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CIRCADIAN RHYTHM OF OUTPUT FROM NEURONES IN THE EYE OF *APLYSIA*

III. EFFECTS OF LIGHT ON CLOCK AND RECEPTOR OUTPUT MEASURED IN THE OPTIC NERVE

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SUMMARY

1. The circadian rhythm of CAP frequency recorded from the optic nerve of isolated eyes at 15 °C was damped out by constant illumination (1100 lux) after several cycles of the rhythm. During illumination (LL) the rhythm was skewed with a rapid rising phase and slow falling phase, and the period was decreased by about 1 h. It is postulated that the circadian clock was stopped by LL at its lowest phase point, and that following cessation of LL, the rhythm was reinitiated from this phase point after a latency of 6–8 h.

2. For light pulses of 80 lux and 1100 lux, the photoresponse of the dark-adapted eye to 20 min light pulses applied beginning at 2 h intervals was not influenced by the circadian clock. At 5 lux there was a periodicity in the magnitude of the photoresponse, in phase with the circadian rhythm of spontaneous CAP production.

3. Small CAPs of non-circadian frequency were recorded together with normal CAPs in about 10% of records of output from isolated eyes. The cells producing the small CAPs had a different temperature sensitivity from those producing normal CAPs. The response of these cells to short light pulses consisted of a phasic burst of activity at light onset, followed by silence during the remainder of the short light pulse, and for 1 or 2 min following cessation of illumination. These small CAPs may be the activity either of H-type receptors or of secondary cells desynchronized from the major population.

INTRODUCTION

The cerebral eyes of *Aplysia californica* appear to serve a double purpose in the normal life of the animal. Each contains a circadian clock which regulates the output of compound action potentials (CAPs) in the optic nerve, and each responds to light stimuli (Jacklet, 1974). The roles of the eye as a circadian clock and as a photoreceptor in the control of the natural behaviour of *Aplysia* are unknown. The effect of the circadian clock on the photoresponse has been noted (Jacklet, 1971) but not

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previously been thoroughly investigated, and it is yet to be proved that the receptor cells in the eye are the mediators of light effects on the circadian clock.

This paper describes the effects of long periods of constant illumination (LL) on the output of the isolated eye, both in terms of possible receptor adaptation, and of damping and stopping of the circadian clock. The response of the eye to repeated, short light pulses was examined to show whether there is a circadian modulation of the photoresponse. Evidence will be presented to demonstrate the presence of 'small' CAPs of non-circadian frequency conducted by the optic nerve.

MATERIALS AND METHODS

Eyes were isolated from specimens of *Aplysia californica* and placed in temperature-controlled chambers of culture medium. Electrical activity from the optic nerves was recorded via tubing electrodes. Details of the culture medium composition, recording techniques, and methods of analysis are given by Benson & Jacklet (1977*a*).

Tungsten microscope lamps were used as light sources, with heat reflecting filters to block radiated heat and neutral density filters to produce different light intensities. Light intensities given in lux are approximations based on measurements made with a light meter, with allowances made for energy loss due to reflection and other factors.

RESULTS

Effects of constant light on the CAP output

Typical examples of the changes in CAP output from the isolated eye when it is subjected to prolonged illumination are given in Fig. 1. The eye was kept in DD for one or two cycles of the circadian rhythm, then illuminated at 1100 lux, and finally returned to DD. The record of CAP output during illumination is given in frequency measurements of CAPs/20 min made approximately every 2 h. Immediately after the beginning of the light pulse near the end of the falling phase of the rhythm, CAP frequency increased by a factor of 4. This high frequency declined in 20 min by 15%, and from this level the frequency continued to oscillate smoothly with a circadian period. All records of this kind of treatment show a relatively high CAP frequency at the beginning of illumination which dropped during the course of 20 min or less to a lower level which was still far above CAP frequencies observed in DD. Change in CAP frequency from this level always followed a circadian periodicity at elevated CAP frequencies. For the duration of the light pulse, the amplitude and period of the circadian rhythm were decreased. At 1100 lux, the period was decreased by approximately 1 h. Decrease in rhythm amplitude resulted from unequal reduction of the rising and falling phases of the rhythm. The rising phase was shortened relatively more than the falling phase, so that the shape of each cycle was skewed. After several days in LL, the rhythm no longer persisted even though CAP frequency continued at a level close to or above the maximum frequency in DD. When the light pulse ended, CAP frequency dropped to zero for an hour or less, and after an interval of as long as 8 h of low frequency CAP output, the rhythm was reinitiated. In all records that showed complete elimination of the rhythm in LL, reinitiation of the rhythm took place from a stable low CAP frequency. This suggests that the loss of rhythmicity

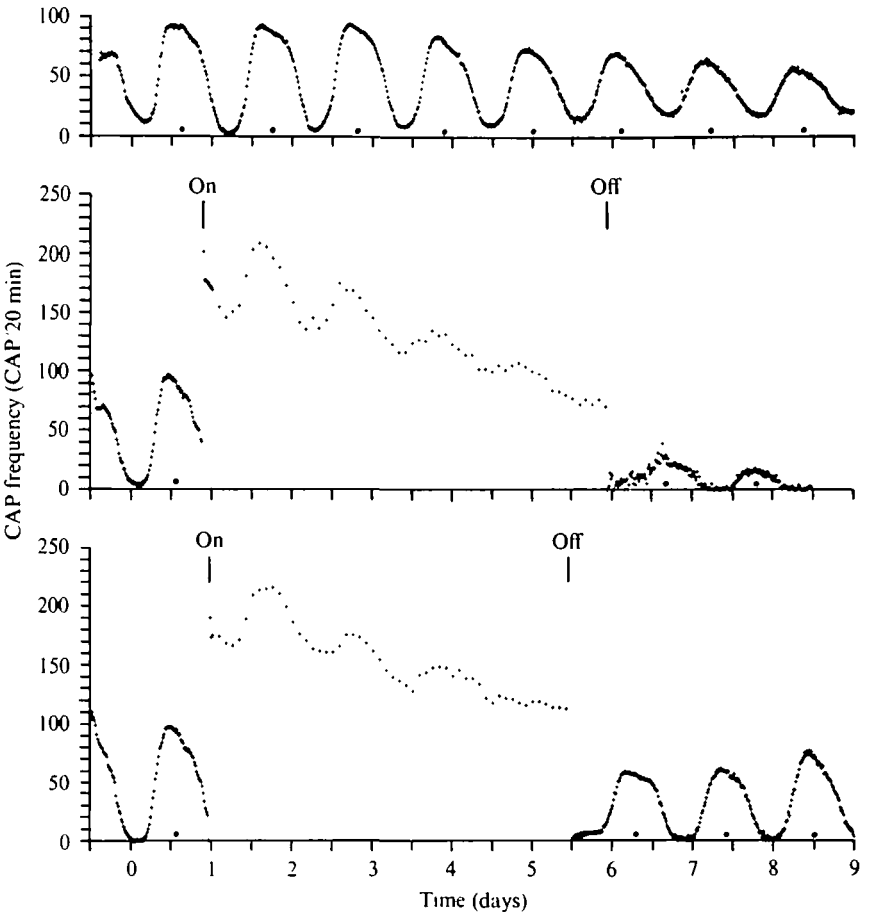

Fig. 1. Clock-stopping effect of prolonged illumination. During the period of illumination, CAP frequencies (recorded here as 20 min samples beginning at 2 h intervals) were greatly increased, but the amplitude of the rhythm decreased until the rhythm damped out and the clock stopped at its lowest phase point. Following cessation of illumination, the rhythm was reinitiated from this phase point after a 6–8 h latency. CAP frequency decreased sharply during the first 20 min of illumination due to light adaptation of the eye. Numerical data for clock-stopping experiments are given in Table 1.

was not simply due to a direct or indirect effect of light on the CAP-producing mechanism or its coupling with the circadian clock, but that the clock itself was stopped at its lowermost phase point. This is confirmed by the records shown in Fig. 1 and by the data for 9 experiments given in Table 1, where the phase of the rhythm subsequent to the light pulse depended on the time of cessation of LL. The first centroid of the reinitiated rhythms always occurred 18–20 h after the cessation of the light pulse. In Fig. 1, the first reinitiated rhythm was approximately 180° out of phase with the other, in which DD resumed 12 h earlier.

An alternative interpretation is that the clock was stopped on its falling phase 6–8 h prior to the lowermost phase point. This would require that upon reinitiation of the rhythm from this phase point, CAP activity be inhibited as a post-illumination effect for exactly the time that the rhythm takes to reach its lowest level. The data show that

Table 1. *Clock stopping effects of* 1100 *lux light pulses*

Record in Fig. 1	Phase of light pulse onset	Projected phase of light pulse cessation	Pulse duration (h)	Hours after pulse cessation to centroid of first post-pulse experimental cycle	Hours after pulse cessation to centroid of first control cycle
3	9·66	12·33	108·0	19·66	14·00
	8·66	11·33	108·0	20·33	15·00
	10·66	19·66	62·0	20·00	6·33
	10·33	19·33	62·0	19·66	6·66
2	8·00	22·00	123·0	18·00	3·00
	5·33	14·00	88·0	18·66	12·33
	5·00	13·66	88·0	18·33	12·66
	5·00	13·66	88·0	19·00	12·66
	4·00	12·66	88·0	19·00	13·66

total inhibition of CAPs after LL lasts 1 h or less, and that for the following 6–8 h CAP frequency is low and quite constant in most records.

Effect of 20 *min light pulses applied every* 2 *h*

Experiments in which isolated eyes were exposed to LL of constant intensity for long periods showed that the light response in these circumstances was modulated by the circadian clock. However, in all records, the CAP frequency immediately following onset of LL was higher than in the subsequent circadian oscillations in LL. If the eyes of *Aplysia* are involved in short-term behavioural responses to visual stimuli from the immediate environment, then the behaviourally significant photoresponses to change in light intensity should also be short-term, and might be expected to be uniform throughout the day for any given light intensity change.

The isolated eye was exposed to a series of 20 min light pulses spaced at 2 h intervals for 2 or 3 cycles of the rhythm. The light response was quantified as the number of CAPs produced during the final 10 min of the light pulse (i.e. during the time when CAP frequency was most uniform). The typical response to white light as measured in the optic nerve consisted of an approximately 1 s burst of high frequency activity at the beginning of illumination, followed by a slightly longer period (2–5 s) with no activity, and finally production of CAPs which rapidly increased to uniform maximum size and constant frequency (Fig. 1 of Jacklet, 1971) proportional to the log of the light intensity (Jacklet, 1969).

Fig. 2 shows typical records of light responses to three intensities of approximately 1100, 80 and 5 lux. At the higher intensities, 1100 and 80 lux, the photoresponse was fairly uniform, possibly with slight evidence of circadian modulation. At the lowest light intensity, 5 lux, there was a strong light response which showed a low amplitude circadian modulation in phase with the circadian rhythm of spontaneous CAP production. These results indicate that the circadian clock can affect the initial output of CAPs in response to light, but only at very low intensities. As shown in the long light pulse experiments described above, CAP output in response to prolonged illumination at intensities of about 1100 lux is clearly circadian in frequency. The absence of

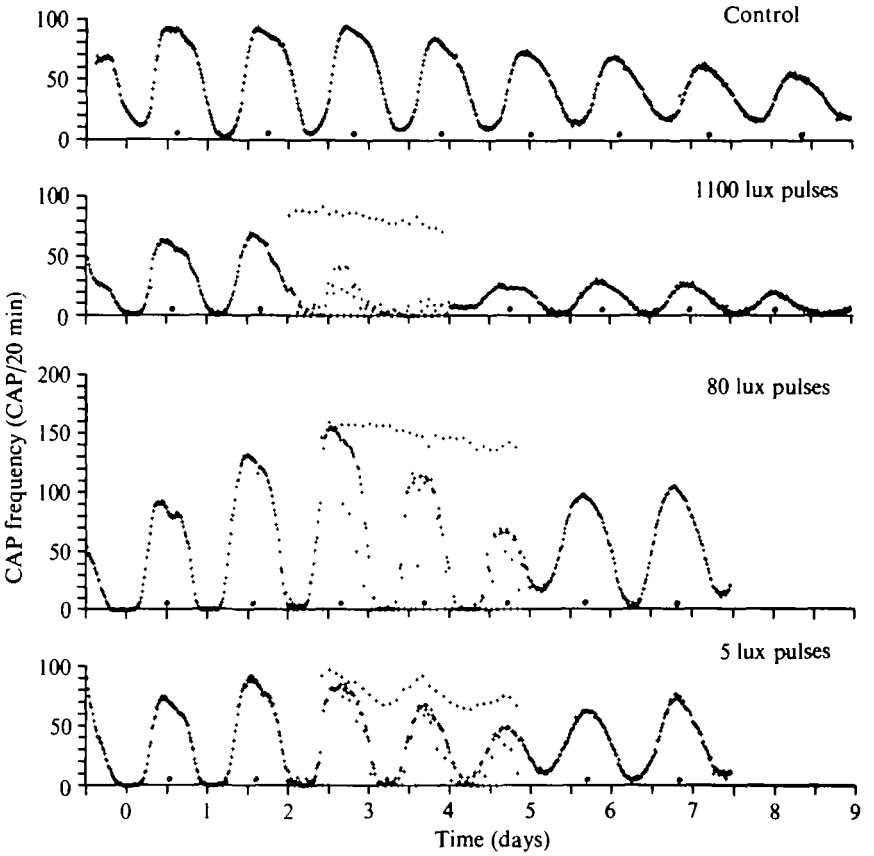

Fig. 2. Effects of trains of 20 min light pulses given at 2 h intervals. The CAP output in response to light pulses (upper line during light pulse trains) is plotted here in terms of the number of CAPs during the final 10 min of each 20 min light pulse. Spontaneous CAP frequencies are in terms of CAPs per 20 min interval. For light pulses of 1100 lux and 80 lux, there was no circadian variation in the photoresponse, but at 5 lux the response varied periodically in phase with the circadian rhythm of CAP frequency in darkness. The light pulses also produced a net phase advance in the rhythms, as can be seen by comparison with the control in the first record.

circadian modulation occurs only in the photoresponse during the first half hour or less of light, when the eye is dark-adapted and the CAP frequency is at its maximum.

A second feature that can be observed in Fig. 2 is that, during the course of the light pulse train, the amplitude of the circadian rhythm decreased. This was not simply a consequence of the silent periods which normally followed each light pulse, because the eye returned to normal CAP production during the 1·7 h between pulses. Intermittent light pulses apparently have a damping effect similar to that of LL. All records show a considerable phase advance after 2 days of multiple light pulse treatment.

Small CAPs from the optic nerve

In more than 10% of 160 records of activity from the optic nerve of isolated eyes in DD, 'small' CAPs were observed among the normal spontaneous CAP output (Fig. 3A). The amplitudes of these small CAPs were about 1/20 those of normal

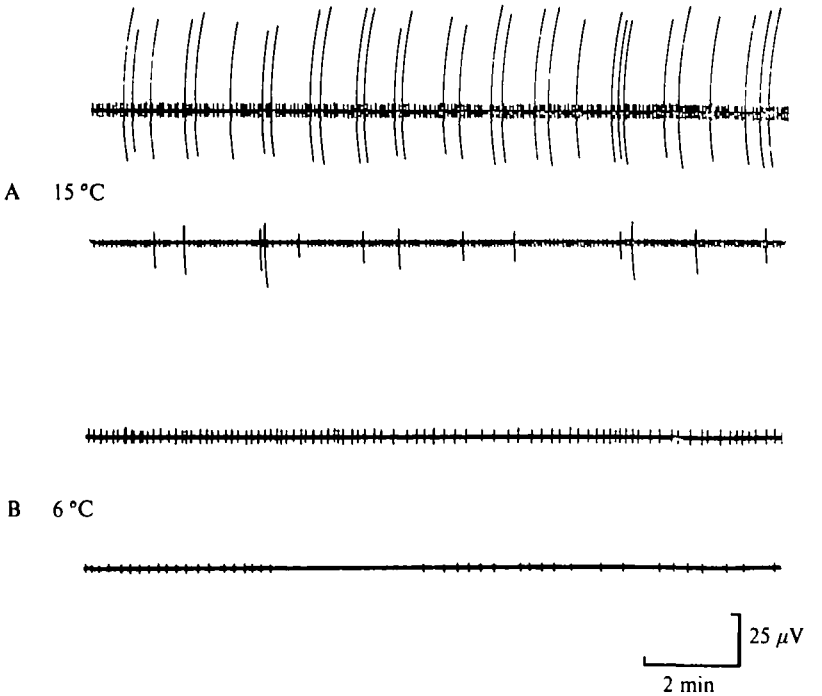

Fig. 3. Records of small CAPs at 15 and 6 °C. (A) Small CAP output from two eyes at different phases of the circadian rhythm of normal CAP frequency, recorded in darkness at 15 °C. (B) Small CAP output from two eyes at 6 °C. The first record is from the same eye as the first record in A. Normal CAP production was completely suppressed by cooling to 6 °C, but small CAPs were still being produced.

CAPs, and they were much more numerous, often five times as frequent as normal CAPs at their maximum firing rate. Because of this high frequency, it was difficult to make long-term measurements of small CAP frequency. The small CAPs may have been generated by cells other than the secondary neurones thought to produce normal CAPs, since they had a different temperature sensitivity. Fig. 3B shows two records of small CAP output of 6 °C, at which temperature production of normal CAPs ceased.

In Fig. 4, the third record illustrates the frequency of small CAPs over two days at 15 °C. The frequency of normal CAPs for the same eye is shown in the second record, and the first record is the CAP output of another eye subjected simultaneously to the same experimental conditions. The frequency of small CAPs at 15 °C changed in a regular manner, but there was no obvious circadian periodicity. Visual examination of other records indicated that such periodicity did not seem to occur in any small CAP output. The irregularity of the normal CAP rhythm in the second record may suggest that the small CAPs were the output of secondary neurones that had become desynchronized from the main population. At 6 °C, small CAP frequency was reduced by a factor of about 5, and no circadian periodicity was present (fourth record, Fig. 4).

Fig. 5 shows the small CAP light response at 15, 11 and 6 °C. At 15 and 11 °C, a brief burst of small CAP activity was followed by silence for the duration of the light pulse. (The burst is obscured in the record by a burst of normal CAPs, but can be

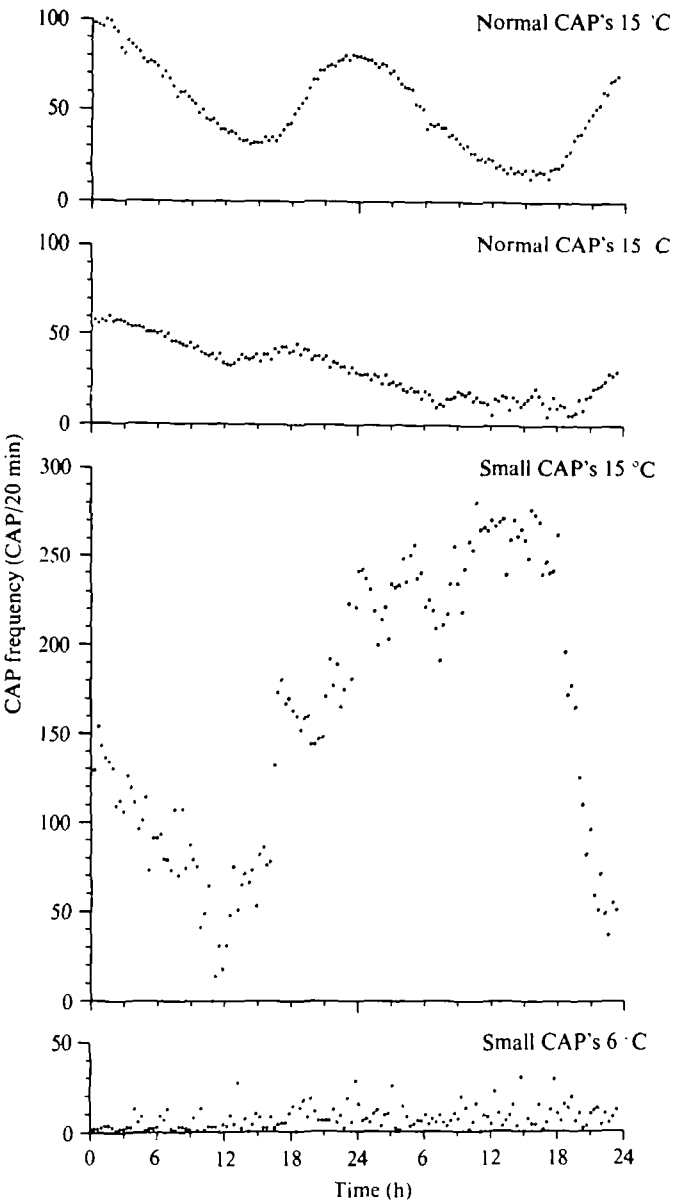

Fig. 4. Frequency of small CAPs in relation to normal CAP frequency. The first and second records show the frequencies of normal CAPs from two eyes in the same experimental chamber at 15 °C in constant darkness. The third record is the frequency of small CAPs recorded from the same eye that produced the normal CAP rhythm shown in the second record. The fourth record illustrates the frequency of small CAPs from another eye kept at 6 °C.

seen in records made at slower chart speeds). Normal CAP activity was increased during the light pulse, as in all eye preparations at these temperatures. No small CAPs were produced for 1 or 2 min after the cessation of the light pulse, and then normal firing resumed, sometimes at a slightly higher frequency for several minutes.

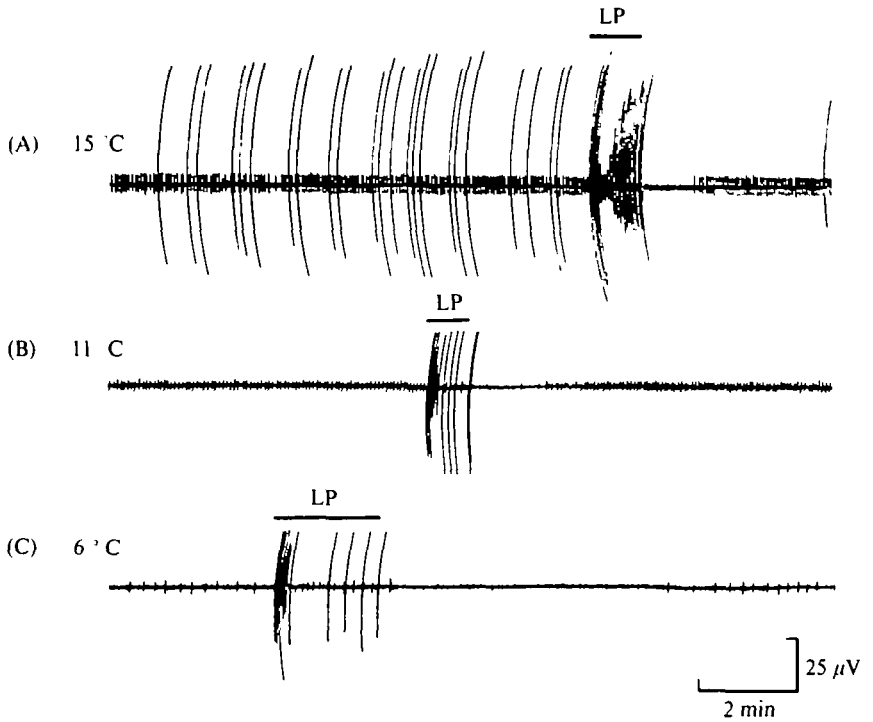

Fig. 5. Light response of small CAP generating cells at 15, 11 and 6 °C. Normal CAP frequency was tonically increased by light after a brief phasic burst and a short latency during which there was no normal CAP activity. Small CAP frequency was greatly increased for the first second of the light pulse obscured by the phasic burst of normal CAP activity, and was then inhibited for the duration of the light pulse and for one or two minutes thereafter. The time course of the small CAP response was lengthened at 6 °C where inhibition of the small CAPs did not occur until after the light pulse had ended.

At 6 °C, small CAP activity did not cease immediately following the initial burst of activity, but decreased in frequency and ceased soon after the end of the short light pulses, to resume again after 1 or 2 min, as at 11 and 15 °C.

DISCUSSION

A circadian rhythm that has been initiated and controlled in LD cycles can then be measured in constant conditions for a length of time that depends on the particular organism, and the light and temperature conditions. Some rhythms persist for weeks or months, while others decay in a few days (Bünning, 1973). Such fade-out often appears to be due to a damping effect of constant light (LL). For example, the circadian variations in growth rate of the fungus, *Neurospora*, were rapidly eliminated by as little as 0·2 erg/cm²/s of blue light (Sargent & Briggs, 1967). Chandrashekeran & Loher (1969) showed that 0·3 lux white light (0·2 erg/cm²/s in the effective band) damped out the circadian rhythm of pupal eclosion in *Drosophila* within 3 days. This was confirmed by Winfree (1974) who found, by systematic testing of various light intensities, that 4 days of LL at only 0·01 erg/cm²/s were sufficient to begin suppr[illegible]sing the *Drosophila* rhythm. It has been reported by Njus & Hastings (1975) that

ght and low temperature had an additive effect on the circadian clock of *Gonyaulax*, and that, combined or individually at sufficient intensities, they could drive the clock to a particular phase point and hold it there. Benson & Jacklet (1977*b*) showed that cold temperature held the *Aplysia* eye rhythm at its lowest phase, from which the rhythm was reinitiated upon return to normal temperature.

Several days of exposure to constant white light at intensities of approximately 1100 lux damped out the rhythm of CAP output from the isolated eye. The phase of the reinitiated rhythm, following return to DD, depended on the time of cessation of LL, so that the light acted ultimately on the clock mechanism itself. It is not known whether the influence of light was mediated via the receptor cells, or whether it directly affected the secondary neurones, which are thought to be the site of rhythm generation. It is postulated that the circadian clock oscillation was driven by light to a stable phase point which coincided with the phase of lowest CAP frequency in the rhythm, and that at the end of a light pulse sufficiently long for complete damping to occur, the rhythm was reinitiated from this point when the eye was returned to DD. This effect of light is similar to the action of prolonged low temperature on the rhythm (Benson & Jacklet, 1977*b*). Low temperatures drive the clock to the same phase point, but reinitiation takes place with no latency. Following prolonged illumination, the CAP frequency remains at a low level for 6–8 h before the rhythm begins again.

The alternative explanation is to hypothesize that the clock stops in the falling phase of its cycle, and that a long-lasting depression in CAP frequency due to LL masks the reinitiated rhythm. However, post-illumination inhibition of CAP activity occurs for only a short time after LL, and subsequent CAP production continues at quite constant low frequency. The combined requirement of a complex fade-out of CAP inhibition, and of uniform CAP depression lasting precisely until the beginning of a new rising phase, irrespective of the duration of LL, suggests that this explanation is unlikely.

The CAP generating mechanism is distinct from the clock oscillation. The CAPs are the effectors by means of which changes in phase, period, and amplitude of the clock are measured but this must be measured in constant conditions after a perturbation. Low temperature reduced CAP frequency, and below about 8 °C CAPs were abolished (Benson & Jacklet, 1977*a*). This means that during very low temperature pulses, the shape of the clock oscillation could not be monitored, and all measurements of the effects of the pulse had to be made on subsequent cycles of the rhythm. On the other hand, light increased the CAP frequency so that it was possible to measure changes in the clock oscillation during the course of a long duration light pulse.

Slow damping of the circadian rhythm of CAP frequency occurred in eyes kept at 9·5 °C, at which temperature CAP production still occurred but at a reduced level (Benson & Jacklet, 1977*a*). In that instance, the amplitude of the rhythm progressively decreased and the period increased. When constant light was applied to the eye, although the CAP frequency was elevated, the amplitude of the rhythm progressively decreased and the period decreased by about 1 h, at 1100 lux. This all but significant period change was in accordance with an earlier study on the effects of light (Jacklet, 1974) and the Circadian Rule which states that the period

length for diurnal animals decreases with increasing light intensity (Aschoff, 1960). In physical systems, any free vibration dies out due to dissipation of energy. However, damping in such a system involves a decrease in amplitude, without necessarily a change in period, especially in pendulum and sinusoidal oscillations (French, 1971). Circadian clocks are not free vibrations, but rather self-sustained oscillators with energy input. This energy input may not be distributed equally through all phases of the oscillation, so that it is more likely to display some of the characteristics of relaxation oscillators which are non-linear.

We have suggested that the effects of low temperature and continuous light are to drive the circadian clock to the same point in its cycle, the phase point of lowest CAP frequency, and that both involve a decrease in rhythm amplitude. The processes differ in that cooling increases the period while light decreases it, and reinitiation of the rhythm is immediate following damping out at low temperature but follows a latency of approximately 6–8 h after continuous illumination. For both cases, most records show a return to normal amplitude after one or two lower amplitude cycles, but the new steady-state period is often slightly increased after cold pulses. The lability of period observed in the *Aplysia* eye clock is characteristic of relaxation oscillators.

When high intensity white light was applied to the eye as a long-duration perturbation, the response in terms of CAP frequency during the first 20 min interval was always at least 15% higher than the subsequent frequency level from which the circadian rhythm continued. This indicates that the eye light-adapted during this 20 min interval, but it is not clear whether slow light-adaptation occurred throughout the light pulse, because any adaptation that may have occurred was obscured by the CAP frequency decrease due to damping of the rhythm.

By applying 20 min light pulses at 2 h intervals, it was shown that the presence of a circadian modulation of the light response, measured in terms of the CAP frequency during the final 10 min of the light pulse, depends on the light intensity. At high intensities, such as an intertidal organism like *Aplysia* would encounter during the daylight hours, the initial light response was not subject to modulation by the circadian clock. At lower intensities (80 lux and 5 lux) there was increasing circadian influence with decreasing light intensity. As shown in the long light pulse experiments described above, CAP output in response to prolonged illumination is clearly under circadian clock control. The absence of circadian modulation of the photoresponse was observed during the first 20 min or so of high intensity light, when the eye is still dark-adapted and the CAP frequency is comparatively high. It is possible that the CAP generating mechanism has a maximum firing rate (Jacklet, 1969), so that when the light stimulus is sufficiently intense, the CAP frequency is held at this maximum until the eye light-adapts, at all points on the circadian cycle. Only at lower intensities would the maximum CAP frequency not be reached and hence the effects of the clock on the photoresponse become visible.

The eye of *Aplysia* is composed of three main cell types: receptor cells, secondary neurons, and support cells (Jacklet, Alvarez & Bernstein, 1972; Jacklet, 1973, 1976). According to Hughes (1970), there are at least two receptor cell types in the eye, one 'ciliated', with equal numbers of cilia and microvilli, and one 'microvillous', with microvilli and an occasional cilium. Jacklet (1969) has characterized electrophysio-

logically three types of neuronal response to light. Intracellular injection of dye (Jacklet, 1976) following recording indicated that two of the cell types (R and H) were located in the receptor cell layer, and one type (D) in the secondary cell region, near the base of the eye. The R type cell responded to illumination with a long-lasting graded depolarization simultaneously with the appearance of CAPs in the optic nerve. In constant darkness (DD), this cell was typically silent during spontaneous CAP activity in the optic nerve. The second receptor type is the H cell, which was usually spontaneously active in DD, and was hyperpolarized by light. The response consisted of an initial depolarization followed by a long-lasting hyperpolarization. There was a burst of activity during the brief depolarization, then all action potential activity ceased. No correlation was found between the spontaneous activity of this cell in DD and the CAPs measured in the optic nerve. The third cell type (D) characteristically depolarized in response to light, with an increase in activity. These action potentials were often correlated with CAPs measured in the optic nerve. This strongly suggests that D cells are secondary neurones. All three cell types were antidromically stimulated via the optic nerve. Receptor cells, as well as secondary neurones, were stained when Procion yellow was driven up the axons of the optic nerve by electric current showing that these cells have axons in the optic nerve.

These results indicate that there are two receptor cell types in the eye of *Aplysia*. Possibly the R and H receptor cells correspond with the rhabdomeric and ciliary cells respectively, as in the pelecypod molluscs, *Pecten* (McReynolds & Gorman, 1970) and *Lima* (Mpitsos, 1973). The hyperpolarizing response of *Pecten* ciliary photoreceptors is due to an increase in potassium conductance (McReynolds & Gorman, 1974). It is interesting that the light-induced hyperpolarization in the giant cell R2 in the PVG of *Aplysia* is also due to an increased permeability to potassium (Brown & Brown, 1972).

'Small' compound action potentials recorded from several eyes exhibited properties which distinguished them from normal CAPs. They averaged 1/20 the amplitude of normal CAPs, and occurred at very high frequencies, often as many as 600 per 20 min interval. They did not appear to show a circadian rhythm, although the frequency often varied during the first 3 or 4 days of the experiment, and then rose to a fairly uniform high level. They were often present after two or more weeks in culture medium, when normal CAPs were often infrequent and irregular in amplitude. The temperature sensitivity of the small CAPs also differed, since they were still frequent, though reduced in number, below 7 °C when normal CAPs are absent in DD.

The light response of the small CAPs was characteristic and somewhat different from the response of the normal CAPs. The time course of this response is remarkably similar to that recorded intracellularly by Jacklet (1976) from H receptor cells in the eye. These cells were spontaneously active and responded to a light pulse with a biphasic response of depolarization followed by long lasting hyperpolarization. There was a burst of activity during the depolarization, followed by inactivity when the cell hyperpolarized. It is possible, therefore, that these receptor cells could be electrotonically or synaptically coupled and fire in synchrony to produce small CAPs. Another possibility is that the small CAPs are generated by desynchronized groups of secondary neurones. The light response of the cells producing the small CAPs has a fairly similar form but different time course to that of the secondary neurones,

and the temperature sensitivities of the two groups are different. It is not clear why small CAPs were recorded in only 10% of the eyes used in these experiments, but if their amplitude is usually very low, tubing electrodes may not be sufficiently sensitive to detect them except in cases of particularly good fit between the optic nerve and the tubing electrode.

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