

Circadian Rhythms in *Limulus* Photoreceptors

I. Intracellular Studies

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ABSTRACT The sensitivity of the lateral eye of the horseshoe crab, *Limulus polyphemus*, is modulated by efferent optic nerve impulses transmitted from a circadian clock located in the brain (Barlow, R. B., Jr., S. J. Bolanowski, and M. L. Brachman. 1977. *Science*. 197:86–89). At night, the efferent impulses invade the reticular, eccentric, and pigment cells of every ommatidium, inducing multiple anatomical and physiological changes that combine to increase retinal sensitivity as much as 100,000 times. We developed techniques for recording transmembrane potentials from a single cell in situ for several days to determine what circadian changes in retinal sensitivity originate in the primary phototransducing cell, the reticular cell. We found that the direct efferent input to the photoreceptor cell decreases its noise and increases its response. Noise is decreased by reducing the rate of spontaneous bumps by up to 100%. The response is increased by elevating photon catch (photons absorbed per flash) as much as 30 times, and increasing gain (response per absorbed photon) as much as 40%. The cellular mechanism for reducing the rate of spontaneous quantum bumps is not known. The mechanism for increasing gain appears to be the modulation of ionic conductances in the photoreceptor cell membrane. The mechanism for increasing photon catch is multiple changes in the anatomy of retinal cells. We combine these cellular events in a proposed scheme for the circadian rhythm in the intensity coding of single photoreceptors.

INTRODUCTION

In many animals, the retina and the brain form a neural loop: the retina transmits information to the brain, and the brain feeds signals back to the retina. The

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central input can modify receptive field characteristics (Miles, 1970; Pearlman and Hughes, 1976), adapt the retina to changes in ambient illumination (Aréchiga and Wiersma, 1969; Fleissner and Fleissner, 1978; Levinson and Burnside, 1981; Yamashita and Tateda, 1981; Brandenburg et al., 1983), and control metabolic processes in the retina (Chamberlain and Barlow, 1979, 1984; Teirstein et al., 1980). In many cases, the efferent input to the retina is generated by an endogenous oscillator with a period of ~24 h, that is, a circadian clock.

A clear example of the modulation of retinal function by a circadian clock in the brain is found in the visual system of the horseshoe crab, *Limulus polyphemus* (Barlow et al., 1977; Barlow, 1983). At night, the clock transmits neural activity via efferent optic nerve fibers to the lateral eyes as well as to all other major photoreceptor organs of the animal (Eisele et al., 1982). Approximately 12 efferent nerve fibers innervate each lateral eye and branch profusely, yielding as many as 10^5 synaptic terminals in the retina (Fahrenbach, 1973). The efferent

TABLE I
Components of Circadian Rhythms in the Limulus Lateral Eye

Retinal property	Day	Night	Reference
Efferent input	Absent	Present	Barlow et al. (1977); Barlow (1983)
Gain	Low	High	This paper; Renninger et al. (1984)
Noise	High	Low	This paper; Barlow et al. (1977); Kaplan and Barlow (1980)
Frequency response	Fast	Slow	Batra (1983); Renninger (1983)
Cell position	Proximal	Distal	Chamberlain and Barlow (1977); Barlow et al. (1980)
Aperture	Constricted	Dilated	Chamberlain and Barlow (1977)
Acceptance angle	6°	13°	Barlow et al. (1980)
Photon catch	Low	High	Barlow et al. (1980)
Pigment granules	Clustered	Dispersed	Barlow and Chamberlain (1980)
Membrane turnover	Trigger	Prime	Chamberlain and Barlow (1979, 1984)
Lateral inhibition	Strong	Weak	Renninger and Barlow (1979); Batra and Barlow (1982)

synapses contact the three types of cells within an ommatidium: pigment cells, reticular cells (photoreceptors), and eccentric cells, which are the second-order neurons that generate optic nerve impulses (Fahrenbach, 1973; Barlow and Chamberlain, 1980). Efferent synapses also terminate on the collateral branches of eccentric cells that mediate lateral inhibitory interactions in the retina (Fahrenbach, 1985).

Table I lists the components of circadian rhythms detected thus far in the lateral eye. The first one, the efferent input, mediates all the others. All except one are endogenous; that is, they continue unabated when the animal is maintained in constant darkness. The one exception is the metabolic event of membrane turnover, which is "primed" by the efferent input and triggered by the first light of dawn. All other components in Table I combine to increase retinal sensitivity at night.

What components of the circadian rhythms in Table I result from physiological changes in the photoreceptor cells? To address this question, we recorded

intracellularly from single photoreceptors in situ for periods of up to 3 d. We found that the efferent signals to the lateral eye modulate early events in the process of photoexcitation. Specifically, the efferent signals decrease noise, the spontaneous quantal events intrinsic to photoreceptors, and increase gain, the membrane potential changes generated in photoreceptors by photon absorption. We combine the circadian changes in noise and gain reported here with those of photon catch reported elsewhere (Barlow et al., 1980) in a proposed scheme for the circadian rhythm in intensity coding of single photoreceptors. To our knowledge, this is the first observation of a direct effect of the central nervous system on photoreceptor function. Preliminary reports of some aspects of this work have appeared (Kaplan and Barlow, 1980; Renninger et al., 1984; Barlow et al., 1985).

MATERIALS AND METHODS

Our procedure for recording transmembrane potentials from single visual cells of the lateral eye in situ has been described elsewhere (Barlow and Kaplan, 1977). Here we will briefly describe several important aspects and modifications of the technique.

All experiments were carried out at the Marine Biological Laboratory, Woods Hole, MA, where freshly collected horseshoe crabs are available throughout the summer. To minimize animal movements during recording periods, we fastened the carapace to a rigid platform immersed in a seawater aquarium. To gain access to ommatidia, we removed a small section of the cornea and inserted through the opening a glass microelectrode filled with 3 M KCl (20–70 M Ω). We advanced the electrode with a Burleigh inchworm (Burleigh Corp., Rochester, NY) in a ventral direction along an axis tilted laterally $\sim 10^\circ$ from the vertical plane of the animal. Visual cells were impaled in ommatidia located ventral into the opening in the cornea. No anesthesia was used.

Detection of circadian changes required stable, long-term recordings of transmembrane potentials from both reticular and eccentric cells in situ. Our previous technique yielded recording periods ranging from 3 to 8 h (Barlow and Kaplan, 1977). In the present study, we found that recording periods were often extended by inserting the microelectrode tip into the retinal tissue and allowing it to stabilize for a number of hours before advancing it further to impale a photoreceptor cell. Cell potentials were recorded differentially (bandwidth, 0–10 kHz), as previously described (Barlow and Kaplan, 1977). The amplitudes of the receptor potentials were digitized at a rate of 1 kHz and then time-averaged with computers (PC/AT, IBM, PDP 11/23, Digital Equipment Corp., Maynard, MA). Time averages were computed with respect to "quiet" resting potentials recorded from cells in darkness between 2000 and 2200 h. During these hours, the cells are nearly free of noise; that is, they generate very few spontaneous quantum bumps (see Fig. 2).

The electroretinogram (ERG) of the lateral eye was recorded using a technique described elsewhere (Barlow, 1983). That reference also gives details of the optical stimulation system for eliciting the ERG as well as the technique for optic nerve stimulation to simulate the endogenous efferent activity generated by the circadian clock. Light stimuli were delivered to the impaled cells either by single light pipes (Barlow, 1969) or bundles of light pipes. For single light pipes, the intensity incident on the ommatidium at the surface of the cornea was 10^{12} photons/s between 400 and 700 nm at the arbitrary zero setting of the optical system, which is indicated by $\log I = 0$ in the figures of this article. For light pipe bundles, the intensity incident on the cornea was 3×10^{15} photons/s \cdot cm 2 , which corresponds to 3×10^{11} photons/s incident on a single ommatidium. Impaled cells were allowed to dark-adapt for at least 2 h before testing.

RESULTS

Daytime and Nighttime Responses

Fig. 1 shows receptor potentials recorded from a dark-adapted reticular cell at various levels of illumination during the day and night. This intracellular recording was stable for a total of 49 h, and the animal remained in darkness throughout the entire period. "Day" responses were recorded in the morning from 0730 to 0800 h, and "night" responses were recorded in the evening from 2200 to 2350 h. Both sets of responses were recorded after the photoreceptor had been impaled for more than 24 h.

At each level of illumination, the nighttime responses were larger than the daytime responses. At low levels of illumination ($\log I = -9$ to -7), all responses are comprised of two components: small potential fluctuations (SPFs; <10 mV), and large potential fluctuations (LPFs; >10 mV). SPFs are the well-known quantum bumps that can be elicited by the absorption of single photons (Fuortes and Yeandle, 1964). LPFs are regenerative events triggered by SPFs (Bayer and Barlow, 1978) that amplify single photon events, enabling them to fire off optic nerve impulses in the next-order cell, the eccentric cell (Dowling, 1968; Barlow and Kaplan, 1977).

At low levels of illumination, both the daytime and nighttime records contained SPFs and LPFs, but more of the discrete events occurred at night. During the day, seven events could be detected in the record at $\log I = -8$, but not all were elicited by photon absorptions. The record taken in darkness ($\log I = -\infty$) during the day suggests that about half of the events at $\log I = -8$ occurred spontaneously. At least 20 discrete events could be detected in the nighttime record at $\log I = -8$, and, judging from the record in darkness, few occurred spontaneously. The nighttime response at $\log I = -8$ more closely resembles the daytime response at $\log I = -7$, a 10-fold-higher intensity. A more detailed analysis of the nighttime increase in the rate of light-evoked bumps indicates that, for a given high intensity, the photoreceptor caught ~ 15 more photons at night.

The photoreceptor responses at intermediate light intensities in Fig. 1 ($\log I = -6$ to -4) are characterized by fluctuating potentials superimposed on a steady depolarizing potential. Such responses result from discrete events, LPFs and SPFs, merging together. In this range of intensities, the nighttime responses were also larger than the daytime responses. For example, the amplitudes and shapes of the nighttime responses at $\log I = -7$ and -6 closely resembled those of the daytime responses at $\log I = -6$ and -5 , respectively. In other words, when nighttime responses are compared with daytime responses having approximately the same photon catch, their waveforms and amplitudes superimpose. Thus, the increased response amplitudes at these light intensities can be largely accounted for by an increase in photon catch.

At high intensities ($\log I > -3$), potential fluctuations were reduced in amplitude and the response waveform was characterized by a large initial transient that decayed to a relatively smooth steady state depolarization. Nighttime responses were also higher than daytime responses, but the matches noted above at lower light levels did not occur. For example, the steady state response at

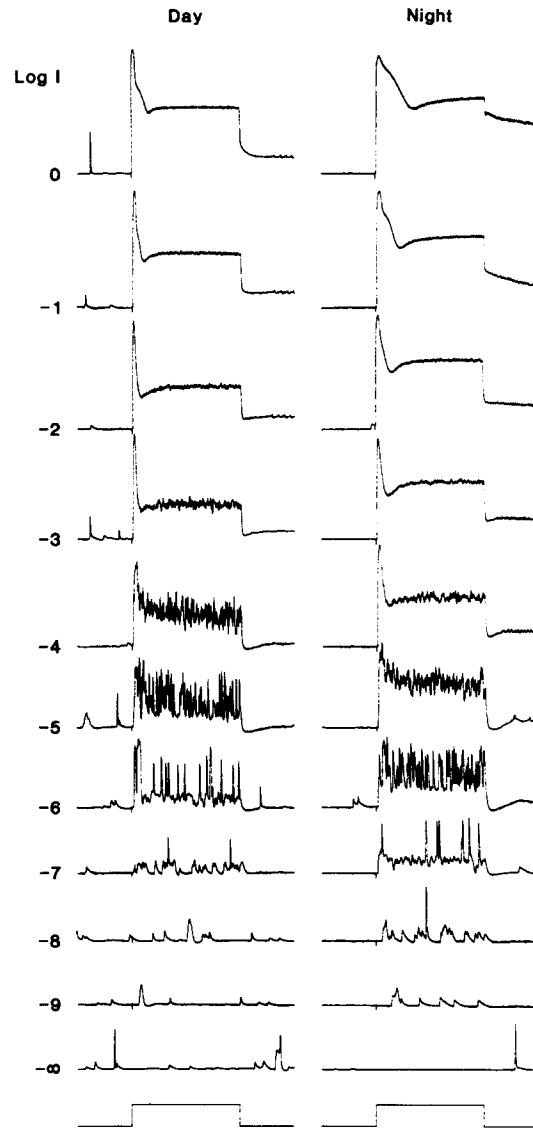


FIGURE 1. Photoreceptor potentials recorded from a single reticular cell in situ during the day and at night. All responses were recorded with the eye fully dark-adapted and after the cell had been impaled for >24 h. The animal remained in darkness throughout the recording period, which extended for 49 h. The responses to low levels of illumination consisted of SPFs and LPFs both day and night. At intermediate levels, the potential fluctuations merged together, producing a noisy receptor potential. The responses to high intensities consisted of an initial transient depolarization followed by a smooth steady state potential. At night, the response amplitude increased and noise decreased. The resting potential was 50 ± 1 mV, and the flash duration was 6 s. The calibration is 10 mV (height of stimulus bar). Light intensity incident on single ommatidium at $\log I = 0$ was 10^{12} photons/s (400–700 nm).

night at $\log I = -3$ was greater than that during the day at $\log I = -2$, a 10-fold-higher intensity. The steady state level of depolarization of 22 mV at $\log I = -3$ at night was about equal to that at $\log I = -1$ during the day. This was the case for all intensities above $\log I = -3$. The circadian changes in photoreceptor response were therefore greater at higher levels of illumination. Such changes cannot be accounted for by an increase in photon catch alone. They appear to reflect an increase in photoreceptor gain (see Discussion).

Fig. 1 also reveals a day-night change in the intrinsic noise of the photoreceptor cell. During the day, spontaneous fluctuations in membrane potential preceded every test flash, whereas at night few occurred. The spontaneous fluctuations included both LPFs and SPFs. All experiments yielded the same result: spontaneous discrete events occurred less frequently at night.

Regarding daily changes in other cellular properties, none were detected in the resting potential of the cell in Fig. 1. Throughout the 49-h recording period, the resting potential remained within the range -51 to -49 mV. In other experiments, resting potentials were recorded in the range -62 to -48 mV and none exhibited endogenous changes with time of day. In addition, no daily changes were detected in the resting membrane conductance of the cell in Fig. 1 or in other experiments.

Circadian Rhythms in Photoreceptor Response and Noise

Fig. 2 shows the daily changes in response and noise recorded from the photoreceptor cell illustrated in Fig. 1. The upper panel plots the amplitude of the receptor potential as a function of the time of day. Each point gives the time-averaged amplitude of the steady state component of the receptor potential in response to a low-intensity flash. Note that the mean amplitude of the response did not remain constant throughout the recording period. 2 h after the cell was impaled (2000 h), the response amplitude increased to a maximum value of 8.8 mV at ~ 2300 h. The amplitude then began to decline, reaching a minimum value of 1 mV at ~ 0900 h on the following day. It remained low during the rest of the daytime hours and increased again at ~ 2000 h of the second day. The cycle detected during the first day was then repeated. We last tested the cell under dark-adapted conditions at 1500 h of the second day. The recording was lost 4 h later, yielding a total recording time of 49 h.

The lower panel in Fig. 2 plots the rate of spontaneous discrete events, both SPFs and LPFs, as a function of time of day. Each point gives the number of events per minute recorded in darkness under dark-adapted conditions. Spontaneous events occurred at a rate of ~ 10 /min at 1800 h 30 min after the cell was impaled. The rate declined steadily for the next 2 h, approaching zero at 2000 h. Over the next 1.5 h, only 21 discrete events occurred, which is an average rate of one every 5 min. At 2200 h, the frequency of spontaneous events increased 25-fold to ~ 5 /min. After midnight, the rate gradually began increasing and, at dawn (0500 h), it rapidly increased to ~ 30 events/min. This high rate continued throughout the day until dusk (1800 h), when, as on the previous day, it declined steadily for 2 h, reaching zero at ~ 2000 h. Few events were again detected from 2000 to 2200 h, and after midnight the rate increased, reaching

a maximum value of $\sim 40/\text{min}$ near midday, several hours before the recording was lost.

The daily changes in photoreceptor noise approximately mirror those of the photoreceptor response. Noise is low when the response is high and vice versa. Both cellular properties exhibit daily rhythms with periods of ~ 24 h, which suggests that both are controlled by a circadian clock. Evidence given below indicates that both are controlled by the same circadian clock.

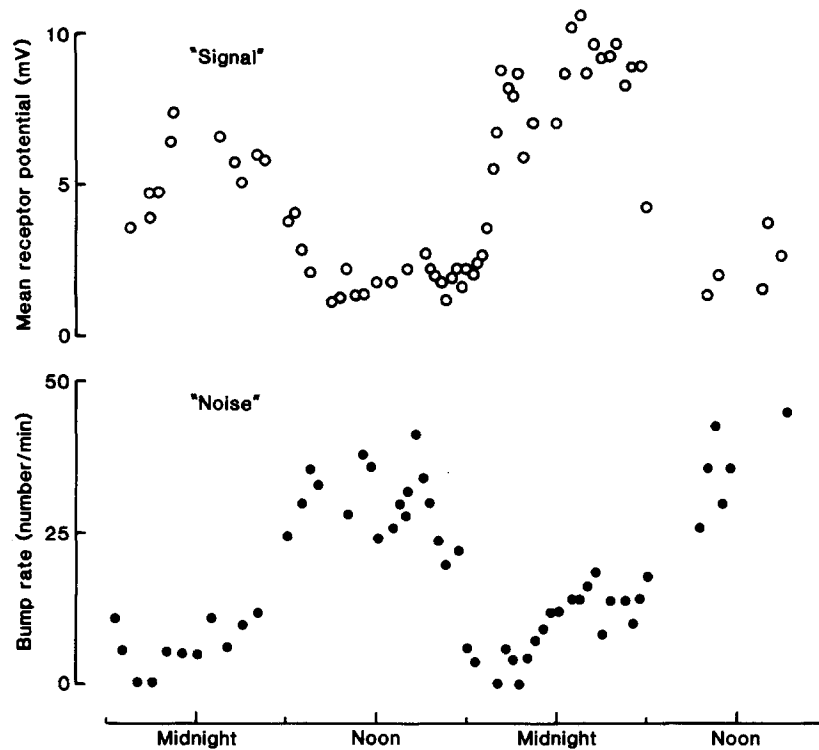


FIGURE 2. Circadian rhythms in response and noise recorded from the photoreceptor illustrated in Fig. 1. The open circles in the upper panel give the mean amplitudes of the receptor potential ("signal") in response to 6-s flashes of dim light ($\log I = -6.0$). The filled circles in the lower panel give the rates of discrete events recorded in darkness ("noise"). Over the 2-d recording period, the measurements of the signal amplitude and noise rate were occasionally interrupted by tests of other cell characteristics.

Circadian Rhythm in Photoreceptor Sensitivity

The sensitivity of a photoreceptor for dim light stimuli can be considered in terms of its signal-to-noise properties. In Fig. 2, the mean receptor potential can be taken as the amplitude of the photoreceptor signal, and the occurrence of spontaneous events can be taken as the photoreceptor noise that masks the signal. For a rough estimate of daily changes in the signal-to-noise properties, we will neglect the short-term temporal effects and assign average values for both

components. During the day, the mean receptor potential for a constant intensity flash ($\log I = -6$) ranged between 1 and 2 mV, with an average value of ~ 1.5 mV. The spontaneous event rate was in the range of 30/min, which, when integrated over time, is equivalent to a mean receptor potential of ~ 0.7 mV. At night, the mean receptor potential to the same dim flash increased about sixfold to an average value of ~ 9 mV during the second night (see the legend to Fig. 2). The spontaneous event rate decreased ~ 10 -fold in the early evening hours, yielding a mean receptor potential of ~ 0.07 mV. From these values, we estimate the signal-to-noise ratio to be ~ 2 during the day and close to 100 at night. The sensitivity of the photoreceptor in Fig. 2 to near-threshold stimuli is therefore ~ 50 times higher at night.

This estimate of the endogenous change in photoreceptor sensitivity assumes that the amplitude of intrinsic noise can be approximated by a steady level of membrane depolarization both during the day and at night. However, photoreceptor noise is comprised of discrete events that occur at random. Moreover, the event rate does not remain constant for more than 1 h; it changes throughout the day and night. In most experiments, spontaneous events occurred at rates of < 1 /min in the late evening hours before midnight. During these periods, photoreceptor sensitivity cannot be realistically evaluated in terms of signal-to-noise concepts. After midnight, the spontaneous event rate increased substantially. Similar changes occurred in the response amplitude, but not to the same extent as for noise. The endogenous changes in the response amplitude were not synchronized with those of noise. The noise decrease did not mirror the response increase. In Fig. 2, the nighttime decrease in the spontaneous event rate preceded by ~ 1 h the increase in the response amplitude. It is apparent that the circadian changes in photoreceptor response and noise are complex. A complete analysis of the temporal changes in photoreceptor sensitivity will require detailed considerations of the discrete properties of light-driven and spontaneous events as well as their temporal characteristics.

Circadian Rhythm in Intensity Coding

Fig. 3 gives the daytime and nighttime intensity-response functions for the data shown in Fig. 1. The amplitudes of the steady state component of the receptor potential are plotted as a function of the logarithm of incident light intensity. Each point is the time-averaged amplitude of the steady state depolarization with respect to the "quiet" nighttime resting potential. Note that the responses are graded over wide ranges of light intensity during both day and night. Both intensity-response functions cover an ~ 9 -log-unit range, but the nighttime function is shifted to the left and has a different shape.

The nighttime function approaches a vertical asymptote, whereas the daytime function increases gradually from a horizontal asymptote set by internal noise. Spontaneous discrete events occur at a rate of ~ 30 /min during the day, which, upon integration, yields a mean receptor potential of ~ 0.7 mV with respect to the cell resting potential recorded in darkness at night. This is the value plotted at $\log I = -\infty$, which indicates darkness. The nighttime function rises from a vertical asymptote because the mean receptor potentials were computed with respect to the nighttime resting potential. At low light levels, the function has a

slope of 1.0 on the log-log coordinates, which indicates that a 10-fold increase in light intensity produced an ~ 10 -fold increase in response. The photoreceptor was functioning nearly as an ideal photon detector in this range, sensing single photons (Kaplan and Barlow, 1976) and linearly summing their responses to produce the receptor potential. The steep slope at night also indicates a high sensitivity of the photoreceptor to small increments of light intensity.

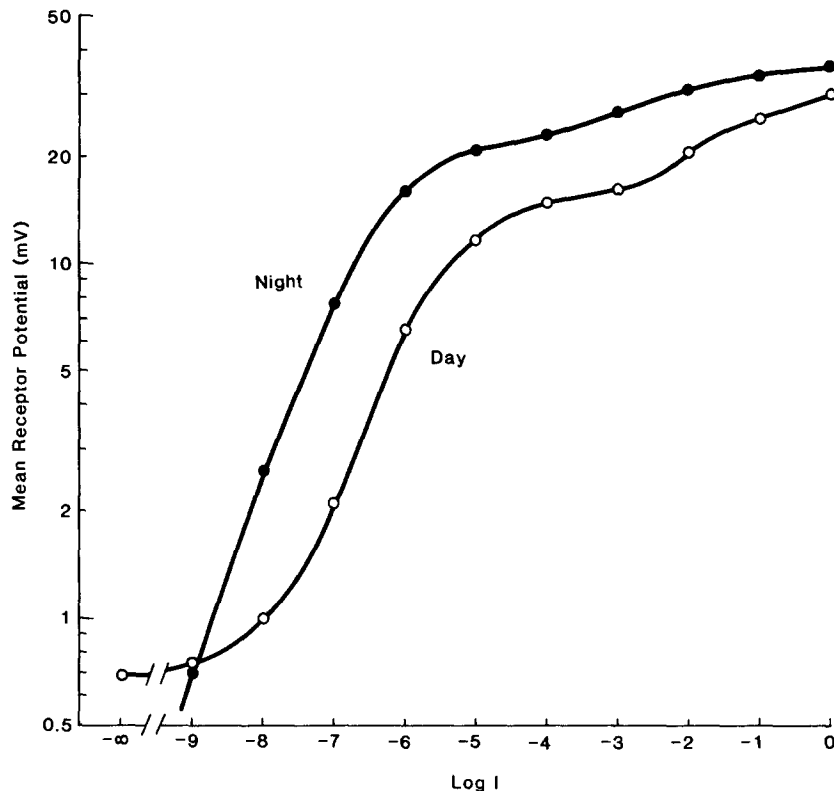


FIGURE 3. Intensity-response functions for the photoreceptor potentials shown in Fig. 1. The mean receptor potential is plotted on the ordinate as a function of log light intensity plotted on the abscissa. All values of the mean receptor potential were computed with respect to the "noise-free" resting potential recorded from the cell at night (see Materials and Methods). Mean values were determined for the entire 6-s period of the flash for the lower light levels ($\log I = -7$ to -9) and for darkness ($\log I = \infty$). For all other responses, mean values were determined for the last 2 s of the 6-s flashes. All tests were made with the cell in the dark-adapted state.

As the light level increased, the daytime characteristics of intensity coding approached those of the nighttime state. From $\log I = -7$ to -6 , the slopes of both functions are about equally steep (slope ≈ 1.0), which indicates that the photoreceptor could also perform as an ideal photon detector during the day, although within a limited intensity range. Subtracting the mean receptor potential owing to spontaneous discrete events (0.7 mV) from the daytime function

extends the range (see Fig. 9 in the Discussion), yielding functions with similar slopes but in different ranges of light intensity (night, $\log I = -9$ to -7 ; day, $\log I = -8$ to -6). Since the response amplitude is nearly proportional to light intensity in both cases, the dark-adapted photoreceptor was capable of operating as an ideal detector at all times, but its performance was degraded by intrinsic noise during the day and shifted to a higher operating range.

The photoreceptor exhibited similar coding characteristics at intermediate light levels, both day and night. From $\log I = -6$ to -2 , both functions flattened and formed plateaus before rising again at higher intensities. The plateau regions in the vicinity of $\log I = -4$ indicate a nearly complete loss of intensity discrimination because changes in light intensity produced very small changes in receptor potential. Similar plateaus have also been found in the intensity-response functions for the optic nerve discharge generated by the second-order cell, the eccentric cell (Barlow et al., 1977). This is understandable since the ionic currents that give rise to the photoreceptor potential are passively conducted from the reticular cell to the eccentric cell, where they form the generator potential for initiating optic nerve impulses (Tomita, 1958; Purple, 1964). The range and shape of the intensity-response function suggest the operation of two receptor mechanisms, one encoding low light levels and the other encoding high light levels (Kaplan and Barlow, 1975; Barlow and Kaplan, 1977). The plateau can be viewed as a transition between the two mechanisms. If this interpretation is correct, then the clock changes the intensity coding characteristics of the cell without significantly affecting its underlying receptor mechanisms.

Above the plateau region, the receptor potential again increased with light intensity before saturating at the highest light levels. Although saturation occurred at higher intensities during the day, very little incremental sensitivity could be detected in the steady state response at the highest test intensity ($\log I = 0$), regardless of the time of day. This intensity marks the upper limit of the dynamic range of the photoreceptor. Note that the transmembrane potential saturated at 28 mV during the day and at 36 mV at night. All experiments yielded about the same difference in saturating potentials. Apparently the circadian clock can modulate the amplitude of the maximum steady state response a photoreceptor is capable of generating. As we discuss below in reference to Fig. 10, this effect appears to reflect a circadian rhythm in the neural mechanisms of light adaptation.

From counting single photons to saturation, the receptor potential is graded over an ~ 9 -log-unit range, both day and night. The circadian clock shifts the range to lower light levels at night and changes the shape of the receptor function such that one cannot overlay the other, regardless of how they are shifted on the log-log coordinates. Such changes in shape can lead to large increases in sensitivity at intermediate light intensities. For example, in Fig. 2, an intensity of $\log I = -2$ produced a steady state response of 20 mV depolarization during the day. To maintain the same response amplitude at night required a 1,000-fold reduction in intensity to $\log I = -5$. The photoreceptor is therefore 1,000 times more sensitive in this intensity range at night. Similar considerations for optic nerve responses yield increases in sensitivity of up to 100,000 times at night (Barlow et al., 1977, 1984a). Such large changes in sensitivity appear to result from a

combination of circadian changes in the anatomy and physiology of the photoreceptor cell (see Discussion).

The data in Figs. 1–3 were recorded over a 49-h period from a single reticular cell. We were successful in recording for such long periods from just two other cells in other animals. Both exhibited circadian properties similar to those shown in Figs. 1–3. In 54 other experiments, we recorded from single cells for shorter periods ranging from 5 to 32 h. In each case, the recording extended over the

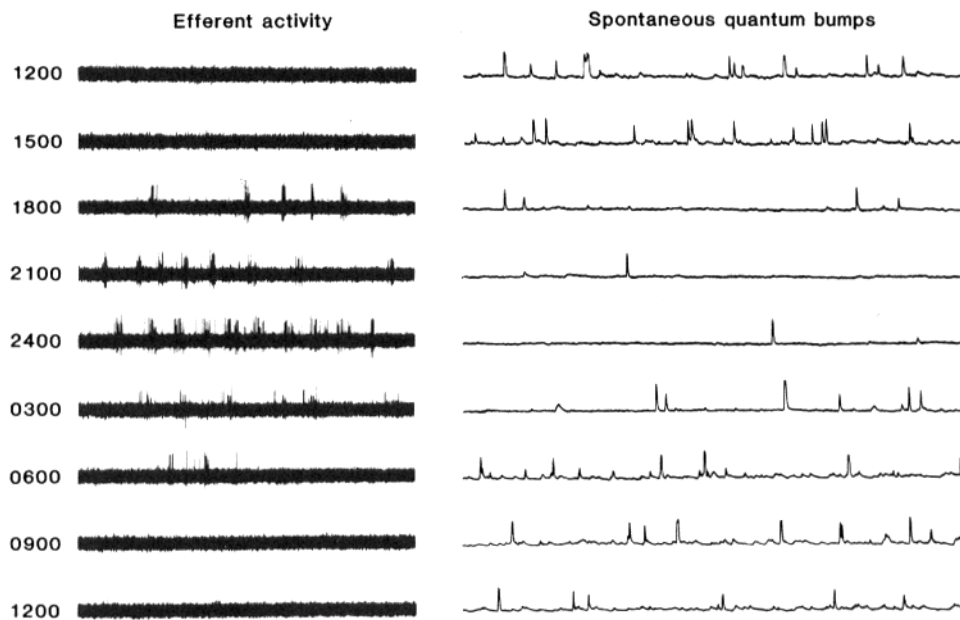


FIGURE 4. Comparison of the efferent input to the lateral eye with the rate of spontaneous quantum bumps recorded from a single reticular cell in situ. The onset of efferent activity at 1800 h corresponds to a reduction in bump rate. As the level of efferent activity increased, the bump rate decreased. After midnight, the process reversed. During daytime hours, no efferent activity was generated by the clock and the bump rate remained at a constant high level. Because it was not possible to record the efferent input and intracellular potentials from the same eye, we used different preparations to collect the two sets of data. However, both experiments were carried out at the same time of year so that the animals would possess circadian rhythms with similar duty cycles (Barlow, 1982, 1983). The duration of each trace of efferent activity is 8 s and that of each reticular cell record is 20 s.

transition period from day to night. Although we could not study the endogenous circadian rhythms of these cells, we could measure short-term changes in some of their properties.

Efferent Optic Nerve Activity Mediates the Circadian Changes in Photoreceptor Physiology

The left-hand panel in Fig. 4 shows samples of efferent optic nerve activity recorded over a 24-h period from the proximal stump of the cut lateral optic

nerve while the animal remained in darkness. The activity began near dusk, became maximal before midnight, diminished in the early morning hours, and stopped at dawn. No activity was recorded during the day. Previous studies indicate that the efferent impulses are generated by a circadian clock located in the brain and are transmitted to the lateral eyes, where they evoke circadian rhythms in ERG and optic nerve responses (Barlow et al., 1977; Barlow, 1983). Do the efferent signals also mediate the circadian rhythms of photoreceptor sensitivity, noise, and intensity coding shown in Figs. 1–3?

The right-hand panel in Fig. 4 indicates that the reduction in photoreceptor noise is related to the appearance of efferent activity. Although the spontaneous discrete events and efferent activity were recorded from different animals, the

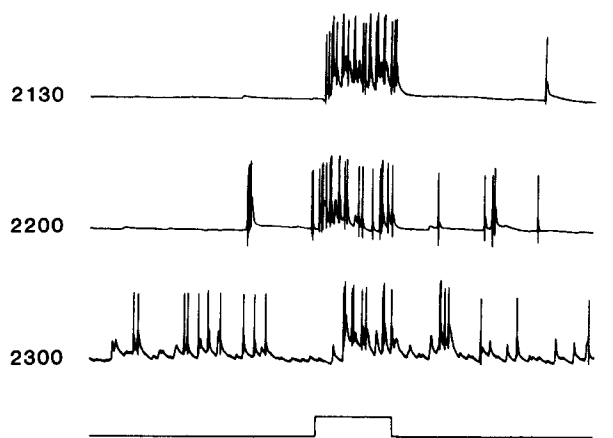


FIGURE 5. Effect of cutting the optic nerve on the membrane potential recorded from an eccentric cell in situ. The cell was impaled at 1600 h and the animal was placed in darkness. At 2130 h, the cell exhibited typical nighttime characteristics: low noise and high response. The optic nerve was cut at 2140 h and, within 20 min, the spontaneous bumps became more frequent and the light response declined in amplitude. By 2300 h, the cells' properties were indistinguishable from those of the daytime state: high noise and low response. Cutting the optic nerve did not affect either the membrane potential or membrane conductance. The flash duration was 4 s and the flash intensity was $\log I = -6$. The calibration mark at the end of the 2300-h record is 10 mV.

experiments were carried out at the same time of the year, so that both animals would possess circadian rhythms having similar characteristics (Barlow, 1982, 1983). Note that the onset of efferent activity (1800 h) coincides with the reduction in the event rate. Note also that the highest level of efferent activity (2400 h) coincides with the lowest event rate. As the level of efferent activity declined in the early morning hours, the event rate increased. It therefore appears that the circadian changes in the spontaneous event rate are mediated by efferent nerve signals. This is indeed the case, as we demonstrate in Fig. 5.

In the experiment illustrated in Fig. 5, an eccentric cell (the second-order cell) was impaled in the lateral eye in the late afternoon, and the animal was placed in darkness at sundown. The recording at 2130 h is typical of the nighttime state

of the lateral eye: low noise in darkness (only two discrete events are clearly discernible), and a high response to dim light ($\log I = -6$). The discrete event occurring before the test flash is an SPF, and that following it is an LPF. The LPF was large enough to trigger a spike in the eccentric cell. Both SPFs and LPFs were decrementally conducted to the eccentric cell from their site of origin

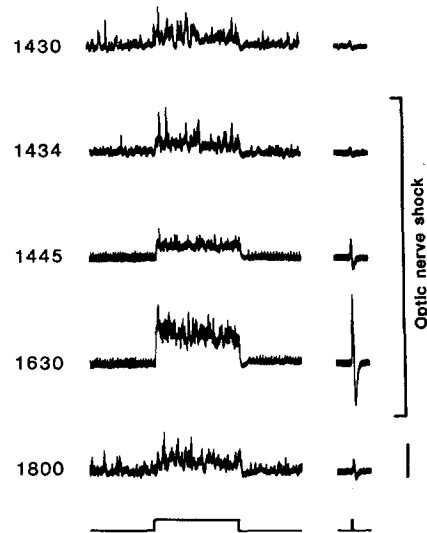


FIGURE 6. Effect of optic nerve shock on the membrane potential of the reticular cell. Intracellular records of responses to 4-s flashes of dim light are shown on the left, and ERGs from the same eye are shown on the right. The distal end of the cut optic nerve was placed in a current-passing electrode at 1250 h, and the reticular cell was impaled at 1330 h. 1 h later, the cell exhibited the typical daytime characteristics of a high spontaneous event rate and a low response amplitude. At 1432 h, current pulses, delivered to the cut nerve at the rate of 4 s^{-1} for 30 s every minute, decreased the spontaneous bump rate and increased the amplitude of the receptor potential and ERG, replicating the nighttime state. 2 h later, the current pulses were turned off and the cell slowly returned to the daytime state. This is one of just a few experiments in which we were successful in maintaining a stable intracellular recording before, during, and after optic nerve shock. Our frequent failures may have been caused by efferent-induced changes in the ommatidial structure (Barlow et al., 1980) dislodging the tip of the electrode. Also, the poor quality of the records relative to those in Fig. 1 may have resulted from the tip of the electrode being located far from the transducing rhabdom in a remote cellular region that experienced minimal structural changes. The scale bar is 10 mV for the intracellular data and $50 \mu\text{V}$ for the ERG records.

in the reticular cells, and thus the generator potential contains a mixture of both types of potentials.

The optic nerve was cut at 2140 h without disturbing the recording or the state of adaptation of the retina. At 2200 h, the response began to exhibit characteristics of the daytime state: a lower light response and a higher rate of spontaneous quantum bumps. At 2300 h, the transition to the daytime state was

complete. The high noise level, together with the lower sensitivity, almost obscured the dim light response. Discrete events of both types are evident, but only the LPFs appear to be large enough to exceed threshold and trigger nerve impulses. Since the record at 2300 h is typical of the daytime state of the retina (Barlow and Kaplan, 1977), we conclude that cutting the optic nerve at night abolished the efferent input to the eye and allowed the retina to return to the daytime state.

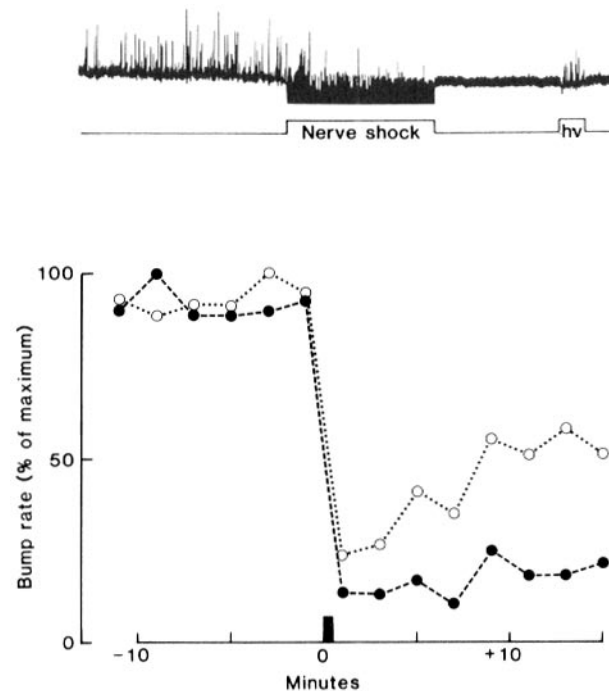


FIGURE 7. Effect of optic nerve shock on the rate of spontaneous discrete events. In the top trace, a 30-s episode of optic nerve shock (4/s) reduced the frequency of bumps recorded from a reticular cell in darkness. The response to a 5-s light stimulus delivered to the eye 25 s after shock offset showed that the transducing mechanism of the reticular cell was not impaired by nerve shock. The lower graph plots the results of two other identical experiments. 30-s episodes of nerve shock reduced the spontaneous bump rate in both experiments by >80%. In all three experiments, the decrease in bump rate recovered to preshock levels within 1 h after shock offset.

Fig. 6 shows that pulses of current delivered to the optic nerve during the day mimic the clock's action on a single photoreceptor. Each of the five intracellular records in Fig. 6 is followed by the ERG, which provides an additional measure of retinal sensitivity. The recording at 1430 h is characteristic of the daytime state of the retina; that is, it contains many discrete events in the dark and a relatively small response to light. After the record was taken at 1430 h, periodic episodes of current pulses were delivered to the distal end of the cut optic nerve

to simulate endogenous efferent optic nerve activity (Barlow, 1983). After 2 h of nerve shock, the receptor potential exhibited all the characteristics of the nighttime state: low noise and high response. Turning off the current pulses at 1630 h allowed the photoreceptor to return to the daytime state (1800 h) and remain in that state until the recording was lost at 2045 h. Shocking the cut optic nerve during the day reproduced the physiological properties that characterize the nighttime state of the photoreceptor.

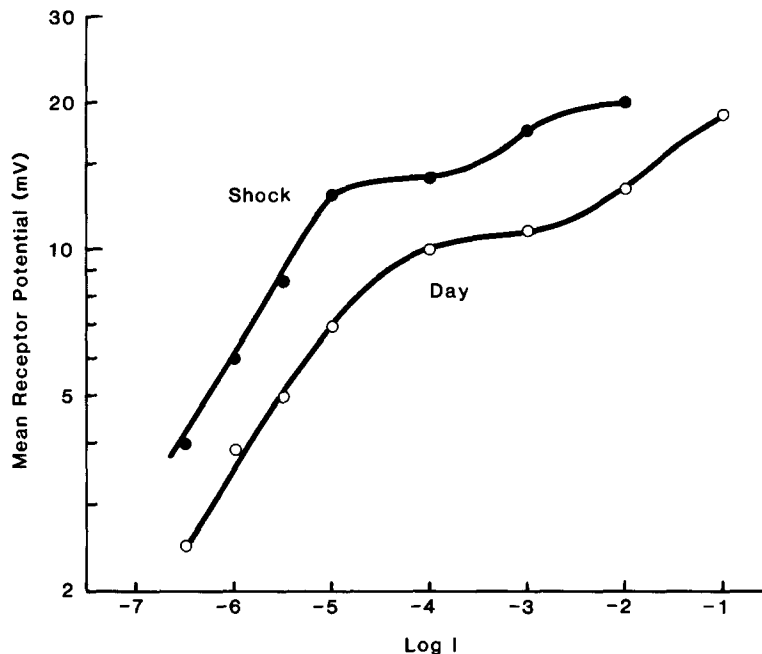


FIGURE 8. Effect of shock in the optic nerve on the intensity coding characteristics of a reticular cell. The daytime intensity-response function was measured according to the same procedure used in the experiment illustrated in Fig. 3, with the exception that the optic nerve was cut and its distal end was inserted in a suction electrode before the cell was impaled. After measuring the "day" function, current shocks were delivered to the cut nerve for 1 h and the measurements were repeated. The similarity of the resulting "shock" function to the "night" function in Fig. 3 indicates that optic nerve shock can mimic the effects of the endogenous efferent input.

Fig. 7 illustrates a more detailed experiment on the effects of optic nerve shock on photoreceptor noise. The trace at the top shows the effect of a 30-s episode of nerve shock on the rate of spontaneous discrete events recorded from a single dark-adapted reticular cell. The negative voltage artifacts of the current pulses hyperpolarized the membrane potential and darkened the trace, but they did not obscure the discrete events. After ~15 s of nerve shock, the event rate was significantly decreased, and by the end of the 30-s episode, no spontaneous bumps could be detected. However, the transduction mechanism that produced the bumps was still intact, as indicated by the response to the brief test flash 25

s after shock offset. The lower graph plots the number of spontaneous events before and after a 30-s episode of nerve shock for two other experiments identical to that of the top trace. In both experiments, the rate of spontaneous events was reduced by >80%. In all three experiments, the reduction in event rate was rapid and occurred without a detectable change in the resting membrane potential of the cell.

Fig. 8 shows that shocking the cut optic nerve during the day can also convert the intensity coding characteristics of the photoreceptor to the nighttime state. The amplitudes of the steady state component of the receptor potential are plotted as a function of log light intensity before and after current pulses were delivered to the cut optic nerve. Note that the daytime intensity-response function closely matches the one plotted in Fig. 2 and that optic nerve shock changed the position and shape of the function, producing one similar to the nighttime function in Fig. 2.

In summary, cutting the optic nerve abolished the effects of the circadian clock on single photoreceptor cells (Fig. 5), and shocking the cut optic nerve during the day reproduced the physiological properties that characterize the nighttime state of the photoreceptor (Figs. 6–8). Specifically, optic nerve shock decreases noise, increases response, and produces an intensity-response function with a characteristic nighttime shape. We therefore conclude that efferent impulses generated by a circadian clock in the brain mediate the circadian changes in photoreceptor physiology reported in this article.

DISCUSSION

A circadian clock in the brain of *Limulus* modulates lateral-eye sensitivity in part by acting directly on the most peripheral cell in the visual system, the photoreceptor. Although the clock also innervates other retinal cells, we show here that some of the major components of the circadian rhythms listed in Table I originate in the photoreceptor cells.

Consider first the physiological components in Table I: gain, noise, frequency response, and lateral inhibition. Figs. 1 and 2 indicate that the endogenous changes in retinal noise initially detected in optic nerve records (Barlow et al., 1977) originate in the photoreceptors. As discussed below, the changes in response amplitude in Fig. 1 reveal a circadian modulation in retinal gain that originates in the photoreceptors. This is also the case for the daily changes in the frequency response of the retina (Renninger, 1983), which were first detected in optic nerve records (Batra, 1983). On the other hand, the circadian rhythm in the strength of lateral inhibition does not originate in the photoreceptors. Lateral inhibition is a property of the eccentric cells, the second-order retinal cells that generate nerve impulses and transmit them to the brain. Efferent terminals make direct synaptic contact with the axon collaterals that mediate inhibitory interactions among eccentric cells (Fahrenbach, 1985).

The anatomical components in Table I include cell position, aperture, acceptance angle, photon catch, and pigment granules. Every night the cells of an ommatidium—the pigment, retinular, and eccentric cells—undergo endogenous retinomotor movements. The pigment cells shift laterally away from the optic

axis of the ommatidium, and the reticular cells and eccentric cell shift distally toward the tip of the corneal lens. In addition, the rhodopsin-containing rhabdom of each reticular cell expands, moves distally, and folds upon itself, yielding a convoluted structure (Barlow et al., 1980; Chamberlain and Barlow, 1984). These anatomical changes widen the acceptance angle of the ommatidium and maximize the photon catch of each photoreceptor (Barlow et al., 1984a; Chamberlain and Fiacco, 1985). The precise role of pigment granules in photoreceptor function is not clear, and thus the effects of circadian changes in their position are not known.

The only component in Table I not yet considered is membrane turnover. This is a massive metabolic event that involves the renewal of most of the rhodopsin-containing membrane in every photoreceptor cell every day. It is primed by the clock's efferent input and triggered by the first light of dawn (Chamberlain and Barlow, 1979, 1984). The renewal of transducing membrane appears to be a ubiquitous photoreceptor property (La Vail, 1976), but its functional role and effect on photoreceptor response are not yet known. Because we carried out our long-term recordings in darkness, membrane turnover should not have affected the circadian phenomena reported in this article.

To summarize, the physiological components in Table I of noise, gain, and frequency response originate in photoreceptors and serve to enhance their response. The anatomical components originate in all three types of ommatidial cells and combine to increase the photon catch of photoreceptors. Other components either do not affect photoreceptor response (lateral inhibition) or have unknown effects (pigment granules and membrane turnover).

Model of Circadian Changes in Photoreceptor Responses

Fig. 9 presents a scheme of how changes in noise, gain, and photon catch may modulate the steady state encoding characteristics of the photoreceptor cell. Changes in the frequency response do not influence steady state responses and thus are not included in this scheme. Their effects on temporal responses will be reported elsewhere (Batra, R., and R. B. Barlow, Jr., manuscript in preparation).

Noise. The clock's efferent input depresses the rate of spontaneous discrete events that occur in darkness. This result is based on long-term intracellular recordings (Figs. 1 and 2) and on the effects of blocking the efferent input (Fig. 5) and stimulating efferent activity with optic nerve shock (Figs. 6–8). Fig. 9 represents the reduction in photoreceptor noise obtained by subtracting the time-averaged value of the receptor potential in the dark (0.7 mV at $\log I = -\infty$) from the daytime responses. The subtractions, indicated by the downward arrows, yield an intensity-response function (dashed line) at low levels of illumination ($\log I = -8.5$ to -7). Quantum bump noise did not significantly affect the amplitudes of receptor potentials of higher light levels. The dashed line segment above $\log I = -6$ will be discussed below.

This is a first approximation of the effect of noise reduction. Note that subtracting a value of 0.7 mV, which corresponds to 30 events/min, assumes that the clock blocked all spontaneous events at night. As shown in Fig. 2, this was the case only for part of the night.

Photon catch. The nighttime increase in photon catch is a second major

cause of increased retinal sensitivity involving photoreceptors. Nighttime changes in the structure of the retina move the photoreceptor cells distally, compress the light-sensitive rhabdom of the cells against the base of the lens, and dilate an aperture formed by pigment cells between the lens and the photoreceptors (Barlow and Chamberlain, 1980; Barlow et al., 1980). These changes increase

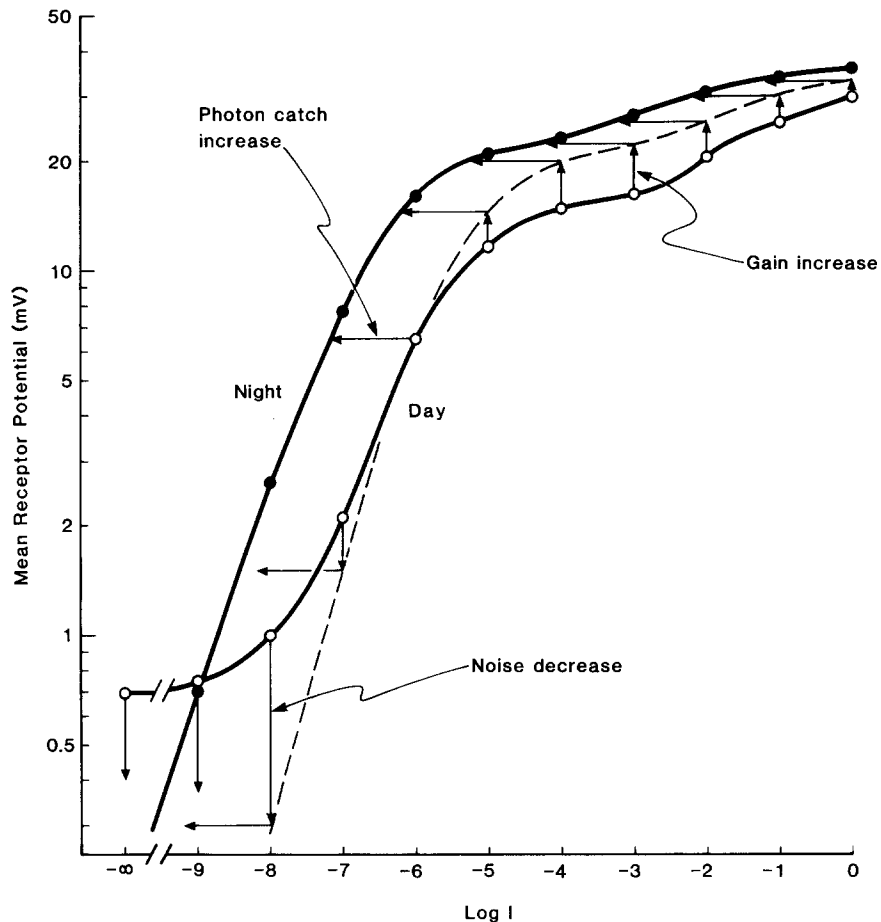


FIGURE 9. Scheme of how circadian rhythms in noise, gain, and photon catch change the intensity coding characteristics of a photoreceptor cell. The intensity-response functions are replotted from Fig. 1. The onset of efferent activity at night causes a rapid increase in gain (upward arrows) and a rapid decrease in noise (downward arrows), yielding an intermediate intensity-response function (dashed line). The intermediate function has not yet been measured in intracellular experiments; however, it has been observed in experiments that recorded responses of single optic nerve fibers (Barlow, R. B., Jr., unpublished observations). After the rapid changes in gain and noise, photon catch (horizontal arrows) increases and moves the intermediate function parallel to the log I axis to produce the nighttime state.

the number of photons incident upon the rhabdom and thus increase retinal sensitivity.

The nighttime increase in photon catch can be measured by the increase in the bump rate at low light levels. For the experiment illustrated in Fig. 1, the bump rate and thus the photon catch increased ~ 16 times (1.2 log units). Catching more photons at every test intensity is equivalent to removing a neutral density filter from the incident light beam. Consequently, we model the increase in photon catch in Fig. 9 by shifting the intensity-response function to the left by 1.2 log units (horizontal arrows). We shifted the function after taking into account the reduction of noise at the lower light levels (dashed line). Note that at these low light levels, the predicted response amplitudes (heads of horizontal arrows) fall short of the actual values. In other words, the combination of noise decrease and photon-catch increase do not account for the entire nighttime transition of the receptor potential. This discrepancy may result from a circadian change in the shape of quantum bumps (Kaplan et al., 1986). At the higher light levels ($\log I \geq -6$), we model the photon-catch increase by placing the heads of the horizontal arrows on the nighttime intensity-response function. The tails of the arrows yield the upper limb of the intermediate function (dashed line). The rationale for this approach is discussed below (see *Gain*). The discrepancy between the intermediate and daytime functions appears to result from a change in photoreceptor gain.

The 1.2-log-unit increase in photon catch in Fig. 9 was based on the rates of light-evoked quantum bumps. Similar changes were observed in measurements of optic nerve and ERG responses. Recordings from single optic nerve fibers indicated that the nighttime decrease in absolute threshold for eliciting one or more nerve impulses with brief flashes of light is 1.3 log units (Westerman, L. A., and R. B. Barlow, Jr., unpublished observations). Also, the clock's input shifts the ERG intensity-response function ~ 1.5 log units to the left on the intensity axis (Barlow, 1983). Taken together, the optic nerve and ERG results indicate that the efferent input to the eye increases the photon catch of single ommatidia within a range of 5–30 times (Barlow 1983; Barlow et al., 1985). This range is consistent with calculations of photon catch based on the optical properties of ommatidia (Chamberlain and Fiacco, 1985).

Gain. The clock's input increases the response amplitude over the entire operating range of the photoreceptor, with particularly large increases at intermediate light intensities. Such increases yield changes in the shape of the receptor potential in Fig. 1 that appear to result from cellular changes other than those of photon catch (refer to text describing Fig. 1). Specifically, they appear to result from an increase in membrane depolarization per absorbed photon, which we define as gain.

In Fig. 9 we attribute the difference between the daytime function and the nighttime function after adjustment for photon catch (dashed line from $\log I = -5$ to 0) to an increase in photoreceptor gain. We represent the gain increase by upward-pointing arrows. Over this 5-log-unit range, the gain change yields an ~ 5 -mV increment in membrane depolarization. The rapid increase in gain and the equally rapid decrease in noise after the onset of efferent activity produced

an intermediate intensity-response function (dashed line) that passed through the heads of the downward-pointing arrows (noise decrease) and the heads of the upward-pointing arrows (gain increase). We have not yet measured the intermediate response function from intracellular experiments; however, it has been observed in experiments that record optic nerve responses (Barlow, R. B., Jr., unpublished observations).

Origin of Circadian Rhythm in Photoreceptor Noise

The circadian rhythm in photoreceptor noise shown in Fig. 2 is one of the most intriguing results of our studies of the *Limulus* eye. We do not know how the clock modulates photoreceptor noise, mainly because the source of the noise itself has not been identified. We shall consider several possible sources, using the schematic diagram of the basic steps in the transduction process in Fig. 10. In the initial step, a photon isomerizes rhodopsin, converting the molecule to an active form (R^*), which then triggers an enzyme cascade that amplifies the weak photon event (Cone, 1973). The last stage of the cascade releases an internal transmitter that increases the conductance of the photoreceptor membrane (Δg). In the final step, R^* inactivates to a thermally stable form of metarhodopsin, the enzyme cascade is turned off, and the membrane conductance returns to the resting state. The net result is a discrete change in membrane potential, the quantum bump. The basic steps are similar to those determined for vertebrate photoreceptors, with the exception that metarhodopsin is unstable in rods and cones (for review see Stryer, 1986).

"Noise" in this transduction process is the production of a quantum bump in the absence of photon absorption. It may occur in several ways: (1) thermal energy may isomerize rhodopsin and trigger the same sequence initiated by photon absorption (Srebro and Behbehani, 1972); (2) R^* may be created by a reversal of the inactivation reaction that produces metarhodopsin (Lamb, 1981; Lisman, 1985); or (3) an enzymatic stage in the amplification cascade may activate spontaneously. All three events would mimic in part the action of light. Is one of them the source of photoreceptor noise?

Past studies on excised lateral eyes have shown that the rate of spontaneous events can be modulated by changes in temperature (Adolph, 1968; Srebro and Behbehani, 1972). Both studies suggest that the spontaneous events result from the thermal isomerization of rhodopsin. Srebro and Behbehani (1972) further suggest that the activation energy measured from the function relating the event rate to temperature change (the Arrhenius relationship) corresponds to the energy barrier separating the *cis* and *trans* isomers of rhodopsin. Baylor et al. (1980) provide a similar interpretation for the source of spontaneous current bumps in vertebrate photoreceptors. Although such interpretations may be correct, it should be noted that the Arrhenius relationship demonstrates only the temperature dependence of the rate of a reaction; it does not necessarily identify the reaction or provide a measure of the height of a specific energy barrier. In a process as complex as visual transduction, any or all of the component steps may be affected by changes in temperature.

In recent studies of the *Limulus* median eye, Lisman (1985) provides evidence

for the production of spontaneous events from the conversion of metarhodopsin back to R^* . He found that a high event rate in darkness correlated with a high concentration of metarhodopsin and that the dark rate declined as metarhodopsin was completely inactivated, presumably by multiple phosphorylations. If this is the case, perhaps the clock's input to lateral-eye photoreceptors speeds up the inactivation of metarhodopsin and thereby lowers the rate of spontaneous events. We carried out a preliminary test of this idea by occluding one lateral eye in each of two animals maintained in dim light for >1 mo. The rationale was that the occluded eye would not produce significant amounts of metarhodopsin, and that after 1 mo in darkness, all metarhodopsin would be converted by metabolic processes to rhodopsin (Lisman and Sheline, 1976). Consequently, if metarho-

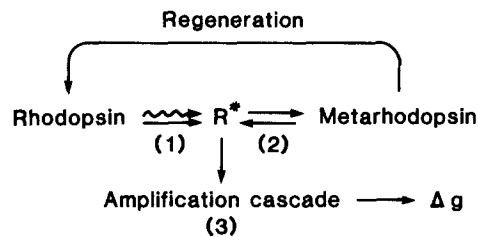


FIGURE 10. A schematic diagram of the transduction process, indicating possible sources of spontaneous quantum bumps. In the initial step of photoexcitation, rhodopsin is isomerized by an absorbed photon to produce the active form, R^* . Rhodopsin may also be isomerized in the dark by thermal energy to produce R^* , as indicated by step 1. In the next step, R^* triggers an amplification cascade, which, judging from vertebrate studies, involves the activation of several enzymes, the last of which releases an internal transmitter that increases membrane conductance (Δg) to produce a quantum bump. The process may be replicated in part by the spontaneous activation of an intermediate step in the cascade, as indicated by step 3. In the final step in the transduction process, R^* is converted to inactive metarhodopsin, but, before complete inactivation, metarhodopsin may spontaneously revert to R^* (Lamb, 1981; Lisman, 1985) and trigger a dark bump, as indicated by step 2. In sum, at least three possible pathways exist that could produce a quantum bump in the dark.

dopsin is the source of spontaneous discrete events, then few if any events would occur in photoreceptors of the occluded eye after 1 mo. However, in the two animals we tested, the occluded eye yielded the same level of spontaneous optic nerve activity as the unoccluded eye. Also, the levels of spontaneous activity recorded from both eyes were characteristic of dark-adapted eyes recorded in previous studies (Kaplan and Barlow, 1975). Although the conversion of metarhodopsin to R^* appears to be a source of noise in median-eye photoreceptors, its role in lateral-eye photoreceptors is not clear.

Another possible source of spontaneous events is the amplification cascade. In their studies of vertebrate photoreceptors, Baylor et al. (1980) reasoned that if intermediate enzymatic steps in the cascade were sources of noise, the resulting spontaneous changes in membrane conductance would be smaller than the light-

induced changes. This may explain the constant low-amplitude noise recorded by Baylor et al. from rods. Regarding *Limulus* photoreceptors, Lisman (1985) reports that spontaneous discrete events in cells of the median eye are smaller than light-evoked potentials, and the same has been found for ventral photoreceptors (Yeandle and Spiegler, 1973; Bayer and Barlow, 1978). However, as we report in another article, light-driven and spontaneous events in lateral-eye photoreceptors are the same size. Thus, we have no evidence that points to the amplification cascade as a source of photoreceptor noise.

Perhaps spontaneous events have more than one source, as suggested by Adolph (1968) and Lisman (1985). If this is the case, then all the sources are sensitive to changes in temperature and all are blocked by synaptic input from the circadian clock. By what mechanism can the clock's input block the effects of thermal energy without influencing those initiated by light? This remains a key question. Perhaps two mechanisms can generate discrete events: one is triggered by light and is quiet in the dark; the other is sensitive to both temperature and efferent input and is noisy in the dark. Whatever the answer, it is clear that neural signals from the circadian clock act at an early stage in the transduction process.

Origin of Circadian Rhythms in Photoreceptor Gain

The clock's input increases the amplitude of the plateau in the receptor potential at intermediate light intensities (Figs. 1 and 3). We propose that this increase in the steady state response represents an increase in photoreceptor gain, the depolarization per absorbed photon. Little is known about the ionic mechanisms underlying the response waveform of lateral-eye reticular cells, but these cells share many physiological properties with ventral photoreceptors (Bayer and Barlow, 1978), which have been studied extensively. Light increases the membrane conductance of ventral photoreceptors to Na ions, producing a large, rapid, transient depolarization of the membrane potential (Millecchia and Mauro, 1969). After the transient changes, other ionic conductance changes may occur to shape the waveform of the receptor potential (O'Day et al., 1982). These conductance changes are voltage dependent (Lisman et al., 1982). Their magnitude and time course depend on the incident light intensity and state of adaptation of the cell.

Voltage-dependent K^+ channels produce a delayed rectifying current that reduces membrane depolarization, producing the steady state plateau of the receptor potential (Pepose and Lisman, 1978). These channels prevent saturation of the receptor potential, compressing the intensity-response function into a small voltage range. They are not activated until the membrane voltage becomes more positive than -40 mV. A voltage-dependent Ca^{2+} conductance can also desensitize ventral photoreceptors during the plateau phase, particularly when the cells are dark-adapted (O'Day et al., 1982). Both voltage-dependent conductances can be viewed as neural mechanisms of light adaptation that reduce photoreceptor gain.

If the reticular cell in the lateral eye possesses similar voltage-dependent conductances, then the clock's input may elevate the response by reducing the

efficacy of the voltage-dependent mechanisms. The proposed gain increase in Fig. 9 occurs for receptor potentials that exceed 10 mV depolarization, which corresponds to a membrane voltage more positive than -40 mV (see the legend to Fig. 1). This is the membrane voltage that activates the voltage-dependent K^+ channels. Perhaps the clock's input partially blocks these channels or the voltage-dependent Ca^{2+} channels. Another possibility is that the efferent input increases the conductance of the light-sensitive Na channels. Whatever the case may be, it appears plausible that the clock modulates the photoreceptor response in part by influencing ionic mechanisms that shape the receptor potential.

Circadian changes in photoreceptor noise and gain may be mediated by the same synaptic mechanism. Octopamine has been identified in the efferent fibers innervating the ventral photoreceptors of *Limulus* (Battelle et al., 1982; Evans et al., 1983). Exogenous octopamine can partially mimic the clock's action in the lateral eye (Kass and Barlow, 1980, 1984). Preliminary experiments with the lateral eye indicate that octopamine activates adenylate cyclase in reticular cells, producing the second messenger cAMP, which mediates the rapid changes in photoreceptor noise and gain reported here (Kass et al., 1984; Pelletier et al., 1984). An additional transmitter(s) may act with octopamine to produce the slow structural changes that increase photon catch (Kass and Barlow, 1984).

Finally, we note that the striking circadian rhythms in photoreceptor sensitivity reported in this article appear to play an important role in the animal's behavior (Barlow et al., 1982). Field studies show that male *Limuli* use visual cues to help them locate and mate with females near the water's edge (Barlow et al., 1983, 1984b, 1986a). Since a significant portion of this activity takes place at night (Lockwood, 1870; Barlow et al., 1986b), the circadian increase in retinal sensitivity may enhance the ability to find mates under the dim illumination of the nighttime sky.

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