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Daily Pattern of Brain Serotonin Levels in the Cockroach, *Leucophaea maderae*

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Abstract

Injections of serotonin can partially mimic the entraining effect of light on the biological clock in the cockroach, *Leucophaea maderae*. To test for a causal linkage between light and afferent input to the clock via serotonergic neurons, endogenous brain levels of serotonin were measured at 4 different times of day using high performance liquid chromatography coupled with electrochemical detection. Levels were determined at light onset, midday, light offset and midnight in roaches maintained under LD 12:12 and 25 ± 2°C. No significant differences in brain levels of serotonin were found among these times of day. Therefore, it seems unlikely that serotonergic neurons play a central role in mediating the entraining effect of light on the biological clock.

Introduction

The cockroach, *Leucophaea maderae*, offers an excellent model organism, analagous to the vertebrate system, in which to study circadian organization. Behavioral (e.g., locomotor activity), physiological (e.g., eye sensitivity to light) and biochemical, (e.g., cytochrome oxidase activity) parameters have been observed to change on a circadian basis. Furthermore, the expression of each of these rhythms is controlled by a pair of oscillators, as demonstrated by an elegant series of lesion studies carried out in *L. maderae* (1). The site of the oscillators that neurally regulate the expression of circadian rhythms of locomotor activity (review; 2), eye sensitivity to length (2, 3) and cytochrome oxidase activity (4) have been localized to the lobula neuropil in the optic lobes of the cockroach brain. Despite the fact that the oscillators which regulate the expression of each of these rhythms reside in the same brain areas, it remains to be demonstrated whether the same cells regulate each of these rhythms. This information is imperative for understanding circadian organization in this model organism. The identification of a neurotransmitter that affects oscillator function (i.e., input) or that is responsible for oscillator output is an important first step in identifying specific cells of the oscillators that regulate these biochemical, behavioral and physiological rhythms. Once a neurotransmitter is identified, it could be radioactively labeled and used to determine selective neuron uptake. Anatomical mapping of the neural pathway to or from the oscillator cells would then be possible.

Serotonin was the neurotransmitter of choice in this study for several reasons. In *L. maderae*, the oscillators can be affected by light input exclusively from the compound eyes (1). A 6-hour light pulse administered to animals freerunning in darkness at circadian time 13–15 hr resulted in a phase delay of approximately 4.0 hr (5). Similarly, 3- or 6-hour perfusions of serotonin in animals freerunning in darkness at circadian time 16–18 hr produced on average a phase delay of 4.0 hours (1). The fact that serotonin injections can partially mimic effects of photic input on the oscillators in *L. maderae* as well as in a mollusc, *Aplysia* (6), coupled with reports that serotonin levels become elevated in the cerebral ganglia of a cricket, *Acheta domesticus* (7), during the dark/light transition of a LD 12:12 hr photoperiodic cycle, led us to hypothesize that brain serotonin levels in *L. maderae* should vary with time of day and variations should correlate with changes in light intensity (i.e., dark/light or light/dark transitions). Therefore, we measured brain levels of serotonin at transition times between daylight and darkness and at midday and midnight in *L. maderae* held under LD 12:12 and 25 ± 2°C conditions using high performance liquid chromatography coupled with electrochemical detection (HPLC-EC) to test this hypothesis.

METHODS AND MATERIALS

Animals.—The cockroaches, *Leucophaea maderae*, used in this study were newly emerged adult males obtained from breeding colonies maintained under LD 12:12 photoperiodic conditions with light onset at 0700 hr or 0600 hr. Roaches from these colonies were transferred to environmental chambers where they were allowed to acclimate to the same photoperiodic conditions as the breeding colonies and 25 ± 2°C for at least one week prior to obtaining brain samples. Several roaches were placed in plastic running wheels in order to monitor the entrainment of the activity cycle to photoperiodic conditions. Each revolution of the wheel closed a magnetic reed switch wired to an Esterline-Angus activity recorder. Closing the switch produced a deflection of an ink pen and thus resulted in a dash on a paper strip chart. The paper chart moved at a constant rate, thereby allowing the daily pattern of activity to be monitored for these representative roaches. The remaining roaches were placed in small plastic cages for easy retrieval. Food (puppy chow) and water (via a petri dish fitted with a cotton wick) was provided for all roaches *ad libitum*.

Surgery.—Brains were surgically removed from roaches entrained to the LD 12:12 photoperiodic schedule at 0600 hr or 0700 hr (light onset), 1200 hr or 1300 hr (midday), 1800 hr or 1900 hr (light offset) or 2400 hr or 0100 hr (midnight). For each of the 6 assays run, 1 roach at each of these times was removed from the plastic holding cages and frozen within 15 s by dropping them into a polypropylene container of petroleum ether chilled to −74°C. Each cockroach was removed from the petroleum ether and taped to the underside of a plastic petri dish lid with its head protruding through a hole. The head was secured with tape placed behind the neck, the lid was placed on a petri dish platform and the head capsule viewed under a dissecting scope. The cuticle between the compound eyes was then removed using a fractured razor-blade scalpel and forceps. Fat and other connective tissues were cleared away using forceps to expose the cerebral ganglia and both optic lobes. The optic nerves were then severed on one side and the corresponding optic lobe was then grasped using forceps. Using the forceps to gently lift up on the brain, iridectomy scissors were used to cut the trachea, the circumesophageal connectives and the optic nerves on the other side, thereby freeing the brain from the head capsule. The entire surgical procedure was performed during a fairly constant time interval of under 2 minutes in order to minimize possible enzyme activity on serotonin. During this time period brain tissue did thaw but remained chilled. The brains were weighed using a Mettler balance and frozen immediately and stored for not more than 1 week in microvials at −74°C until prepared for analysis using HPLC-EC.

Preparation for HPLC-EC Analysis.—Brain samples from each of the 4 sampling times were simultaneously thawed. Each brain was transferred to a Potter Elvehjem ground glass tissue homogenizer and 100 µl of mobile phase (i.e., that used in the HPLC analysis) plus 100 µl (0.48 µg/ml) of internal standard (i.e., Nω-methyl-5-hydroxy-tryptamine) were added. The brain tissue was homogenized for 30 s. The homogenate was transferred to a chilled microvial and centrifuged at 20,200 × G for 30 min using a Sorval refrigerated centrifuge at 4°C. The supernatant was transferred to a clean microvial using a micro-pipette and 0.8 ml of (8:1 v/v) heptane : chloroform was added. This microvial was placed on a vortex mixer for 30 s. After the 2 phases separated, the lower aqueous phase was removed using a 1 cc syringe equipped with a 26 gauge needle. After withdrawal, the needle was subsequently replaced with a 45 µm syringe filter and the aqueous phase filtered into a new microvial and stored over ice until injected into the HPLC.

HPLC Conditions.—HPLC grade methanol and 1-heptanesulfonic acid (sodium salt), were obtained from Aldrich Chemical Company. Ethylenediamine-tetraacetic acid (EDTA), serotonin creatinine sulfate monohydrate (5-hydroxytryptamine) and the oxalate salt of Nω-methylserotonin (N_ω-methyl-5-hydroxytryptamine) were obtained from Sigma Chemical Company. All other reagents were obtained from Fisher Scientific Company.

The mobile phase was prepared by mixing 18.00 g of sodium dihydrogen phosphate, 200 ml of HPLC grade water, 6.0 mg EDTA, 300 ml of HPLC grade methanol, 10 ml of ethyl

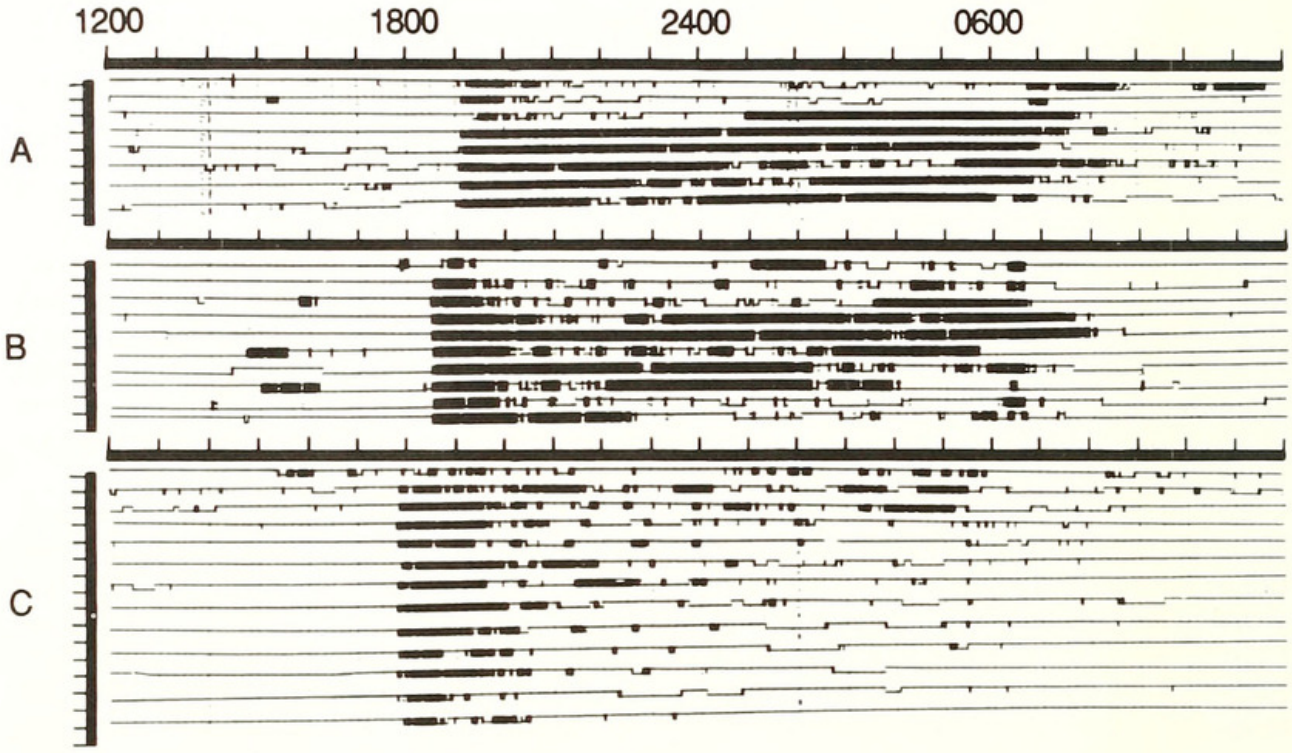

FIG. 1. Representative locomotor activity patterns of three *L. maderae* acclimated simultaneously to the same photoperiodic (LD 12:12) and temperature (25 ± 2°C) conditions as those roaches from which brains were removed for HPLC-EC analysis of serotonin. Light onset was at 0700 hr (roaches A and B) or 0600 hr (roach C).

acetate and 0.20 g 1-heptanesulfonate. This solution was filtered through a 0.45 μm nylon filter (Micron Separations, Inc.), transferred to a 2 liter volumetric flask, the pH adjusted to 4.0 using phosphoric acid and diluted to the mark with HPLC grade water. This solution was filtered through a 0.45 μm membrane filter and degassed under vacuum prior to use.

Standard stock solutions of serotonin and Nω-methylserotonin were prepared by dissolving 1.0 mg of standard into a 25 ml volumetric flask with mobile phase. Standards of 0.016 to 1.2 μg/ml serotonin were prepared from the stock solution. An internal standard of Nω-methylserotonin at a concentration of 0.16 μg/ml was prepared from its corresponding stock solution. Working standards were prepared by adding 1.00 ml of internal standard, Nω-methylserotonin, to 1.00 ml of each standard of serotonin.

A Varian model 5000 HPLC, equipped with a 10 μl injection loop and a reverse-phase Brownlee™ analytical C_{18} column with 5-μm particle size, was used for all separations. The detection system was Bioanalytical Systems Model LC-4B amperometric controller, LC-17A flow cell, Model MF-1000 glassy carbon working electrode and Ag/AgCl reference electrode. A flow rate of 1.8 ml/min was used for all runs. Detector voltage was operated at +0.75 V vs. Ag/AgCl.

Data Analysis.—Calibration curves were prepared by plotting the peak height ratio of serotonin to Nω-methylserotonin versus nanograms of serotonin injected. Data were subjected to a linear least squares regression analysis in order to generate an equation with which brain serotonin levels could be quantified. The correlation coefficient was 0.999. Calibration curve data points represented the average value determined in triplicate.

Mean brain levels of serotonin determined in triplicate for 6 roaches at each of the 4 times of day were compared using a one-way analysis of variance at the 95% confidence level.

RESULTS

Animals appeared to be entrained to the light/dark cycle (LD 12:12) in that representative animals held in the running wheels initiated activity at the same time each evening (Fig. 1).

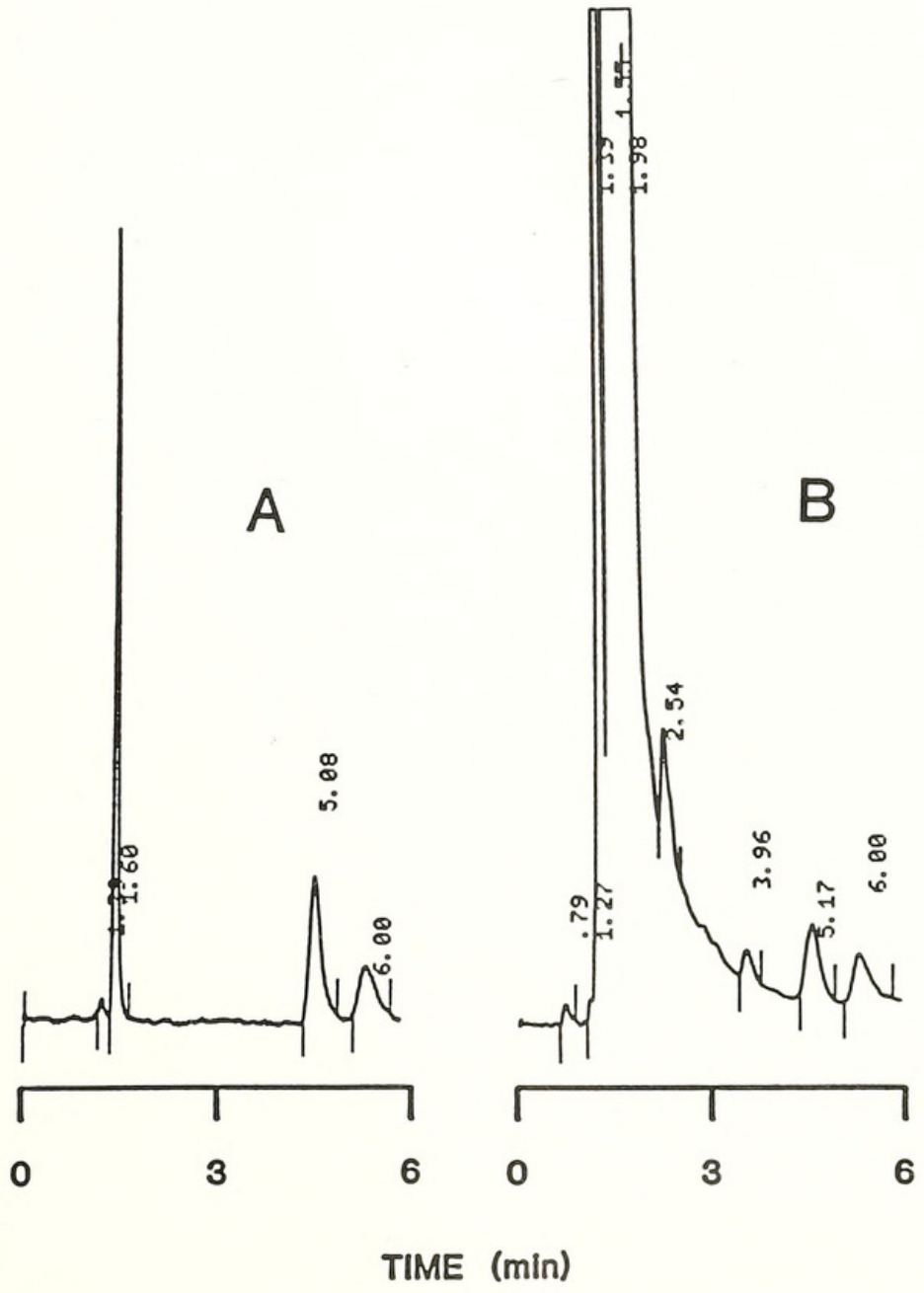

FIG. 2. Typical HPLC-EC chromatographs resulting from 10 μl injections of a standard solution (A) and a brain extract (B). The serotonin peak eluted at approximately 5.0 min and the internal standard (Nω-methylserotonin) at 6.0 min.

The assay procedure using HPLC-EC gave good resolution of serotonin from both the internal standard and other compounds extracted from brain samples (Fig. 2).

No temporal variation in brain serotonin levels was detected in comparisons made among samples collected at the four times of day (Table 1). The lowest levels of 3.2 ± 0.9 ng/brain detected at the dark/light transition (i.e., lights on) were not significantly different from the highest levels of 4.4 ± 0.7 ng/brain detected at the light/dark transition (i.e., lights off).

DISCUSSION

Serotonin levels in the brain of this cockroach species do not appear to vary on a temporal basis. Despite the fact that the existence of a daily rhythm in brain serotonin levels can not be disproved based on data obtained from only 4 times of day, it seems highly unlikely

TABLE 1. Levels of serotonin determined for six brains taken from male *Leucophaea maderae* acclimated to LD 12: 12 at 25 ± 2°C at four times of day.

	Lights on	Midday	Lights off	Midnight	ANOVA[1]
Serotonin (ng/brain)	3.2 ± 0.9[2]	3.6 ± 0.7	4.4 ± 0.7	3.5 ± 0.8	N.S.
Brain wt. (mg)	2.5 ± 0.2	2.4 ± 0.2	2.6 ± 0.2	2.4 ± 0.1	N.S.

[1] One-way analysis of variance at the 95% confidence level. N.S. = no significant differences found among treatment groups.
[2] Mean ± standard error.

when considered in conjunction with data from HPLC-EC studies carried out in another cockroach, *Periplaneta americana* (8), and the scorpion, *Leiurus quinquestriatus* (9) in which no daily variation was detected. In addition, the levels of serotonin detected in this cockroach species (3.2 to 4.4 ng/brain) are slightly lower but consistent with levels reported in *P. americana* (5.8 ng/brain, (10); 6 ng/brain, (11); 4.8 ng/brain, (8)), another cockroach species.

Serotonergic neurons do not appear to be centrally involved in mediating the entraining effect of the light/dark cycle on the oscillators. Serotonin levels did not change significantly in response to daylight, darkness, the dark/light or light/dark transitions. A significant change in levels would be expected if serotonergic neurons were directly involved in transferring input from photoreceptors to the clock. This idea is tempered by the fact that data regarding release, re-uptake rates and metabolism of serotonin are not available. The lack of pronounced changes in serotonin levels does not rule out the possibility that serotonin plays a modulating role in affecting input to the clock from photoreceptors, especially in light of the slight tendency toward increasing levels during daylight hours. The idea that serotonin might modulate the activity of other input(s) to the oscillators would be consistent with data indicating the phase shifting influence of serotonin injections observed in this cockroach species (5) that partially (i.e., phase delay only) mimic photoperiodic entrainment. If serotonin modifies the flow of neural information along entraining pathways from the photoreceptors to the oscillators, then alterations in serotonin levels by injection could modify the flow of information to the clock, particularly under constant conditions of darkness. Information regarding release, re-uptake rates and metabolism of serotonin is necessary to help resolve this problem.

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