0270-6474/81/0108-0835$02.00/0

The Journal of Neuroscience
Vol. 1, No. 8, pp. 835–839
August 1981

IN VITRO RESETTING OF THE CIRCADIAN CLOCK IN THE *APLYSIA* EYE

I. Importance of Efferent Activity in Optic Nerve[1]

ROBERT G. PRICHARD[2] AND MARVIN E. LICKEY

Department of Psychology, University of Oregon, Eugene, Oregon 97403

Abstract

A transition from constant light (LL) to constant darkness (DD) will reset the circadian rhythms of most organisms to a phase that normally occurs near dusk. We tested the circadian oscillator in the *Aplysia* eye for this property. The test was run *in vitro* under two conditions. Using the two eyes of a single animal, one eye was left attached to the cerebral ganglion by its intact optic nerve and the other was detached by cutting its nerve. The amount of LL preceding LL/DD was the independent variable; the phase of the two eyes was the measured variable. When LL < 12 hr, neither the cut nor the attached eye was reset by LL/DD. When $12 \leq$ LL ≤ 24 hr, the attached eye was reset but the detached eye was not. Thus, for these durations of LL, resetting by LL/DD was found to be dependent on the integrity of the optic nerve. When LL was about 21 hr, the differential response of the two eyes caused them to be about 12 hr out of phase. When LL was between 27 and 75 hr, LL/DD reset both eyes but there was a low amplitude cyclic modulation of about ±3 hr in the phase to which they were reset by LL/DD. This modulation shows that LL did not stop the eye clock but rather that LL/DD reset its phase while it was free running in LL.

Circadian rhythms can be demonstrated in the physiological functions of nearly all animals. In only a few cases, however, has a circadian oscillator been localized to a discrete bit of tissue. One of these is the *Aplysia* eye. Several laboratories have studied the *Aplysia* eye (for review, see Eskin, 1979), and it has been found to exhibit many of the formal properties of circadian rhythms in intact organisms. The ocular oscillator is especially well suited for investigating the physiological basis of circadian pacemaking because it produces a high amplitude rhythm of optic nerve activity that free runs for many cycles *in vitro* (Jacklet, 1969a).

The *Aplysia* eye also is well suited for study as a component of a circadian system. The eye is connected to the cerebral ganglion by an optic nerve that contains efferent fibers as well as afferent ones (Eskin, 1971). Several studies have suggested that activity in the optic efferents can influence the clockwork in the eye, at least *in vivo* (Block et al., 1974; Hudson and Lickey, 1980; Lickey and Wozniak, 1979), and the efferents may serve to couple the ocular oscillator with photoreceptors and circadian oscillators that exist outside of the eye (reviewed by Lickey et al., 1976; Page and Hudson, 1980). The implication of these studies is that the eyes are components of a circadian system that contains multiple photoreceptors and oscillators.

The purpose of the present experiments was to pursue the study of the *Aplysia* eye as a component of a circadian system. Since most of the *Aplysia* nervous system can be removed from the animal along with the eyes, we asked whether efferent activity in the optic nerve could influence the phase of the eye clock after an *in vitro* manipulation of the light regimen. We arrived at the procedure of resetting the eye clock by transitions from constant light (LL) to constant darkness (DD). LL to DD transitions (LL/DD) have long been known to be effective in setting the phase of circadian rhythms (Pittendrigh, 1966), a point that has been established for organisms as diverse as single celled algae (Sweeney and Hastings, 1957) and hamsters (Bruce, 1960). In all organisms studied so far, an LL to DD transition will initiate or reset a circadian rhythm to a phase point that corresponds to subjective dusk. In the present studies, we found that the *Aplysia* eye also conforms to this rule. More interestingly, we also found that efferent activity in the optic nerve participates in the resetting process.

[1] We thank Colin S. Pittendrigh and David Hudson for discussions and Steven O. Hiaasen and Joan A. Wozniak for technical assistance. This work was supported by Public Health Service Grant NS 12374 and National Science Foundation Grant 77-28251 to M. E. L.

[2] To whom correspondence should be addressed.

Materials and Methods

Aplysia californica were kept in 380-liter living tanks containing Instant Ocean at 15°C. Upon arriving at the laboratory, the animals lived for at least 5 days in light cycles of 12 hr of light (500 lux) and 12 hr of darkness (L*D* 12:*12*). An experiment began by transferring an animal to another 15°C tank in which it was exposed to LL (500 lux). The transfer occurred during the lighted portion of the light cycle. After 1 to 75 hr in LL, measured from the last dawn, we removed the eyes and central ganglia and placed them in a Petri dish for recording the compound action potential (CAP) rhythm by attaching suction electrodes to the optic nerve. The Petri dish contained about 100 ml of artificial seawater (formula of Jacklet, 1973) supplemented with 10,000 units each of penicillin and streptomycin. The pH of the medium was adjusted to 7.8 to 8.0 with NaOH. The eyes from two animals were run simultaneously in the same dish. One eye of each animal was detached from the cerebral ganglion by cutting its optic nerve. The other eye remained attached to the cerebral ganglion and was recorded *en passant*. Immediately after cutting the nerve, the LL/DD transition was produced by covering the recording chamber with a light-tight metal box. The box was left in place for the remainder of the experiment. The time required for setting up two pairs of eyes, from the moment the animals were removed from the aquarium to the moment of LL/DD, was about 0.5 hr. Recording was terminated after three or four cycles of the CAP rhythm had been expressed. The data were analyzed by plotting the frequency of the CAPs as a function of time in 0.5-hr bins. We used the midpoint of the rising portion of the rhythm as the phase reference point.

Some experiments were performed with animals that had had one optic nerve cut in an operation 1 or 2 days before the start of the experiment. Muscle contraction was prevented during the operation by injecting an isotonic solution of $MgCl_2$ (25% of body weight). We then made a small incision in the body wall above the cerebral ganglion, allowing us to locate and cut one optic nerve. The incision was closed with sutures. Other experiments required that LL be continued *in vitro* for up to 21 hr. In these experiments, the recording chamber remained illuminated for a variable number of hours after the eyes were placed in the Petri dish. The intensity (500 lux) and light source (fluorescent) were similar to that which had prevailed in the living tanks.

Results

Eye rhythms of attached and detached eyes after 12 hr of LL. The activity in the optic nerve of a detached eye consists of compound action potentials (CAPs) made up of hundreds or perhaps thousands of afferent axons firing in synchrony (Jacklet, 1969b). Figure 1*A* shows the typical short term pattern of CAPs produced by a detached eye. The circadian rhythm shown in Figure 1*B* is a modulation of CAP frequency that is accomplished by changes in both the number of spikes per burst and the duration of the interburst interval. The rhythm shown is from an animal that received 12 hr of LL before the eyes and brain were removed and placed in darkness. (Note that recording after 12 hr of LL is equivalent to recording the eye rhythms after exposure to L*D* 12:*12*.)

As shown by Eskin (1971) and Luborsky-Moore and Jacklet (1976), an eye attached to the cerebral ganglion produces an irregular pattern of CAPs, and the irregularity is caused by activity in the efferent fibers in the optic nerve. Typical short term patterns from an attached and detached eye are shown in Figure 1, *A* and *C*. Small

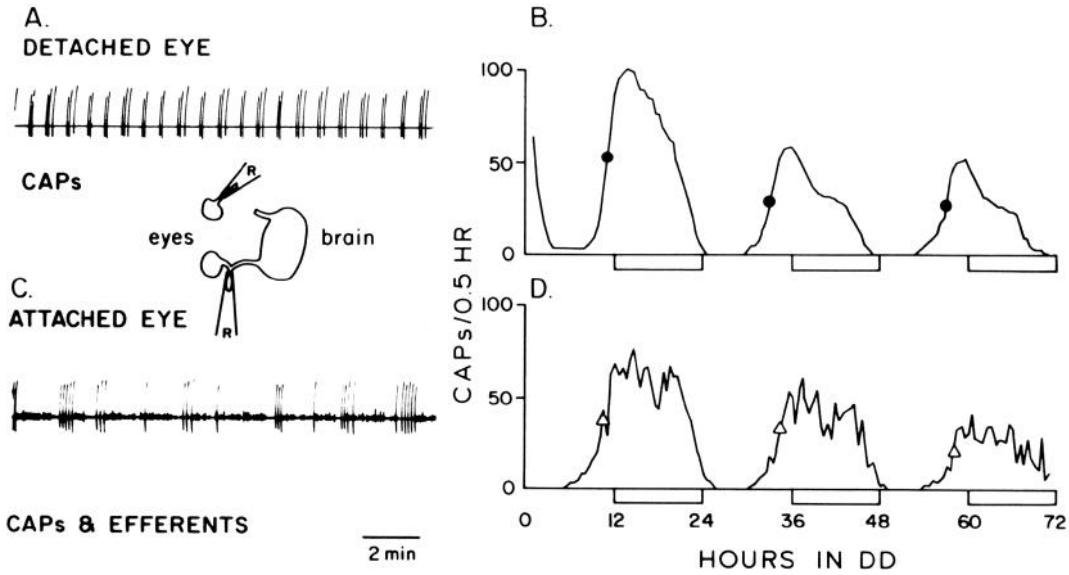

Figure 1. Compound action potentials (CAPs) and circadian rhythm of CAP frequency produced by attached and detached eyes. *A*, CAPs produced by an eye that is detached from the cerebral ganglion; *B*, CAP rhythm of a detached eye after 12 hr of LL; *C*, CAPs of an eye that is attached to the cerebral ganglion; *D*, CAP rhythm of an attached eye after 12 hr of LL. ●, Phase reference points from detached eyes; △, phase reference points from attached eyes; *R*, suction electrode. *Rectangles* on the *base line* show projected days of previous L*D* 12:*12*.

spikes from the efferent fibers are visible clearly in the recording from the attached eye (Fig. 1*C*). The circadian rhythm of an attached eye is also more irregular than that of a detached eye, but the irregularity, itself, does not affect the phase of the rhythm. As seen in Figure 1, *B* and *D*, the mid-rise points of the attached and detached eye rhythm occurred nearly simultaneously near projected dawn and they did not drift out of phase with each other during the time of recording. This timing of the ocular rhythm concurs with previous observations of rhythms from both attached and detached eyes when animals are maintained in L*D* 12:*12* (Jacklet, 1969a; Block and Page, 1979).

The effect of LL/DD after 1 to 75 hr of LL. The focus of attention in our experiments is the phase of rhythms from attached and detached eyes following LL/DD. Both the absolute phase and the phase difference ($\Delta\Psi$) between attached and detached eyes were affected by changing the duration of LL. The relation between eye phase and LL duration is shown in Figure 2. For simplicity, only the mid-rise points are shown and each *point* represents the mean of two to six eyes. When LL < 12 hr, neither the attached nor detached eyes were reset by LL/DD. The mid-rise points typically occurred a few hours before the projected dawn of the prior LD, as they do following L*D* 12:*12*. It appears, therefore, that LL/DD has little effect on either attached or detached eyes when the duration of light is less than 12 hr.

When $12 \leq LL \leq 24$ hr, LL/DD had a different effect on attached eyes than on the detached eyes. The attached eyes were reset by LL/DD to a new phase near subjective dusk; their mid-rise points occurred about 12 + *n*24 hr after LL/DD. The detached eyes were much less affected or slightly advanced by the LL/DD. The free running period of most diurnal circadian rhythms is shortened by LL (Aschoff, 1960), and therefore, some advancement would be expected even in the absence of any special action by LL/DD. The differing response of the attached and detached eyes when $12 \leq LL \leq 24$ caused large phase differences between them. The maximum possible phase difference (about 12 hr) occurred following LL = 21 hr.

When $LL \geq 27$ hr, the LL/DD reset both the attached and detached eyes about equally to a new phase near subjective dusk. All of the mid-rise points were about 12 + *n*24 hr after LL/DD regardless of the old phase at which LL/DD occurred. It is important also to note that there was an approximately ±3-hr oscillation of the reset phase around the central value of about 12 + *n*24 hr after LL/DD.

Resetting by LL/DD after cutting one optic nerve in vivo. It was possible that the phase difference between attached and detached eyes following $12 \leq LL \leq 24$ was due to unequal experimental treatment of the two eyes prior to LL/DD. The detached eye had its optic nerve cut just prior to LL/DD, and the trauma associated with cutting the afferent or efferent axons might have prevented a normal sensory response to LL/DD in the detached eye. To test this possibility, we conducted experiments on animals that had had one of their optic

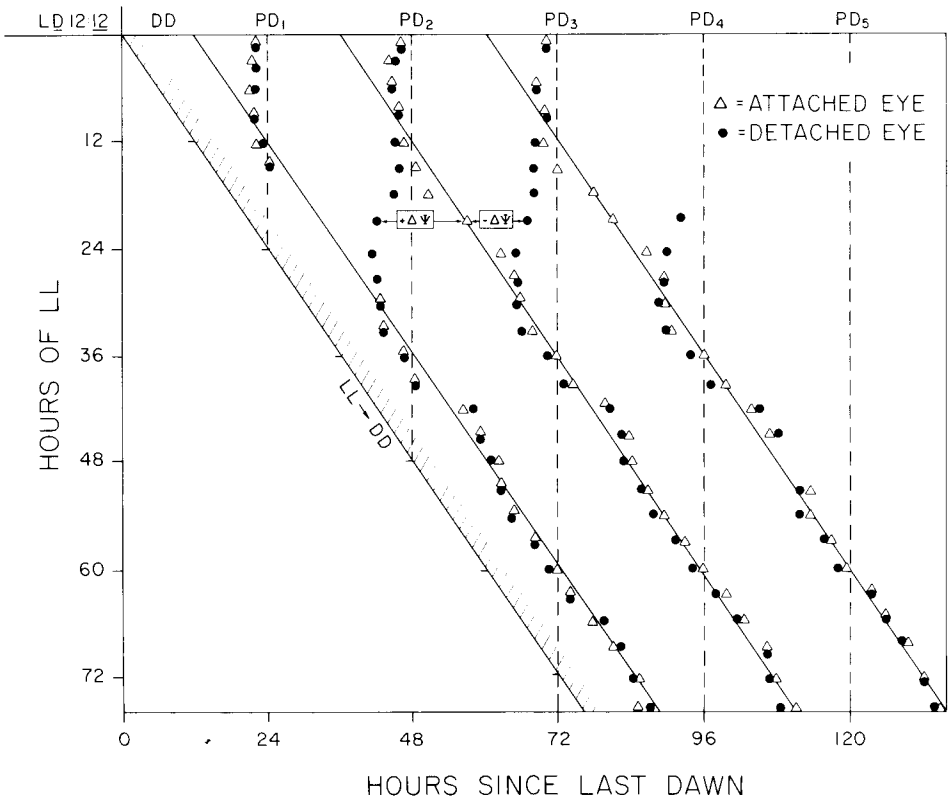

Figure 2. Phase of attached (△) and detached (●) eyes following 1 to 75 hr of LL. Each *point* is a mean of two to six eyes. The CAP frequency curves are omitted for clarity. *Dashed vertical lines* are the projected dawns (*PD*) of prior LD. *Solid diagonal lines* are the subjective dawns if eyes are reset by LL/DD. Note phase difference ($\Delta\Psi$) between attached and detached eyes when $12 \leq LL \leq 24$.

nerves cut *in vivo* several days prior to treatment with eye removal and LL. The LL duration was either 21 or 24 hr. Experiments using control animals were run at the same time according to the standard protocol in which the optic nerve of the detached eye was severed less than 10 min before LL/DD. There was little difference between the results from the two groups (Table I). Cutting the optic nerve several days before LL/DD did not eliminate the $\Delta\Psi$; the detached eyes' failure to be reset by LL/DD evidently is not due to cutting the optic nerve near the time of LL/DD.

Extended LL in vitro. Since eye removal occurred very near the time of LL/DD, there was ambiguity about whether the eye removal or LL/DD was the stimulus responsible for resetting the eye rhythms. Eye removal was performed under bright illumination and the eyes were placed in a different ionic environment (artificial seawater) for recording. These procedures could be responsible for the reset.

To resolve this question, we tested seven animals in which eye removal and LL/DD were separated temporally. The duration of LL *in vivo* was 48 hr or more. All eyes were detached at the time of eye removal, and LL/DD was postponed for 3 to 20.5 hr by exposing the eyes to additional light (500 lux) *in vitro*.

The mid-rise of the eye rhythms typically occurred about 12 hr after LL/DD but at arbitrary times after eye removal (Fig. 3). Therefore, LL/DD, not eye removal, is the effective stimulus for resetting.

Low amplitude modulation of reset phase. As mentioned above, there was a low amplitude cyclic modulation of the phase around the central value of about 12 + *n*24 hr after LL/DD (Fig. 2). To emphasize this modulation, the data of Figure 2 have been replotted in Figure 4 on slightly different coordinates. The ordinate is the duration of LL and the abscissa is the time since LL/DD. The *points* give the phase of individual attached and detached eyes on the first cycle in DD after 27 to 75 hr of LL. The *vertical dashed line* is placed at 12 hr after LL/DD as a reference. The amplitude of the modulation is seen clearly to be about ±3 hr. The complex time course and the lack of many cycles prevent us from precisely measuring the period of the cyclic modulation, but the available evidence suggests that it is approximately 24 hr. There was a tendency for damping as exposure to LL increased. The cyclic modulation in the effect of LL/DD shows that the state of the eyes is changing in LL. If it were not changing, one would expect the invariant stimulus (LL/DD) always to have the same effect on the eyes.

TABLE I

Phase difference ($\Delta\Psi$) between attached and detached eyes

LL Duration	Precut Nerve[a]	Standard Protocol[a]
hr		
21	$+\Delta\Psi$ 6.7 ± 1.7	6.8 ± 5.5
	$-\Delta\Psi$ 18.5 ± 2.1	15.8 ± 7.3
24	$+\Delta\Psi$ 21.2 ± 3.0	20.3 ± 1.5
	$-\Delta\Psi$ 3.2 ± 2.8	4.0 ± 2.0

[a] Mean and standard deviation of either three or four animals. The complementary values for $\Delta\Psi$ are given. Positive values were calculated as if the attached eye were delayed; negative values were calculated as if the attached eye were advanced.

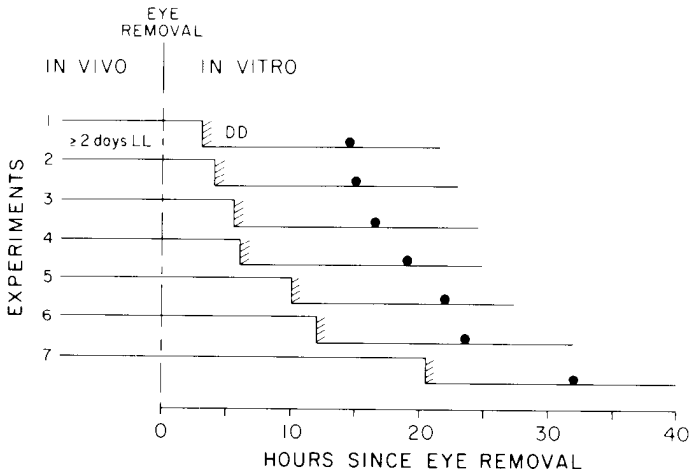

Figure 3. Phase of detached eye rhythms with respect to eye removal and LL/DD when LL is continued *in vitro* for 3 to 20.5 hr.

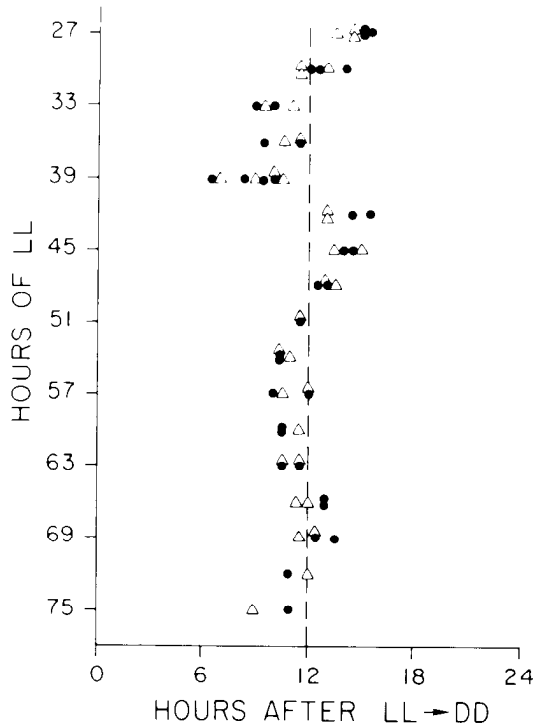

Figure 4. Phase of attached (△) and detached (●) eyes on the first cycle in DD after 27 to 75 hr of LL. The *dashed vertical line* marks 12 hr after LL/DD.

Discussion

The basic result that we describe is the resetting of the ocular rhythm by LL to DD transitions *in vitro*. As in other circadian systems assayed *in vivo*, LL/DD resets the circadian clock to a phase that corresponds to the time of dusk when the system is entrained by L*D* 12:*12*. Further, we have measured the minimum duration of LL that is required as a precursor for resetting by LL/DD. For eyes that are attached to the cerebral ganglion, as little as 12 hr of LL is sufficient; for eyes that are isolated from neural inputs, about 27 hr is required. There is a low amplitude modulation of the phase of reset eyes. This modulation is a cyclic function of the duration of LL, and although precise estimates are impossible with

the data at hand, the period of the modulation appears to be in the circadian range.

An interesting question to which our results may be addressed is whether LL to DD transitions determine the phase of circadian systems by restarting a clock that has been stopped by the action of LL or by changing the phase of a clock that is still in motion at the time of dark onset. Our data favor the latter interpretation. The principal evidence is the existence of the low amplitude modulation of phase that occurs with increasing duration of LL (Figs. 2 and 4). This orderly variation in response to the invariant stimulus (LL/DD) demonstrates that the eye clock undergoes an orderly change of state with the passage of time in LL. This change of state is cyclical with a period that is probably circadian. We therefore suspect that the circadian clock continues to run in the presence of LL and that the onset of darkness shifts the phase of the moving clock.

On the basis of observations somewhat different from ours, Benson and Jacklet (1977) favored the alternative interpretation, i.e., that LL stops the *Aplysia* eye clock at a phase point corresponding to dusk and that the onset of darkness restarts the stopped clock. Their interpretation was based on the fact that the eye rhythm damps out after 63 hr or more of LL and that rhythmicity is restored beginning near subjective dusk when the preparation is darkened. In our experiments, however, LL/DD was effective after as little as 12 hr of LL (27 hr with detached eyes), and our light intensity was only half of that used by Benson and Jacklet. It appears to us, therefore, that, even though circadian oscillations often do damp out in extended LL, the resetting action of LL/DD can occur before complete damping has occurred. It is possible that the resetting action of LL/DD is unrelated, mechanistically, to the damping action of LL.

Perhaps the most interesting question raised by our results concerns the function of the optic nerve and cerebral ganglion in the mechanism of resetting by LL/DD. After some durations of LL ($12 \leq LL \leq 24$), an eye that is attached to the cerebral ganglion via an intact optic nerve is reset by LL/DD, whereas an eye deprived of this input is not. The implication is that some form of efferent neural signaling originating in the cerebral ganglion participates in the resetting. Hormonal communication between the eye and brain probably is not involved since both the attached and detached eyes are in a common bathing medium.

There are several possibilities for the role of the optic nerve and cerebral ganglion in the nerve-dependent resetting that we observed after 12 to 24 hr of LL. One is that the cerebral ganglion contains photoreceptors with axons in the optic nerve. Signals from such photoreceptors might summate with signals from the ocular photoreceptors, rendering the eye clock more responsive to LL/DD. Another possibility is that the cerebral ganglion contains a circadian clock that drives the eye clock via efferent fibers in the optic nerve. The ganglion clock might require only 12 hr of LL as a precursor for resetting, while the eye clock requires 27 hr. Yet a third possibility is that all of the photoreceptors, oscillators, and coupling mechanisms required for resetting are present in the eye, but, in order for them to respond fully after only 12 hr of LL, they must be activated properly by some modulatory substance secreted by the terminals of the optic efferents. These possibilities have been investigated and are reported in two subsequent papers (Prichard and Lickey, 1981a, b).

References

Aschoff, J. (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor Symp. Quant. Biol. *25:* 11–28.

Benson, J. A., and J. W. Jacklet (1977) Circadian rhythm of output from neurons in the eye of *Aplysia*. III. Effects of light on clock and receptor output measured in the optic nerve. J. Exp. Biol. *70:* 183–194.

Block, G. D., and T. L. Page (1979) Effects of efferent activity on entrainment of the *Aplysia* eye. Comp. Biochem. Physiol. (A) *62:* 635–638.

Block, G. D., D. J. Hudson, and M. E. Lickey (1974) Extraocular photoreceptors can entrain the circadian oscillator in the eye of *Aplysia*. J. Comp. Physiol. *89:* 237–249.

Bruce, V. G. (1960) Environmental entrainment of circadian rhythms. Cold Spring Harbor Symp. Quant. Biol. *25:* 29–48.

Eskin, A. (1971) Properties of the *Aplysia* visual system: *In vitro* entrainment of the circadian rhythm and centrifugal regulation of the eye. Z. Vgl. Physiol. *74:* 353–371.

Eskin, A. (1979) Circadian system of the *Aplysia* eye: Properties of the pacemaker and mechanisms of its entrainment. Fed. Proc. *38:* 2573–2579.

Hudson, D. J., and M. E. Lickey (1980) Internal desynchronization between two identified circadian oscillators in *Aplysia*. Brain Res. *183:* 481–485.

Jacklet, J. W. (1969a) A circadian rhythm of optic nerve impulses recorded in darkness from the isolated eye of *Aplysia*. Science *164:* 562–564.

Jacklet, J. W. (1969b) Electrophysiological organization of the eye of *Aplysia*. J. Gen. Physiol. *53:* 21–42.

Jacklet, J. W. (1973) The circadian rhythm in the eye of *Aplysia*. Effects of low calcium and high magnesium. J. Comp. Physiol. *87:* 329–338.

Lickey, M. E., and J. A. Wozniak (1979) Circadian organization in *Aplysia* explored with red light, eye removal and behavioral recording. J. Comp. Physiol. *131:* 169–177.

Lickey, M. E., G. Block, D. Hudson, and J. T. Smith (1976) Circadian oscillators and photoreceptors in the gastropod *Aplysia*. Photochem. Photobiol. *23:* 253–273.

Luborsky-Moore, J. L., and J. W. Jacklet (1976) *Aplysia* eye: Modulation by efferent optic nerve activity. Brain Res. *115:* 501–505.

Page, T. L., and D. J. Hudson (1980) Circadian organization in *Aplysia californica*. Fed. Proc. *38:* 2580–2582.

Pittendrigh, C. S. (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: A model for the photoperiodic clock. Z. Pflanzenphysiol. *54:* 275–307.

Prichard, R. G., and M. E. Lickey (1981a) *In vitro* resetting of the circadian clock in the *Aplysia* eye. II. The critical period for optic nerve activity. J. Neurosci. *1:* 840–845.

Prichard, R. G., and M. E. Lickey (1981b) *In vitro* resetting of the circadian clock in the *Aplysia* eye. III. Location of photoreceptors that signal continuous light to continuous darkness. J. Neurosci. *1:* 846–848.

Sweeney, B. M., and J. W. Hastings (1957) Characteristics of the diurnal rhythm of luminescence in *Gonyaulax polyedra*. J. Cell. Comp. Physiol. *49:* 115–126.