Effects of Circadian Rhythm and cAMP on Retinomotor Movements in the Green Sunfish, *Lepomis cyanellus*

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The photoreceptors and retinal pigmented epithelium (RPE) of teleosts undergo diurnal changes in position in response to day/night changes in light conditions. These position changes, called retinomotor movements, may also persist under conditions of constant darkness. In this study, the authors have compared the retinomotor movements of rods, cones, and RPE under conditions of constant darkness and constant temperature in the green sunfish, *Lepomis cyanellus*. In this species, cones undergo circadian cycles of retinomotor movements in constant darkness but rods and RPE do not. Also cone contraction commences in early morning before the expected time of light onset, thus suggesting that circadian rhythms may play an important regulatory role in these cells even under cyclic light conditions. Since treatments that elevate cAMP previously have been shown to induce dark-adaptive retinomotor positions, the authors also have compared the effects of exogenous cAMP analogs on retinomotor positions of rods, cones, and RPE pigment in cultured green sunfish retinas. The authors found that concentrations of cAMP analogs required to produce extreme dark-adaptive retinomotor positions were at least fivefold higher for cones than for rods and RPE.

In teleost retinas, changing light conditions are accompanied by movements of the photoreceptors and the pigment granules of the retinal pigmented epithelium (RPE). These movements have been called retinomotor or photomechanical movements. Under normal cyclic lighting conditions, photoreceptors and RPE exhibit a diurnal cycle of retinomotor movements: at night the rods are short, the cones are long, and the pigment granules are aggregated near the base of the RPE cells; while in the day rods are long, cones are short, and pigment granules disperse into the RPE apical projections. In the absence of environmental light or temperature signals, fish retinas may continue to exhibit cycles of retinomotor movements that reflect the previously entrained light/dark cycle. Thus, retinomotor movements are regulated not only by light but also by a circadian rhythm.

The persistence of cyclic retinomotor movements in constant darkness has been described in several species of fish and has been called an “endogenous retinomotor rhythm.” In most species examined, cones exhibit some form of retinomotor rhythm in constant darkness, but the amplitude of movement and the pattern of the cycle vary among species. Reports of circadian RPE movements are rare: in most species the RPE pigment maintains a stationary dark-adapted (DA) position in constant darkness. Less is known about circadian movements of fish rods. Because they are difficult to discern in paraffin sections, fish rod movements have been analyzed and reported for only a few species. Modern techniques of plastic embedding now make analysis of rod position possible in retinas with the tiny rods characteristic of most fish species.

In the present study, we have compared the retinomotor movements of rods, cones, and RPE under conditions of constant darkness and constant temperature in the green sunfish, *Lepomis cyanellus*. This fish was selected because its large retinomotor excursions facilitate quantitative analysis and because considerable information has been accumulated about the mechanisms and regulation of its retinomotor movements. We have reported previously that treatments elevating cAMP induce dark-adaptive retinomotor positions in cones, rods, and RPE of three different fish species. These observations, together with reports that retinal cAMP is higher in darkness, led us to suggest that changes in cyclic AMP levels may be regulating retinomotor positions in cyclic light conditions and in constant darkness. As a start toward understanding this regulatory process, we have also com-
pared the effects of cAMP analogs on the retinomotor positions of rods, cones, and RPE in green sunfish retinas cultured in the light.

Materials and Methods

Green sunfish were obtained from Funetz Fish Farm (Sebastopol, CA), maintained in our laboratory at 22°C, and, for at least one month before use, entrained to a 12-hr light/12-hr dark cycle, with light onset at 6:00 AM and offset at 6:00 PM. All experiments were performed during the summer. At 6:00 PM on the first day of the experiment, fish of uniform size were placed in permanently dark, aerated tanks maintained at 22°C. Fish were killed for histologic preparation of their retinas by spinal cord section at 3-hr intervals up to 48 hr. More sample times were considered near the time of light offset and around the times of subjective dawn and dusk since these are the times when retinomotor movements are most extensive during the normal light/dark cycle. A minimum of three fish were used at each sample point. Posterior eyecups were prepared from both eyes, using dim red light to minimize retinomotor movements during dissection (which required less than 3 minutes for each fish). Fish were exposed to red light only after sacrifice. Eyecups were immersed in 2% glutaraldehyde (TAAB; Reading, UK) in 0.1 M phosphate buffer (pH 7.0), containing 0.5% cetyl pyridinium chloride (CPC) (Sigma; St. Louis, MO) to enhance the adhesion of RPE to neural retina. The addition of CPC greatly decreased the incidence of retinal detachment during processing. The eyecups remained in fixative at 22°C in the dark for 4–20 hr before further dissection. A specified region of the posterior pole dorsal to the optic nerve was dissected from each eyecup to ensure comparison of similar retinal samples for each eye, taking care to keep the RPE attached. These retinal blocks were postfixed in 1% OsO4, dehydrated in ethanol, embedded in Epon, and sectioned into 2-μm sections. Blocks were oriented so that photoreceptors were sectioned longitudinally. Sections were stained with 0.5% Toluidine Blue in 50% ethanol containing 0.5% Borax, and examined by bright field light microscopy. Measurements were made using a x 40 objective and an ocular micrometer.

Measurements

Cones: Changes in cone lengths were determined by measuring cone myoid length, which is the distance between the base of the cone ellipsoid and the outer limiting membrane (OLM). Twenty representative cone myoids were measured for each retina, including double and single cones. Means for each retina were averaged to yield sample time means; thus n = numbers of animals examined.

Rods: Rod myoid length is difficult to measure since the ellipsoid tapers into the myoid in long rods; therefore, rod inner segment length was used for analysis.7 Rod inner segment length was determined by measuring the distance from the base of the rod outer segment to the OLM. In green sunfish, the rods are arranged in tiers, so length changes in only the shortest rod tier were recorded. Twenty representative rods were measured for each retina.

RPE: The position of the RPE granules was recorded by determining the RPE index, ie, the distance from Bruch’s membrane to the vitreal-most edge of the RPE pigment expressed as a percentage of the total distance from Bruch’s membrane to the OLM. The mean distance from Bruch’s membrane to the OLM was 154 ± 3 μm (n = 90). Ten measurements were made for each retina. For rods, cones, and RPE, data are presented as mean ± standard error of the mean; n refers to number of animals examined.

Retinal Cultures for CAMP Dose Response

Organ cultures of light adapted (LA) retinas with RPE attached were prepared as described elsewhere.8 Half retinas were cultured in 0.3 ml Hank’s balanced salt solution containing increasing concentrations of dibutyryl cAMP (dbcAMP) and 0.5 mM iso-butyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor. Cultures were gassed with 100% oxygen at 21°C on a rotary shaker at 50 rpm for 2 hr. After culture, retinas were either (1) fixed in 6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), and then chopped in 25-μm slices for examination by Nomarski optics to make measurements of the RPE and cones; or (2) fixed in 2% glutaraldehyde in 0.1 M phosphate buffer and prepared for embedding, sectioning, and measurement of rods in the same way as those retinas taken during the constant dark experiment.

Results

The morphologic appearances of representative retinas from this study are shown in Figure 1. Figures 1A and 1B illustrate normal dark- and light-adapted positions in retinas fixed directly from fish maintained under cyclic light conditions. Under conditions of constant darkness, rods and RPE remained in their normal DA positions (Compare Figs. 1A, C, and D). Cones, however, continued to exhibit cycles of movement in constant darkness, though they did not achieve the fully LA and DA positions of the normal diurnal cycle (Figs. 1A, 1B). In constant darkness, cones assumed intermediate lengths that were shorter at noon (Fig. 1C) and longer at midnight (Fig. 1D).

Quantitative descriptions of the changes in retinomotor position observed before and during 48 hr of
Fig. 1. Light micrographs of 2 µm plastic sections from green sunfish retinas fixed at the following times: A, dark-adapted retina in normal L/D cycle, 6 hr after lights out; B, light-adapted retina in normal L/D cycle, fixed at noon; C, dark-adapted retina in constant darkness, fixed at noon, 18 hr after light offset; D, dark-adapted retina in constant darkness, fixed at midnight, 30 hr after light offset. In C both RPE and rods are in dark-adapted retinomotor positions, but cones have approached their light adapted positions. In D cones, rods, and RPE are all in fully dark-adapted positions. (Small black arrows = OLM; large black arrows = base of cone ellipsoid; white arrows = base of rod outer segment.)

Constant darkness are shown in Figure 2. Normal LA positions for rods, cones, and RPE are indicated in the 5:45 PM sample before initial light offset at 6:00 PM. Under cyclic light conditions these LA positions are held throughout the light period (data not shown). After light offset, both rods and RPE moved rapidly to their DA retinomotor positions (rates are indicated in Table 1) and remained there for the rest of the sample period in constant darkness. Minor rod and RPE changes in retinomotor positions at subjective dawn and dusk represented less than 10% of the excursions seen in these cells under cyclic light conditions; dawn and dusk positions were not significantly different from the positions held at midnight in the first night of darkness.

Cones, on the other hand, continued to exhibit large retinomotor excursions in the absence of light cues (Fig. 2). Immediately following light offset on the first night, cones elongated at 1.13 µm/min (Table 1) to achieve their longest DA retinomotor positions by 9:00 PM (Fig. 2). Early in the first morning of constant darkness, in anticipation of the time of expected light onset, cones began to contract toward their light-adapted retinomotor positions (Fig. 1). The rate of this predawn contraction (0.25 µm/min) was much slower than that observed when dark-adapted fish are abruptly transferred from dark to light (1.5 µm/min)11 (Table 1). At the time of light onset, cone myoids had already contracted 75% of the way toward the fully LA positions. By noon, cone myoids in constant darkness had contracted maximally to 15 µm and, thus, had undergone 80% of their normal diurnal excursion (compare Figs. 1B and 1C). At approximately 8:00 PM (2 hr after subjective dusk) in the second night of darkness, cones began to elongate, at 0.13 µm/min (Table 1). This rate is an order of magnitude slower than that observed immediately after light offset (Table 1). Cones elongated slowly to reach a maximum length of 70 µm at 3:00 AM (65% of their normal nighttime excursion). At midnight, after 30 hr in constant darkness, the cones myoids were 50 µm long (Fig. 1D).

These cyclic movements of contraction and elon-
Fig. 2. Retinomotor movements of cones, rods, and RPE observed in green sunfish retinas under conditions of constant darkness. Upper horizontal bar illustrates lighting conditions during experiment; the lower bar illustrates previous entrainment cycle. Open squares indicate maximal LA position, filled squares indicate maximal DA position. RPE index represents the % of the distance from the Bruch's membrane to OLM occupied by pigment granules. For these fish, OLM to BM distance was 154 ± 3 μm (n = 90). Each point represents mean ± SEM for three to six fish.
gation continued through the second day of constant darkness, but the individual variation increased. At 6:00 PM on the second day (48 hr in darkness, subjective dusk) measured cone lengths ranged from 98.5 μm in one fish to 28.8 μm in another fish, with five others spread out in between. Thus, mean values are not very informative. Since cones of 98.5 μm are essentially maximally elongated, it does not seem likely that amplitudes of movement are decreasing in the second cycle. It seems more likely that the wide range of values results because different animals have different cycle periods that increasingly grow out of phase by 48 hr. Clearly it is not possible from our results to conclude whether amplitudes of movement are decreasing with time in constant darkness. Our results do suggest, however, that rates of cone movement became progressively slower with increased time in darkness (Table 1).

Figure 3 illustrates dose–response curves for the effects of varying cAMP concentration on rod, cone, and RPE retinomotor positions in green sunfish retinas cultured for 2 hr in vitro. The phosphodiesterase inhibitor IBMX was present at constant concentration (0.5 mM) in all preparations. This concentration of IBMX caused no movement in the absence of dbcAMP. The lowest threshold for initiating RPE dark-adaptive retinomotor movement was approximately an order of magnitude lower than that required for initiating cone movement. Maximal dark-adapted positions were achieved by rods and RPE at dbcAMP concentrations in the mM range; extreme cone positions on the other hand, were not achieved until dbcAMP concentration was fivefold higher. Also, for rods and RPE, levels of dbcAMP below 10^{-4} M caused a significant change in cell length or melanin granule position. Cones on the other hand, were not affected unless dbcAMP levels were higher than 10^{-4} M.

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<tr>
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<th>Cone elongation (μm/min)</th>
<th>Cone contraction (μm/min)</th>
<th>Rod contraction (μm/min)</th>
<th>RPE aggregation (μm/min)</th>
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<tr>
<td>First night</td>
<td>1.13 ± .18</td>
<td>1.62 ± .19</td>
<td>5.19 ± .40</td>
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<td>n = 9</td>
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<tr>
<td>First morning</td>
<td>0.25 ± .02</td>
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<td>n = 6</td>
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<tr>
<td>Second night</td>
<td>0.13 ± .02</td>
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<td>n = 11</td>
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<td>Second morning</td>
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<td>n = 3</td>
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Rates were calculated from linear portions of curves in Figure 2. Rates for cone elongation at subjective dusk in constant darkness were much slower than the rates (1.2 μm/min) that were measured previously for in vivo cone elongation induced by light offset or intraocular injections of dbcAMP. Rates for cone contraction at subjective dawn in constant darkness also were slower than the rates (1.5 μm/min) that were measured previously for in vivo cone contraction after light onset.

**Discussion**

Under conditions of constant darkness and constant temperature, green sunfish cones continued to exhibit cycles of retinomotor movements appropriate to subjective day and night. Green sunfish rods and RPE, on the other hand, assumed dark-adapted positions throughout the period of constant darkness and showed no circadian movement. The persistence of cone movements in constant darkness now has been reported for 12 species of teleost. In only two species have workers found an absence of circadian movement in constant darkness: in salmon and tench, a nocturnal species that has stationary cones even under cyclic light conditions. The occurrence of RPE circadian retinomotor movements in constant darkness shows more species variation. Circadian RPE move-
ments have been reported to be present in three species but not present in five species. Circadian retinomotor movements in rods have been examined in only three fish species before our study: in all three, the rods exhibited reduced excursions when compared with the normal light/dark cycle.

An examination of these various studies of teleost circadian retinomotor movements provides several interesting generalizations. First, circadian retinomotor movements appear to be most consistently found and most dramatic in cones but can also be found in rods and RPE of some species. Second, the pattern of circadian retinomotor movements usually mimics the diurnal movement cycle seen under cyclic light (trout being the only reported exception to this generalization). Third, circadian retinomotor movements often appear to proceed in constant darkness with reduced amplitude of excursion compared with diurnal excursions in cyclic light. Only Astyanax and Nannacara appear to retain full excursion. However, the apparent reduction in excursion in most species may result because cycle periods vary from fish to fish, and the pooled samples grow increasingly out of phase. This suggestion is consistent with our finding that some animals exhibited extreme DA retinomotor positions on the second night of constant darkness. Fourth, circadian rod, cone, or RPE retinomotor movements can clearly occur in the absence of movements by the other cell types. Finally, constant light abolishes the circadian rhythm of retinomotor movements in all but two species examined. The only two species that show persistent circadian movements in constant light are Nannacara and Cichlasoma; even in these tropical cichlids, movements in constant light are greatly damped compared with those in constant darkness. Green sunfish do not show retinomotor cycles in constant light; rods, cones, and RPE retain light adapted positions during subjective night (Ackland and Darry, unpublished observations).

Our study revealed that in green sunfish, cones begin to contract in anticipation of the expected time of light onset in the first night in darkness; they had, in fact, contracted 75% of the way toward the fully LA position by the expected time of light onset. This observation suggests that cone contraction occurs primarily in response to circadian rather than light signals, at least under our maintenance conditions, ie, with rectangular light cycles having abrupt light onset and offset. Similar anticipation of light onset by cones and RPE retinomotor movements can be detected in published retinomotor studies of four other fish examined under laboratory rectangular light cycle conditions.

Light-adaptive cone movements that anticipated dawn were also seen in the wild in grunts sampled from a coral reef. A similar anticipation of dawn was observed in the circadian rhythms of swimming activity in lake chubs. These observations suggest that even in the wild sunfish, cone contraction may be activated predominantly by circadian signals, rather than light. What could be the functional advantage of these predawn anticipatory cone movements? If cones are inactive in the elongated state, for example, because the cable properties of their long myoids attenuate the signal from outer segment to synapse, they may be nonfunctional at propagating light signals unless contracted to some necessary extent. Since the rate of cone contraction is relatively slow (1–1.5 μm/min), contracting a green sunfish cone from its 90 μm DA myoid length to the 5 μm LA length would take approximately an hour. Thus, if movement were activated only by light, contraction might take a full hour after light reached threshold intensity at dawn. During this whole time, light intensity would be increasing, rods saturated, and the fish might still be functionally cone blind. Clearly, a fish with long cones very likely would be eaten at dawn by a fish with short cones, if he had to rely only on light to activate cone myoid contraction.

All fish species studied so far reveal reproducible patterns of retinomotor movements in constant darkness. Since these patterns entail the assumption of reproducible intermediate retinomotor positions that are different from fully LA or fully DA positions, the circadian regulatory mechanism cannot simply be an on/off switch but must be able to dictate a graded motile response. Similar graded effects on retinomotor position have been obtained by varying adaptational light intensities near threshold, varying exogenous cyclic nucleotide, and calcium concentrations in cultured retinas.

In earlier studies, we have shown that treatments elevating cAMP induce dark-adaptive retinomotor movements of rods, cones, and RPE in a dose-dependent fashion. Since cones continue to exhibit cycles of movements in constant darkness, Burnside et al have suggested that these circadian movements might be mediated at least, in part, by cyclic changes in cone cAMP levels. Since green sunfish rods and RPE both respond to exogenous cAMP analogs with intermediate retinomotor positions, similar to those seen in cones, it is perhaps surprising that they do not exhibit circadian cycles of retinomotor movement in constant darkness. At least two possible explanations might be suggested: (1) that cytoplasmic cAMP levels undergo circadian cycles in cones, but not in rods and RPE or (2) that cAMP levels cycle in all three cell types but even the lowest dark cAMP levels in rods and RPE are high enough to maintain them in maximal DA retinomotor positions. The dbcAMP dose–response curves we report here indicate that in green sunfish, much lower (fivefold) exogenous dbcAMP concentra-
tions are necessary to produce maximal DA retinomotor positions in rods and RPE than in cones. These results are, thus, consistent with the second suggested mechanism but of course do not prove it. They might for example, reflect the presence of a more active phosphodiesterase in cones than rods and RPE.

We have shown that in the green sunfish Lepomis cyanellus cones continue to exhibit circadian cycles of retinomotor movements in constant darkness while rods and RPE do not. We also have shown that much higher (fivefold) exogenous dbcAMP concentrations are required to produce fully DA retinomotor positions in cones than in rods and RPE. These findings are compatible with the idea that cycles in cytoplasmic cAMP level play a role in regulating circadian, as well as diurnal, cycles of retinomotor movement.

**Key words:** cone, rod, retina, cAMP, circadian rhythm

**References**