IN VITRO RESETTING OF THE CIRCADIAN CLOCK IN THE APLYSIA EYE

III. Location of Photoreceptors That Signal Continuous Light to Continuous Darkness¹

ROBERT G. PRICHARD² AND MARVIN E. LICKEY

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

Abstract

As shown in the previous two papers (Prichard, R. G., and M. E. Lickey (1981a, b) *J. Neurosci.* 1: 835-839; 840-845), the circadian rhythm of compound action potentials (CAPs) from the *Aplysia* eye can be reset *in vitro* by single transitions from continuous light to continuous darkness (LL/DD). The magnitude of the reset depends on the duration of LL that precedes the LL/DD. When LL duration is 18, 21, or 24 hr, the magnitude of the reset is different if the eye is neurally attached to the brain than if it is neurally detached. The brain gives rise to optic efferent fibers that terminate in the retina. In the present paper, we explore how the optic efferents contribute to resetting by asking whether LL/DD is sensed by the photoreceptors known to exist in the brain or those in the eye. Eye-brain preparations were placed in a special recording chamber in which the eye and brain could be isolated photically from each other while one optic nerve remained intact. The LL/DD then was delivered to the eyes and to the brain at different times. During the separate illumination, neural communication between the eye and brain was prevented by reversible nerve blockade. The magnitude of the resulting resets always corresponded to the duration of LL that was delivered to the eyes. Photoreceptors in the brain did not contribute effectively to resetting. Also, resetting was not disturbed by nerve blockade that began before LL/DD and lasted for more than 6 hr after LL/DD. This implies that information about the timing of LL/DD was stored in the eye for more than 6 hr before it was expressed in the form of a reset. We conclude that the efferent fibers in the optic nerve do not instruct the eye about the magnitude of the reset. Instead, they activate or modulate circadian functions that are latent in the eye itself.

In the two preceding papers on the circadian rhythm in the *Aplysia* eye (Prichard and Lickey, 1981a, b), we described how the rhythm can be reset *in vitro* by single transitions from continuous light to continuous darkness (LL/DD). When the duration of LL is 12 to 24 hr, activity of the efferent fibers in the optic nerve is required for such resetting (Prichard and Lickey, 1981a). Resetting results in a phase difference, $\Delta\psi$, between a cut and an attached eye. The required nerve activity occurs neither at the time of LL/DD nor during the first few hours thereafter. Rather, it occurs about 28 hr after the start of LL. This long delay between the effective photic stimulus and the effective nerve activity suggested that the nerve activity is probably not the output of photoreceptors that

sense the occurrence of LL/DD (Prichard and Lickey, 1981b). Instead, there is probably a long term nonsensory process interpolated between the time of LL/DD and the time of nerve-dependent resetting (NDR).

Photoreceptors located somewhere in the preparation must signal LL/DD, however, because the time of LL/DD determines the new phase that is produced by nerve-dependent resetting. The cerebral ganglion contains photoreceptors (Block and Smith, 1973) as does the eye, and the experiments reported in this paper were performed to determine whether the eye or the brain contains the photoreceptors that signal LL/DD. In the course of the work, we also obtained information bearing on the location of the long term process that stores information about the time of LL/DD.

The strategy was to present one LL/DD transition to the eyes after one duration of LL and a second LL/DD transition to the brain after a different duration of LL. Since the duration of LL that precedes LL/DD determines the magnitude of the reset ($\Delta\psi$), we reasoned that the resulting reset of the attached eye would tell us which

¹ We thank Colin S. Pittendrigh and David Hudson for discussions and Steven O. Hiasen 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.

of the two LL/DD stimuli was effective in evoking the resulting reset. The photoreceptors would be located in the structure exposed to the effective stimulus.

Materials and Methods

Aplysia californica were maintained in the laboratory and the eye rhythms were recorded as reported previously (Prichard and Lickey, 1981a). The methods of data analysis and the determination of the phase reference points have been described also (Prichard and Lickey, 1981a).

These experiments required that extra LL be applied separately to the eyes and cerebral ganglion *in vitro*. This was accomplished with the special photic isolation chamber shown in Figure 1. The apparatus was milled from black Plexiglas and was designed to fit inside of the normal 100-ml recording chamber. Either the eye or cerebral ganglion of an intact optic nerve preparation could be placed in the inner compartment. The optic nerve was threaded through a slot (A) in the wall between the inner and outer compartment. Light leaks through the slot were prevented by sealing the nerve in place with petroleum jelly that had been made opaque by the addition of fine graphite particles. Artificial seawater (ASW) was circulated between the inner and outer compartments by a small air lift pump. The circulation pattern is indicated by the arrows (B) and the circulation openings were constructed with multiple bends that excluded exterior light. With the black Plexiglas lid (F) in place, the inner compartment was dark and photically isolated from the outer compartment. The parts of the preparation in the outer compartment were given extra LL *in vitro*. Light was supplied by a microscope illuminator that was shielded with 12 inches of Plexiglas to remove most of the infrared radiation.

The walls of the photic isolation chamber contained a narrow channel (C) that exposed about 2 mm of optic nerve to a solution that could be changed independently

of the circulating ASW. While extra LL was being applied to the exposed part of the preparation, Na⁺-free, Ca²⁺-free ASW was perfused through the channel in order to block optic nerve action potentials. Perfusion was accomplished by supplying Na⁺-free, Ca²⁺-free ASW to a reservoir (D) at one end of the channel and removing it by suction (E) at the other end. As reported in the previous paper (Prichard and Lickey, 1981b), Na⁺-free, Ca²⁺-free ASW was made by substituting sucrose for the Na⁺ and Ca²⁺ and most of the Cl⁻ ions in ASW.

The animals received 18 hr of LL *in vivo* before the eyes and cerebral ganglion were removed and placed *in vitro* for recording. Two preparations were run in each experiment. The eyes (one cut, one attached) of one preparation were placed in the dark compartment while the brain was exposed to extra LL in the outer compartment. The brain of the other preparation was placed in the dark compartment, while the eyes (one cut, one attached) received the extra LL. In all cases, the tissue in the dark compartment received an LL/DD transition after 18 hr of LL. The tissue outside received an extra 6 hr of LL *in vitro* for a total LL duration of 24 hr. Just before the dark compartment was covered, producing the LL/DD transition for the tissue inside of the dark compartment, a sucrose block was applied to the two intact optic nerves via the channel in the wall dividing the eyes and the brain. Shortly after the *in vitro* light was terminated, the nerve block was reversed without disturbing the preparation, restoring neural communication between the eye and the brain. The eye rhythms were recorded for the next three cycles in DD. The experiment was repeated three times.

Results

The results are shown in Figure 2. Control experiments in which the eye and brain received LL/DD simultaneously after 18 hr of LL are shown at the top of the figure (A). Control experiments in which the eye and brain received LL/DD simultaneously after 24 hr of LL are shown at the bottom (D). For simplicity, only the first cycle phase reference points are shown. It is seen that the magnitude of the reset is dependent on the duration of LL, replicating our previous results (Prichard and Lickey, 1981a).

Preparations in which the brains received 24 hr of LL, while the eyes received 18 hr are shown in B. The phases of the attached eyes were nearly the same as obtained when the entire preparation had received LL/DD after 18 hr of LL. The effective LL/DD signal appears to have been the one received by the eyes.

The preparations in part C were paired with the ones from part B, but, in this case, the eyes received 24 hr of LL and the brains received 18 hr of LL. The phases of the attached eyes were nearly the same as those that occurred when the entire preparation received 24 hr of LL. Again, the effective LL/DD stimulus appears to have been received by the eyes.

Discussion

The attached eyes were reset to the phase specified by the LL/DD stimulus applied to the eyes. In contrast, the LL/DD applied to the cerebral ganglion did not influence the phase of the attached eyes. Cerebral photoreceptors,

Figure 1. Recording chamber used to block conduction in optic nerve. A, Slots for the optic nerves; B, circulation channel between the inner and outer compartments; C, channels for perfusing a short stretch of nerve; D, tubes for introducing Na⁺-free, Ca²⁺-free ASW and normal ASW into the nerve block channel; E, suction; F, cover for the inner compartment.

Figure 2. Phase of attached (Δ) and detached ($\bullet$) eyes when LL/DD was applied to the eyes and brain at different times. A and D, Controls in which LL/DD was applied to the eyes and brain at the same time following 18 and 24 hr of LL; B, the eyes received LL/DD after 18 hr of LL and the brain received LL/DD after 24 hr of LL; C, the eyes received LL/DD after 24 hr of LL and the brain received LL/DD after 18 hr of LL. NB, Optic nerve blockade; PD, projected dawn of the previous light cycles. Preparations run concurrently are indicated by the number near detached eye mid-rise points.

if they are involved in determining the magnitude of resetting, are dominated completely by photoreceptors located in the eye.

In the preceding paper (Prichard and Lickey, 1981b), we showed that an attached eye is not reset immediately after LL/DD; instead, the reset is delayed until after the critical period for nerve-dependent resetting. The information about the timing of LL/DD therefore must be stored by some intervening mechanism at least until the critical period. The present results indicate that the intervening storage probably occurs in the eye. When the eyes and the brain received LL/DD after 18 and 24 hr of LL, respectively (Fig. 2A), communication between the eye and brain was prevented by nerve block during the entire time intervening between the LL/DD applied to the eye and the LL/DD applied to the brain. The ocular photoreceptors sensitive to LL/DD thus were not able to signal the brain that LL/DD had occurred until at least 6 hr after the event. It is almost certain, therefore, that the mechanism storing temporal information about LL/DD is located in the eye, and we conclude that the brain does not instruct the eye about the timing of LL/DD or the magnitude of the reset.

Implied by this conclusion is that the eye itself has access to two different phases during the time between LL/DD and the critical period. One, "old phase," is determined by the previous light cycle. If the optic nerve is cut or blocked during the critical period, this phase is expressed on subsequent cycles of the rhythm. The second phase, "new phase," is also available to the eye. This phase will be expressed if the nerve is attached and unblocked through the critical period.

In the previous paper (Prichard and Lickey, 1981b), we showed that the optic nerve did not convey the photic

output of photoreceptors. The experiments reported here show that the photoreceptors that sense LL/DD are not in the cerebral ganglion. What, then, is the role of the efferent fibers in the nerve-dependent resetting of an attached eye? As pointed out previously (Prichard and Lickey, 1981b), a remaining possibility is that the brain is responsible for setting the time of the critical period. It is equally possible, however, that the compound action potential rhythm itself could perform this function. Experiments are under way to determine how the time of the critical period is established, but in lieu of results, our present suspicion is that all of the photoreceptors, oscillators, and coupling mechanisms required for resetting are present in the eye itself. Accordingly, the eye, which often has been thought of as a unitary circadian oscillator, emerges as a complex circadian system, perhaps, though not necessarily, containing more than one oscillator. At the critical time, the efferent fibers provide important activation or modulation that is essential for the normal operation of the eye system. Without the efferent input, the system changes its circadian properties and responds differently to an LL/DD transition and perhaps other photic signals as well.

References

- Block, G. D., and J. T. Smith (1973) Cerebral photoreceptors in *Aplysia*. *Comp. Biochem. Physiol. (A)* 46: 115-121.
- Prichard, R. G., and M. E. Lickey (1981a) *In vitro* resetting of the circadian clock in the *Aplysia* eye. I. Importance of efferent activity in optic nerve. *J. Neurosci.* 1: 835-839.
- Prichard, R. G., and M. E. Lickey (1981b) *In vitro* resetting of the circadian clock in the *Aplysia* eye. II. The critical period for optic nerve activity. *J. Neurosci.* 1: 840-845.