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Extraocular Photoreceptors and Oscillators Can Control the Circadian Rhythm of Behavioral Activity in *Aplysia*

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Summary. The daily rhythm of behavioral activity in *Aplysia* does not require the eyes as essential photoreceptors or essential driving oscillators; activity can be diurnal in lightcycles (Figs. 1, 2) and can freerun in constant darkness (Fig. 2) after the eyes have been surgically removed. The eyes, however, do play a role in modulating the activity rhythm; eye removal may change the temporal distribution of diurnal activity; reduce the punctuality of activity onsets, increase the amount of nocturnal activity, and decrease the total amount of activity (Fig. 1). These results, together with previously published ones, make it unlikely that a circadian oscillator known to reside in the eye and another one located in the abdominal ganglion are the sole sources of behavioral rhythmicity in *Aplysia*.

Introduction

The marine gastropod, *Aplysia*, shows a diurnal circadian rhythm of behavioral activity (Kupfermann, 1967; Strumwasser, 1967). There is also a circadian rhythm in the frequency of compound action potentials recorded from the optic nerve when the eye and optic nerve are isolated in organ culture (Jacklet, 1969). Photoreceptors sufficient for entraining this ocular oscillator must reside within the eye itself because the rhythm can be entrained by lightcycles applied to the eye *in vitro* (Eskin, 1971). There is yet another circadian oscillator in the abdominal ganglion. It governs the frequency of ongoing spikes in the indentifiable neuron R15 as well as in other neurons in the ganglion (Strumwasser, 1965, 1967; Lickey, 1969). Photoreceptors in the eye are known to be unnecessary for its entrainment because lightcycles exert phase control over the abdominal oscillator after the eyes have been surgically removed (Lickey and Zack, 1973; Lickey, Zack, and Birrell, 1971). The oscillator in the abdominal ganglion can not be the only one which drives behavioral activity because freerunning and photic entrainment persist unimpaired after the abdominal ganglion has been removed (Strumwasser, Schlechte, and Bower, 1972).

These facts led us to ask whether ocular photoreceptors are necessary for diurnal behavior and whether the ocular oscillator is necessary for behavioral freerunning. We have found that the eye is unnecessary as either an oscillator or a photoreceptor.

Methods

Aplysia were obtained from Pacific Bio-Marine Supply Co., Venice, California. In the laboratory they lived in a 7500 liter recirculating aquarium system containing natural seawater at 14.0 ± 0.5°C. The aquarium system has been fully described elsewhere (Lickey, Emigh, and Randle, 1970).

At irregular intervals, varying between five days and one month, the animals were fed either fresh *Ulva* gathered along the Oregon coast, or dried seaweed called "Laver" which was imported from Korea and obtained from a wholesaler of Oriental foods. The *Aplysia* gained weight and remained vigorous for up to one year.

Behavioral activity was detected and recorded by a method similar to that of Kupfermann (1967). *Aplysia* lived individually in 38 liter glass aquaria which received branch circulation from the main seawater system. Each aquarium contained eight (but in one case, three) Plexiglas rods suspended vertically about 1.5 cm from the walls. A mercury switch attached to each rod activated a multichannel event recorder whenever the rod was deflected by 5 to 10°. One pen on the event recorder corresponded to one aquarium. Our apparatus did not detect locomotion across the bottom of the aquarium, but since *Aplysia* seldom crawl across the bottom (Kupfermann, 1967; Strumwasser, 1967) very little locomotor activity went undetected. Some types of activity other than locomotion probably were detected also, e.g., deflection of rods by head movements while the animal was standing still. Each aquarium was inside a light proof enclosure which was painted white on the inside. It was illuminated by either fluorescent or incandescent light bulbs held about 10 cm above the water surface. The fluorescent fixtures contained one or two 8 W cool-white bulbs; the incandescent fixtures contained one or two 7.5 W bulbs. Light intensity could be attenuated to any desired value below full output by applying electrician's tape (fluorescent) or black paint (incandescent) to some portion of the bulb's surface. Intensities during the lighted portion of the lightcycle varied from less than 1 lux to about 1400 lux for different animals. Light intensity was measured with an incident light meter, and the value reported is for the brightest point on the water surface. Four activity recording aquaria were operated simultaneously and each one could be illuminated independently of the other three. The lightcycle was always 12 hr of light alternating with 12 hr of darkness ($L\underline{D}$ 12:$\underline{12}$), but the time of light onset varied from one tank to the next. The data were analyzed in traditional manner by constructing activity records from the event recorder chart (see Richter, 1965). Eyes were surgically removed according to procedures described by Lickey and Zack (1973), and they did not regenerate. Twelve *Aplysia* were studied. The experiments were carried out from January through October.

Results and Discussion

The *Aplysia* were typically diurnal both before and after their eyes were removed. Three of the twelve were, at best, poor runners and perhaps arhythmic; none were nocturnal. Although we are not certain why some *Aplysia* should be poor runners, two of the three arhythmic animals were exposed to a light intensity of only 170 lux and they might have been diurnal had the intensity been increased. In any case, the poor runners were not useful to us in evaluating the role of the eyes for the daily rhythm, and we did not study them in detail.

A feature typical of most activity records was a period of inactivity during the last half of the dark period followed by a sharp onset of activity near light onset (Figs. 1 and 2). Some preparations also showed a burst of activity near dusk (Fig. 1C). In agreement with Kupfermann (1967), we found behavioral activity to be sensitive to the degree of aeration or turbulence in the seawater; accidental failure of circulation nearly invariably led to inactivity. *Aplysia californica* (Fig. 1A, B; Fig. 2) and *Aplysia vaccaria* (Fig. 1C) appear to be substantially similar in the temporal distribution of activity except that *vaccaria* more frequently show a burst of movement following dusk than do *californica*. The rhythm can be entrained by either cool-white fluorescent light (Fig. 1A, C) or incandescent light (Fig. 1B; Fig. 2) at intensities as low as 170 lux (Fig. 1B, C; Fig. 2). We have not made a detailed study of either the absolute threshold or the action spectrum for entrainment.

The locomotor rhythm can also freerun after the eyes have been removed. A freerun, in the presence of a "lightcycle" from completely black painted incandescent bulbs, is shown in Fig. 2. In this case, the endogenous period was greater than 24 hr; other *Aplysia* have shown freerunning periods of less than 24 hr. Therefore, the oscillator in the eye cannot be the only one governing the circadian rhythm of behavorial activity in *Aplysia*. Also, nonvisible radiations from the light sources are ineffective as exogenous time signals in eyeless *Aplysia*.

Although the eyes are not essential for either diurnal activity in lightcycles or freerunning in constant darkness, it is equally clear that they participated in controlling the rhythm. Activity records frequently showed changes in the temporal distribution of activity following eye removal (Fig. 1A, B). In some cases, eye removal eliminated a prominent feature of the animal's activity pattern (Fig. 1A). In several animals there was more nocturnal activity post-operatively than pre-operatively (Fig. 1A, B). Frequently there was greater variability in the timing of activity onsets post-operatively (Fig. 1A, B) and a reduction in the total amount of daily activity (Fig. 1A, B, C). The present data, however, do not justify any general or precise statement about the role of the eyes in controlling the rhythm.

We also point out that we do not have unequivocal evidence that the oscillator governing freerunning in eyeless *Aplysia* is entrained by the lightcycle when the animal is behaving diurnally. It is remotely possible that eyeless *Aplysia* are diurnal because they are reflexively active in response to light and that the entrainment of the circadian oscillator, as expressed in constant darkness, does not occur.

The evidence does, however, demonstrate (1) extraocular photoreceptors can sustain diurnal activity in LD, (2) extraocular oscillators

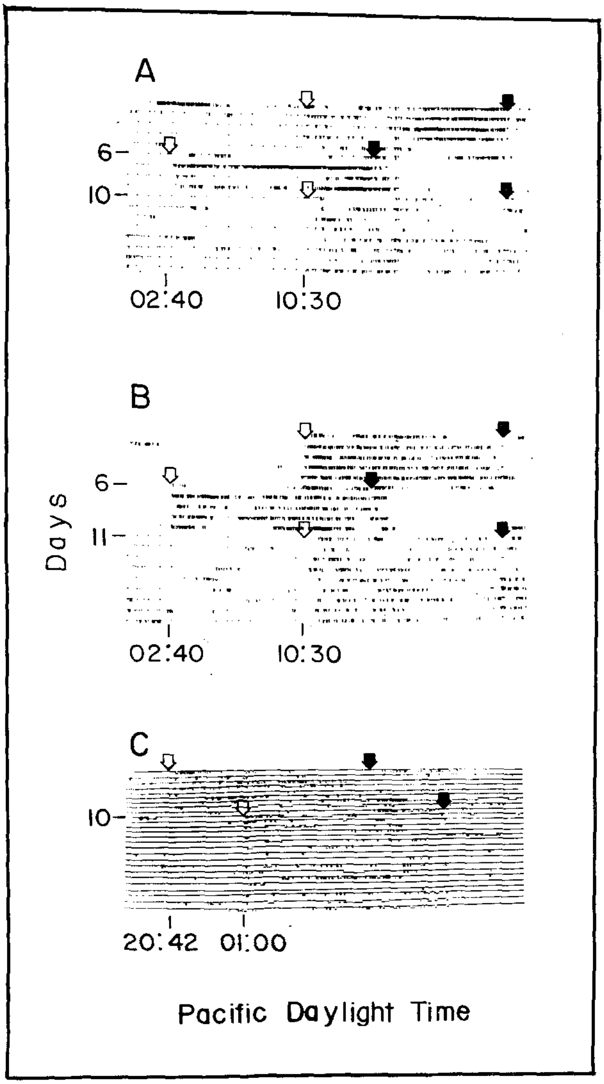

Fig. 1 A—C. Diurnal rhythm of behavioral activity before and after eye removal. Behavioral activity signified by vertical marks on horizontal lines. Each line represents one calendar day; first day of experiment at top and successive days listed in order below. Open arrows: light onset. Closed arrows: light offset. A, B, and C show records from three different *Aplysia*. A. *Aplysia californica*. LD 12:12, 1400 lux: 0 lux, fluorescent. Days 1–5: eyes intact; activity is diurnal. Days 6–9: time of light onset advanced 7 hr, 50 min: activity onset shows similar advance requiring about one day for completion. Both eyes removed just before light offset on day 9. Day 10–end: time of light onset delayed 7 hr, 50 min; activity remains diurnal in now eyeless animal. Note the change in temporal profile and the reduction in amount

can sustain circadian freerunning in DD and (3) removal of the eyes has a marked but difficult to characterize effect on the diurnal rhythm.

The evidence given in this paper and preceding reports allows us to draw the following four conclusions about the mechanisms of circadian organization in *Aplysia*. First, the eyes are not the only photoreceptors and oscillators controlling rhythms of neural and behavioral activity in *Aplysia*. Second, the eyes may participate in controlling these rhythms since the variability circadian timing appears to be increased, in unperdictable ways, by eye removal. Third, a conclusion which is implied by the first two, there is a multiplicity of oscillators and a multiplicity of photoreceptors which govern circadian timing. The eye itself contains oscillators and photoreceptors sufficient for controlling an ocular circadian rhythm *in vitro* (Eskin, 1971) yet the rhythms of behavorial activity and neural activity continue to function on the basis of extraocular photoreceptors and oscillators. Furthermore, a recent report by Strumwasser, Schlechte, and Bower (1972) leads us to suspect that there may be extraocular oscillators in addition to the one in the abdominal ganglion. Fourth, separate oscillators may be entrained independently by different photoreceptors. The ocular oscillator is entrained by ocular photoreceptors (Eskin, 1971) while the extraocular oscillator(s), e.g. the oscillator in the abdominal ganglion, can be entrained by extraocular photoreceptors.

Several cadidates for extraocular photoreceptors and oscillators are suggested by the literature. It has been argued that the ordinary wrinkled skin of the gastropod *Helix* contains photoreceptors (Bullock and Horridge, 1965), and it has been recently reported that the isolated siphon of *Aplysia* contracts in response to light after it has been completely severed from the central nervous system (Lukowiak and Jacklet, 1972; Newby, 1972). It is also well known that light applied directly to the *Aplysia* central nervous system can cause various neurons to

of activity following eye removal on day 9. B. *Aplysia californica*. Light: LD 12 : 12, 170 lux: 0 lux, incandescent. Day 1–5: eyes intact; activity is strongly diurnal. Days 6–10: time of light onset advanced 7 hr, 40 min; after one day of adaptation, activity is again strongly diurnal. Both eyes removed just before light offset on day 10. Day 11: time of light onset delayed 7 hr, 40 min; activity adapts and remains diurnal in the now eyeless animal. Note the changed temporal profile of activity, the decrease in total amount of activity, the increase in irregularity of activity onsets, and the increase in nocturnal activity following eye removal. C. *Aplysia vaccaria*. Only three rods in aquarium. LD 12 :12 170 lux: 0 lux, fluorescent. Days 1–9: eyes intact; locomotion predominately diurnal but with burst of activity following dusk on days 1 and 7. Both eyes removed just before light offset on day 9. Day 10–end: time of light onset delayed 4 hr, 18 min; activity rapidly adapts and remains diurnal

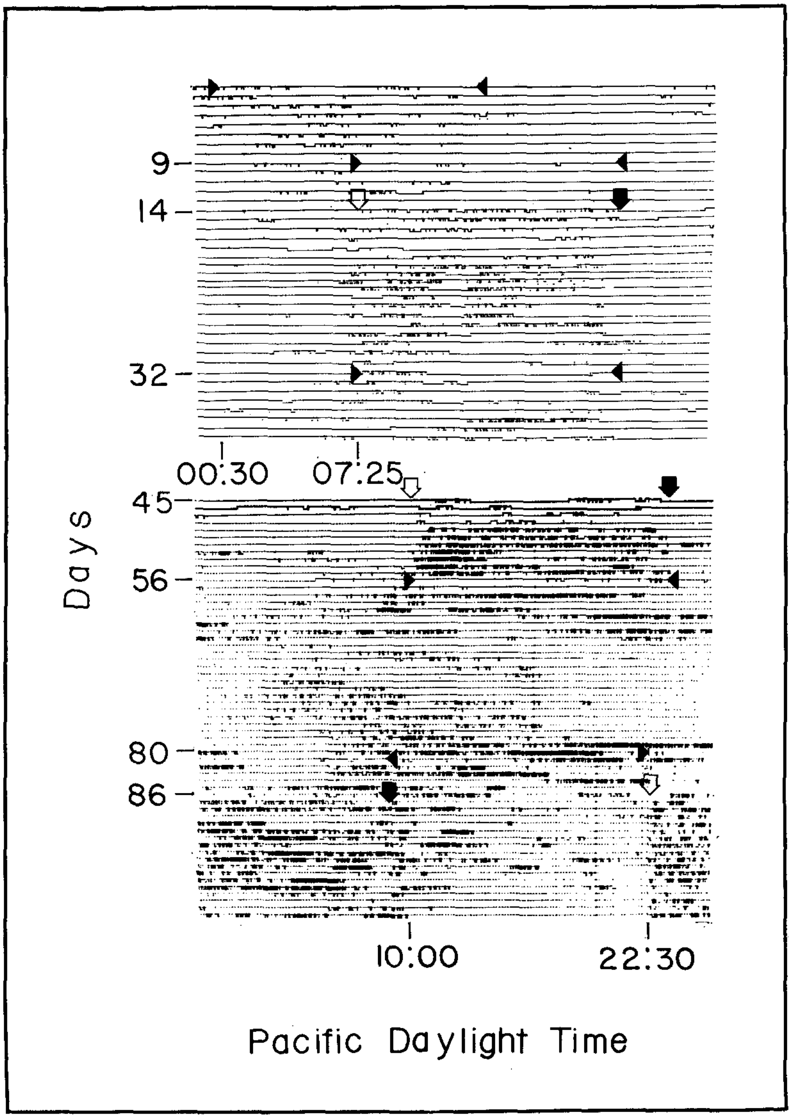

Fig. 2. Diurnal and freerunning activity before and after eye removal. *Aplysia californica*. LD 12:12 either 170 lux: 0 lux from one unpainted 7.5 W incandescent bulb (called "LD") or 1 lux: 0 lux from two black painted 7.5 W incandescent bulbs (called "pseudo LD"). Closed triangles indicate time of "light" from black painted bulbs, triangle pointing right = onset, toward left = offset. Other symbols as in Fig. 1. Days 1–8: eyes intact; activity appears to freerun in pseudo LD. Days 9–13: activity fails to follow phase shift in pseudo LD. Days 14–22: switch to LD; activity becomes diurnal. Days 23–31; eyes removed shortly before light offset on day 23; activity remains diurnal. Days 32–39: return to pseudo LD;

either hyperpolarize or depolarize (Arvanitaki and Chalazonitis, 1961). There is ample precedent in other species that photoreceptors and oscillators governing circadian rhythms may reside within the central nervous system (Menaker, 1971; Nishiitsutsuji-Uwo and Pittendrigh, 1968; Stephan and Zucker, 1972). In *Aplysia,* the circadian oscillator in the abdominal ganglion is similar to the oscillator governing locomotion in that in does not require the eyes as essential photoreceptors for entrainment (Lickey, Zack, and Birrell, 1971; Lickey and Zack, 1973). The oscillator in the abdominal ganglion cannot be the only one driving behavioral activity, however, because Strumwasser, Schlechte, and Bower, (1972) have shown that the locomotor rhythm functions nearly normally with respect to both freerunning and entrainment after the abdominal ganglion has been surgically removed.

These results emphasize that experiments on the physiological organization of activity and rest in complex animals can not presume the existence of a single photoreceptor or single "clock". As has long been suggested (Pittendrigh and Bruce, 1959) but seldom directly demonstrated, the problem is one of unraveling the relationships between a multiplicity of coupled oscillators and photoreceptors.

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activity appears to start freerunning but equipment failure on day 39 terminates data collection before definitive evidence of freerunning can be obtained. Days 45–55: resume in LD with new version of apparatus which is more sensitive to locomotor movements; activity is diurnal. Days 56–79: return to pseudo LD; a clear freerun develops. Days 80–85: activity does not follow a phase shift in pseudo LD. Days 86–end: LD restored, activity again becomes diurnal

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