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

II. The Critical Period for Optic Nerve Activity[1]

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Abstract

When constant light is 18, 21, or 24 hr, a constant light to constant darkness (LL/DD) transition *in vitro* results in a phase difference ($\Delta\Psi$) between an eye that is attached to the cerebral ganglion and its partner that is detached by cutting its optic nerve just prior to the LL/DD. This $\Delta\Psi$ develops during the first 24 hr after LL/DD as the result of efferent activity in the optic nerve of the attached eye. By cutting the nerve of the originally attached eye at various times after LL/DD, we determined when the phase resetting nerve activity occurs. This experiment was carried out following LL = 18, 21, and 24 hr. Optic nerve activity at the time of and for several hours after LL/DD did not produce nerve-dependent resetting (NDR). Instead, there was a restricted critical period, 2 to 3 hr long, during which the effective nerve activity occurred. Following 24 hr of LL, a 2-hr window of optic nerve activity during the critical period could produce NDR, but a 2-hr window outside of the critical period was ineffective. The resetting effect of nerve activity was produced suddenly as if NDR were an all-or-nothing event. Following 18, 21, and 24 hr of LL, the critical period occurred 10, 7, and 4 hr, respectively, after LL/DD. In each case, this was about 28 hr after the last dawn of the light cycles to which the animal had been exposed prior to LL. We conclude that the critical period is timed by a mechanism that is not reset by LL/DD.

In another paper (Prichard and Lickey, 1981a), we describe the resetting of the *Aplysia* eye clock by transitions from constant light to constant darkness (LL/DD). It was observed that, following some durations of LL, a transition reset an eye that was left attached to the cerebral ganglion, while its detached partner was not reset. Thus, under some circumstances, the optic nerve is involved in resetting the eye clock. We hypothesized that the efferent fibers in the optic nerve (Eskin, 1971) are the critical neural component in this nerve-dependent resetting (NDR).

The present paper reports experiments designed to investigate the characteristics of NDR and specify when, after LL/DD, the relevant nerve activity occurs. The time course of NDR is important for answering questions about the type of information supplied by the optic nerve that results in resetting. The strategy was to limit optic nerve activity to specific times after LL/DD by the use of a reversible blockade of nerve action potentials and surgical nerve cuts.

Materials and Methods

Aplysia californica were maintained in the laboratory and the eye rhythms were recorded as described in the preceding paper (Prichard and Lickey, 1981a). Data collection and the determination of the phase reference point of the eye rhythm have been described also.

Optic nerve activity was blocked in some experiments by the use of a special recording chamber in which we exposed about 2 mm of the optic nerve to Na^+-free, Ca^{2+}-free artificial seawater (Tauc and Epstein, 1967; Kupfermann et al., 1970). Sucrose was substituted for the Na^+ and Ca^{2+} ions to maintain osmolarity. The solution contained the following concentration of ions: 10 mM KCl, 26 mM $MgSO_4$, 22 mM $MgCl_2$, 880 mM sucrose, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer. The HEPES was prepared with KOH instead of NaOH, and the pH of the solution was adjusted to 7.8 to 8.0 with KOH.

Results

Following LL durations between 12 and 24 hr, there is a phase difference ($\Delta\Psi$) between an attached and detached eye due to the fact that the attached eye is fully reset by LL/DD, while the detached eye is unaffected or only slightly phase advanced. Figure 1 shows a typical record from a pair of eyes treated with 21 hr of LL. Both

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eyes were clearly out of phase by the second cycle *in vitro*. They were presumably in phase just before the LL/DD transition because, prior to this time, both were attached and subject to identical photic and physiological conditions. During the first cycle *in vitro*, the rhythm of the attached eye was abnormally complex and its phase was ambiguous. The phase shift to the reset condition was occurring during this time. In the experiments below, we determine more precisely the time of occurrence of the nerve activity that leads to the phase shift of an attached eye.

The general approach was to limit the amount of time in which the attached eye could interact with the cerebral ganglion to see when sufficient interaction had occurred to produce the $\Delta\Psi$. The eye-brain interaction was terminated at the end of the time by cutting the optic nerve. The first experiment is shown in Figure 2. At the *bottom* of the figure are the results of experiments in which one optic nerve was left intact for the duration of the experiment. Three cycles of the rhythm were recorded, but, for simplicity, the phase reference points are shown only for the first cycle. The *cross-hatching* indicates the time

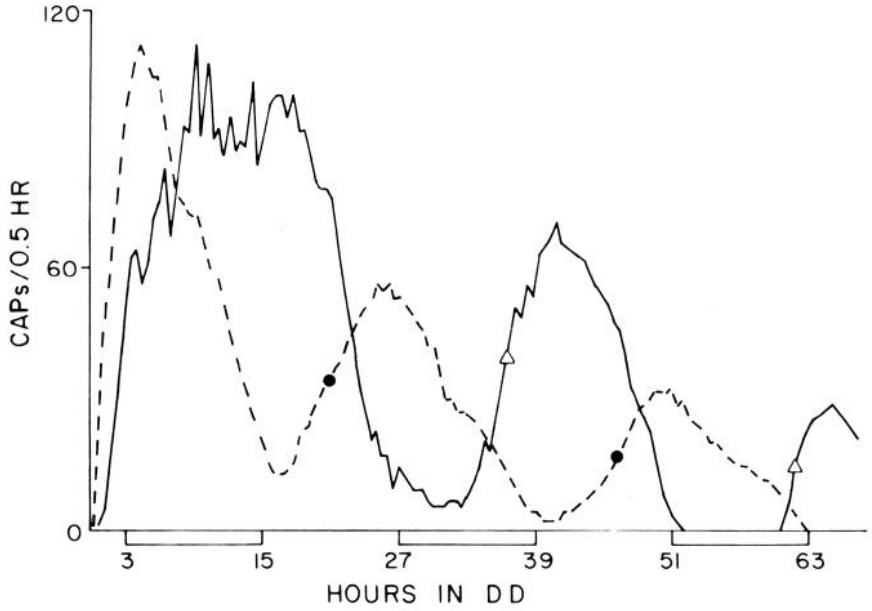

Figure 1. Compound action potential (CAP) rhythm of attached and detached eyes following 21 hr of LL. ●, Phase reference points of detached eye; △, phase reference points of attached eye. *Rectangles* on the *base line* show projected days of previous light cycles.

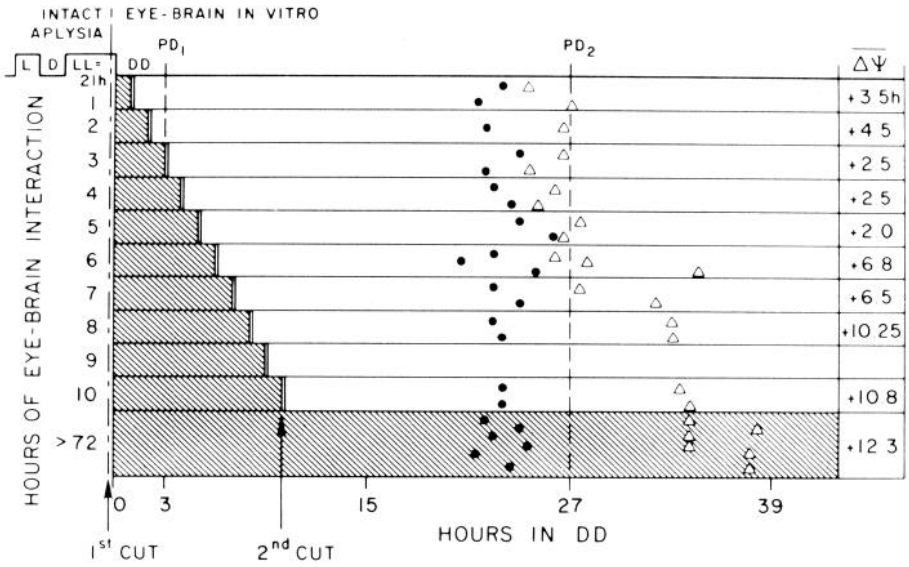

Figure 2. Phase of attached (△) and detached (●) eyes after varying durations of attachment following LL = 21 hr. The time during which the attached eye could communicate with the cerebral ganglion is shown by the *cross-hatched area* on the *left*. The mean $\Delta\Psi$ for all animals treated similarly is shown on the *right*. *PD*, projected dawns of prior light cycles.

during which the optic nerve was intact. In the rest of the experiments in Figure 2, the two eyes received the same initial treatment (LL = 21 hr; one attached eye, one detached eye), but the originally attached eye's optic nerve was cut after a variable time *in vitro*. This nerve cut required that the preparation be illuminated for about 2 min. Such brief light pulses had no apparent effect on the phase of the eye rhythm. Also, in several preparations, the nerve was cut by a snare so that illumination was not necessary. The results from the snare experiments were the same as those obtained using brief illumination.

When the second eye was detached 1 to 5 hr after LL/DD, little phase difference ($\Delta\Psi$) developed between the two eyes. Since NDR normally produces a $\Delta\Psi$ of 12.3 hr after LL = 21 hr, it seems safe to conclude that NDR did not occur. When the attached eye's nerve was cut 6 to 7 hr after LL/DD, large $\Delta\Psi$ values began to appear. When the nerve was cut 8 to 10 hr after LL/DD, only large $\Delta\Psi$ values occurred.

These results suggest that nerve activity at the time of LL/DD, while perhaps necessary, is not sufficient in itself to produce NDR. NDR did not occur unless nerve activity was allowed for at least 6 hr after LL/DD. We next performed experiments to see if the time of necessary nerve activity was the same following 24 and 18 hr of LL.

Figure 3 shows the results of cutting the attached eye's optic nerve at various times after LL/DD when LL = 24 hr. A group of comparison preparations in which the nerve was not cut at all is again shown at the *bottom* of the figure. (It will be noted that the phase difference between a cut and attached eye can be considered as either a large delay or a small advance. For ease of exposition, we have chosen to refer to all nerve-dependent $\Delta\Psi$ values as delays.)

When the nerve was cut 1 to 3 hr after LL/DD, only very small $\Delta\Psi$ values developed. Large $\Delta\Psi$ values first appeared after as little as 4 hr of attachment to the cerebral ganglion, and only large $\Delta\Psi$ values were observed after 7 to 15 hr of attachment. NDR occurs sooner after LL/DD when LL = 24 hr than when LL = 21 hr.

Figure 4 shows the time course for the development of NDR after 18 hr of LL. Small $\Delta\Psi$ values were observed for 4, 6, and 8 hr of attachment; other small attachment times were not run in order to conserve animals. When the nerve was left intact for about 10 hr, the $\Delta\Psi$ increased nearly to the value that was observed in the comparison group where the nerve was not cut at all. Following LL of 18 hr, the full $\Delta\Psi$ is only about 6 hr.

Figure 5 summarizes the results of the time course experiments following 18, 21, and 24 hr of LL. The mean phase difference, $\Delta\Psi$, is plotted against the time of the second optic nerve cut following 18, 21, and 24 hr of LL. Each value of $\Delta\Psi$ is the mean $\Delta\Psi$ obtained from all pairs of eyes receiving the same LL pretreatment and same duration of optic nerve attachment. Note that the intermediate values of $\Delta\Psi$ shown following 21 and 24 hr of LL are not typical of individual cases but are the result of averaging large and small $\Delta\Psi$ values.

The important point of these results is that the NDR does not always occur at a fixed time after LL/DD. Following 18 hr of LL, NDR was not observed unless the optic nerve was intact for about 10 hr. Only about 6 to 7 hr of attachment were required after 21 hr of LL, and only 4 hr were required after 24 hr of LL. In each case, NDR required that the nerve be attached until about 28 hr after the start of LL.

These results suggest two hypotheses about the timing of the nerve activity that is responsible for NDR. (*1*) The occurrence of NDR may require efferent activity only during the brief span of time when the $\Delta\Psi$ occurs or (*2*) it may require the continuous presence of nerve activity from the time of LL/DD to the time of the phase shift. To decide between these alternatives, we performed ex-

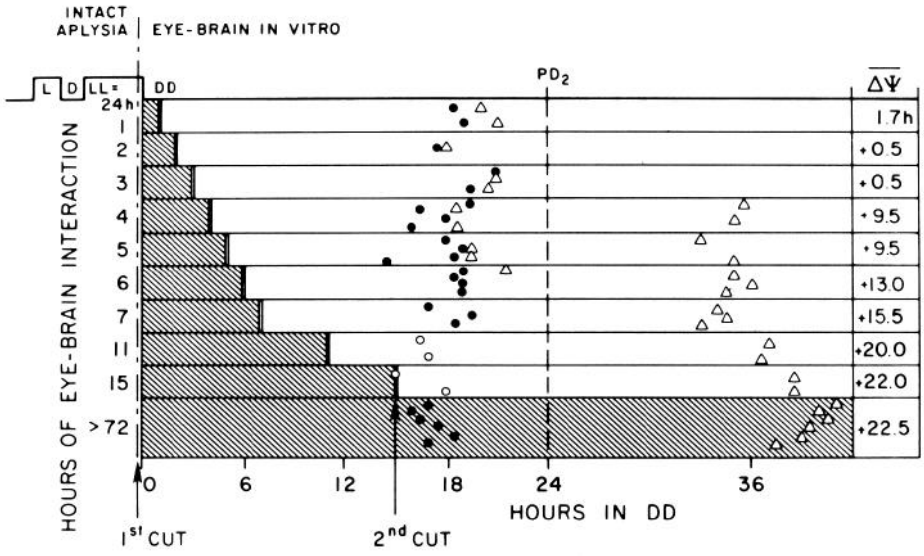

Figure 3. Phase of attached (△) and detached (●) CAP rhythms after varying durations of attachment following LL = 24 hr. *Open circles* (○) give the mid-rise points of the second cycle *in vitro* for those eyes whose first mid-rise points could not be measured. Other symbols are as in the previous figures.

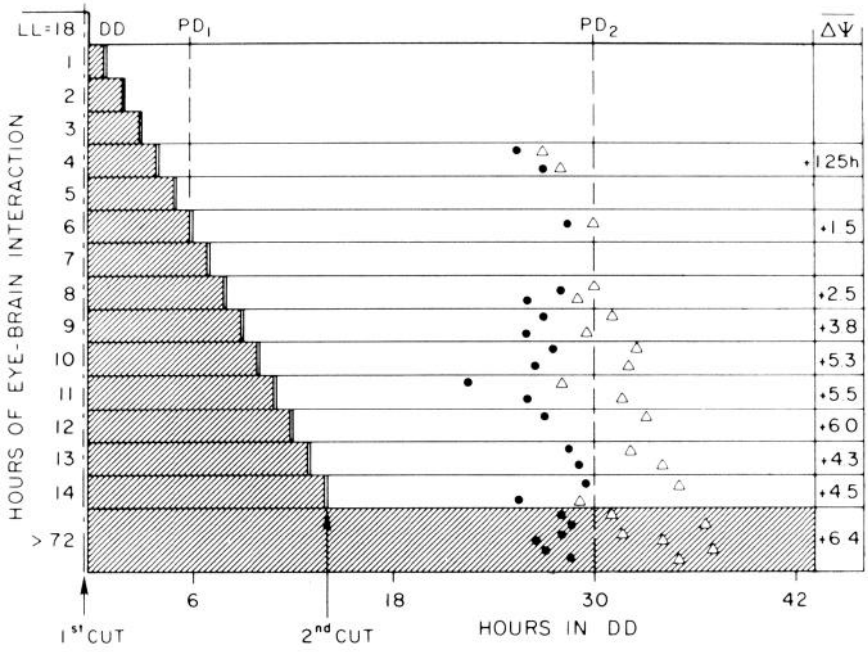

Figure 4. Phase of attached (△) and detached (●) eyes after varying durations of attachment following LL = 18 hr. Other symbols are as in the previous figures.

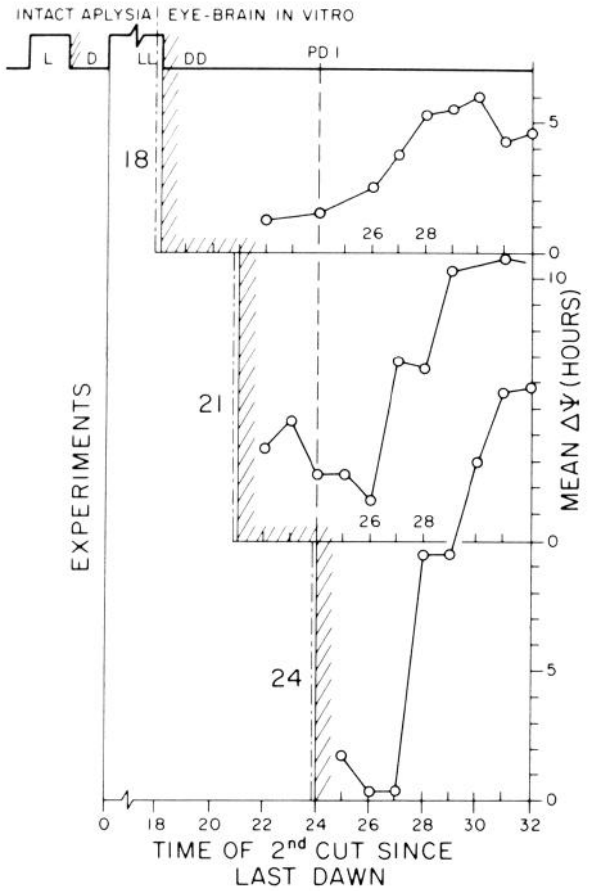

Figure 5. Time course of development of ΔΨ following 18, 21, and 24 hr of LL. *Points* show mean ΔΨ for each duration of attachment after three durations of LL pretreatment. *PD1* is the first projected dawn of the previous light cycles.

periments using reversible nerve blockade. This technique allowed us to test the effectiveness of nerve activity at selected intervals after LL/DD.

In these experiments, the animals received 24 hr of LL before the eyes and cerebral ganglion were removed for recording. One optic nerve was cut and the other was left intact. Both optic nerves were placed across a narrow channel of a special recording chamber and sealed with petroleum jelly. Compound action potentials (CAPs) were recorded from the eyes in the normal manner where the nerves emerged from the channel. Just before LL/DD, the channel was filled with Na^+-free, Ca^{2+}-free artificial seawater to block the flow of action potentials. We know that the nerve block was effective because the CAPs failed to cross the channel. At a variable time after LL/DD, the Na^+-free, Ca^{2+}-free solution was replaced with artificial seawater to unblock the nerves. The interaction between the attached eye and the brain was terminated finally 7 hr after LL/DD by cutting the nerve. The phase of the rhythm in both eyes then was evaluated as usual during the following two or three cycles.

The first experiment in this series compared the ΔΨ produced by an unblocked nerve that was cut after 7 hr with the ΔΨ produced by a blocked nerve that was cut after 7 hr (Fig. 6, *A* and *B*). Large ΔΨ values occurred only when the nerve was unblocked; NDR produced a ΔΨ of about 15 hr sometime within this first 7 hr *in vitro*.

When nerve activity was allowed only during the 4th through 7th hr after LL/DD, large ΔΨ values occurred (Fig. 6*C*), indicating that activity only during these 4 hr was capable of producing NDR. When nerve activity was allowed only during the 6th and 7th hr, large ΔΨ values failed to occur. When nerve activity was allowed only during the 4th and 5th hr, two of the three pairs of eyes had large ΔΨ values. A 2-hr "window" of optic nerve

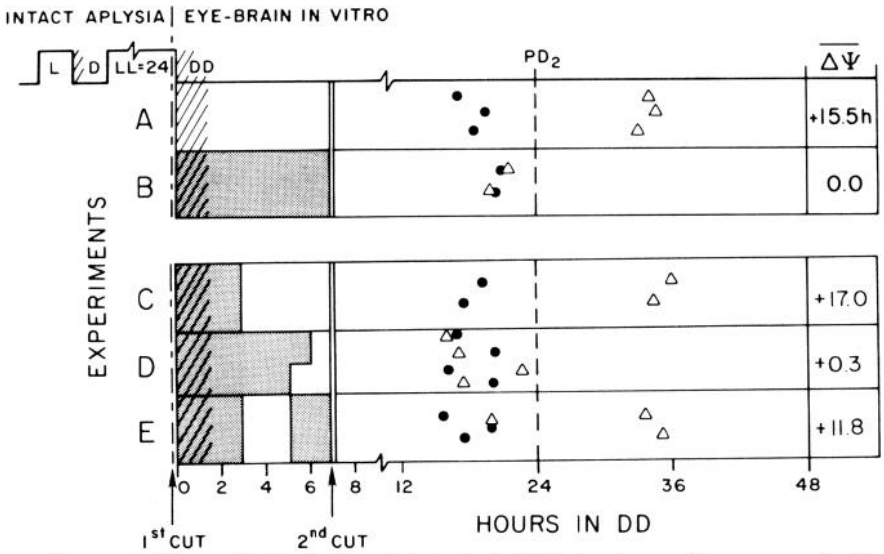

Figure 6. Phase of attached and detached CAP rhythms with communication between eye and brain restricted to selected portions of the first 7 hr in DD. *Shaded areas* show the times when the intact optic nerve was blocked with Na^+-free, Ca^{2+}-free artificial seawater. *A*, No restriction of eye-brain communication; *B*, optic nerve blocked for the entire 7 hr; *C*, first 3 hr blocked; *D*, first 5 or 6 hr blocked; *E*, entire 7 hr blocked except for a 2-hr "window" during the 4th and 5th hr in DD. Other symbols are as in the previous figures.

activity during the 4th and 5th hr is apparently capable of producing NDR, while a similar window 2 hr later is not. This result agrees well with the results of Figure 3 in which large separations suddenly appeared, in an all-or-nothing way, when the nerve was left intact until the 4th and 5th hr after LL/DD.

Discussion

Several characteristics of NDR emerge when the time course of the relevant optic nerve activity is determined after 18, 21, and 24 hr of LL. First, nerve-dependent resetting of the CAP rhythm does not occur immediately after LL/DD. Depending on the amount of LL, there is a delay of 4 to 10 hr before nerve activity resets the CAP rhythm. Second, optic nerve activity at the time of the LL to DD transition and for several hours thereafter is not required for NDR, at least after 24 hr of LL. Third, the relevant optic nerve activity in each case occurs about 28 hr after the start of LL. Fourth, the reset produced by NDR is nearly all-or-none.

One hypothesis about the mechanism responsible for NDR that is not supported by these results is that cerebral photoreceptors signaling LL/DD reset the attached eye. Optic nerve activity for the first few hours after LL/DD was not sufficient to produce NDR (Fig. 5). While very small $\Delta\Psi$ values often were observed when the nerve was cut a few hours after LL/DD, in no case did the magnitude of these phase shifts approach the magnitude of the full reset. If NDR were a response to the output of cerebral photoreceptors, one would expect the sensory information to be sent to the eye at the time of LL/DD, but, in fact, optic nerve activity at the time of LL/DD was not required for NDR (Fig. 6, *C* and *E*).

Several of the above results indicate that a timing mechanism is involved in NDR. The results of cutting the optic nerve at later and later times after LL/DD indicate that NDR does not occur gradually over a wide span of time. Rather, it occurs suddenly during a restricted period a few hours long. Furthermore, the effective nerve activity occurs at a fixed time with respect to the beginning of LL regardless of LL duration. Thus, there appears to be a *critical period* for optic nerve activity. This interpretation is supported by the nerve blockade experiments following 24 hr of LL. A brief 2-hr window of optic nerve activity, if placed appropriately, will produce NDR, whereas the same window will not produce NDR at other times.

It is tempting to hypothesize that the critical period is produced by a timed burst of activity in the efferent fibers, but, as yet, we have no data that this is the case. There was no change in the amount or pattern of efferent activity recorded during the critical period that could be detected by a casual inspection of polygraph records. The fact that the critical period always occurs about 28 hr after the start of LL indicates that its timing mechanism is not reset by LL/DD. The CAP rhythm also is not reset by LL/DD (until after the critical period), and it could be the timer for the critical period.

An interesting characteristic of NDR is that the reset is nearly all-or-none. Following 21 and 24 hr of LL, attached eyes were reset hardly at all or almost completely as the optic nerve was cut at later and later times (Figs. 2 and 3). Resets of intermediate size were not observed. The case for all-or-none resetting is less clear following 18 hr of LL. With 18 hr of LL, however, the average magnitude of the full reset is only 6 hr, and the all-or-nothing character could be obscured easily by random variations of a few hours.

While NDR is probably not the result of a direct photoreceptor input to the eye clock, it is apparent that photoreceptors located somewhere, in either the eye or brain, do sense LL/DD. Otherwise, NDR would not

occur. The fact that the actual resetting of the attached eye does not take place immediately indicates that a slowly acting intervening mechanism is at work between LL/DD and NDR. The locations of the intervening mechanism and the photoreceptors that sense LL/DD are investigated in the next and final paper (Prichard and Lickey, 1981b).

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