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Circadian Rhythm of Neuron R15 of *Aplysia californica: In Vivo* Photoentrainment

(effects of dissection time/role of eyes in photoentrainment)

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ABSTRACT **(1) The neuron R15 in the parietovisceral ganglion of *Aplysia* has a circadian rhythm of spiking activity when recorded in the isolated ganglion. The rhythm is entrained *in vivo* by light–dark cycles. (2) The phase of the R15 rhythm is a function not only of the entraining light schedule, but also of the time of dissection. Changes in the dissection time during the light portion of the light–dark cycle yield little change in the subsequent R15 peak time. Dissections during the dark portion produce peak times that vary with dissection time with a slope that is approximately one. (3) The circadian rhythm of R15 can be phase-shifted *in vivo* by changes in the phase of the entraining light–dark cycle in one to two weeks. R15 neurons of blinded *Aplysia*, however, show little or no phase shift in this time. (4) It is concluded that the eyes are important as receptors for the photoentrainment of the R15 rhythm *in vivo*, but that neural connections from the eyes to R15 are not required.**

The neuron R15 in the parietovisceral ganglion (PVG) of *Aplysia californica* has been shown to have a circadian rhythm (CR) of spiking activity *in vitro* when recordings are made from the ganglion in natural sea water (1, 2). The phase is measured by the time of maximum spiking frequency during the first 24 hr after dissection. R15 produces another cycle of spike activity, but of diminished amplitude, in the second 24 hr period of *in vitro* recording but it is evident from the published figures that this system is heavily damped (1, 2). The time of peak activity is reported to be predictable on the basis of the light–dark (LD) schedule to which the *Aplysia* had been exposed prior to dissection. However, the time of peak has varied considerably from report to report. The phase has been reported to be primarily at onset of projected light (1) but also, in some animals, at offset of projected light (2, 3). For example, in Table I of Lickey (2) six of 11 R15s studied between October and mid-May had a phase angle of circadian time (CT) 2336 ± 0206 (mean ± SD) while the remaining five had a phase angle of 1230 ± 0124; in other words the two phase angles in this population of 11 R15s were either at projected dawn or dusk. However, other phase angles of R15 have been reported and claimed to be dependent on season; for example mid-day/midnight peaks were reported to occur in the period end of April through October (2). Slightly later phase angles for R15 were reported in more recent papers (4, 5). It is obviously disturbing to consider R15 a circadian system if the phase angle can vary so much, particularly since the system also damps out rapidly *in vitro*.

In this report, data will be presented that may explain some of the variability seen in R15 peak timing in these previous papers: phase is influenced by dissection time. The nature of this relationship is one of the subjects of this paper. Our studies show that the phase angle of the R15 rhythm is dependent on both the LD cycle and the time of dissection of the PVG. The question of the role of the eyes in the photoentrainment of the R15 rhythm is re-examined in the light of these new findings. The eyes are found to be important for entrainment to LD cycles in our studies, in contrast to previous reports (4, 5).

Abbreviations: LD, light–dark; CR, circadian rhythm; CT, circadian time: for LD 12:12 (hr:hr), lights on = CT 0000 (first two digits, hours; last two digits, minutes) and lights off = CT 1200 regardless of the position of these events with respect to the solar day; PDT, Pacific daylight time (all times given, other than circadian time, are in PDT); PVG, parietovisceral ganglion; the term projected light schedule is used to denote the light schedule that the animal had been exposed to prior to dissection.

METHODS

Aplysia californica were kept in a community tank in a closed recirculating natural sea water system (1500 gallons). The temperature was maintained at 14 ± 0.5°. The light schedule in this system was LD 12:12 (180 lux:0 lux), with lights-on at 0800 hr Pacific daylight time (PDT) (CT 0000) and lights-off at 2000 hr PDT (CT 1200). All animals spent at least 1 week in these community tanks before any experimental manipulation was performed.

At dissection, the PVG was isolated from the rest of the nervous system and pinned out on a silastic platform in either a 10 ml or a 100 ml dish. This dish was filled with Millipore-filtered (0.22 μm) natural sea water. The temperature at the level of the ganglion was kept at 15 ± 1°. All recordings were made in constant light at approximately 75 lux. The spike activity of R15 was recorded for the following 24–30 hr by conventional intracellular techniques using glass microelectrodes filled with 0.6 M K_2SO_4 (blinding studies) or extracellularly (all other runs). Extracellular recording was accomplished by placing a 50–150 μm tip diameter fire-polished glass pipette filled with sea water on the connective tissue over the R15 soma. The extracellular spikes were amplified by Tektronix 122 preamplifiers, and monitored on a Tektronix 5103 oscilloscope and recorded on a Grass model 7 polygraph. Extracellularly recorded spikes usually ranged from 30 to 100 μV in amplitude.

Spikes were counted either by hand from polygraph recordings, or with a Sodeco counter driven by a standardized pulse triggered by the spikes. The spikes were counted in 10 min

bins, and these were averaged over 30 min. The maximum spike frequency over a 24 hr period is, therefore, reported to the nearest half hour. While a moving average reduces the apparent noise in the 24 hr graphs, it does not significantly alter the estimate of the timing of the phase angle and hence is not used as a method in this paper.

Photoreceptor for Entrainment. Experimental animals were taken from the community tanks, and one of three procedures was carried out: blinding, sham-blinding, or no operation. Blinding was accomplished by seizing the skin of the animal near the eye with rat-tooth forceps, lifting up slightly, and cutting off the eye along with a small patch of skin. The underlying muscular body wall was left essentially intact. Microscopic examination of the removed piece of skin and autopsy (at the time when the PVG was removed) were used to verify removal of the entire eye. The sham blinding operation consisted of removal of a similar sized piece of skin just anterior to the eye.

After the appropriate operation, the experimental animals were placed in individual 57 liter aquaria, isolated from each other and from the rest of the sea water system. The temperature was less precisely controlled than in the main system. During the lighted portion of the light-dark cycle, the temperature rose to between 15 and 15.5°; the night temperature fell to 13.5 to 14°. The lights used were 15 W fluorescent bulbs placed about 15 cm above the water surface. Light intensity in these aquaria varied from about 250–2000 lux, depending on position and orientation of a light meter placed in the tank. The lighting schedule was LD 12:12, as in the main system, but 6 hr advanced. Lights-on occurred at 0200 hr, and lights-off at 1400 hr. Experimental *Aplysia* were sacrificed after 7–15 days in these aquaria.

All final dissections, both for control animals from the main system (dawn = 0800 hr) and the experimentals from the individual aquaria (dawn = 0200 hr), were performed within 1 hr of 0900 hr but most were within 15 min. Therefore, dissections for controls were at CT 0100, and for experimentals at CT 0700. These experiments were run from June through August, 1973. The assessment of seasonal differences in R15 timing was performed on five animals from the community tank dissected at CT 0100 in January and February, 1974. The effect of dissection time on R15 timing was assayed on 17 animals dissected between CT 1000 and CT 2200 in the period June through August, 1974. The ± values following means are standard deviations throughout this paper. In order to determine whether animals from community tanks differed in R15 timing from those in isolated aquaria, we dissected additional animals from community tanks at CT 0100 (n = 2) and CT 0700 (n = 6) in February, 1975.

RESULTS

(a) *Relation Between Time of Dissection and Time of Peak.* Dissection times and dates, and times of maximum R15 spiking in the 24 hr immediately following dissection, are given in Table 1 for experiments conducted between June 1973 and August 1974. These data were used to generate graphs of R15 peak time versus dissection time (Fig. 1A and B). More than half of the data was obtained using extracellular recording. We were unable to observe systematic differences in peak time between intracellular and extracellular recordings. Furthermore, when the community tank animals (n = 6) were compared with those in isolated aquaria (n = 10) at CT 0700, the means of R15 peaks were not significantly different (community tank average, 1645 ± 0120; isolated aquaria average, 1436 ± 0222).

TABLE 1. *Dates, dissection time, and time of maximum spiking of R15 neuron*

Date	Dissection time	Peak time	Date	Dissection time	Peak time
06/11/73-I	0100	1630	07/19/74	1000	1700
06/13/73-I	0100	1830	07/20/74	1000	1630
06/15/73-I	0100	1430	08/13/74	1000	2200
06/26/73-I	0100	1330	07/12/74	1300	2200
07/04/73-I	0100	1200	07/13/74	1300	1830
01/17/74	0100	1700	07/15/74	1300	2130
01/29/74	0100	1730	07/25/74	1600	2200
02/11/74	0100	1030	08/17/74	1600	0300
02/14/74	0100	1700	08/18/74	1600	2000
02/16/74	0100	1630	06/01/74	1900	0600
06/24/73-I	0700	1330	06/02/74	1900	0500
06/28/73-I	0700	1600	06/19/74	1900	0430
06/30/73-I	0700	1330	06/20/74	1900	0300
07/15/73-I	0700	1400	06/21/74	1900	0700
07/17/73-I	0700	1700	07/17/74	2200	1100
07/29/73-I	0700	1230	07/23/74	2200	0700
08/02/73-I	0700	1330	08/15/74	2200	0530
08/07/73-I	0700	1600			
08/08/73-I	0700	1900			
08/09/73-I	0700	1100			

All times are circadian times; lights-on = 0000; lights-off = 1200. I = intracellular; remainder are extracellular.

Several features of the R15 peak times and graphs should be noted. First, although CT 0100 dissections were performed during the summer of 1973 and the winter/spring of 1974, no seasonal change in the timing of the peaks was observed (summer average, CT 1500 ± 0233 hr; winter average, CT 1542 ± 0255 hr). This is contrary to the findings of Lickey (2) that summer animals yield peaks near the middle of the projected day or night (near CT 0600 or CT 1800) while winter ones yield peaks near CT 0000 or CT 1200.

Second, the time of peak spiking activity is strongly dependent on dissection time, with, in these experiments at least, all peaks occurring 4–18 hr after dissection. With this large number of experiments (n = 37, Table 1), the probability of omission of any peaks in 10 hr of the day strictly by chance is vanishingly small ($P = 10^{-14}$).

Third, the timing of the peaks is nevertheless predictable on the basis of the predissection light schedule, when dissection time is also taken into account. This is most easily seen with dissections performed at CT 0100 and CT 0700. In these two cases, the average R15 peak times were CT 1521 and CT 1436, respectively. The peaks averaged 1421 hr after CT 0100 dissections, but only 0736 hr after CT 0700 dissections. The peak times based on the predissection light schedule were nearly the same (difference = 45 min; $t = 0.7$; $P > 0.4$), but the intervals between dissection and peak were widely different (difference = 6:45 hr; $t = 6.0$; $P \ll 0.001$). However, at a different time of dissection, CT 1900, for example, both the time of R15 peak and the interval between dissection and peak are significantly different from those of CT 0100 dissections (time of peak: difference = 1015 hr; t = 8.0;

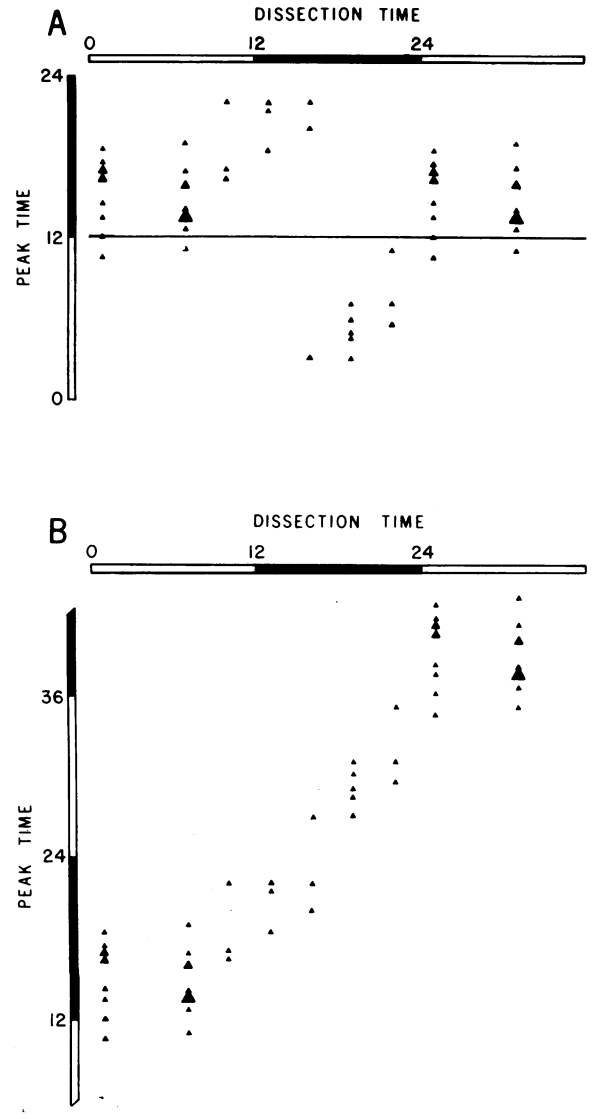

FIG. 1. (A) Peak spiking activity of R15 in the isolated PVG as a function of the time of dissection within the light-dark cycle. The dark bars represent the dark portion of the LD cycle (projected dark for the peak time axis). Medium and large triangles indicate two and three data points at the same time, respectively. Note that the early day dissection points are repeated to emphasize the near zero slope during this time.

(B) Peak spiking activity of R15 in the isolated PVG as a function of the time of dissection. Instead of being restricted to one 24 hr cycle starting at dawn, the ordinate has been extended and phase-advanced data points have been plotted by adding 24 hr to their values. Again, note that the early day dissection points are repeated.

$P < 0.001$; interval: difference = 0415 hr; $t = 3.3$; $P < 0.01$). Thus the peak spiking time can be adequately predicted only by taking both dissection time and lighting schedule into account.

Fourth, there is a linear relation between peak time and dissection time when dissections are performed during the period of darkness. The slope of the regression line fit to the points between dissections at CT 1300 and CT 2200 is 1.3 (95% confidence interval 0.85–1.75). When dissections are made in the first half of the day there is no evidence of this dependency on dissection time.

(*b*) *Photoreceptor for Entrainment In Vivo.* Four groups of five animals each were used. The dissections for animals from the control group in the main sea water system were at CT 0100. Dissections for the three experimental groups (blinded, sham-blinded, and no operation) in the 6 hr phase advanced individual aquaria were at CT 0700.

The five controls comprise five of the ten CT 0100 dissection points of Fig. 1, and their average time of R15 peak spiking activity was CT 1500 ± 0233 hr (−2300 hr PDT; minus sign indicates a peak occurring on the solar day of dissection). The intact and sham-operated animals comprise the CT 0700 dissection points of Fig. 1. The R15s from these animals peaked at CT 1448 ± 0136 hr (−1648 PDT) and CT 1424 ± 0309 hr (−1624 PDT), respectively. In this region of the peak time–dissection time curve, changes in the dissection time produce essentially no change in the peak time. The change in peak time in relation to the solar day is almost exactly the same as the change in the phase of the entraining light cycles (Table 2).

If the blinded *Aplysia* also entrained their R15 rhythms, then the peaks should have occurred at about CT 1500, or −1700 hr PDT. The actual peak times averaged CT 1954 ± 0147 hr (−2154 PDT). These peaks clearly differ from those of the intact or sham-operated *Aplysia* entrained in an identical manner to the same light schedule. In fact, the blinded peak solar time average was very nearly the same as the solar time average of the unshifted controls. Examples of the shifted animals' R15 rhythms are given in Fig. 2.

Although the timing of the R15 activity peak is different for blinded *Aplysia* as compared to sighted animals, the average spike frequency is not. The average spike rates for the first 24 hr of recording were: blinded, 20.0 spikes/min; intact, 19.2 spikes/min; sham-blinded, 22.9 spikes/min. It will be remembered that these animals were all isolated in individual aquaria.

DISCUSSION

(*a*) *Peak Time–Dissection Time Relation.* Since most of the past reports make little mention of the time of dissection, it is impossible to tell with certainty whether some or most of the R15 rhythms reported would agree with the peak time-dissection time curve shown in Fig. 1. However, the graphs of spiking activity included in some of the previous papers do show the entraining LD schedule and the time of impalement of R15 with the intracellular electrode. If it is assumed that the experiments were performed without interruption, then the impalement of the neuron should have followed dissection rather quickly. Granted this assumption, we can then examine some of the past experiments in the light of the present data.

Strumwasser (1) shows activity graphs of three R15s from animals previously exposed to 9, 3, and 2 days of entrainment, respectively. In all of these experiments, impalement occurred between about CT 1330 and CT 1700, that is, 1.5–5 hr after dusk. Allowing 1–2 hr between dissection and impalement, the dissections would have taken place CT 1200 to CT 1600. Fig. 1 would predict R15 peaks at about CT 1800 to CT 0200. The actual peaks in these graphs (1) occurred approximately at CT 0130, CT 2330, and CT 0000. These are entirely in the last half of, but within the range of, the prediction.

Turning to Lickey (2), we again find three R15 activity graphs for LD 12:12 entrained *Aplysia*. Impalements ranged from CT 1300 to CT 1700, implying dissections from about

TABLE 2. *Changes in peak time*

	Control[b]	Normal shift[c]	Blind shift[c]	Sham shift[c]
Time of peak[a] (PDT)	+0030	−1530	−2030	−1430
	+0230	−1800	−2130	−1530
	−2230	−1530	−2400	−1800
	−2130	−1600	−2000	−2100
	−2000	−1900	−2330	−1300
Mean ± SD	−2300 ±0233	−1648 ±0136	−2154 ±0147	−1624 ±0309
Predicted peak[d]	—	−1700	−1700	−1700

[a] Positive times are those peaks that occurred on the solar day after dissection; negative times are those which occurred on the solar day of dissection. All times in Table 2 are PDT.

[b] Control light schedule: LD 12:12; lights-on at 0800 hr; lights-off at 2000 hr.

[c] Shifted light schedule: LD 12:12; lights-on at 0200 hr; lights-off at 1400 hr.

[d] Predicted peak time based on a 6 hr advance over the control peak average, since dissections for both control and experimental tanks were performed in the flat region of the peak time–dissection time curve (Fig. 1).

CT 1100 to CT 1600. Fig. 1 predictions would call for peaks between about CT 1700 and CT 0200. Actual peaks were CT 0300, CT 2200, and CT 0500. These peaks are slightly later than the prediction. Strumwasser ran his experiments at about 11.5°, and Lickey at 12–13°, as compared to the 15° for the experiments of Fig. 1. The uncertainty of dissection time in these previous reports and the lower temperatures of the experiments are likely factors contributing to these deviations.

It is of interest to note that the published spiking records of R15s taken from *Aplysia* kept in constant light prior to dissection do not fit into the peak time-dissection time curve. The peaks occurred about 21 hr (ref. 2, Fig. 3) and no more than 2–3 hr (ref. 1, Figs. 5 and 6) after dissection with the latter two having the appearance of the dissection occurring during an ongoing peak which continues after impalement. No peaks in these experiments occur with either this long or this short an interval between dissection and peak. This indicates that the peak time–dissection time relation depends on the presence of an LD cycle, and that it is not the dissection per se, but the time of dissection within the LD cycle, that produces the relation.

The CRs of almost any organism free-running in constant darkness can be phase-shifted by a short (minutes to a few hours) pulse of light. The amount and direction of the phase shift is dependent on the phase of the free-running rhythm at which the light pulse is applied. Only small changes in phase result from light pulses applied during the subjective day. The largest changes are seen for pulses given during the subjective night, with delays (activity peak or onset occurs later) induced early in the subjective night, and advances induced late in the subjective night. A changeover from delays to advances occurs somewhere near the middle of the subjective night (see Fig. 1 in ref. 6).

As can be seen from the description above, the peak time–dissection time curve for R15 is very similar to a phase response curve (Fig. 1A). Unfortunately, the phase of the

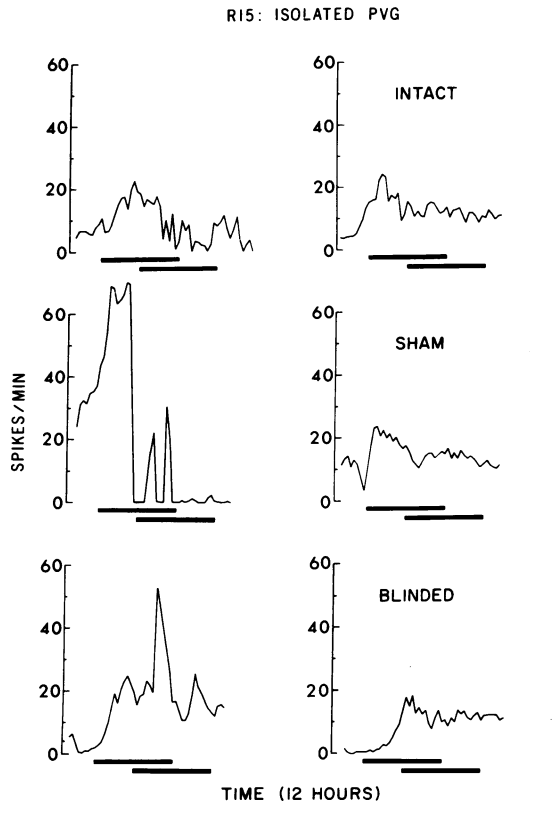

FIG. 2. Spike activities of R15s in isolated PVGs taken from intact (top), sham-blinded (center), and blinded (bottom) *Aplysia*. Two examples are shown in each category. Bars (12 hr long) represent two lighting regimes: upper bar on each graph denotes projected night of the postoperative schedule; lower bar denotes projected night of the preoperative schedule. Animals were kept on the postoperative schedule at least 1 week prior to final dissection.

R15 rhythm cannot be determined *in vivo* prior to dissection. The resemblance may, therefore, be only coincidental; however, the implication remains that the events involved in dissection can act as a phase-setting stimulus for the rhythm of R15. Stimuli that occur during dissection include cutting of nerves, removal of the PVG from components of the blood, and exposure to light.

(*b*) *Photoreceptor for Entrainment In Vivo.* Blind *Aplysia* do not appear to be able to photoentrain their R15 rhythms. The average peak activity of the R15s of blinded animals occurs at a significantly different time than the peaks of the intact (difference = 0506 hr; $t = 4.8$; $P < 0.01$) and the sham-operated phase-shifted animals (difference = 0530 hr; $t = 3.4$; $P < 0.01$). Interestingly, the R15 peaks of the blinded animals were not very different from the controls from the main sea water system, but they are shifted in the same direction as the light cycle phase shift, by about 1 hr. This may imply that the blinded animals are still weakly coupled to the light, but that the phase angles of their R15s are different than the phase angles of sighted animals. Alternatively, R15s in blinded animals may be free-running very close to 24 hr *in vivo*. Although the R15 rhythm damps out *in vitro* in the isolated PVG (1, 2), this may not happen *in vivo*. The temperature cycle in these aquaria (see *Methods*) may also be

able to influence the rhythms of the R15s of the blinded *Aplysia*.

A major difference in protocol between the present experiments and those of Lickey *et al.* (4) and Lickey and Zack (5) is that our animals were isolated during entrainment and the Lickey animals were not. This suggests that social stimuli from normal animals may be able to entrain the R15 rhythm of blinded *Aplysia*. This has been found to be true for blind mice (7) and for men kept under constant conditions (8). Birds can entrain to sound stimuli under constant conditions of light, food, and temperature (9, 10), which may also be considered to be "social entrainment." Perhaps the locomotor rhythms of the normal *Aplysia* in Lickey's experiments could have entrained the R15s of the blinded animals. Another possibility is pheromone or metabolite release with a circadian period by the normal animals.

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