neurosci* 26: 5190–5197, 2006.

**Wong RO.** Retinal waves and visual system development. *Annu Rev Neurosci* 22: 29–47, 1999.

**Wong RO, Meister M, Shatz CJ.** Transient period of correlated bursting activity during development of the mammalian retina. *Neuron* 11: 923–938, 1993.

**Wong WT, Myhr KL, Miller ED, Wong RO.** Developmental changes in the neurotransmitter regulation of correlated spontaneous retinal activity. *J Neurosci* 20: 351–360, 2000.

**Yamada ES, Dmitrieva N, Keyser KT, Lindstrom JM, Hersh LB, Marshak DW.** Synaptic connections of starburst amacrine cells and localization of acetylcholine receptors in primate retinas. *J Comp Neurol* 461: 76–90, 2003.

**Zheng JJ, Lee S, Zhou ZJ.** A developmental switch in the excitability and function of the starburst network in the mammalian retina. *Neuron* 44: 851–864, 2004.

**Zheng JJ, Lee S, Zhou ZJ.** A transient network of intrinsically bursting starburst cells underlies the generation of retinal waves. *Nat Neurosci* 9: 363–371, 2006.

**Zhou ZJ, Zhao D.** Coordinated transitions in neurotransmitter systems for the initiation and propagation of spontaneous retinal waves. *J Neurosci* 20: 6570–6577, 2000.

Downloaded from jn.physiology.org on October 18, 2009