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Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach

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Abstract

The olfactory response in antennae of the cockroach, *Leucophaea maderae*, was investigated by measuring electroantennograms (EAGs) in restrained animals. The amplitude of the EAG response to pulses of ethyl acetate, octanol, or fenchone, exhibited a robust, light entrained, circadian rhythm that persisted at least 14 days in constant darkness. Dilution-response curves measured at the peak and trough of the rhythm indicated there was a 10-fold change in sensitivity. The EAG rhythm was abolished by severing the optic tracts, while entrainment was abolished by ablation of the compound eyes. The results indicate that the circadian system modulates olfactory sensitivity in the antennae and that the rhythm is driven by a circadian pacemaker in the optic lobes that is entrained by photoreceptors in the compound eyes.

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1. Introduction

Many animals rely heavily on olfactory cues for initiating and directing a variety of behaviors including reproductive activity, intra-specific competition, foraging, and host identification. Behavior is often also temporally regulated, and scattered evidence from insects has accumulated over the past three decades that suggests the circadian clock may play an important role in the regulation of insect olfactory behavior. For example, behavioral studies on moths (Payne et al., 1970; Riddiford, 1974; Linn et al., 1996) have shown daily rhythms (in a light cycle) in the male's response to female pheromone. In the cockroach, *Periplaneta americana*, males maintained in a light cycle show a daily rhythm in sensitivity to female sex pheromone as measured by locomotor activity (Hawkins and Rust, 1977) or wing raising behavior (Zhukovskaya, 1995), with peak sensitivity approximately 30 times greater in the early part of the night than in the morning hours (Zhukovskaya, 1995).

These observations, though limited, suggest that circadian clocks may play a role in regulating olfactory behavior in insects and raise the question of where in the olfactory pathway rhythmicity is imposed. Information is currently limited to a single study on the fruit fly, *Drosophila melanogaster*, that suggests it may occur as early as the primary sensory neurons (Krishnan et al., 1999). In these experiments, animals were exposed to a pulse of odorant and the olfactory response was assessed by measuring the amplitude of the electroantennogram (EAG). When the average amplitudes of the EAGs measured at different times of day in different flies were compared, it was found that responses of flies tested near the middle of the night were significantly higher than those of flies tested at other times of day, even when flies were maintained in constant darkness. The results indicated that a circadian clock regulates the response of the olfactory system at the level of olfactory reception in the antennae.

Cockroaches offer several potential advantages for the study of circadian regulation of olfaction. One major advantage is that EAG responses can be measured in individual animals, restrained in constant darkness, for at least 14 days. This avoids complications that occur when rhythms are only measurable in populations, where

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each animal can only be sampled at one time of day and variability from one individual to the next in phase, period, or amplitude can hinder analysis. Further, there has been a substantial amount of work on both the physiology and behavior of olfaction in cockroaches (e.g., Sass, 1978; Bell, 1982; Boeckh and Ernst, 1987; Seelinger, 1990; Fujimura et al., 1991; Kapitskii and Gribakin, 1992; Getz and Akers, 1997), and the circadian system is well understood in terms of anatomical and physiological organization (Page, 1990; Page, 2001). We report the discovery of a circadian rhythm of EAG amplitude in the cockroach *Leucophaea maderae*. Evidence is presented that the rhythm is driven by a circadian pacemaker in the optic lobe and that the photoreceptors for entrainment by light are located in the compound eyes.

2. Materials and methods

2.1. Animals

Experiments were performed with male cockroaches, *Leucophaea maderae*, from laboratory colonies maintained in a 24-h light cycle (12 h of light alternated with 12 h of darkness, LD 12:12) at 25 °C. For entrainment to light cycles that were at a different phase from the colony room, animals were housed in groups in light-tight plywood boxes maintained in a constant-temperature room. Each box was equipped with a 4-W fluorescent lamp controlled by an electronic timer. Light intensity on the floor of the boxes was approximately 100 lux. Food and water were available ad lib.

2.2. EAG recording

EAGs were recorded from restrained animals using a method similar to that of Kapitskii and Gribakin (1992). Animals were anesthetized briefly with CO₂ and taped to the underside of a petri dish lid with the head protruding through a hole in the lid. A piece of tape behind the head prevented the animal from pulling it back through the hole. The antenna was threaded through small loops in two silver wires. One wire, near the distal end of the antenna served as the active electrode while the second wire, located a few mm from the scapus, served as the indifferent electrode. Contact between the antenna and the wire was made with EKG electrode cream which surrounds and stabilizes the antenna and wire. For long-term recording of circadian rhythms, animals were maintained in light-tight boxes within a temperature controlled incubator. Recordings were stable with this system for at least 14 days and gave signals that were indistinguishable from those recorded by the method of a saline-filled micropipette placed over the tip of the antenna with a reference electrode inserted into either

the head capsule or base of the antenna. We have used both methods to record rhythms. The signal from the antenna was led to an amplifier (Gain: X100, Bandpass: 0.1–200 Hz) then to an oscillographic recorder or computerized data acquisition system (Superscope II).

2.3. Stimulus

A half cylinder fashioned from Tygon tubing (0.9 cm diameter) was taped over the antenna. The stimulus delivery system consisted of a constant stream of humidified air flowing in one end of the half cylinder (at the distal end of the antenna) with a vacuum tube at the other to remove any odorants. The air stream (~250–300 ml/minute) was bubbled through de-ionized water, run through a carbon filter, a flow gauge, and then into a solenoid controlled valve that switched between two output ports. Stimulus chambers were vials whose tops were fitted with two 16 gauge hypodermic needles—one for flow into the chamber and the other for flow out. One port from the solenoid valve served to provide a stream of clean air through a blank (empty) stimulus chamber throughout the inter-stimulus interval. On command from a programmable timer the solenoid was activated, directing the stream of air through the active stimulus chamber containing the odorant. Outputs from the blank and active stimulus chambers were combined to a single line that blew over the antenna. The system allowed for scheduled odorant pulses that were controlled by an electronic timer. Duration of the stimulus pulses ranged from 0.5 to 3 s as noted in the results. Odorants (ethyl acetate, octanol, and fenchone) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). For measuring dilution-responses curves, dilutions were made with mineral oil and pulses were repeated at three minute intervals beginning with the most dilute solution.

2.4. Surgery

Surgical procedures were carried out with CO₂ anesthesia. For optic tract sectioning, access to the brain was obtained by removing a small square of cuticle from the head capsule. Special care was taken to avoid damage to the antennae, antennal nerves, and accessory antennal heart; however, the surgery did require the removal of the ocelli. After removal of the ocellus, and reflection of the trachea, the optic tract was visible and was cut with fine micro-scissors. Success of the surgery could be visually confirmed at the time of the operation. In sham surgery, each step, including the removal of the ocelli, exposure of the optic tract, and insertion of the scissors for sectioning, was reproduced as faithfully as possible. After surgery, the cuticle was replaced and sealed in place with low-melting point wax.

Photoreceptors of the compound eyes were ablated by

cutting along the margin of the eye, removing the cuticle of the eye's surface, and using forceps to clean away any remaining photoreceptors. Sham surgery on a second control group involved removing only a small portion of the eye, near the antenna. After the lesion was made, the cuticle was replaced and held in place with a small amount of melted wax.

2.5. Olfactory behavior

To evaluate an animal's ability to carry out olfactory mediated behavior, individuals were maintained, freely moving, in petri dishes (150 mm in diameter) in constant dim red light. The lid for each dish had six small, equally spaced, holes drilled around the circumference. *Leucophaea* are strongly attracted to apples and, typically, when exposed to a piece of apple will rapidly initiate an olfactory searching behavior. To quantify the efficacy of the olfactory response, small disks (5 mm diameter, 2 mm thick) of apple were dropped in the hole furthest away from the animal's position in the dish and the behavior was observed for 5 min. To quantify the response, the latency to the animals' contact with the apple was measured in seconds. Animals that did not respond within the 5 min period are reported as non-responders. For purposes of analyzing the data by a non-parametric statistical test (Kruskal-Wallis One Way Analysis of Variance) non-responders were all assigned a latency of 300 seconds. As a control for response to any small mechanical disturbance or visual stimulus, an identical test was done to evaluate the response to a similar sized piece of tygon tubing.

2.6. Activity recording

Circadian rhythms of locomotor activity were monitored as described in previous publications (e.g., Page, 1982). Briefly animals were housed in running wheel cages whose rotation activated a magnetic reed switch. Switch closures were counted by a computerized data acquisition system (Data Sciences, Inc.) which accumulated counts in 10-min bins. Data were plotted as actograms in the conventional fashion for visual analysis. Activity monitors were housed in temperature-regulated incubators and activity was recorded at a constant temperature of 25 ± 0.5 °C.

2.7. Data analysis

Graphical and statistical analysis was carried out with SigmaPlot and SigmaStat software (Jandel Scientific). Because there are no strong statistical tools for evaluating the presence or absence of periodicity in short data sets of only a few days as presented here, judgement of the presence or absence of rhythmicity was made by subjective evaluation of the graphed data. In general the

presence or absence of a rhythm was quite clear (see Figures) though in a few cases (as noted) the results were not clear cut. In these cases the presence or absence of a rhythm is reported as questionable.

3. Results

3.1. EAGs in *Leucophaea*

EAGs recorded from the antennae of *Leucophaea* were similar in waveform to those reported for the cockroach *Periplaneta americana* (e.g., Kapitskii and Gribakin, 1992). The amplitude of the EAG was typically somewhat smaller than EAGs we and others have recorded from *Periplaneta*. The responses to the three odorants used in the present study were reproducible and dose-dependent with a peak amplitude on the order of 0.7–1.0 mV. Fig. 1 shows the EAG waveform and

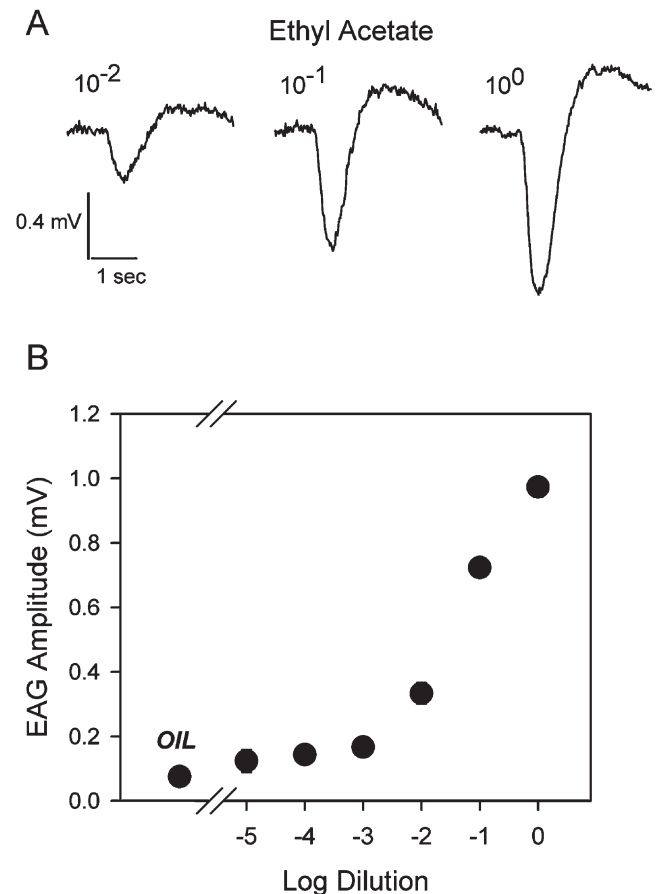


Fig. 1. EAGs in *Leucophaea*. A. Shows electroantennograms recorded from *Leucophaea* in response to 1-sec pulses of three different dilutions of ethyl acetate in mineral oil. Dilutions are indicated above each trace. Scale: 0.4 mV, 1 sec. B. Dilution-response curve: Amplitude of response from the animal whose EAGs are shown in A. Each point represents the average of three responses given at 3 min intervals. In each case the standard deviation of the mean is smaller than the data point in the graph, indicating the reproducibility of response and complete recovery from desensitization within the 3-minute interval.

dilution response curve measured with ethyl acetate as the odorant. Repeated stimulus pulses (ethyl acetate, 1 sec) at intervals of 1 minute or less, resulted in some decrement in amplitude due to adaptation, while stimuli spaced at longer intervals showed recovery from adaptation was complete in under three minutes. Dilution-response curves were measured to the three odorants utilized in the experiments reported here. None of them saturated the EAG response at the highest concentrations (e.g., Fig. 1).

The amplitude and waveform of the EAG were unaltered by removal of the antenna from the body of the animal. We recorded EAGs in response to pulses of ethyl acetate given every 5 min from intact, restrained animals. One minute prior to the fourth stimulus pulse, we simply cut the antenna at its base and recorded from the detached, isolated antennae. EAGs could be recorded from isolated antennae for 1–2 h before the amplitude began to exhibit significant decline. As shown in Fig. 2B, EAGs recorded within 1 min after being detached

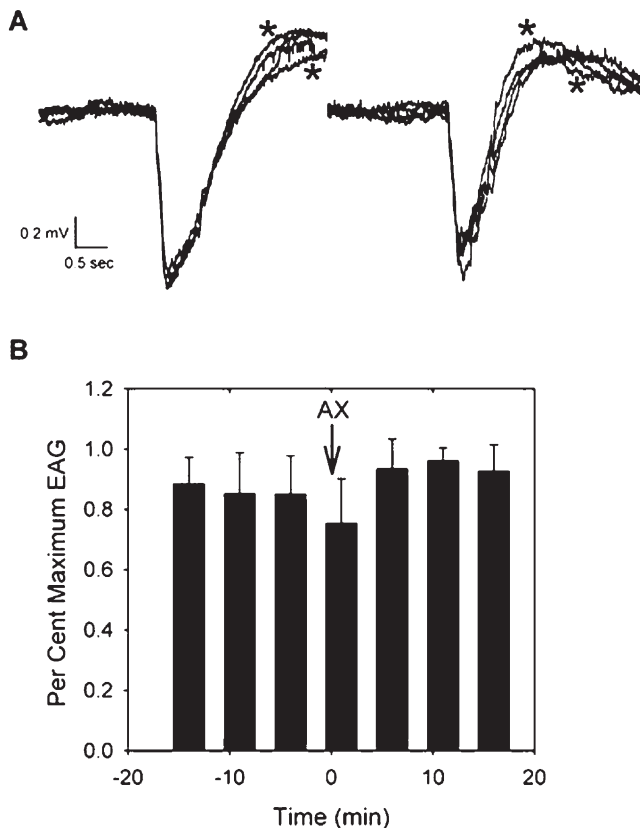


Fig. 2. Effects of detaching the antennae on the EAG. A. EAGs in response to stimulus pulses given at 5 min intervals from two different animals before (marked by asterisks) and after the antennae were detached from the body. There are four superimposed traces in each of the two records. In each case, traces recorded 9 and 4 min prior to severing the antenna at its base and traces recorded 6 and 11 min after the antennae were detached are shown. B. Shows average relative amplitudes of EAGs recorded before and after the antennae were detached (mean \pm s.e.m.; $N = 6$). The results indicate that detaching the antenna has little or no effect on amplitude or waveform.

exhibited a slightly reduced amplitude compared to the control. The antennae recovered within 6 min, and after recovery, the average amplitude was slightly, but not significantly, larger than any of the control EAGs (Fig. 2B). There were no detectable differences in the waveforms of EAGs recorded from attached and detached antennae (Fig. 2A). These results indicate that the EAG in *Leucophaea* is purely antennal in origin.

3.2. EAG amplitude rhythms

In initial experiments, EAGs were recorded in response to a 3-sec pulse of ethyl acetate given once per hour to 19 intact, restrained cockroaches. EAGs of these animals, which had been entrained to LD 12:12 light cycles, were monitored for periods of 3–14 days in constant darkness. In 13 of the animals, the amplitude of the EAG response to the food-related odorant showed a robust rhythm that persisted in constant darkness and constant temperature (Fig. 3A,B). In three of the remain-

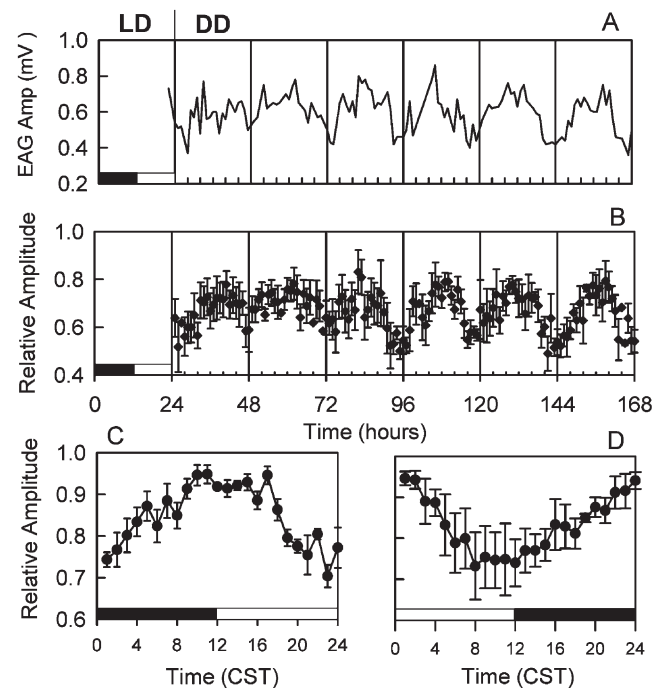


Fig. 3. Freerunning EAG amplitude rhythms. A. Freerunning rhythm of EAG amplitude in response to 3-sec pulses of ethyl acetate given once per hour for six consecutive days. Recording was done in constant darkness and constant temperature (25 °C). B. Average (\pm s.e.m.) of EAG amplitudes in response to 3 sec pulses of ethyl acetate, normalized for each animal to the maximum EAG recorded, for five animals freerunning in DD. In both panels, the light cycle to which the animals had been entrained prior to recording is illustrated by the light and dark bars on the first 24 h of the graph (LD 12:12, lights on at 12:00 noon). C, D Average of the first three cycles of EAG responses for five animals from each of two light cycles (illustrated by the bars at the bottom of each graph) that were 12 h out of phase. In both conditions, peaks were near subjective dawn and troughs were near subjective dusk indicating entrainment of the rhythm by the LD cycle to which the animals were exposed prior to recording.

ing six animals the presence of a rhythm was, subjectively, questionable, and for the last three animals there was no evidence of rhythmicity. Similar rhythms were observed using a 10^{-2} dilution of ethyl acetate in mineral oil; however, since undiluted stimuli did not saturate the response, long-term recording was generally carried out without dilution of the odorant to ensure a constant stimulus without concern for changes in dilution over time.

In these experiments the animals had all been entrained to one of three LD 12:12 light cycles prior to recording in constant darkness (lights-on at 7:00, 12:00, or 24:00). The time of the minimum of the EAG response was dependent on the prior LD cycle and consistently occurred near the time of lights-off of the light cycle to which the individual animal had been entrained prior to recording (Fig. 3B). To evaluate the phase relationship between the EAG amplitude rhythm and the prior light cycle more carefully, the relative amplitude (normalized to the maximum amplitude for each individual) of the hourly EAGs were averaged over the first three cycles of constant darkness for ten animals, five from each of the two light cycles that were 12 h out of phase. The results clearly showed that the freerunning rhythms from the two light cycles were in anti-phase and that the peak response occurred near subjective dawn, a time when *Leucophaea* is normally quiescent, and showed a minimal response at subjective dusk, the time of peak locomotor activity (Fig. 3C,D).

We wanted to determine if the rhythm in the antenna revealed by stimulation with ethyl acetate affected responses to other, chemically distinct, odorants. The responses to pulses of fenchone, a mono-terpene and to octanol, an alcohol, were also monitored for several days in constant darkness. Roaches exhibited robust rhythms in response to both odorants with waveforms and freerunning periods similar to those observed with ethyl acetate as a stimulus (Fig. 4). For fenchone, three of five animals exhibited clear rhythms while for octanol three of three animals were rhythmic.

In order to obtain an objective estimate of the period (τ) of the freerunning EAG response rhythms we determined both the time of peak EAG amplitude and the time of the minimum EAG amplitude in each cycle for all animals for which we had six or more days of data. The timing of the minima appeared to be more reliable as a phase reference point (Fig. 4). Regressions calculated through the minima for six animals that showed rhythms in response to ethyl acetate yielded an average period estimate of 23.1 ± 0.56 h. For fenchone, $\tau = 23.5$ ($N = 2$) and for octanol $\tau = 23.7$ ($N = 2$). The data indicate that the freerunning period is consistently less than 24 h, and exhibits a value similar to the rhythm of locomotor activity (Barrett and Page, 1989). As with ethyl acetate, the peak response to both octanol and fenchone occurred near subjective dawn while the minimum was

at subjective dusk (Fig. 4). The results of these experiments show unequivocally that there is a circadian rhythm in the EAG response that can be entrained by light and that the rhythm free-runs in constant darkness with a period slightly less than 24 h.

3.3. Rhythm in sensitivity

The amplitude of the EAG rhythm in cockroaches represents a 30–40% change in response. To determine the magnitude of the change in sensitivity this represents, dilution-response curves to ethyl acetate were measured in four animals at or near both the peak and the trough of the rhythm on the third to fifth day in constant darkness (Fig. 5). In all four animals the dilution-response curves measured at the trough (subjective night) matched the curve for the peak (subjective day) shifted along the log-dilution axes. In three animals a shift of 0.7 log units appeared to be the best fit (by eye), while in a fourth the best fit was a 1.0 log unit shift of the curve. The results indicate that the rhythm in EAG response represents a 5–10 fold change in sensitivity.

3.4. Pacemaker localization

In the cockroach, it is well-established that the circadian pacemaker that regulates the locomotor activity and ERG amplitude rhythms is located in the optic lobe (Page, 1982, 1990; Wills et al., 1986). Experiments were carried out to determine if the rhythm in olfactory response in the antenna is also driven by the optic-lobe pacemaker. EAG responses to 2-sec pulses of ethyl acetate were recorded after bilateral section of the optic tracts (OTX), which neurally isolates the optic lobe from the central nervous system and abolishes the circadian rhythm of locomotor activity.

Importantly, the surgery does not impair acute olfactory behavior or significantly affect the average EAG amplitude. To evaluate behavior, we examined the ability of animals to respond to the presence of a small disk of apple (see Materials and methods). Both intact ($N = 7$) and optic-tract sectioned ($N = 8$) animals were tested on two consecutive days, 9 and 10 days after surgery. Every animal in both groups exhibited rapid onset of searching behavior (antennal waving, directed locomotor activity) and began feeding on the apple in at least one of the two trials (Table 1). Based on latency to contact with the apple pieces, there were no significant differences associated with either treatment (control or optic-tract sectioned) or day of testing ($p = 0.56$). In contrast, in trials involving pieces of tygon tubing, none of the animals exhibited directed locomotion toward the stimulus, and in cases where contact with the tubing was made within the 5 min trial period (Table 1), it appeared to be accidental contact during random locomotor activity. In order to show that the behavior was depen-

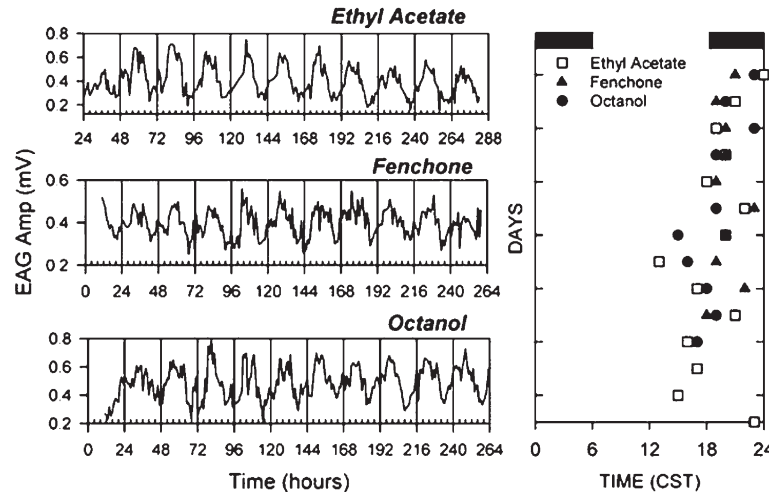


Fig. 4. Freerunning EAG amplitude rhythms to three distinct odorants. **Left:** Plots of EAG amplitude recorded over 11 days in constant darkness (DD) in three animals. The panels show rhythms in EAG response to 3 sec pulses of three different odorants, indicated at the top of each record, given once per hour. **Right:** Data show the time of the daily minimum EAG response from individual animals to ethyl acetate (squares), octanol (circles) and fenchone (triangles). The time of the minimum value for each day is plotted for 10–14 consecutive days. The bar at the top of the figure shows the light cycle to which animals had been entrained prior to recording. Regressions calculated for each animal give unbiased estimates of the periods of the freerunning rhythms (EA—23.6 h; Octanol—23.5 h; Fenchone—24.0 h). For all three odorants the time of the minimum is similar and projects to a time near the light-dark transition.

dent on the antennae, in a separate experiment tests of olfactory behavior were made on seven animals in which the antennae had been removed. These animals exhibited substantially lower levels of exploratory behavior compared to the control or optic-tract sectioned animals, and none of the animals exhibited any movement toward, or made contact with, the apple stimulus within 5 min after its introduction into the chamber (Table 1). These results indicate that the olfactory pathways in the antennae and central nervous system that mediate olfactory behavior are not significantly damaged by section of the optic tracts.

To examine the effects of optic-tract section on the EAG response rhythms, animals were subjected to optic-tract section or to sham surgery and returned to LD 12:12 for 7–10 days for recovery from surgery. The EAG was then recorded in constant darkness for 5–7 days. In the nine animals in which the optic tracts were severed bilaterally there was no evidence of rhythmicity (Fig. 6 c,d). In general, in these animals, the EAG was relatively constant in amplitude over the 24-h day. In contrast, four of five sham operated animals showed clear circadian rhythms in EAG response (Fig. 6a,b). Mean amplitudes calculated over the second and third day of recording, though slightly smaller in the OTX group (0.42 ± 0.15 mV, mean \pm SD) than in the sham operated animals (0.55 ± 0.21 mV), were not significantly different between the two groups. The results suggest that the circadian pacemaker in the optic lobes drives the rhythm of olfactory response in the antennae and that the pathway for circadian regulation involves neural connections between the optic lobe and the mid-brain.

Since these recordings were made 7–10 days after the surgery, it is possible that a rhythm could persist for the first several days after the surgery, perhaps driven by a damped peripheral oscillator as has been suggested for *Drosophila* (Krishnan et al., 2001). To check this we recorded for 5–7 days from another group of seven animals after optic-tract section and from seven animals subjected to sham surgery with recording beginning within 24 hours of surgery. EAG amplitudes recorded in the first 2–3 days after surgery were more variable from one hour to the next than for the animals that had been given a recovery period after surgery. Because this would make low amplitude circadian rhythms more difficult to detect in the optic-tract sectioned animals, the data were smoothed a bit by calculating a 3-point moving average (Fig. 6e–h). Five of the seven sham-operated animals were clearly rhythmic during the first days after surgery but even after smoothing, we were unable to detect evidence of rhythmicity in any of the optic tract sectioned animals. Average EAG amplitudes calculated over the second and third day of recording were nearly identical for the two groups — 0.64 ± 0.17 mV for the group with severed optic tracts and 0.69 ± 0.17 for the sham operated group.

The results show that connections between the optic lobes and the midbrain are necessary for the expression of the normal, sustained rhythm in EAG amplitude. Neither the average EAG amplitude nor the ability to carry out simple olfactory behavior is affected by the surgery, and only the temporal modulation is disrupted. These data strongly suggest that, like the locomotor activity rhythm and ERG rhythms (Page, 1990), the driving oscillator for the EAG amplitude rhythm is located

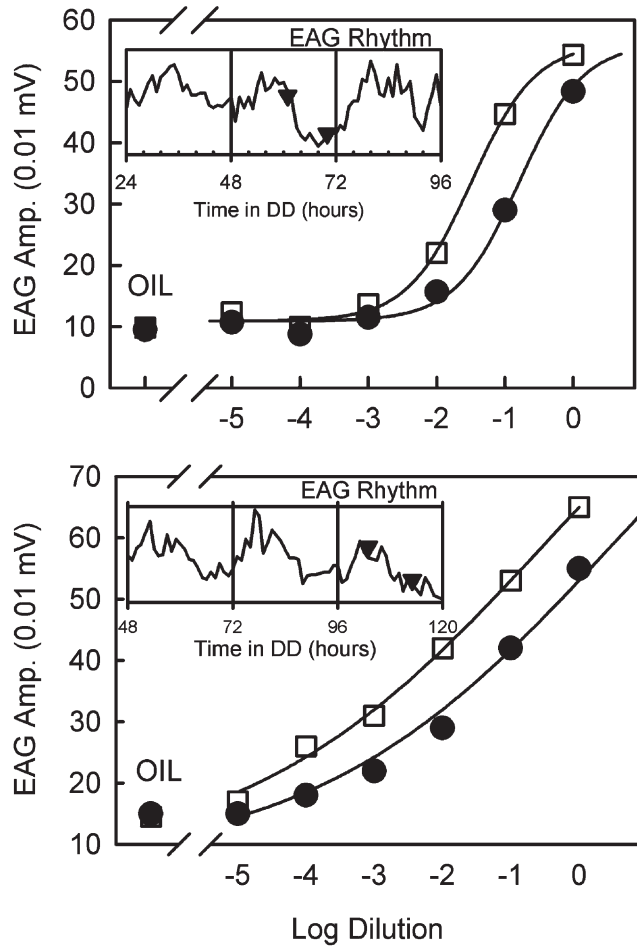


Fig. 5. Dilution-response curves to ethyl acetate diluted in mineral oil and measured in two animals at peaks (open squares) and troughs (closed circles) of the EAG rhythms (insets) monitored in constant darkness. The times at which dilution response curves were measured is shown by the triangles plotted on the EAG rhythm data (Day 3 in DD in the top panel and Day 4 in DD in the bottom panel). A sigmoidal curve was fit by regression to the data obtained at the peak of each rhythm. The same curve shifted along the dilution axes by 0.7 log units fits the data from the trough of the rhythm in the top panel, while a shift of 1.0 log unit is required in the bottom panel.

in the optic lobes. It should be noted, however, that although the data provided no evidence of an oscillation after optic-tract section, it is difficult to conclusively rule out the presence of a rapidly damped oscillator outside the optic lobes that could transiently drive rhythmicity.

3.5. Photoreceptors for entrainment

The circadian pacemaker in the optic lobe that regulates locomotor activity is entrained by light via photoreceptors in the compound eyes (Roberts, 1965; Page, 1990). Therefore, if the suggestion that the same optic-lobe pacemaker drives the EAG rhythm is valid, then the eyes should also be necessary photoreceptors for entrainment of the EAG rhythm. In order to determine whether the eyes were necessary, we ablated the photo-

receptors of the compound eyes in one group of animals and performed sham surgery on the second control group. The animals were then placed in a light cycle for 8–10 days that was phase delayed by 10 h (lights-off at 04:00 CST) from the cycle the animals had been entrained to prior to surgery (lights-off at 18:00 CST). During this 8–10 day period, locomotor activity rhythms were recorded as an independent check on the success of the surgery. As expected, locomotor activity rhythms of sham operated animals invariably entrained to the new LD cycle while ablation of the compound eye prevented entrainment of the locomotor activity rhythms as has been shown several times previously (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968; Page, 1978).

Both groups were then placed in DD and the phase of the EAG amplitude rhythm was assayed. We predicted that the EAG rhythms of the sham operated group would entrain to the new cycle (nadir of the EAG rhythm at the new time of the light to dark transition) while those of the experimental group would freerun with a period of slightly less than 24 h (nadir near the time of L to D transition of the light cycle prior to surgery). Evaluation of phase on the day of entry into constant darkness was accomplished by calculating regressions through daily minima for 3–7 days of rhythmicity. The results showed that ablation of the compound eyes abolished entrainment to the phase shifted light cycle while the control animals readily entrained (Fig. 7). The projected phases of the EAG rhythm in the sham animals was 3.7 ± 3.83 (CST, mean \pm SD, $N = 6$) while for eyeless animals it was 17.5 ± 3.81 (CST, $N = 6$). The phases of the two groups were clearly different ($p < 0.001$, t-test) and the phase for each group was similar to the predicted phase based on the hypothesis that the compound eyes are the sole photoreceptors for entrainment. The results show that photoreceptors in the eyes are necessary for entrainment and that neither extra-retinal receptors nor the ocelli (which remained intact) are sufficient.

4. Discussion

The data presented here demonstrate a persistent circadian rhythm in the olfactory response in individual cockroaches as measured by the amplitude of the EAG. They further show that this rhythm is driven by the optic lobes and that photoreception for entrainment occurs in photoreceptors of the compound eyes. While these results represent only the second demonstration of a circadian rhythm in an olfactory system, the fact that both cockroaches and the evolutionarily distant fruit fly (Krishnan et al., 1999) exhibit circadian modulation of antennal sensitivity suggests that circadian control of olfaction may be widespread, at least among the insects. In addition, the results raise several interesting questions

Table 1

Effects of optic-tract section (OTX) or antennal ablation (ANTX) on behavioral responses to apple or tygon tubing

Stimulus	Group	Responders ^a	Non-responders ^a	Responder Latency (seconds) (Mean±sd)
Apple	Intact-Day1	7	0	24.4 ± 13.01
	Intact-Day2	6	1	18.3 ± 25.46
	OTX-Day1	6	2	21.2 ± 15.25
	OTX-Day2	7	1	15.1 ± 8.21
	ANTX-Day1	0	7	
	ANTX-Day2	0	7	
Tubing	Intact-Day1	2	5	135 ± 77.8
	Intact-Day2	4	3	125 ± 70.0
	OTX-Day1	2	6	200 ± 70.7
	OTX-Day2	1	7	170

^a Responders are those animals that contacted the stimulus within the 5 min test period. Non-responders are animals that did not contact the stimulus within 5 minutes.

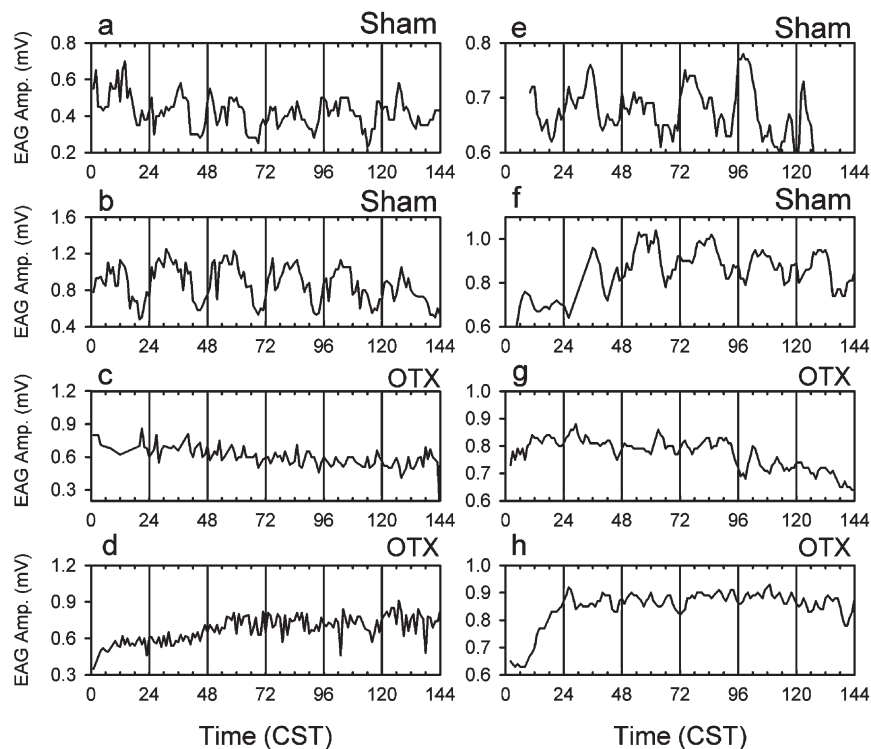


Fig. 6. Effect of sham surgery (a,b,e,f) and optic-tract section (c,d,g,h) on the freerunning rhythm of EAG amplitude recorded in constant darkness. The animals in the left panels (a–d) were given 7–10 days to recover from surgery before recording while records for those in the right panels (e–h) begin within 24 h of surgery. Data in e–h were smoothed with a 3 point moving average. Rhythmicity is apparent in the sham operated animals while it is absent following optic tract section. Stimulus: ethyl acetate, 2 sec pulses.

about the functional significance of the circadian modulation of olfactory response, the mechanism by which the response is regulated, and the organization of the circadian system.

4.1. Functional significance

One question concerns the functional impact of the rhythm in the antennae. Of particular interest is the fact

that the phase of the rhythm is such that minimum sensitivity in the EAG occurs at the time of the roach's maximum locomotor activity. A similar phase relationship between activity and EAG amplitude was reported for fruit flies which are day active, but exhibit maximal EAG responses in the middle of the night (Krishnan et al., 1999). In this case, the authors speculate that the olfactory system may be sensitized at night to function as an alarm system during a time when the fly is quiescent.

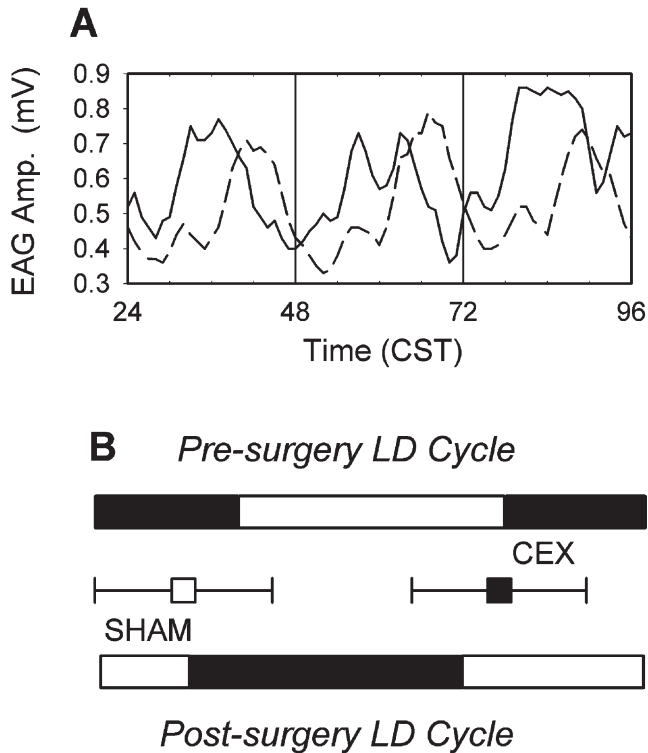


Fig. 7. Compound eyes are necessary for entrainment. A. Examples of EAG amplitude rhythms recorded in constant darkness from sham-operated control (dashed line) and eye-ablated (solid line) animals following a 10 h phase delay in the light cycle. Only the rhythm from the sham-operated animal was delayed. B. Average phases (mean \pm SD) of animals subjected to sham surgery (open squares) or compound eye ablation (CEX, filled squares) following exposure to a phase-shifted light cycle. The top bars indicate the light cycle prior to surgery and the bottom bars show the light cycle to which the animals were exposed immediately after surgery. The sham animals shifted phase and are entrained by the post-surgery LD cycle while the CEX animals are in a phase appropriate to the LD cycle experienced prior to surgery and did not respond to the shift in the LD cycle.

ent. This explanation is probably not tenable for the cockroach. Behavioral studies on pheromone sensitivity in *P. americana* (Zhukovskaya, 1995) and threshold sensitivity to a food-related odor (ethyl acetate) in *L. maderae* (Karasiewicz and Page, unpublished) indicate that the animals are at least 30–100 fold more sensitive, behaviorally, to odors presented in the early night than in the early day. One possible explanation for this apparent paradox is that central processing of olfactory input or the excitability of central elements involved in olfactory behavior are also regulated by the circadian system such that the animals are more sensitive, behaviorally, to olfactory input at dusk despite a reduction in antennal sensitivity.

An alternative possibility is that EAG amplitude does not accurately reflect the responses of the olfactory sensory neurons. One problem is the lack of a complete understanding of the relationship between the EAG and the olfactory receptor neuron (ORN) response. In the

cockroach, available data suggest that the EAG is an accurate reflection of activity in the receptors. Kapitskii and Gribakin (1992) present a model (based on data from *P. americana*) in which the EAG arises from two types of stimulus-induced voltage changes in the antennae—the ORN receptor potential and the electrical potential that exists between the sensilla lymph and the hemolymph—the transepithelial potential (TEP). The peak of the EAG in the model is particularly dependent upon the receptor current, and Kapitskii and Gribakin (1992) suggest that the kinetics of the EAG reflects the kinetics of the receptor current within an accuracy of several percent. In support of this view, Mayer (2001) found a strong correlation between EAG amplitude and spike frequency of ORNs in the moth *Tricoplusia ni*. These and other studies would suggest it is a reasonable assumption that an increase or decrease in the EAG reflects an increase or decrease in ORN response. On the other hand, there are indications that the EAG or TEP response may, in some aspects of antennal receptor physiology in some insects, diverge from the ORN response as measured by spike frequency. For example, studies on olfactory responses in moth antennae showed that while octopamine caused a substantial increase in the impulse frequency of ORNs, there was no detectable effect on the TEP (Pophof, 2000; Grosmaître et al., 2001).

It seems reasonable to suggest that interpretation of the physiological and behavioral significance of the circadian rhythm in the EAG will ultimately depend on concomitant studies of ORN responses. This is feasible in *Leucophaea* where it is possible to record continuously from individual olfactory sensilla in restrained animals for several days (Page, unpublished). At the same time, it is a safe assumption that the circadian changes in the EAG are reflective of significant physiological changes in the olfactory system in the antenna, and that the EAG will be a useful tool for investigations into the origin (though perhaps not the functional importance) of those changes.

4.2. Mechanism of regulation

At this point it is unknown what specific processes are modulated to cause a daily change in the amplitude of the EAG. Conceptually it could involve any number of elements including components of the sensory transduction cascade (reviewed in Krieger and Breer, 1999) or the structural organization of the ORNs or accessory structures. As an example, circadian rhythms in the visual system of *Limulus*, which are driven in part by octopamine, appear to involve a wide variety of changes in receptor structure, transduction mechanisms, and accessory pigment position (Barlow, 1983; Barlow et al., 1985). One approach to this problem will be the identification of the proximate circadian signal that causes

changes in olfactory response. It is notable that no antennal efferents to the ORNs have been described in cockroaches, indicating the rhythm driven by the optic lobe is likely to be regulated by a factor in the blood.

The antenna is supplied by a single, large hemolymphatic vessel (Kapitskii and Gribakin, 1992). Circulation through the vessel is driven by a myogenic, accessory heart composed of two ampullae, located at the base of the antennae (Pass, 1985), which receive a rich innervation from terminals of neurosecretory axons that contain octopamine (Pass et al., 1988a, Pass et al., 1988b). This is of particular interest because octopamine has been reported to modulate the EAG response to the sex pheromone periplanone A in *P. americana* (Kapitskii and Zhukovskaya, 2000), the responses of pheromone sensitive ORNs in two species of moths (Pophof, 2000; Grosmaître et al., 2001), and rhythmic moth olfactory behavior (Linn et al., 1996). These observations suggest that octopamine is particularly interesting as a candidate signal molecule that conveys clock information to the olfactory sensory system.

4.3. Circadian organization

The results presented here bear on an additional question which concerns the organization of animal circadian systems and the extent to which a single “master clock” orchestrates the timing of most or all of the rhythms within the individual. In the cockroach, the optic lobe pacemaker has been shown to be necessary for sustained oscillations in locomotor activity (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Page, 1982), retinal sensitivity as measured by the ERG (Wills et al., 1986), and now, olfactory response in the antennae. Alone these data would suggest that the optic lobe functions as a master clock in the circadian system. On the other hand, following the imaginal molt, cockroaches exhibit a rhythm in the cuticle deposition that appears to be completely independent of the optic lobe (Lukat, 1978; Weber, 1985). Other work on both insects and mammals has demonstrated the existence, in a wide variety of tissues (including fruit-fly antennae) of peripherally driven circadian oscillations in transcription of reporter genes coupled to the promoter of the “clock” gene *period* (Plautz et al., 1997; Yamazaki et al., 2000). There is also evidence from insects (some of it nearly 30 years old) for autonomous peripheral oscillations in physiology (reviewed in Giebultowicz, 2000; Page, 2001) such as the cuticle deposition rhythms (Lukat, 1978; Weber, 1985), the release of sperm from the testis (Riemann et al., 1974; Giebultowicz et al., 1988), or the production of ecdysone in the prothoracic glands (Mizoguchi and Ishizaki, 1982; Vafopoulou and Steel, 1998). The data leave no doubt that there are circadian oscillators distributed throughout the body, and that in some cases at least, these oscillations can autonomously drive physiologi-

cally relevant rhythms. What is not very well understood is the relationship between the various oscillatory systems within the individual and what rules might govern which rhythms need to be driven or synchronized by the central “master” pacemaker, and which might be allowed autonomy. Speculation at this point may be premature, but the data in the cockroach suggest that those rhythms that are directly engaged in regulation of behavior (locomotor activity, visual sensitivity, olfactory response) are all under control of a single pacemaking system in the optic lobes while the one autonomous rhythm, that of cuticle deposition, would appear to have no clear behavioral relevance.

It is also notable that while there have been many studies on rhythms in visual sensory reception in a wide variety of vertebrates and invertebrates (Underwood et al., 1997), little attention has been paid to the potential for circadian regulation of other sensory systems. The discovery that the olfactory system is regulated by the circadian system suggests that circadian control of sensory responses may be more widespread among sensory systems than has been previously recognized. This, in turn, indicates that a complete understanding of the functional importance of circadian organization will depend on understanding the extent to which circadian clocks not only regulate physiological and behavioral output, but also directly modulate sensory input.

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