Circadian Rhythms in the Green Sunfish Retina

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ABSTRACT We investigated the occurrence of circadian rhythms in retinomotor movements and retinal sensitivity in the green sunfish, *Lepomis cyanellus*. When green sunfish were kept in constant darkness, cone photoreceptors exhibited circadian retinomotor movements; rod photoreceptors and retinal pigment epithelium (RPE) pigment granules did not. Cones elongated during subjective night and contracted during subjective day. These results corroborate those of Burnside and Ackland (1984. *Investigative Ophthalmology and Visual Science*. 25:539–545). Electroretinograms (ERGs) recorded in constant darkness in response to dim flashes (λ = 640 nm) exhibited a greater amplitude during subjective night than during subjective day. The nighttime increase in the ERG amplitude corresponded to a 3–10-fold increase in retinal sensitivity. The rhythmic changes in the ERG amplitude continued in constant darkness with a period of ~24 h, which indicates that the rhythm is generated by a circadian oscillator. The spectral sensitivity of the ERG recorded in constant darkness suggests that cones contribute to retinal responses during both day and night. Thus, the elongation of cone myoids during the night does not abolish the response of the cones. To examine the role of retinal efferents in generating retinal circadian rhythms, we cut the optic nerve. This procedure did not abolish the rhythms of retinomotor movement or of the ERG amplitude, but it did reduce the magnitude of the nighttime phases of both rhythms. Our results suggest that more than one endogenous oscillator regulates the retinal circadian rhythms in green sunfish. Circadian signals controlling the rhythms may be either generated within the eye or transferred to the eye via a humoral pathway.

INTRODUCTION

Circadian rhythms in retinal morphology and physiology have been detected in many visual systems. They appear to be characteristic of invertebrate visual systems (e.g., see Jacklet et al., 1982; Barlow, 1983), but a number of examples exist for vertebrates as well. A circadian clock participates in controlling the disk-shedding process of rods in rat retinas (LaVail, 1976), the functional dominance of rods and cones in pigeon retinas (Barattini et al., 1981), and the activity of

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serotonin *N*-acetyltransferase in chicken and *Xenopus* retinas (Hamm and Menaker, 1980; Besharse and Iuvone, 1983). These rhythms persist in constant darkness with a "free-running" period of ~24 h.

A circadian oscillator also influences the retinomotor movements of teleost photoreceptors (John et al., 1967; Ali, 1975; Levinson and Burnside, 1981; Douglas and Wagner, 1982). In green sunfish, *Lepomis cyanellus*, photoreceptors and pigment granules of the retinal pigment epithelium (RPE) undergo movements in response to both light and circadian signals (see reviews by Burnside and Nagle, 1983; Burnside and Dearry, 1986). Light onset induces cone myoid contraction, rod myoid elongation, and pigment granule dispersion. Light offset induces opposite movements. In green sunfish kept in constant darkness, only cones continue to move, elongating in a scleral direction during subjective night and contracting in a vitreal direction during subjective day. Rods and RPE pigment remain stationary in their dark-adapted positions during constant darkness (Burnside and Ackland, 1984). Such retinomotor movements may change the amount of light absorbed by the photoreceptors and thereby change the retinal sensitivity. If so, the circadian rhythm in cone movement should produce a corresponding rhythm in the retinal sensitivity in fish maintained in constant darkness.

We therefore examined the possible contribution of circadian retinomotor movements to retinal sensitivity in green sunfish. Electroretinogram (ERG) responses were recorded from green sunfish maintained in constant darkness to test for circadian changes in retinal sensitivity. Since only cones exhibit circadian retinomotor movement in this species, we examined the spectral sensitivity of the ERG for evidence of changes in cone responses during subjective day or night. To evaluate the possible role of efferent input, we tested the effect of optic nerve section on both ERG responses and cone retinomotor movement. We report here that the ERG amplitude of the green sunfish does indeed exhibit a circadian rhythm. After optic nerve section, the circadian rhythms of the ERG amplitude and of cone retinomotor movement are damped but not blocked. Our results suggest that more than one circadian mechanism modulates retinal sensitivity and that the circadian signals regulating retinal sensitivity and cone retinomotor movement are either generated within the eye or transmitted to the eye by a humoral agent.

MATERIALS AND METHODS

Experimental Animals

Experiments were conducted on green sunfish, *Lepomis cyanellus*. In vivo, these fish exhibit extensive cone, rod, and RPE pigment retinomotor movements (Burnside and Ackland, 1984). The fish used in these experiments were 8–10 cm in overall length. They were maintained in aerated filtered tap water (~18°C and pH ~5) under a natural light/dark cycle (dawn, ~6 a.m.; dusk, ~7 p.m.) for at least 3 wk before being used.

Electroretinography

ERGs were recorded from green sunfish following the method of Barlow (1985). A fish was briefly anesthetized with tricane methanesulfonate (Ayerst Chemical Co., New York)

and then spinalized ~1 cm rostral to the dorsal fin by inserting and withdrawing a 22-gauge syringe needle. The fish was then immobilized in a Plexiglas holder by inserting four pairs of pins in the musculature along the length of the body (see Fig. 1). The fish-holder was immersed in an aquarium located in a light-proof, shielded cage so that the fish's eyes remained above the water level. Fish recovered from anesthesia and respired

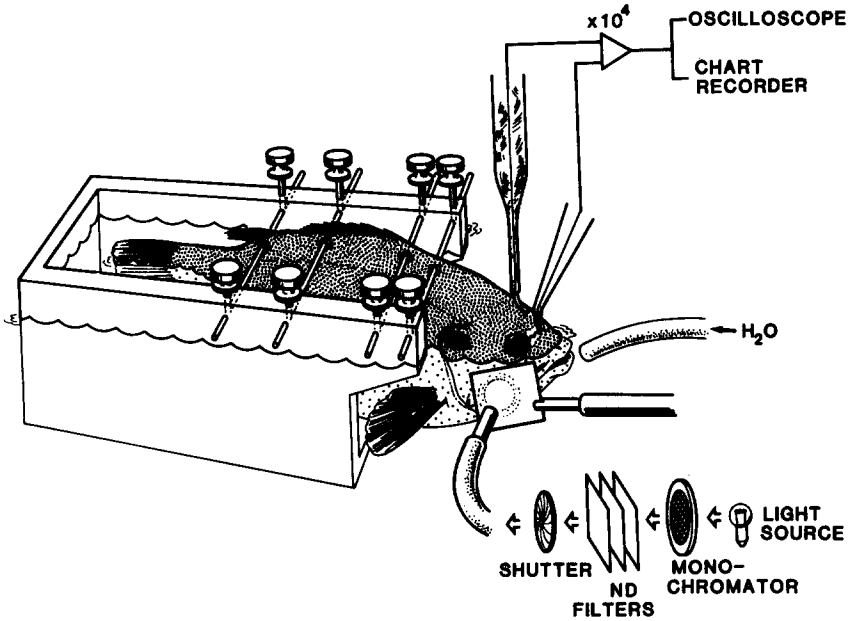


FIGURE 1. Experimental arrangement of fish and ERG stimulation and recording apparatus. A fish was first lightly anesthetized and then spinalized ~1 cm rostral of the dorsal fin. The fish was immobilized in a Plexiglas holder by the parallel insertion of four pairs of pins along the length of the body. The fish-holding apparatus was placed in an aquarium so that water circulated through the fish's mouth and over the gills, but the eyes remained above the water level. An oscillating pump delivered freshly aerated water directly in front of the fish's mouth. Fine tubing attached to a peristaltic pump delivered a slow stream of water to the fish's head to prevent drying (not shown). Light stimuli were projected onto a white Teflon screen (shown reduced in size) ~1 cm in front of the fish's right eye by a large fiberoptic bundle. This technique produced a diffuse uniform illumination of the entire eye. The ERG was recorded differentially between a conductive thread electrode contacting the cornea and a wick electrode contacting the body of the fish. These electrodes were connected to a differential amplifier (gain, 10^4; bandpass, 3–300 Hz). The amplifier output was displayed on an oscilloscope and a chart recorder.

independently. Thus, they were not anesthetized during the course of an experiment. However, since fish were immobilized, filtered aerated water was delivered in front of the mouth to assist ventilation. Water was continuously recirculated by an oscillating pump (model 11968-000, Gorman-Rupp Industries, Bellville, OH) and flowed out of a tube ~1 cm in front of the fish's mouth at a rate of ~500 ml/min. Previous work (Hoffert and Ubels, 1979*a*) had shown that the amplitude of the ERG b-wave recorded in vivo

from dark-adapted trout at 20°C was insensitive to changes in the ventillatory flow rate between 50 and 625 ml/min. To prevent drying of the exposed area of skin, a slow stream of water was directed to the dorsal surface of the animal through fine tubing attached to a peristaltic pump (junior cassette model, Manostat Corp., New York; not shown in Fig. 1). Fish could be maintained in a viable condition in this system for 48 h.

The ERG was recorded between a conductive thread electrode (Dawson et al., 1979) in contact with the cornea and an indifferent electrode in contact with the skin near the eye. These electrodes, together with a ground electrode immersed in the aquarium, were connected to a differential amplifier (gain, 10^4; bandpass, 3–300 Hz; Electronics Laboratory, The Rockefeller University, New York). The ERG amplitude was defined as the potential difference between the trough of the a-wave and the peak of the b-wave. In all experiments, fish remained in constant darkness throughout the recording period except for brief flashes presented periodically to elicit ERGs. On several occasions, movements of the head resulted in baseline noise and disruption of ERG recordings. These movements may have represented animal discomfort. Consequently, these experiments were immediately terminated and the fish was killed.

Light stimuli from a quartz fiberoptic bundle were projected onto a white Teflon diffusing screen (2 × 2 cm) placed ~1 cm in front of the right eye. The size of the screen is reduced in the drawing in Fig. 1 to avoid obscuring the eye and electrode. This technique produced a diffuse uniform illumination of the entire eye. The other end of the fiberoptic bundle was plugged into either a tungsten or xenon light source. The output of the tungsten lamp passed through a Bausch & Lomb (Rochester, NY) model 2 high-intensity visible monochromator (grating no. 33-86-76, 350–800 nm, 19.2-nm bandwidth). The output of the xenon lamp passed through an Oriel Corp. (Stamford, CT) ultraviolet-visible monochromator (grating no. 7242, 200–700 nm, 10-nm bandwidth). The latter equipment was used for spectral sensitivity studies. Monochromatic light then passed through calibrated quartz neutral density (ND) filters (Oriel Corp.) and a Uniblitz shutter (Vincent Associates, Rochester, NY) before being focused with a quartz lens onto the end of the fiberoptic bundle. The intensity of illumination at the cornea was measured for each light source and test wavelength with a calibrated silicon photodiode (PIN 10UV, United Detector Technology, Santa Monica, CA). The time interval between stimuli was controlled by a computer (AIM-65, Rockwell International, El Paso, TX), and the stimulus duration was controlled by a pulse generator (model G88, Grass Instrument Co., Quincy, MA). Unless specified otherwise, the light stimuli used to elicit ERGs were 10 ms in duration and had a wavelength of 640 nm. This wavelength was chosen in order to maximize cone contribution to the ERG since only cones exhibit circadian retinomotor movements in green sunfish. However, it seems probable that our ERG responses contain contributions from both cones and rods. Responses were displayed on an oscilloscope (model 502A, Tektronix, Inc., Beaverton, OR) and chart recorder (Brush model 220, Gould, Inc., Cleveland, OH).

Microspectrophotometry

Visual pigments in green sunfish photoreceptors were characterized with a photon-counting microspectrophotometer (PMSP) (MacNichol, 1978). After 45 min of dark adaptation, fish were killed, their eyes were enucleated and hemisected, and the retinas were isolated from eyecups under infrared illumination as previously described (Dearry and Burnside, 1984). A small piece of retinal tissue (~1 mm^2) was cut and transferred in a Ringer solution to a coverslip. The tissue was surrounded by a ring of silicone oil and another coverslip was placed on top (see Collins and MacNichol, 1984). This coverslip sandwich was positioned in the PMSP. Under infrared illumination, the outer segments

of various photoreceptor types were located and transverse absorption spectra were recorded (see Levine and MacNichol, 1979).

Measurement of Retinomotor Positions

Burnside and Ackland (1984) reported that cones undergo circadian retinomotor movements in one population of green sunfish, but Eckmiller and Burnside (1983) failed to detect circadian cone movements in another population. Both of these investigations maintained fish on an artificial 12-h-light/12-h-dark cycle in the laboratory, whereas we maintained fish under ambient natural lighting. In view of these differences, we thought it necessary to examine the occurrence of circadian retinomotor movements in the population of green sunfish used for our electrophysiological experiments.

For these experiments, fish were placed in constant darkness at 6 p.m. and two fish were killed at each of several time points over a 32-h period. Under infrared illumination, eyes were removed and hemisected, and the posterior eyecups were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. A central region of each eyecup dorsal to the optic nerve was removed and postfixed in 1% OsO_4, dehydrated in ethanol, embedded in Epon, and sectioned into 2-μm sections (see Burnside and Ackland, 1984). Sections were stained with 0.5% toluidine blue and examined by bright-field light microscopy. These plastic-embedded sections were required in order to visualize rod inner segments.

In other experiments in which rod retinomotor movements were not analyzed, glutaraldehyde-fixed central regions of posterior eyecups were chopped into ~20-μm-thick slices as previously described (Dearry and Burnside, 1984). These slices were then examined under a light microscope using Nomarski interference contrast optics.

All measurements of retinomotor positions were made using an ocular micrometer. The cone myoid length, the distance (in microns) between the outer limiting membrane (OLM) and the base of a cone ellipsoid, was determined for 20 representative cells in each retina. The rod inner segment length, the distance (in microns) between the OLM and a rod-connecting cilium, was recorded for 20 representative cells in the shortest rod tier in each retina. The pigment index, the percentage of the distance from Bruch's membrane to the OLM occupied by pigment granules, was measured in 10 positions per retina. Data are presented as means ± standard error of the mean, where n refers to the number of fish. The significance of the difference between means was determined using a two-tailed Student's t test.

RESULTS

Circadian Rhythm of Cone Retinomotor Movement

Cones of green sunfish kept in constant darkness underwent circadian retinomotor movements. Cones contracted during subjective day and elongated during subjective night (Fig. 2). Rods and RPE pigment remained in their dark-adapted retinomotor positions throughout the period of constant darkness. Our results thus substantiate those of Burnside and Ackland (1984) and demonstrate that cone retinomotor movement in green sunfish possesses a circadian rhythm.

Circadian Rhythm of the ERG Amplitude

The amplitude of the ERG recorded from green sunfish kept in constant darkness was higher during subjective night than during subjective day. Representative ERGs recorded from a sunfish eye in situ in response to a 640-nm flash of 10 ms duration are shown in Fig. 3. The ERG appeared after a latency of ~100 ms

from the onset of the flash and consisted of a small corneal-negative potential (a-wave) followed by a large corneal-positive deflection (b-wave). The b-wave response recorded at night (11 p.m.) was approximately fourfold larger than that recorded at midafternoon (4 p.m.) (Fig. 3).

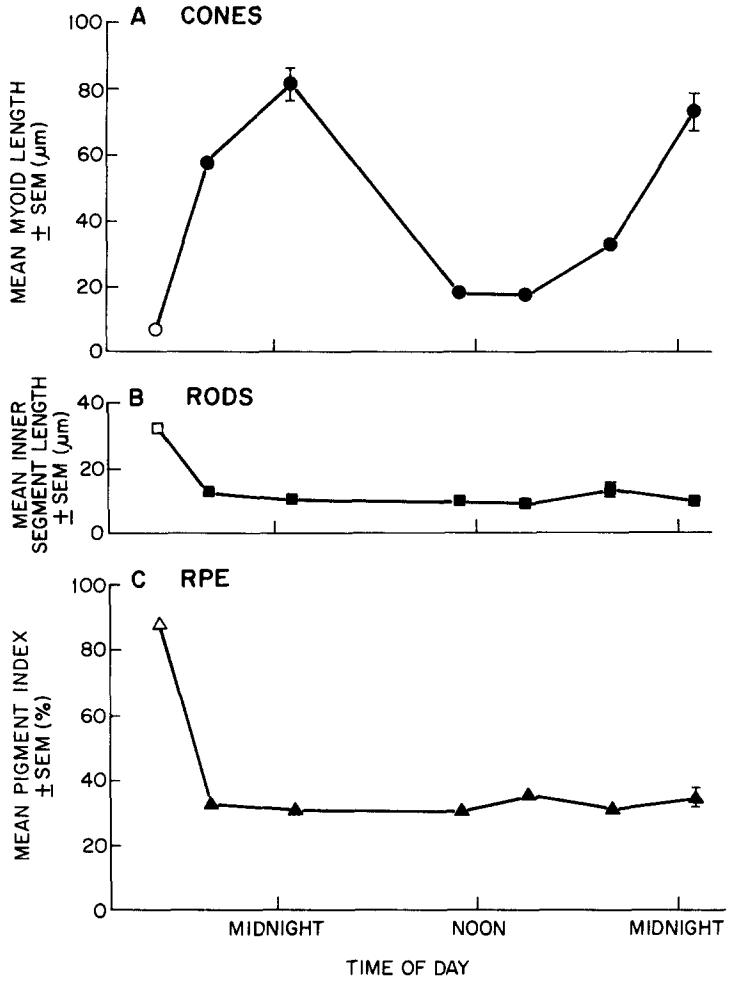


FIGURE 2. Circadian rhythm of cone, but not rod or RPE, retinomotor movement in green sunfish maintained in constant darkness. Control fish were killed 1 h before light offset (open symbols). Experimental fish were kept in constant darkness beginning at 6 p.m. Two fish were killed at each of the indicated times. Manipulations on dark fish were performed with the aid of an infrared image converter. The retinal cones of fish kept in constant darkness (filled circles in *A*) contracted during subjective day and elongated during subjective night. Rods (filled squares in *B*) and RPE pigment (filled triangles in *C*) remained in their dark-adapted retinomotor positions throughout the period of constant darkness.

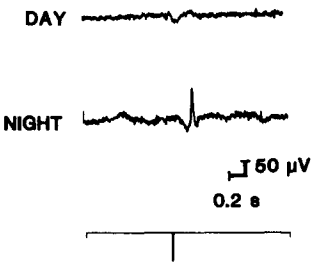


FIGURE 3. ERGs recorded from a green sunfish during subjective day (4 p.m.) and subjective night (11 p.m.). The fish remained in constant darkness. The stimulus monitor (bottom trace) indicates a 10-ms light flash (downward deflection). The amplitude of the ERG b-wave (upward deflection) elicited by a constant-intensity stimulus was greater during subjective night than subjective day.

These changes in ERG amplitude exhibited an endogenous rhythm with a period of ~24 h. ERG responses recorded from a fish over a 48-h period of constant darkness are shown in Fig. 4. On the first day of the experiment, the ERG amplitude began to increase at 6 p.m., reached a maximum level about midnight, remained elevated until 7 a.m., and then began to decrease to a low daytime level. The cycle continued on the second day when the amplitude again began to increase at about 6 p.m. The maximum amplitude was higher during the second night, and the ERG amplitude again declined around 8 a.m. The

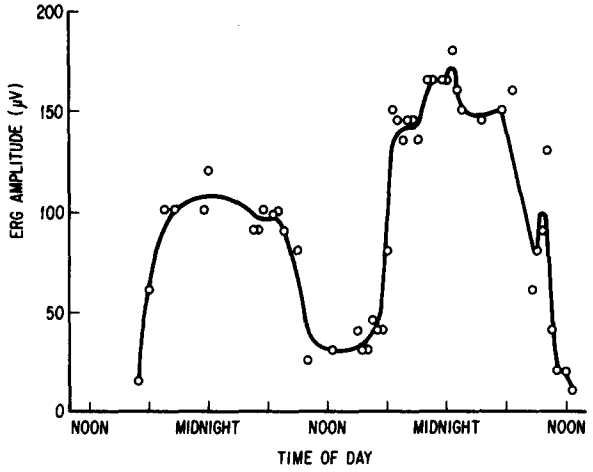


FIGURE 4. Circadian rhythm of ERG b-wave amplitude. A fish was maintained in constant darkness except for 10-ms light stimuli of constant intensity. The response amplitude increased at expected dusk, remained high during subjective night, decreased at expected dawn, and was relatively low during subjective day. The solid line in this and subsequent ERG vs. time graphs was generated by a smoothing algorithm implemented by convolution with weights obtained from a low-pass Blackman window having a cutoff frequency of 0.3 (see Blackman and Tukey, 1958). This operation eliminates all frequencies with periods shorter than 100 min.

recording was lost shortly after noon of the second day. Similar free-running rhythms were recorded for periods of 2 d from four fish and for shorter periods from five fish.

This pattern of high ERG amplitude during the night and low amplitude during the day was observed in all experiments. However, most fish also exhibited a secondary rise in ERG amplitude near subjective midday. Examples of this phenomenon recorded from three different fish are shown in Fig. 5. The secondary peak occurred from about 10 a.m. to noon. It was equal in amplitude to that recorded during the preceding night for the fish in *A* but not for the fish in *B* or *C*. After this midday peak, the response amplitude fell to a lower level during the remaining subjective day.

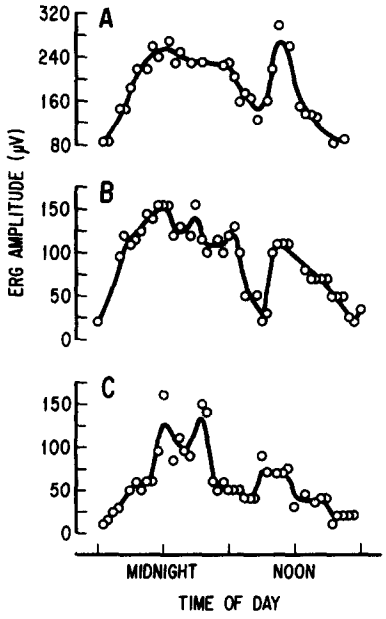


FIGURE 5. Secondary midday peak in ERG circadian rhythm. The figure shows ERGs recorded from three different fish (*A*, *B*, and *C*) over a 24-h period of constant darkness. As in Fig. 4, the response amplitude increased at expected dusk, remained high during subjective night, and decreased at expected dawn. Each of these fish also exhibited a secondary increase in response amplitude around 10 a.m. to noon. The response amplitude thereafter declined to a lower subjective day level.

Circadian Rhythm of Retinal Sensitivity

The above results demonstrate that endogenous changes occur in the ERG amplitude recorded from the dark-adapted sunfish eye. The corresponding changes in retinal sensitivity can be determined from the function that relates response amplitude to light intensity. Fig. 6 plots log ERG amplitude on the ordinate as a function of log light intensity on the abscissa. "Day" data were recorded at 4 p.m.; "night" data were recorded at 1 a.m. Both sets of measurements were carried out with the eye in the dark-adapted state.

The daytime data (open circles) in Fig. 6 were fitted with a smooth curve by eye. The same curve was shifted 0.6 log units to the left and 0.15 log units vertically, approximately overlaying the nighttime data (filled circles). Such a lateral shift to the left is equivalent to removing a 0.6 ND filter from the incident light beam, thereby increasing by a factor of 4 the number of photons absorbed by the photoreceptors. The small vertical shift (0.15 log units) of the intensity-response function corresponds to an ~40% increase in retinal gain (response per absorbed photon).

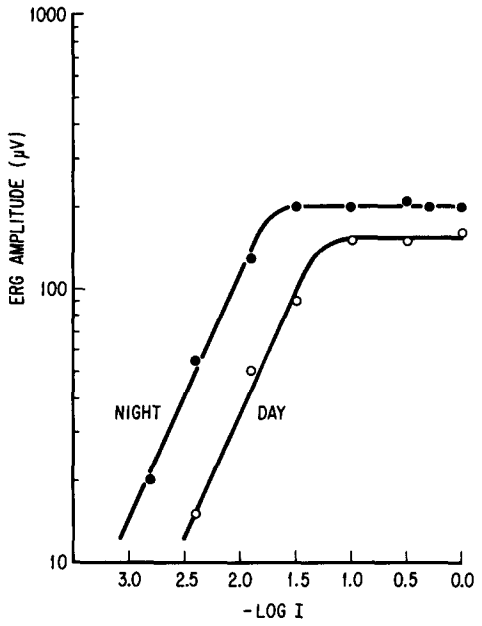


FIGURE 6. Circadian rhythm of retinal sensitivity. Intensity-response functions of the ERG recorded from a fish during subjective night (1 a.m.) and subjective day (4 p.m.). Each response was evoked by a 10-ms flash while a green sunfish was maintained in constant darkness. The ERG b-wave amplitude is plotted on a log scale (ordinate) as a function of log light intensity (abscissa). Both sets of data were fitted by the same curve moved 0.6 log unit on the abscissa and 0.15 log unit on the ordinate. The lateral shift corresponds to a fourfold increase in retinal sensitivity during subjective night.

During both day and night, the ERG amplitude was graded with light intensity over a range of ~1.5 log units. At low levels of illumination, the ERG amplitude was linearly related to test flash intensity. Because in this range a 10-fold increase in intensity produced a 10-fold increase in amplitude, it is possible to use changes in the ERG amplitude in this range as a measure of changes in retinal sensitivity. For example, at $\log I = -2.4$, the ERG amplitude increased from 13 μV during the day to 55 μV at night. Because the response remained within the linear range, we can conclude that the sensitivity of the retina increased fourfold from day to night. Similar experiments with other fish yielded 3–10-fold increases in retinal sensitivity.

ERG Spectral Sensitivity and Photoreceptor Visual Pigments

The above results indicate that retinal sensitivity to a stimulus having a wavelength of 640 nm varies as a function of time of day. Because only cones exhibit endogenous retinomotor movements in green sunfish maintained in constant darkness (Fig. 2), we reasoned that changes in retinal sensitivity might also vary as a function of wavelength. To test this hypothesis, we measured the ERG spectral sensitivity during subjective day and subjective night. We also recorded absorption spectra from green sunfish photoreceptor outer segments to check for possible correlations between visual pigments and spectral sensitivity.

Relative sensitivity was determined from intensity-response functions measured for light flashes over the range of 360–680 nm using a criterion response of 50 μV (see Barlow, 1985). Fig. 7 plots log relative sensitivity as a function of

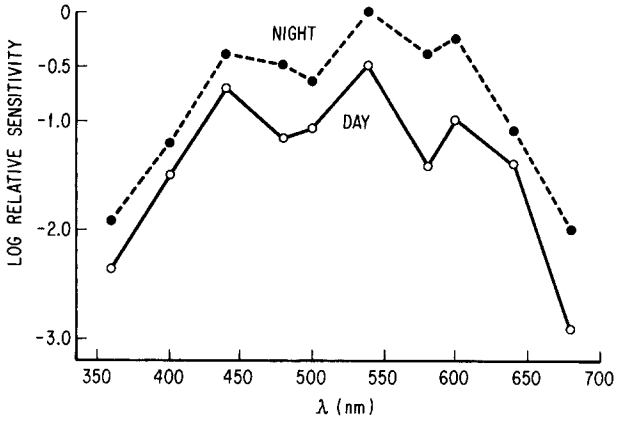


FIGURE 7. Spectral sensitivity of green sunfish ERG recorded during subjective night and subjective day. The sensitivity is the inverse of the relative light intensity of a 10-ms flash that elicited a 50-μV b-wave criterion response. Data were normalized with respect to the response recorded at 540 nm during subjective night. Both night and day curves had peaks at 440, 540, and 600 nm. Most of the difference in day vs. night sensitivity was in the green-to-red region of the spectrum.

wavelength for a dark-adapted sunfish eye during the day and during the night. Both sets of data were recorded from the same fish, which remained in darkness throughout the experiment. The maximal spectral sensitivity occurred near 540 nm both day and night. Secondary peaks were detected at 440 and 600 nm. Sensitivity was greater at night over the entire range of wavelengths tested. The average increase in nighttime sensitivity for all wavelengths tested was 0.6 ± 0.3 log units. Changes in sensitivity were greater in the green-to-red region (550–600 nm) with a 1.0-log-unit increase measured at 580 nm.

The maximal sensitivity at 540 nm may correspond to two visual pigments: one in rods and the other in single cones. Fig. 8*A* shows the absorption spectrum of sunfish rods (λ_{max} = 525 nm), and Fig. 8*C* shows the absorption spectrum of single cones (λ_{max} = 532 nm). We recorded spectra from more than 100 single cones from 10 different retinas. Although single cone outer segments varied from 3 to 6 μm in basal diameter and from 6 to 20 μm in axial length, all

contained the same pigment. No single cones were found that absorbed in the blue or red regions of the spectrum. The secondary peak in ERG sensitivity at 600 nm may be related to the pigment found in double cones. Fig. 8*E* shows the absorption spectrum obtained by averaging spectra from both outer segments of 22 double-cone cells. Each member of the double-cone pairs absorbed in the red with $\lambda_{max} = 621$ nm. Although the spectral sensitivity curve in Fig. 7 contains a secondary peak in the blue at 440 nm, no comparable blue-absorbing pigment was detected in any photoreceptor with microspectrophotometry. Because of the reported small sizes of blue- and ultraviolet-sensitive cones in other fish (e.g., Hárosi and Hashimoto, 1983), we purposefully scanned the outer segments of many small single cones from all retinal regions. However, microspectrophotometry is necessarily a selective assay, and we may have missed a small population of blue-absorbing cones. Alternatively, the 440-nm peak in the ERG spectral sensitivity could be due to a summation of responses generated by the secondary absorption bands of the green and red photopigments. It seems unlikely that green sunfish are sensitive to ultraviolet light since neither PMSP nor ERG recordings detected the presence of a pigment absorbing in the 350–400-nm range.

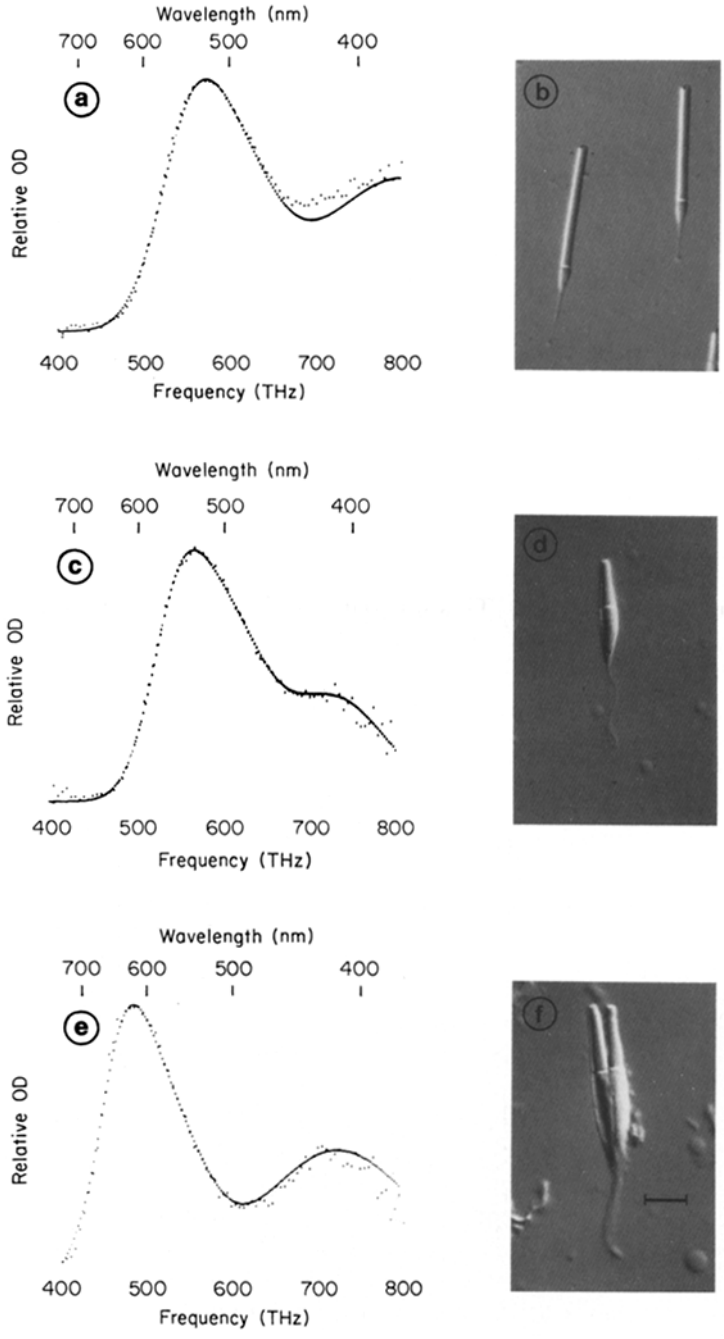
Effect of Optic Nerve Section on the Circadian Rhythm of the ERG Amplitude

Efferent nerve fibers have been identified in the optic nerves of several teleost fish (Ebbesson and Meyer, 1981; Munz et al., 1982; Springer, 1983; Stell et al., 1984). In some invertebrates, the activity of such efferent fibers mediates a circadian rhythm of the ERG amplitude (e.g., in horseshoe crab, Barlow, 1983; in orb weaving spider, Yamashita and Tateda, 1981; in scorpion, Fleissner and Fleissner, 1978). To test whether efferent fibers to the retina also have a role in producing the circadian rhythm of the ERG amplitude in green sunfish, we sectioned the right optic nerve.

After anesthesia, the optic nerve was exposed by cutting the connective tissue around the dorsal half of the eye and gently rotating the eye forward and down. The optic nerve forms an anastomosis with the ophthalmic artery near the eye, but toward the rear of the orbit the two usually separate and the optic nerve can be cut in this region. Accidental puncture of the ophthalmic artery led to rapid, profuse bleeding in the orbit, and the experiment was terminated. After optic nerve section, operated fish were allowed 4 h to recover before recording ERGs as described above.

Sectioning the optic nerve changed the waveform of the ERG response. After the operation, the corneal-negative a-wave was larger and the corneal-positive b-wave was smaller than for intact fish (Fig. 9). Similar waveforms were recorded from three operated fish. Although partial retinal hypoxia cannot be eliminated as a possible cause of this change in waveform, our ERG responses did not resemble those recorded from dark-adapted hypoxic trout by Hoffert and Ubels (1979*b*). Since higher intensities were required to elicit responses from operated fish (see Figs. 9 and 10), it is apparent that sectioning the optic nerve reduced the overall retinal sensitivity. The change in the ERG waveform may reflect the higher light intensities used with the operated animals, but this possibility was not tested.

Sectioning the optic nerve did not abolish the ERG circadian rhythm. The response amplitude was still higher during the night (1 a.m.) than during the day (4 p.m.) (Fig. 9). The ERG amplitude recorded from an operated fish is displayed



as a function of time in constant darkness in Fig. 10 (squares, solid line). Although the other eye could have served as a useful control during these experiments, our apparatus did not permit simultaneous recordings from both eyes. For comparison, we have replotted the data from the intact fish of Fig. 4 (dashed

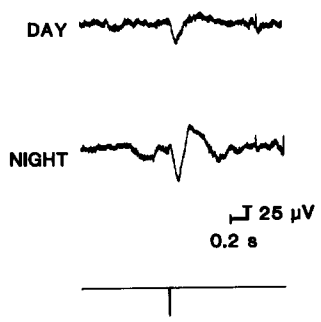


FIGURE 9. ERGs recorded from a green sunfish having a sectioned right optic nerve during subjective day (4 p.m.) and subjective night (1 a.m.). The fish remained in constant darkness. The stimulus monitor (bottom trace) indicates a 10-ms light flash (downward deflection). The amplitude of the ERG b-wave (upward deflection) elicited by a constant-intensity stimulus was greater during subjective night than during subjective day.

line). In both experiments, ERG amplitudes were higher during subjective night than during subjective day. This was true for all three fish with unilateral optic nerve section. It should be noted that the intensity of the light flash used to elicit ERGs from operated fish was 10-fold greater than for intact fish. Such intensities yielded ERGs of about the same amplitude in both cases during the day, but at

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FIGURE 8. (*opposite*) Microspectrophotometry of green sunfish photoreceptor outer segments. Absorption spectra were recorded on the MacNichol (1978) PMSP. (*a*) Rod absorption spectrum. Since rod outer segments in this fish have a diameter of only 2 μm (see *b*), spectra were recorded from a retinal area of massed rod outer segments having the same orientation. Four records were averaged to produce the absorption spectrum indicated. Data were fitted by eye to a template formed by generating the sum of three Gaussian functions (Hárosi, 1976). All absorption curves are normalized and indicate relative density as a linear function of frequency in terahertz (lower abscissa) and as a nonlinear function of wavelength in nanometers (upper abscissa). The rod absorption maximum was 525 ± 1 nm. (*c*) Single-cone absorption spectrum. Spectra from 10 cells such as *d* were averaged to produce the curve shown. Similar spectra were obtained from 107 single cones from 10 retinas. All single cones had an absorption maximum of 532 ± 1 nm. (*e*) Double-cone absorption spectrum. Absorption spectra were recorded from each outer segment member of double-cone pairs. Spectra from 22 cells such as *f* were averaged to produce the curve shown. Similar spectra were obtained from 60 double-cone pairs (i.e., 120 cells) from 10 retinas. Each member of all double-cone pairs had an absorption maximum of 621 ± 1 nm. The bar in *f* is 10 μm and applies to each photomicrograph; all photoreceptors shown were from the same retina.

night the responses from operated fish were always smaller. None of the operated fish exhibited a secondary midday rise in ERG amplitude as seen in Fig. 5.

Effect of Optic Nerve Section on the Circadian Rhythm of Cone Retinomotor Movement

Since the circadian rhythm of the ERG amplitude persisted after unilateral optic nerve section, we tested whether the circadian rhythm of cone retinomotor movement could also survive such treatment. Right optic nerves were cut as described above and, after 4 h, fish were placed in constant darkness. Fish were

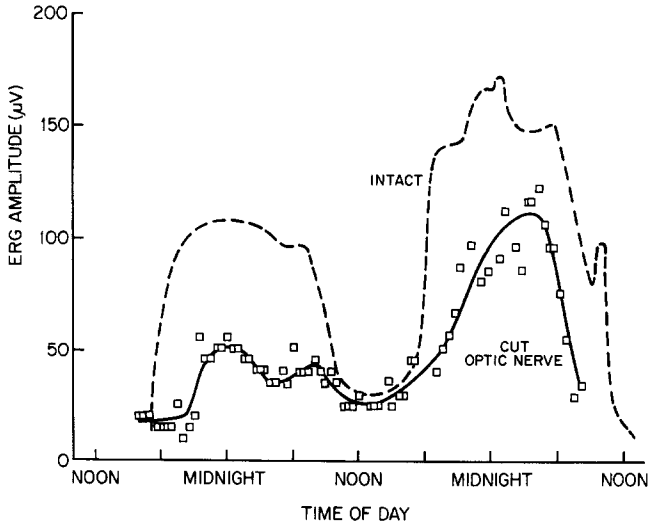


FIGURE 10. Unilateral optic nerve section does not abolish the circadian rhythm of the ERG in green sunfish. The right optic nerve of a fish was cut and responses to 10-ms stimuli were recorded while the fish was in constant darkness (squares with a solid line). Responses from the intact fish of Fig. 4 are shown for comparison (dashed line). The stimulus intensity was 10-fold higher for the fish having a cut optic nerve. Both fish exhibited an ERG circadian rhythm, although the response amplitude of the fish having a cut optic nerve did not increase as much during subjective night as did that of the intact fish.

killed at selected time intervals over a 42-h period. Cone and RPE pigment retinomotor positions were analyzed in both a left eye having an intact optic nerve and a right eye having a cut optic nerve.

Cutting the optic nerve blocked cone elongation during the first hour of darkness (Figs. 11 and 12). Eyes fixed immediately before light offset had a mean cone myoid length of 3.8 ± 0.4 μm (n = 6). After 1 h of darkness, cones of intact eyes elongated to 65.7 ± 7.7 μm (n = 5). However, eyes having a cut optic nerve in the same fish had a mean cone myoid length of 6.6 ± 0.7 μm (n = 5). To rule out possible anesthetic or surgical effects, we performed sham operations; i.e., we executed identical anesthetic and surgical procedures but did not actually cut optic nerves. In these fish, cones of intact and sham-operated eyes elongated to 63.3 ± 5.9 (n = 3) and 61.9 ± 3.2 μm (n = 3), respectively, after 1 h of darkness

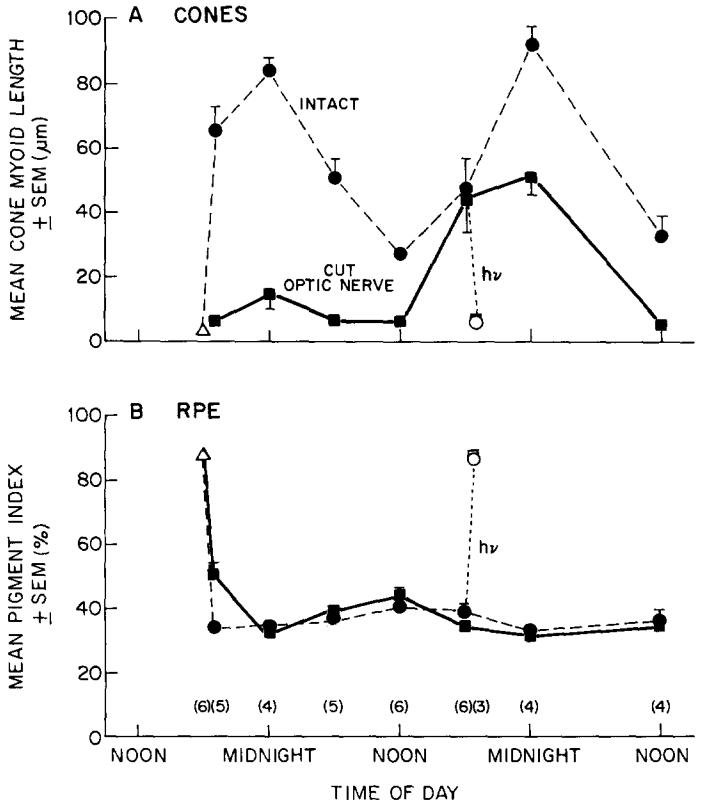
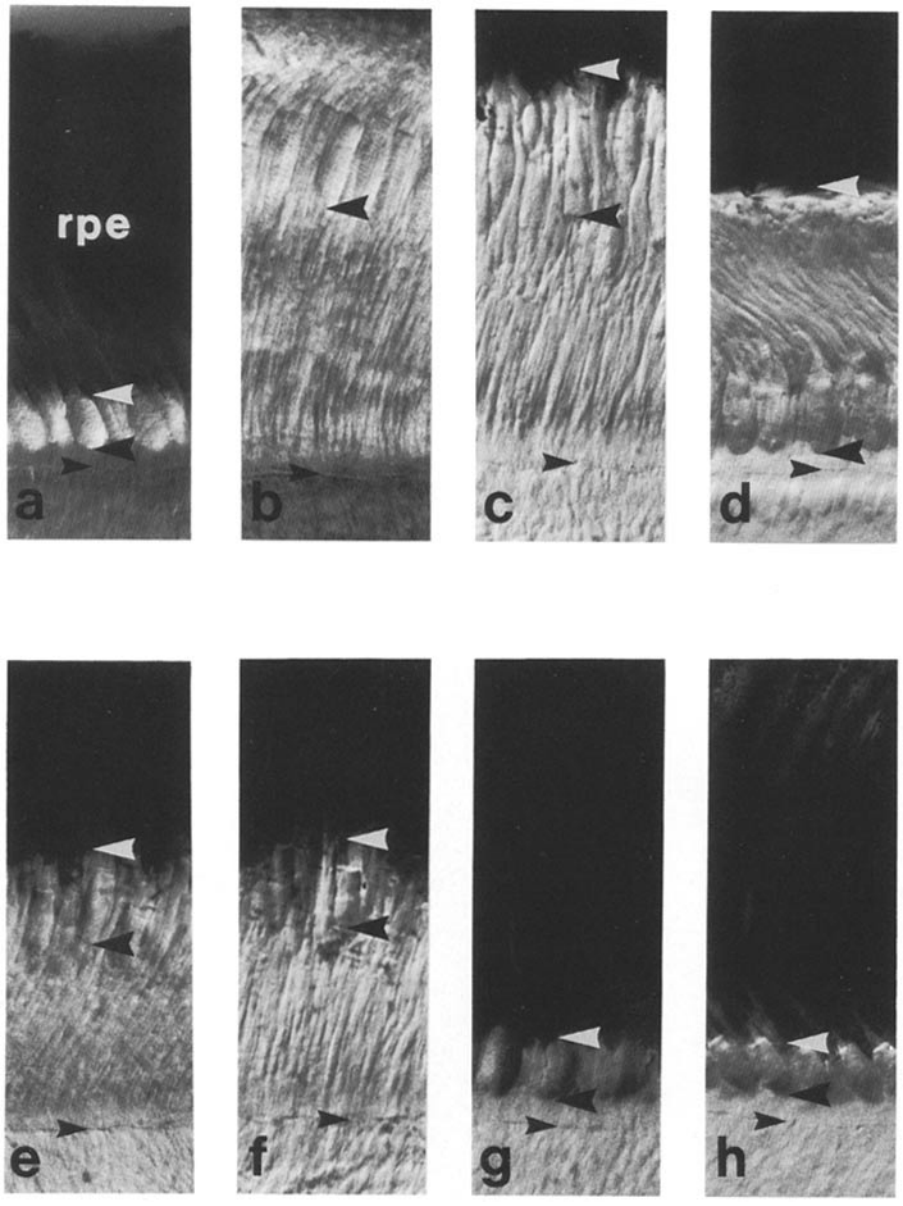


FIGURE 11. Effect of unilateral optic nerve section on circadian- and light-induced cone (*A*) and RPE pigment (*B*) retinomotor movements. Control fish were killed immediately before light offset (open triangles). The right optic nerve of experimental fish was cut ~4–5 h before light offset, and these operated fish were then placed in constant darkness at 6 p.m. At the indicated times, operated fish were killed and eyecups from the left intact eye (circles connected by dashed lines) and from the right cut optic nerve eye (squares connected by solid lines) were fixed under infrared illumination. After 24 h in constant darkness, three operated fish (open circles and squares) were subjected to 1 h of illumination (white light of 200 fc, or 8.85×10^{14} photons/s·cm^2 at 546 nm, at the water surface). Cone and RPE pigment retinomotor positions were determined as described in the Methods. The *n* values indicated above the abscissa represent the number of fish killed at a given time and thus correspond to the number of intact and cut optic nerve eyes examined at each time point. In intact eyes, cones underwent circadian elongation and contraction during subjective night and day, respectively. In eyes with cut optic nerves, cone elongation was blocked during the first hour of darkness. Thereafter, cones underwent circadian elongation and contraction, but the extent of elongation during subjective night was significantly less than that observed in intact eyes. Cutting the optic nerve partially inhibited RPE pigment aggregation during the first hour of darkness; otherwise, the pigment of cut optic nerve and intact eyes remained aggregated during constant darkness. Light onset produced full light-adaptive cone contraction and pigment dispersion in both cut optic nerve and intact eyes.

(see Fig. 12). These results suggest that the lack of dark-induced cone elongation in cut optic nerve eyes was not a result of surgical trauma. Thus, in fish having unilateral optic nerve section, cone elongation proceeded normally in the intact eye but was completely blocked in the operated eye for the first hour of darkness. These findings indicate that cone elongation can occur independently in each eye and suggest that an efferent input to the retina may have a role in dark-induced cone elongation.



Circadian cone movements were observed in both intact and cut optic nerve eyes over the 2-d period of constant darkness, but cone myoid lengths in cut optic nerve eyes were substantially shorter than those in intact eyes (Fig. 11). After 6 h of darkness, cones of cut optic nerve eyes elongated to 14.3 ± 3.7 μm ($n = 4$). This value is significantly longer than the cone lengths observed after 1, 12, or 18 h of darkness in these eyes ($P < 0.05$). This result suggests that the circadian rhythm of cone retinomotor movement is considerably damped but is still expressed during the first cycle after optic nerve section. Cone elongation in cut optic nerve eyes was more pronounced during the second subjective night than during the first night, but cones did not elongate to their maximally dark-adapted lengths. During subjective day, cones of these eyes contracted to their fully light-adapted positions, whereas cones of intact eyes remained partially elongated (see Fig. 11). As a consequence, cones of cut optic nerve eyes were shorter than those of intact eyes at any given time point, but the circadian cone excursion (the distance traversed between subjective day and night) was approximately equal in both eyes during the second cycle. Thus, the circadian rhythm of cone retinomotor movement continued in the absence of efferent input.

Cutting the optic nerve partially inhibited dark-induced pigment aggregation during the first hour of darkness ($P < 0.01$; Figs. 11 and 12). After 6 h, pigment in cut optic nerve eyes aggregated to its fully dark-adapted position. RPE pigment migration did not exhibit a circadian rhythm in fish kept in constant darkness, and there were no other significant differences in pigment distribution between intact and cut optic nerve eyes.

Dark-induced rod contraction was also partially inhibited by optic nerve section. After 1 h of darkness, eyecups from two fish were fixed, embedded in plastic, and sectioned. Rods of intact eyes contracted to 10.1 ± 0.3 μm ($n = 2$), whereas rods of cut optic nerve eyes had a length of 21.5 ± 1.1 μm ($n = 2$). After 18 h of darkness, rods in the intact eye of one fish had a length of 11.2 μm, and those of the cut optic nerve eye had a length of 10.5 μm. Thus, rods

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FIGURE 12. (*opposite*) Light micrographs of 20-μm-thick retinal slices obtained with Nomarski interference contrast optics. The OLM and the base of a cone ellipsoid are indicated by small and large black arrows, respectively. The cone myoid length corresponds to the distance between these arrows and is equal to 5 μm in *a*. The apical-most pigment granules of the retinal pigment epithelium (rpe) are indicated by white arrows. × 468. Eyecups were fixed from fish killed immediately before light offset (*a*), after 1 h in the dark (*b–d*), 24 h in constant darkness (*e* and *f*), or 24 h in darkness followed by 1 h in light (*g* and *h*). Optic nerves were cut in *d*, *f*, and *h*; *c* was a sham operation; other eyes were intact. The optic nerve section blocked cone elongation and partially inhibited pigment aggregation during the first hour of darkness (*d*); intact (*b*) and sham-operated (*c*) eyes exhibited normal dark-adaptive retinomotor movements during this time. After 24 h in constant darkness, intact (*e*) and cut optic nerve (*f*) eyes exhibited approximately equal extents of dark-adaptive movements. After this time, light onset produced full light-adaptive cone contraction and pigment dispersion in both intact (*g*) and cut optic nerve (*h*) eyes.

and RPE pigment, unlike cones, reached their fully dark-adapted retinomotor positions after optic nerve section when fish were given sufficient time in the dark.

Since cutting the optic nerve blocked dark-induced cone elongation, we also examined whether optic nerve section might block light-induced cone contraction. Fish with unilateral optic nerve section were kept in constant darkness for 24 h and then transferred to light for 1 h. Light onset produced full cone myoid contraction and RPE pigment dispersion in both intact and cut optic nerve eyes (Figs. 11 and 12). Thus, light can elicit appropriate cone and RPE retinomotor movements independently of efferent input to the retina.

DISCUSSION

Circadian Rhythm of ERG in Green Sunfish

The ERG of the green sunfish exhibits an endogenous rhythm in amplitude. When fish were maintained in constant darkness, the ERG amplitude was high during subjective night and low during subjective day. Changes in the ERG amplitude reflect changes in the sensitivity of the retina. The nighttime increase in the ERG amplitude represents a 3–10-fold increase in retinal sensitivity.

The endogenous rhythm in the ERG amplitude appears to be controlled by a circadian oscillator. Relatively short recording periods did not allow us to carry out all the experiments necessary to establish the existence of an underlying circadian clock. However, the ERG rhythm free runs in constant darkness with a period of ~24 h. Therefore, it seems reasonable to conclude that the rhythmic changes in ERG amplitude are generated by a circadian oscillator. To our knowledge, this is the first observation of a circadian rhythm in electrophysiological response recorded from the teleost retina.

Circadian rhythms in ERG amplitude have been reported for several other vertebrate retinas. In pigeon, the ERG amplitude varied with wavelength from day to night, which suggests an endogenous change from cone to rod vision (Barattini et al., 1981). In rabbit, the ERG amplitude was higher at night (Brandenburg et al., 1983), whereas in lizard, the ERG amplitude was higher during the day (Fowlkes et al., 1984). Our results with green sunfish are most similar to those reported for rabbit. At night, retinal sensitivity was up to 8-fold greater for rabbit and 3–10-fold greater for green sunfish. In addition, our results are similar to two recent behavioral measurements of circadian changes in visual sensitivity. Bassi and Powers (1985) reported that goldfish visual sensitivity was 3-fold greater during subjective night, while Terman and Terman (1985) found that rat visual sensitivity was ~10-fold greater during subjective night. Together, these results indicate that retinal sensitivity possesses an endogenous circadian rhythm and that in most vertebrates examined, sensitivity is 3–10-fold greater during the night.

Relationship of ERG Responses to Retinomotor Movements

Our observations suggest that the circadian rhythm of green sunfish ERG amplitude is not solely a function of photoreceptor retinomotor movements. If

cone contraction toward the OLM during the day and rod contraction during the night serve to increase photon absorption by the respective photoreceptors, then cone sensitivity would be expected to be greater during the day and rod sensitivity greater during the night in fish kept under diurnal light/dark conditions. Since only cones exhibit endogenous movements in green sunfish kept in constant darkness, one would expect cone sensitivity to be greater during subjective day and rod sensitivity to remain essentially unchanged. We detected significant increases in retinal sensitivity during the night in all fish and secondary increases during midday in most fish. In eyes having cut optic nerves, the extent of circadian cone retinomotor movement was reduced by 90% during the first cycle and the daytime increase in the ERG amplitude was not observed. Thus, the daytime increase in the ERG amplitude may be associated with circadian cone contraction, but it seems unlikely that the nighttime increase in the ERG amplitude is related to retinomotor movements.

Our results also indicate that extensive cone myoid elongation during the night does not significantly attenuate the contribution of the cone system to the ERG response. In green sunfish kept in constant darkness, cone myoids elongated from a length of ~20 μm during subjective day to a length of 80–100 μm during subjective night. It has been proposed that lengthening of the cone myoid decreases the response of the cones in the dark (Miller and Snyder, 1972). However, our spectral sensitivity function suggests that cones contribute to ERG responses recorded from dark-adapted green sunfish during subjective day and night. This result may at first seem unusual because the spectral sensitivity of the ERG b-wave in dark-adapted humans primarily reflects rod function with little or no contribution from cones (Riggs et al., 1949; Crescitelli and Dartnall, 1953). However, studies of ERG spectral sensitivity in dark-adapted goldfish, carp, lemon shark, and Japanese dace have also demonstrated clear deviations from a pure rod function (Burkhardt, 1966; Witkovsky, 1968; Cohen et al., 1977; Barlow, 1985). In addition, Powers and Easter (1978) found that the spectral sensitivity of goldfish measured behaviorally with stimulus intensities at absolute threshold peaked in the green (530–570 nm) with shoulders in both the blue (440 nm) and red (640 nm). They concluded that goldfish remain mesopic at absolute threshold. Allen and Fernald (1985) have reported that cone inputs also influence the scotopic behavioral action spectrum in the cichlid fish *Haplochromis burtoni*. Our results similarly suggest that cones contribute to ERG spectral sensitivity in dark-adapted green sunfish and that extensive cone myoid elongation at night does not inactivate the cone system.

Location of Circadian Oscillators

Unilateral optic nerve section did not abolish the circadian rhythms of the ERG amplitude or of cone retinomotor movement in green sunfish. After this operation, the nighttime amplitude of both rhythms was considerably diminished during the first cycle and then increased during the second cycle. Despite this temporal correlation, we know of no causal relationship in the re-establishment of these rhythms. The capacity of both rhythms to continue after optic nerve section is consistent with the idea that the circadian oscillators regulating these

processes reside within the eye. We do not exclude the possibility that a centrally located oscillator may influence these rhythms via a humoral pathway. However, work from other laboratories has suggested that intraocular oscillators control endogenous retinal rhythms in several vertebrate species. For example, intraocular oscillators control photoreceptor shedding in rat and *Xenopus* (Goldman et al., 1980; Flannery and Fisher, 1984) and retinal *N*-acetyltransferase activity in *Xenopus* (Besharse and Iuvone, 1983). Terman and Terman (1985) reported that the circadian rhythm in rat visual sensitivity persisted after lesioning the suprachiasmatic nucleus (SCN), the putative location of the circadian oscillator in the mammalian central nervous system (see Groos, 1982; Moore and Card, 1985). Intraocular circadian oscillators have also been detected in invertebrates, e.g., *Aplysia* (Jacklet, 1969) and *Bulla* (Block and Wallace, 1982).

Extraocular oscillators may also participate in controlling retinal circadian rhythms. The circadian rhythm of the ERG amplitude in rabbits was abolished by bilateral section of the cervical sympathetic nerves (Brandenburg et al., 1981), which suggests that the retinal rhythm is mediated by the SCN. Optic nerve section prevented re-entrainment of the rat shedding rhythm to a change in the light/dark schedule (Tierstein et al., 1980), which suggests that central input modifies the endogenous oscillator within the eye. In the *Limulus* eye, optic nerve section abolished the circadian rhythms in structure and sensitivity (Barlow et al., 1977, 1980). Thus, circadian oscillators can be located in the eyes and in the central nervous systems of both vertebrates and invertebrates. Our results with green sunfish suggest that the circadian signals controlling cone retinomotor movement and retinal sensitivity are either generated within the eye or transmitted to the eye by a humoral agent.

Role of Efferent Input and Nature of Retinal Circadian Signals

Although circadian cone movements continued in cut optic nerve eyes, efferent input to the retina may have a role in the occurrence of dark-adaptive retinomotor movements at light offset. Cutting one optic nerve blocked cone elongation and partially inhibited rod contraction and RPE pigment aggregation in the eye with a cut nerve during the first hour of darkness. Since the two retinas of an individual operated fish responded independently to darkness, it seems unlikely that a humoral agent directly induces dark-adaptive retinomotor movements in green sunfish. Instead, neural input from the brain may normally participate in producing dark-adaptive movements. Dearry and Burnside (1984) demonstrated that darkness was able to elicit appropriate retinomotor movements in vitro only when isolated retinas were cultured in the presence of 10^{-5}–10^{-7} M free Ca^{++}. Darkness was unable to induce dark-adaptive movements in the presence of 10^{-3} M free Ca^{++}. These results suggest that efferent input may promote dark-adaptive movements by inhibiting a Ca^{++}-dependent process. Since dark-adaptive movements eventually occurred in cut optic nerve eyes, our experiments further suggest that a circadian signal may partially reproduce the effect of efferent input.

In contrast to its effect on dark-induced movements, cutting the optic nerve did not interfere with light-induced cone contraction and pigment dispersion.

This result is consistent with the previous experiments of Easter and Macy (1978), who found that cutting the optic nerve did not impair light-induced retinomotor movements in *Cichlasoma*. In vitro, light onset initiated light-adaptive rod and cone movements in isolated whole retinas or isolated photoreceptors of green sunfish (Dearry and Burnside, 1985, 1986*a*). These observations suggest that light can act independently of efferent input to evoke appropriate retinomotor movements.

Why did cutting the optic nerve inhibit dark- but not light-induced retinomotor movements? Several observations suggest that the absence of efferent input may enhance retinal dopamine release and that dopamine subsequently inhibits dark-induced retinomotor movements. Dearry and Burnside (1986*a*) demonstrated that dopamine can induce light-adaptive retinomotor movements in cones, rods, and RPE cells. They also found that dopamine inhibited dark-adaptive movements induced by forskolin in isolated light-adapted retinas (Dearry and Burnside, 1985). Moreover, cones of isolated dark-adapted retinas incubated for 30 min in constant darkness contracted to their light-adapted positions (Burnside and Basinger, 1983; Dearry and Burnside, 1984). This spontaneous, light-independent cone contraction was blocked either by lowering the extracellular Ca^{++} level to $\leq 10^{-6}$ M or by adding a D2 dopamine antagonist to the medium (Dearry and Burnside, 1984, 1986*a*). These observations suggest that Ca^{++}-dependent dopamine release is enhanced upon isolation of retinas from dark-adapted green sunfish. Isolated goldfish retinas may also spontaneously release endogenous dopamine in a Ca^{++}-dependent manner (O'Brien and Dowling, 1985). In addition, cutting the optic nerve has been reported to increase dopamine levels in rat retinas (Osborne and Perry, 1985). A similar increase in dopamine levels in green sunfish retinas after cutting the optic nerve may inhibit dark-induced retinomotor movements.

Increased dopamine release after optic nerve section may also have a role in eliciting light-independent photoreceptor shedding and in inhibiting retinal degeneration. Stimulation of shedding by enucleation alone has been observed in *Xenopus* and rat eyes (Goldman and O'Brien, 1978; Flannery and Fisher, 1979; Tamai and O'Brien, 1979; Philp and Bernstein, 1981), whereas light-evoked shedding in rats was reportedly inhibited when dopamine synthesis was blocked (Reme et al., 1986). Furthermore, retinal degeneration in rats caused by constant light exposure was inhibited either by treatment with bromocriptine, a D2 dopamine agonist (Bubenik and Purtill, 1980), or by cutting the optic nerve (Bush and Williams, 1986). Although the precise role of dopamine in photoreceptor shedding and retinal degeneration has not been elucidated, these results are consistent with the idea that isolation of retinas from efferent input increases retinal dopamine release. A higher level of retinal dopamine may explain our finding that cone myoids were shorter in cut optic nerve eyes than in intact eyes. In addition, elevated levels of dopamine could account for a lower-amplitude ERG response in cut optic nerve eyes since dopamine and apomorphine, a D2 dopamine agonist, have been found to decrease the ERG b-wave amplitude in cats and rabbits, respectively (Jagadeesh and Sanchez, 1981; Dawis and Niemeyer, 1986). Together, these findings suggest that efferent activity may inhibit

dopamine release and that efferent activity may be modulated by changes in ambient lighting.

Dopamine has previously been proposed to be a messenger of both light and circadian signals in green sunfish retinas (Dearry and Burnside, 1985, 1986*a*). Since dopamine induces light-adaptive cone contraction and decreases the ERG b-wave amplitude (see above), our results are consistent with the notion that dopamine levels in green sunfish retinas are elevated during subjective day. A circadian rhythm in retinal dopamine content with higher levels during the day has been observed in rats maintained in constant darkness (Wirz-Justice et al., 1984). Our data further suggest that dopamine release is modulated by intraretinal or humoral mechanisms as well as by the activity of efferent fibers, or that dopamine is not the sole circadian messenger in green sunfish retinas. Since the circadian rhythms of the ERG amplitude and cone retinomotor movement continued in the absence of efferent input, dopamine release may be directly controlled by an endogenous oscillator in the retina or by some other retinal or humoral agent. For example, it has been reported that melatonin inhibited electrically evoked release of [^{3}H]dopamine from rabbit retinas (Dubocovich, 1983). In several vertebrates, the retinal melatonin content and the activity of serotonin *N*-acetyltransferase, the rate-limiting enzyme in melatonin synthesis, exhibit a circadian rhythm (see review by Besharse, 1982). Maximal levels of both have generally been found to occur during the night. In chicks, the nighttime increase in ocular melatonin concentration was not abolished by optic nerve section (Reppert and Sagar, 1983). In *Xenopus*, the rhythm of *N*-acetyltransferase activity persisted in eyecups maintained in vitro (Besharse and Iuvone, 1983). Thus, vertebrate eyes may contain a circadian clock regulating retinal melatonin levels. Although Dearry and Burnside (1986*b*) did not detect an effect of melatonin on retinomotor movement in green sunfish retinas, recent results (Pierce and Besharse, 1986) suggest that the effect of melatonin on such movement in *Xenopus* retinas is dependent upon light intensity. Melatonin induced dark-adaptive cone elongation in this species only under low ($\leq 10^{-6}$ W/cm^2) levels of illumination. Thus, melatonin may be an effective inhibitor of dopamine release during subjective night. In turn, dopamine may inhibit melatonin synthesis during subjective day (Iuvone and Besharse, 1986). These results suggest that melatonin and dopamine act as antagonistic circadian signals in vertebrate retinas.

Summary

In conclusion, our physiological and anatomical observations are consistent with the following scheme. (*a*) At least two circadian mechanisms modulate retinal sensitivity in green sunfish. (*b*) One mechanism, possibly related to circadian cone retinomotor movement, increases retinal sensitivity during the day. (*c*) The endogenous rhythm of cone movement is not mediated by efferent optic nerve fibers. (*d*) A second circadian mechanism unrelated to cone retinomotor movement increases retinal sensitivity at night. (*e*) The second mechanism is also not mediated by efferent optic nerve fibers. (*f*) Efferent optic nerve fibers have a role in controlling dark-induced cone elongation. This scheme provides a framework for further examining the origin of circadian rhythms and the identity of circadian messengers in the green sunfish retina.

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