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Regulation of serotonin levels by multiple light-entrainable endogenous rhythms

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Summary

This study examined whether serotonin levels in the brain of the American lobster, *Homarus americanus*, are under circadian control. Using high-performance liquid chromatography and semi-quantitative immunocytochemical methods, we measured serotonin levels in the brains of lobsters at six time points during a 24-h period. Lobsters were maintained for 2 weeks on a 12 h:12 h light:dark cycle followed by 3 days of constant darkness. Under these conditions, brain serotonin levels varied rhythmically, with a peak before subjective dusk and a trough before subjective dawn. This persistent circadian rhythm in constant darkness indicates that serotonin levels are controlled by an endogenous clock. Animals exposed to a shifted light cycle for >10 days, followed by 3 days in constant darkness, demonstrate that this rhythm is light entrainable. Separate analyses of two pairs of large deutocerebral neuropils, the accessory and olfactory lobes, show that serotonin levels in these functionally distinct areas also exhibit circadian rhythms but that these rhythms are out of phase with one another. The olfactory and accessory lobe rhythms are also endogenous and light entrainable, suggesting the presence of multiple clock mechanisms regulating serotonin levels in different brain regions.

Key words: *Homarus americanus*, Crustacea, neurogenesis, 5-hydroxytryptamine, central nervous system.

Introduction

Circadian, or near-24-h, rhythms of biological processes that persist in the absence of environmental cues, have been described in organisms ranging from photosynthetic bacteria to humans. The molecular clocks that control these rhythms are highly conserved across a broad range of species (King et al., 1997; Sun et al., 1997; Tei et al., 1997). Such clocks provide critical controls allowing organisms to adapt to daily cycles in environmental conditions. The interaction of endogenous clocks and external signals, such as temperature, time of feeding and light, coordinate physiological functions so that they occur at a particular phase of each day.

In the present study, we have asked whether serotonin levels in the lobster brain are under circadian control. We are interested in this question because serotonin regulates a variety of functions in crustaceans (Beltz and Kravitz, 2003). Among these actions, serotonin increases transmitter release from both excitatory and inhibitory nerve terminals and enhances the contractility of muscle fibers (Dudel, 1965; Florey and Florey, 1954; Glusman and Kravitz, 1982), modulates the sensitivity of sensory neurons (Pasztor and Macmillan, 1990), increases the frequency and intensity of the heartbeat (Battelle and Kravitz, 1978; Florey and Rathmayer, 1978) and serves as a modulator of segmental reflexes in the walking system (Gill and Skorupski, 1996). Because serotonin plays so many roles, it is important to understand the factors that influence its synthesis and availability.

Serotonin has also been implicated as a regulator of life-long neurogenesis in the lobster brain (Benton and Beltz, 2001; Beltz et al., 2001), and the generation of new neurons in this system is under circadian control, with a peak rate at dusk. This finding may be correlated with the habits of lobsters, which are most active around dusk (Weiss, 1970; Cooper and Uzmann, 1980; Chabot et al., 2001). This rhythm of neuronal proliferation is regulated by an endogenous oscillator that is entrained by the light:dark (L:D) cycle (Goergen et al., 2002). We have proposed that light may provide a dominant coordinating signal for the many factors, including serotonin, that influence the persistent generation of new neurons. If this hypothesis is correct, then serotonin levels should cycle over a ~24-h period, be entrainable by light and maintain a fixed phase relationship with the rhythm of neurogenesis.

In the present study, we measured serotonin levels at various times of day in the brains of lobsters that were maintained in several different light regimes. These studies show that serotonin levels undergo circadian variations that are light entrainable and controlled by an endogenous clock. We also examined serotonin levels independently in functionally distinct brain regions (Fig. 1) and show that the serotonin rhythms observed in these areas have contrasting phases, each

of which is light entrainable. This indicates that separate mechanisms control serotonin metabolism in different parts of the brain, with light as a common zeitgeber. These findings are important because they suggest that serotonin may be involved in the light-activated cascade of events that culminates in the circadian regulation of a variety of physiological functions in crustaceans, including the rate of neuronal proliferation in the brain.

Materials and methods

Animal rearing and experimental conditions

Juvenile lobsters (*Homarus americanus* Milne-Edwards; 2–4 cm total body length) were obtained from the New England Aquarium (Boston, MA, USA). Animals were kept on a 12 h:12 h L:D cycle in individual containers (5×5 cm) that floated freely on the surface of a tank of circulating artificial seawater (14°C). Holes in the containers ensured circulation of water. Because the rate of neurogenesis and endogenous levels of various hormones fluctuate during the molt cycle (Harrison et al., 2001; Gorissen, 2002), the lobsters were molt staged (Aiken, 1973) and only intermolt animals were included in the experimental groups. Animals were fed brine shrimp, *Artemia salina*, three times a week 1.5 h before dusk; food was always supplied on the same days of the week.

All animals were entrained to a 12 h:12 h L:D cycle for a minimum of 2 weeks. The light intensity at the surface of the water was 6–11 μEinsteins m^{-2} s^{-1}. Groups of lobsters were exposed to four different experimental protocols, all based on a 12 h:12 h L:D schedule, and brain levels of serotonin were then assessed over a 24-h period by high-performance liquid chromatography (HPLC). Group 1 was exposed to the 12 h:12 h L:D cycle. Group 2 was exposed to the same L:D cycle as Group 1 but, following the 12 h:12 h L:D exposure (entraining light regime), animals were transferred to constant darkness for 3 days without food (D:D conditions). Group 3 was exposed to an L:D regime that was phase-shifted by 4 h relative to Group 2, followed by D:D for 3 days prior to HPLC measurements. A fourth group of animals (Group 4) was subjected to the same light regime as Group 2, but in these animals the olfactory lobes (OLs), accessory lobes (ALs) and the brain remainder were separated from one another and the individual areas then assayed for serotonin content.

Changes in brain serotonin levels measured in Group 1 (L:D) did not show a clear diurnal rhythm and were not repeatable over eight trials (see Discussion). All HPLC data reported in this paper, therefore, are from animals in Groups 2, 3 and 4, which were first entrained to an L:D regime and then exposed to 3 days of D:D prior to HPLC assessments. Exposure to constant darkness prior to HPLC measurements was aimed at preventing possible masking effects of sudden intensity changes in light, and of feeding, on serotonin release and also demonstrated that the rhythms observed are endogenous (Chiu et al., 1995). The phase-shift in the conditioning light regime for Group 3 relative to Group 2 tested whether serotonin levels are light entrainable.

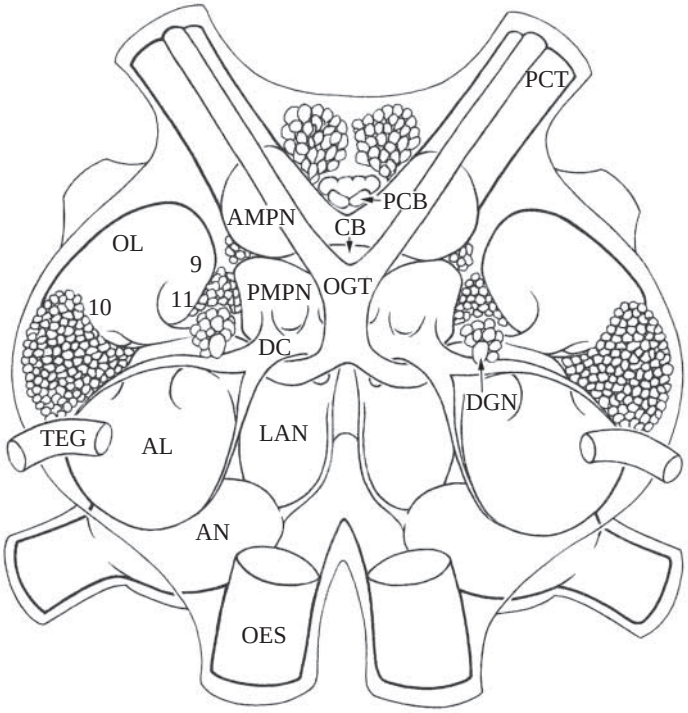

Fig. 1. Diagram of the lobster brain, reconstructed from serial sections, showing the location of the neuropils and cell somata relevant to this study. Accessory lobe (AL); anterior median protocerebral neuropil (AMPN); antenna II neuropil (AN); central body (CB); deutocerebral commissure (DC); dorsal giant neuron (DGN); lateral antennular neuropil (LAN); oesophageal connectives (OES); olfactory globular tract (OGT); olfactory lobe (OL); protocerebral bridge (PCB); protocerebral tract (PCT); posterior median protocerebral neuropil (PMPN); tegumentary nerves (TEG); soma cluster 9 (9); soma cluster 10 (10); soma cluster 11 (11). Terminology of Sandeman et al. (1992).

Dissection

Following the entraining light regime and 3 days in D:D, four animals from each of Groups 2–4 were assayed for serotonin levels by reverse-phase HPLC every 4 h over a 24-h period. Animals were retrieved from their containers using night-vision goggles (Bushnell Corporation, Overland Park, KS, USA) and placed on ice in the dark. The eyestalks were quickly removed in dim light in order to minimize light activation of serotonergic pathways. Brains were then dissected in cold lobster saline [462 mmol l^{-1} NaCl, 16 mmol l^{-1} KCl, 34 mmol l^{-1} $CaCl_2$, 17 mmol l^{-1} $MgCl_2$, 11 mmol l^{-1} α-D(+)-glucose and 10 mmol l^{-1} Hepes buffer (pH 7.4)].

HPLC methods

Following dissection, tissues were transferred to microcentrifuge tubes containing 50 μl of 0.1 mol l^{-1} perchloric acid. Samples were diluted with 200 μl of mobile

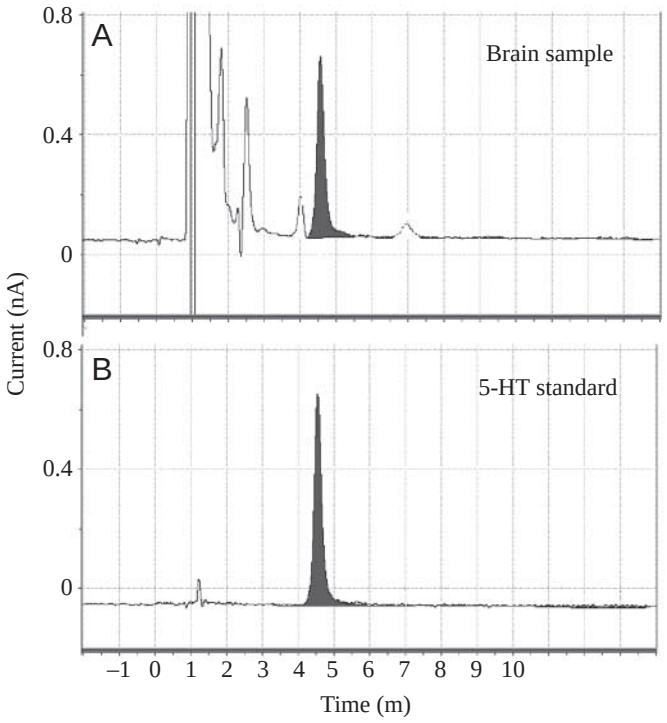

Fig. 2. HPLC chromatograms (A/D Instruments Inc.; PowerChrom version 2.2.4) showing the peaks contained in a brain sample (A) compared with a serotonin (5-HT) external standard (B).

phase [20 mmol l^{-1} anhydrous monobasic sodium phosphate, 1.85 mmol l^{-1} heptanesulfonic acid sodium salt, 0.27 mmol l^{-1} anhydrous EDTA and 16% MeOH:4% acetonitrile (v/v) as organic modifiers (Sigma, St Louis, MO, USA)] and homogenized manually with a pestle. The final solvent buffer was adjusted to pH 3.25 with concentrated phosphoric acid. Homogenates were transferred into Eppendorf tubes containing a 0.45 μm filter insert (VWR, West Chester, PA, USA) and centrifuged for 15 min at 20 000 ***g*** and 21°C. The clear supernatants were transferred into autosampler microvials [Bioanalytical Systems Inc. (BAS), West Lafayette, IN, USA] and sealed with Teflon caps. 10 μl samples were applied to a C18 reverse-phase column (Alltech Associates Inc., Deerfield, IL, USA; 3 μm, 100×4.6 mm) *via* a BAS autosampler (Samplesentinel). Eluted compounds were detected electrochemically with a BAS liquid chromatography system consisting of a CC-5 liquid chromatography module, a PM 80 solvent delivery system and an LC 4C amperometric detector. The detector potential was set at 625 mV and the detection limit was in the range of 2 nA. Recovery rates were close to 100% and no further corrections were applied. An A/D converter (A/D Instruments Inc., Colorado Springs, CO, USA) and a strip-chart program with integrated chromatography software (A/D Instruments Inc.; PowerChrom version 2.2.4) were used to measure peak amplitude (Fig. 2).

HPLC data analysis

The data for the chronograms were obtained from serially independent measurements of serotonin levels in groups of animals killed and assayed at each time interval. A requirement for serially independent measurements of this nature is that the individuals in the groups are as similar to one another, and to those in the other groups, as possible. To ensure this equality, we used animals that had been reared under the same conditions of temperature and nourishment and were as close in size as practically possible. The mean body length (rostrum to telson) of all animals that were used in the results presented here was 4.49±0.056 cm (mean ± S.E.M.; N=72). Furthermore, in a previous study we found that the volume of the lobster brain is linearly correlated with body volume for the size range that we used (Helluy et al., 1995). Given that the individual animals, and therefore their brains, were very similar in size, we were able to simply use the serotonin content in picomoles per brain in the chronograms without normalizing these values to brain mass or dimensions, which were very difficult to assess for these small tissue volumes. When normalization of values to tissue mass was attempted, standard errors became larger, indicating that the normalization procedure was introducing additional variability into the results. Raw data are therefore reported for all results.

Immunocytochemistry

Methods for processing brains as whole mounts for serotonin immunocytochemistry were taken from Beltz and Burd (1989). Juvenile lobsters were maintained either on a 12 h:12 h L:D cycle for at least two weeks (Group 1 protocol) or on a 12 h:12 h L:D entraining light regime followed by 3 days in D:D (Group 2 protocol). Animals were retrieved from their containers, placed on ice in the dark, and the eyestalks removed in dim light. Brains were then dissected at 3 h prior to subjective dawn (N=4) and 3 h prior to subjective dusk (N=4) and fixed in cold 4% paraformaldehyde for ~18 h. Tissues were then rinsed five times in 0.1 mol l^{-1} phosphate buffer (PB; pH 7.4) and incubated in 0.1 mol l^{-1} PB with 0.3% Triton X-100 (PBTx) for 45 min, followed by incubation in rabbit anti-serotonin antibody (1:1000; DiaSoren, Stillwater, MN, USA) for 48 h at 4°C. Following six rinses in PBTx, goat anti-rabbit Alexa 488 (1:50; Molecular Probes, Eugene, OR, USA) was applied for 36 h, after which samples were rinsed six more times in PB. Preparations were mounted in Gel Mount (BiØmeda Corp., Foster City, CA, USA) and visualized using a Leica TCS SP confocal microscope (Leica Microsystems, Germany).

In order to assess relative levels of serotonin immunoreactivity in the samples, all brains were screened during a single session and the initial laser and filter settings on the confocal microscope were maintained at the same levels throughout the entire analysis. Leica confocal quantitative software (version 2.0) was used for semi-quantitative analyses of the intensity of serotonin labeling in the OLs and ALs. These measurements were done by comparing the intensity of fluorescence (serotonin immunoreactivity) in the OLs and ALs for each time point, relative to background levels in the brain. A t-test was used for statistical analysis (SPSS Inc., Chicago, IL, USA).

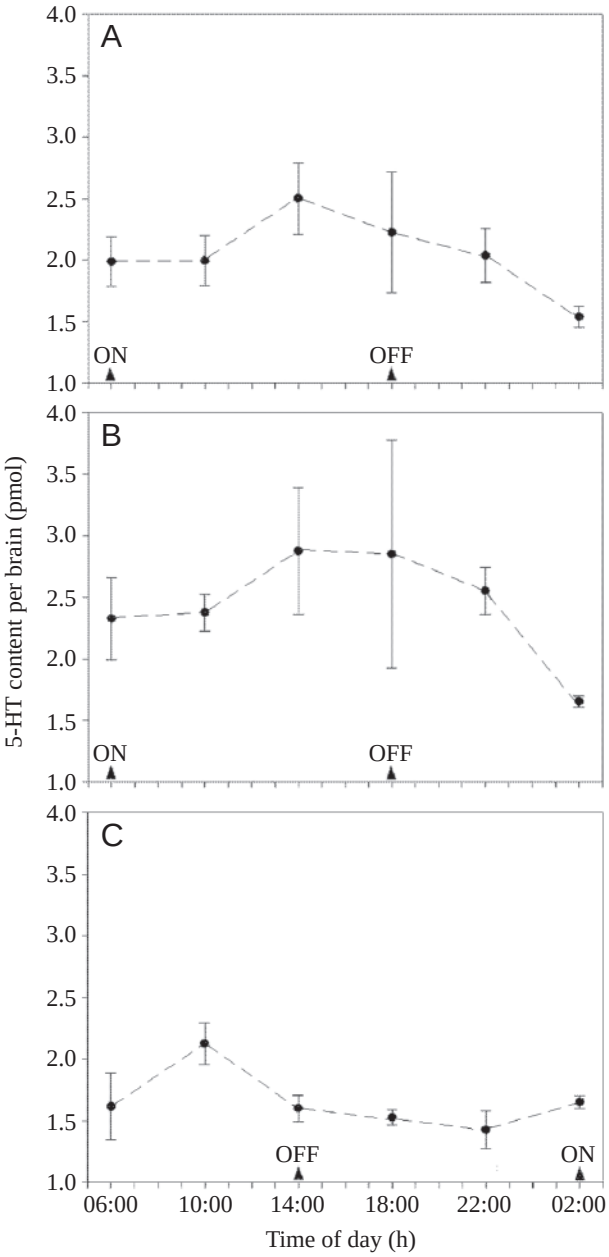

Fig. 3. Chronograms in which the serotonin (5-HT) levels in lobster brains measured by HPLC are shown over a 24-h period, plotted as means ± S.E.M. All three graphs show the measurements taken after the animals had been in darkness (D:D) for 3 days without food. The timing of light-on and light-off experienced by the animals during their entraining L:D periods is marked by the black triangles. Note that in C light-off has been shifted by 4 h in time and occurred at 14:00 h. (A) Pooled data from B and C. (B) Serotonin levels in the brains of juvenile lobsters that had been maintained for 2 weeks on an entraining 12 h:12 h L:D cycle. (C) Serotonin levels in brains of animals that had been subjected to a phase-shifted light cycle. In pooling the data, we matched the subjective light-on and light-off transitions of the two groups. The pooled data (A) show a pre-dusk peak and pre-dawn trough [*N*=8 for each time point; the peak prior to subjective dusk is significantly different (*P*=0.039) from the trough prior to subjective dawn]. (B,C) Plotting the data for the two groups separately illustrates the phase shift of the peak and trough in the animals that had been subjected to the shifted light regime (*N*=4 for each time point).

Results

HPLC measurements of brain serotonin levels

Rhythmic changes in serotonin levels are observed in the brains of lobsters subjected to D:D conditions for 3 days following maintenance in L:D conditions for at least 2 weeks (Group 2). These brains show a consistent pattern in serotonin measurements, with the major peak in serotonin levels occurring prior to subjective dusk and a trough in serotonin before subjective dawn (Fig. 3). Imposing a shift in the L:D cycle before entering 3 days in D:D (Group 3) results in a phase shift of the peak and trough (Fig. 3B,C), indicating that brain serotonin levels are light entrainable. Visual inspection of the chronograms suggests that in constant darkness the changes in serotonin levels precede the time at which the light changed during the entraining light regime. We used a three-way ANOVA to test this possibility and found that when D:D data from the normal and time-shifted groups (2 and 3) are pooled, there is a significant interaction (*P*=0.039) between an 'anticipatory' rise or fall in serotonin levels and the occurrence of the light-off or light-on, respectively (Figs 3A, 4). These data confirm the existence of a pre-dusk maximum and pre-dawn minimum in the chronograms.

Serotonin levels in the OLs and ALs

Experiments were also conducted to determine whether the OLs and ALs, two midbrain regions with distinct functions and intense serotonergic innervation, individually exhibit rhythmic changes in serotonin levels. The OLs in Group 2 animals show a clear rhythm in serotonin levels (Fig. 5A,D), with a peak before dusk and a sharp decline at dusk to a level that is maintained throughout the subjective night phase and into the subjective daytime hours. The most distinctive aspect of the OL serotonin rhythm is the peak that occurs prior to subjective dusk, a feature that is also evident in the fluctuation of serotonin content in the whole brain (Fig. 3). A similar pattern in serotonin levels was measured in the brain remainder (Fig. 5B,E), which was comprised of the protocerebral and tritocerebral areas and medial deutocerebral regions such as soma clusters 9 and 11, the lateral antennular neuropil and the olfactory globular tract (see Fig. 1). The ALs, however, show a rhythm in serotonin levels that is distinct from that of the OLs and the brain remainder. High levels of serotonin were measured in the ALs beginning in the hours before dusk, as in the OLs; however, high levels of serotonin were also measured throughout the night, with a drop at subjective dawn (Fig. 5C,F).

If the levels of serotonin measured in each one of the OLs, ALs and the brain remainder are all added together for each of the six time points, the result provides a measure of the relative proportions of serotonin found in the different brain regions over time (Fig. 6). These histograms illustrate that the

Table 1. *The results of densitometric measurements of immunofluorescence in the accessory lobe (AL) and the olfactory lobe (OL)*

	Staining intensity (15:00 h)	Staining intensity (03:00 h)	*P*-value
D:D			
ALs	178.21±48.54	124.39±51.74	0.050
OLs	169.62±55.94	106.48±52.03	0.042
L:D			
ALs	78.62±97.70	12.38±4.70	0.158
OLs	64.87±78.50	14.82±7.13	0.180

Labeling in both lobes was more intense at pre-dusk (15:00 h) than at pre-dawn (03:00 h) after the animals had been exposed to constant darkness for 3 days (D:D). No significant difference was detected between pre-dusk (15:00 h) and pre-dawn (3:00 h) immunofluorescence in the AL and OL of animals living under 12 h:12 h L:D conditions. *N*=4 per time point.

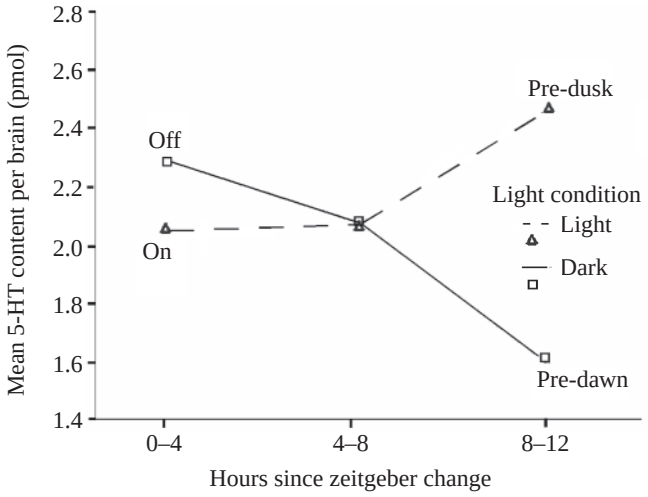

Fig. 4. An 'anticipatory' rise or fall in the level of serotonin in the brains of lobsters is revealed when the means of serotonin (5-HT) content in whole brains (data from Fig. 3A) are pooled and plotted against the hours since light-on or light-off in a three-way ANOVA. Little change in serotonin levels follows light-on or light-off either in the 0–4 or 4–8 h time periods after they have occurred. In the 8–12 h period, however, the levels in the animals approaching dusk increase and those approaching dawn decrease. These changes occur well before the light-on or light-off transition.

serotonin content in the OLs and ALs accounts for ~70% of total brain levels. As in the whole-brain studies (Fig. 3), the pre-dusk peak is the most striking feature of Fig. 6. Examination of the components of this pre-dusk peak shows that serotonin levels in the ALs rise by 18%, the OLs by greater than twofold (123%), and the brain remainder by roughly threefold (197%) over the pre-dawn values. These sharp increases in serotonin coincide with the period of arousal and increased activity as lobsters undertake their nocturnal foraging and social activities.

Immunocytochemistry

We tested whether the brain serotonin levels measured by HPLC are also detectable immunocytochemically. Whole mounts of brains dissected from animals reared in the same conditions as Groups 1 and 2 and killed 3 h before subjective dawn and dusk (the times when the serotonin trough and peak were measured in the OLs and ALs by HPLC, respectively; see Fig. 5) were used for these studies. Densitometric measurements of the intensity of serotonin labeling in the confocal images reveal detectable differences between the pre-dawn and pre-dusk time points of both the Group 1 (L:D) and Group 2 (D:D) animals (Table 1). The intensity of labeling in the Group 1 brains was highly variable. Therefore, although the means for pre-dawn and pre-dusk brains are different, the standard errors are large and the differences are not statistically significant (see Table 1, L:D). For the Group 2 (D:D) brains dissected at pre-dusk (15:00 h), we detected a more intense labeling of the OLs and ALs than in the same regions of the brains of pre-dawn (03:00 h) lobsters. Statistical analyses of the densitometric measurements for pre-dawn *vs* pre-dusk ALs (P=0.050) and pre-dawn *vs* pre-dusk OLs (P=0.042) indicated significant differences. Taken together with the HPLC measurements, the immunocytochemical measurements confirm that the levels of serotonin in the ALs and OLs of lobster brain fluctuate rhythmically, being relatively low before dawn and high before dusk.

Discussion

The present study was undertaken in order to learn whether serotonin levels in the lobster brain are under circadian control and to understand these findings in the context of serotonin's actions in the crustacean nervous system. One of our major interests is the serotonergic regulation of neurogenesis in the lobster brain. We have shown that serotonin levels regulate the rate of generation of new neurons in this system (Beltz et al., 2001; Benton and Beltz, 2001) and that neurogenesis is also under circadian control (Goergen et al., 2002). We hypothesized that if serotonin is involved in the circadian pathway that regulates neurogenesis, brain levels of this molecule should also cycle diurnally, be light entrainable and show a fixed relationship to the neurogenic rhythm. In keeping with this model, our results demonstrate that serotonin levels in the brains of lobsters have diurnal fluctuations that are sustained by a light-entrainable endogenous rhythm.

In other crustaceans such as the fiddler crab *Uca pugilator* and the crayfish *Procambarus clarkii*, fluctuating serotonin levels have mostly been described for eyestalks (Fingerman and Fingerman, 1977; Fingerman et al., 1978; Escamilla-Chimal et al., 2001; Fanjul-Moles and Prieto-Sagredo, 2003). Castanon-Cervantes et al. (1999) are the only authors to our knowledge who have assessed serotonin levels over 24 h in the brain of another crustacean. These authors have shown that in

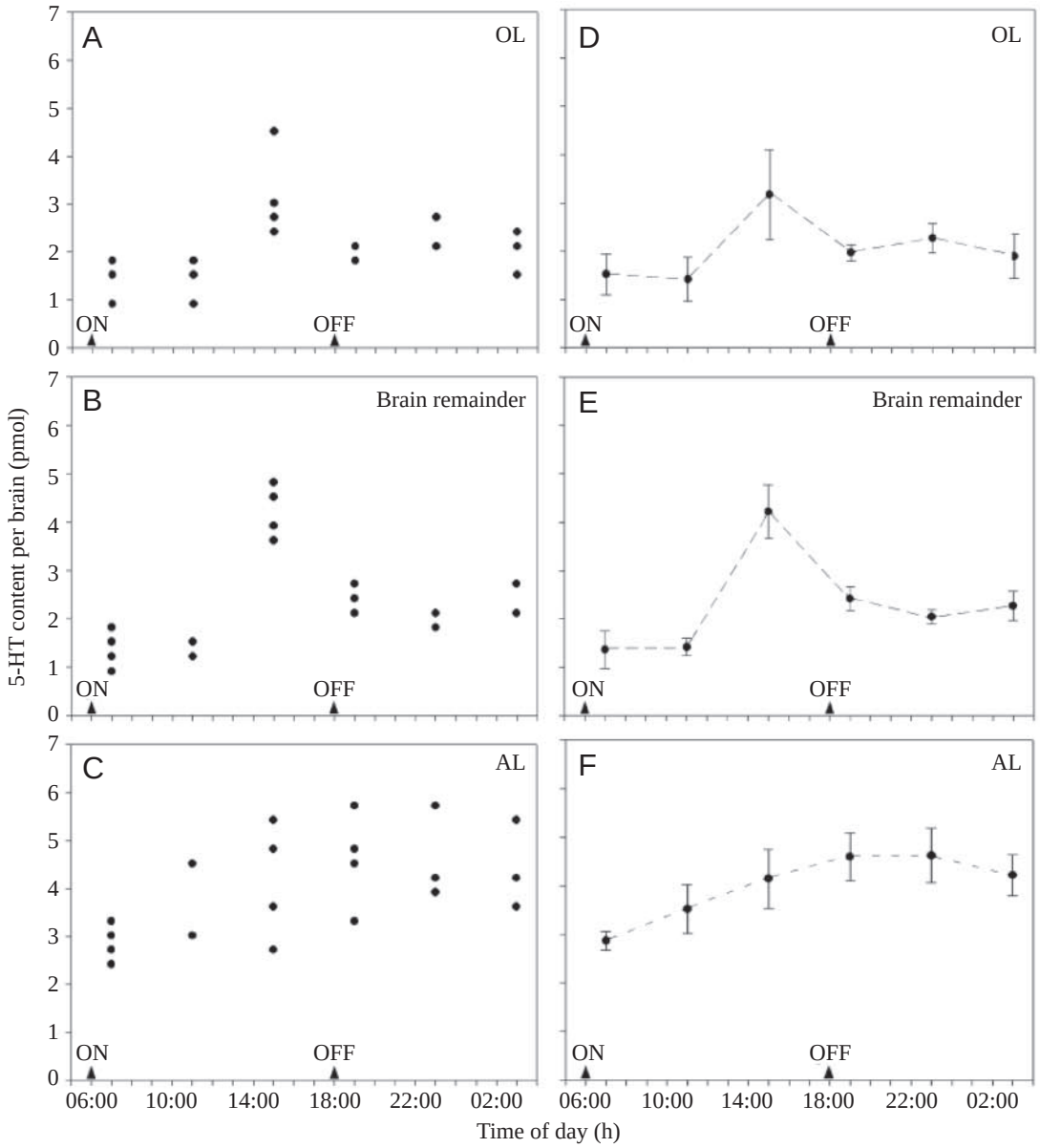

Fig. 5. Separate analyses of serotonin (5-HT) levels in individual regions of the brains of juvenile lobsters that were entrained to a 12 h:12 h L:D light regime followed by 3 days in D:D. Chronograms on the left show the raw data collected from four individuals at each time point (values for some measurements were the same and so appear as a single point). Chronograms on the right show the arithmetic means (± S.E.M.) of the data. All measurements were made from animals that had been in constant darkness for 3 days. The most distinctive aspect of the serotonin levels of the olfactory lobe (OL) and the brain remainder (consisting of the protocerebral and tritocerebral areas, the medial deutocerebral regions, the lateral antennular neuropil, the medial antennular neuropil, the olfactory globular tract and the antenna II neuropil) is the abrupt rise to a peak that occurs prior to subjective dusk followed by an exponential-like decay (A,B,D,E). By contrast, serotonin levels in the accessory lobes (ALs) rise during the subjective day to a high around dusk, are sustained throughout the night hours and fall at subjective dawn (C,F) (N=4 for each time point). t-tests between the peaks and troughs in serotonin levels show significant differences in each of these brain regions (AL, P=0.016; OL, P=0.029; brain remainder, P=0.00034).

post-embryonic *P. clarkii*, brain serotonin levels show a bimodal rhythm with a major peak at night and a minor peak during the day when held in constant light. A trimodal rhythm becomes evident in adults, reflecting an endogenous rhythm peaking every 8 h (Castanon-Cervantes et al., 1999).

The lack of a clear diurnal rhythm in serotonin levels in lobsters maintained in 12 h:12 h L:D conditions (see Materials and methods) was contrary to the reports cited above. We reasoned that masking, a situation where an endogenous rhythm is obscured by transient changes in the molecule being measured due to direct stimulation by light-activated neural pathways, was a potential cause of our result. Alternatively,

serotonin content in different parts of the brain may cycle at different times and thus obscure a distinct single rhythm. This is one possible interpretation of the trimodal rhythm reported in adult crayfish by Castanon-Cervantes et al. (1999).

Constant darkness reveals an endogenous, light-entrainable, diurnal serotonergic rhythm

To exclude the influence of masking from our study, lobsters were entrained to a 12 h:12 h L:D cycle for at least 2 weeks (the entraining light regime) and then introduced to D:D conditions where no direct light stimulation or feeding occurred. At the end of the third day in D:D, serotonin levels were measured at six time points during a 24-h period. Using this D:D protocol, an endogenous diurnal serotonergic rhythm was revealed (Fig. 3). The most reliable characteristics of this whole-brain rhythm are a pre-dusk serotonin peak, with a decrease in serotonin levels to a pre-dawn serotonin trough. Altering the light cycle for a group of lobsters so that light-off was shifted by 4 h (to occur at 14:00 h clock time; light-on at 02:00 h clock time; see Fig. 3B,C) had the effect of shifting the serotonin peak and trough accordingly, showing that the characteristic components of the whole-brain rhythm are light entrainable (Fig. 3C).

A visual inspection of the chronograms in Fig. 3 showed that they are not sinusoidal, and hence analysis with the single cosinor is inappropriate (Nelson et al., 1979; Reinberg and Smolensky, 1983; Minors and Waterhouse, 1988; De Prins and Waldura, 1993), excluding a mathematical dissection of the chronograms to reveal sub-rhythms hidden within them. However, due to the highly modular construction of the crustacean brain, we could physically dissect the brains into three regions: the OLs, the ALs and the brain remainder (medial regions of the protocerebrum, deutocerebrum and tritocerebrum), all of which contain measurable levels of serotonin.

Serotonergic rhythms in the olfactory and accessory lobes

Regional separation of the brain into the OL, AL and brain remainder and assaying these individually reveals that not only are the changes in serotonin levels in these areas out of phase with one another but also that the nature of the specific patterns of these changes is characteristically different. The changes in the serotonin levels in the OL and the brain remainder, for example, are abrupt. That is, the levels of serotonin in these areas of the brain during the interval preceding the peak are clearly different from the levels at the peak itself (paired *t*-test; OL, P=0.016; brain remainder, P=0.0002). Serotonin levels then decline in an exponential fashion (Fig. 5D,E). By contrast, a comparison of these two measurements in the ALs shows that they change slowly and monotonically (paired *t*-test; AL, P=0.242; Fig. 5F). The combination of the gradual rise and extended plateau of serotonin level in the ALs with the peaks and troughs of the serotonin levels in the OLs and brain remainder explains the relative flatness of the whole-brain chronogram and histogram (Figs 3A, 6). If we assume that changes in serotonin levels in the brain are indicative of changes in the releasable pool of this transmitter, then availability of serotonin in the OL and the brain remainder changes in a pulse-like fashion in comparison with the AL.

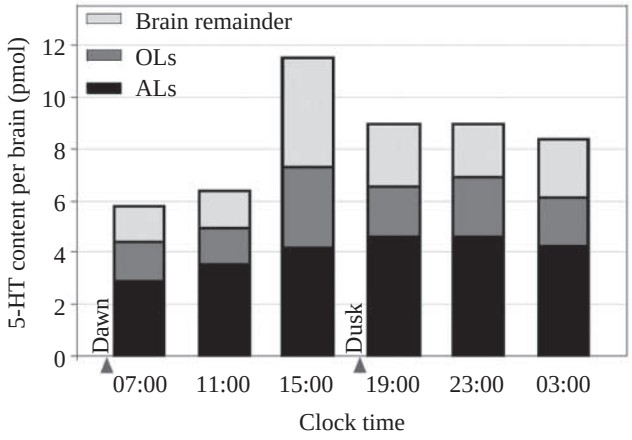

Fig. 6. Combined serotonin levels in various areas in the brains of juvenile lobsters. The levels of serotonin in all the accessory lobes (ALs; black), olfactory lobes (OLs; dark gray) and brain remainders (light gray) are added together for each of the six time points (data from Fig. 5A–C). As in the whole-brain studies (Fig. 3), the pre-dusk peak is the most pronounced feature. Comparisons of the changes in serotonin content in individual brain areas at the pre-dusk peak (11:00 h compared with 15:00 h) show that serotonin levels in the ALs rise by 18%, the OLs by 123% and the brain remainder by 197% during this period.

The contrasting diurnal rhythms in serotonin levels that were measured in the OLs, ALs and brain remainder (Fig. 5) are of particular interest because these regions are functionally distinct. The paired ALs in crustaceans receive no primary sensory input but rather receive projections of local interneurons in clusters 9 and 11 that carry higher-order visual, mechanosensory and olfactory information (Sandeman et al., 1995; Wachowiak et al., 1996; J. M. Sullivan and B. S. Beltz, unpublished results). The AL output is carried by the axons of the cluster 10 projection neurons, which continue to proliferate throughout the animal's life (Harzsch et al., 1999; Benton and Beltz, 2002) and which project to the hemiellipsoid bodies located in the lateral protocerebrum (Sullivan and Beltz, 2001). This connectivity pattern and the fact that multimodal inputs project to this region suggest that the ALs are involved in higher-order integration (Sandeman et al., 1995; Sullivan and Beltz, 2001). The OLs, on the other hand, are innervated by olfactory receptor neurons from the first antennae (antennules). Their output, like that of the ALs, is carried by the axons of cluster 10 neurons that project to the lateral protocerebrum *via* the olfactory globular tract (Fig. 1). However, the olfactory projection neurons are different from those that innervate the ALs in that they target neuropil regions of the medulla terminalis. Therefore, the output pathways from the OLs and ALs project to separate, largely non-overlapping regions of the lateral protocerebrum (Sullivan and Beltz, 2001), further evidence of the distinctive functions of these regions.

While the connectivity and functions of the OLs and ALs

are distinct, both regions nevertheless receive a massive serotonergic innervation from the same neuron – the ipsilateral dorsal giant neuron (DGN; Sandeman and Sandeman, 1987, 1994; Benton and Beltz, 2001). The input–output relationships of the DGN in these regions are not known, but in both regions this neuron innervates each and every glomerulus (Benton and Beltz, 2001). The OLs and ALs are also innervated by relatively few, smaller serotonergic interneurons whose cell bodies are located in clusters 9 and 11 (Beltz, 1999). However, as the predominant serotonergic input to both areas is from the DGN, our results suggest that the contrasting rhythms in serotonin content measured in these areas may reflect a differential regulation of serotonin metabolism in the OL and AL arbors of the same neuron. Many years ago, we proposed that the DGNs, by virtue of their massive axonal arbors projecting to functionally distinct areas, were likely to engage in localized signaling within discrete areas of the OLs and ALs, thereby 'multitasking' (Sandeman et al., 1993). This concept takes on new meaning if neuronal activity is able to regulate serotonin levels in these areas.

The logical extension of this idea is that local activity patterns imposed by differential inputs to the OLs and ALs may be able to influence the synthesis, degradation, uptake and release of serotonin in these regions. Serotonin levels in the OL arbors of the DGNs could thereby be altered by chemosensory stimulation when lobsters become aroused and begin to forage during the hours just prior to dusk (Weiss, 1970; Ennis, 1983; Cooper and Uzmann, 1980; Arechiga et al., 1993; Chabot et al., 2001). Zimmer-Faust et al. (1996) have shown, using behavioral tests, that the activity state does influence the sensitivity of spiny lobsters and crabs to food odorants in the water and that such sensitivity is much higher during periods when physical activity is high. The implication from this work is that the change in the responsiveness is centrally and not peripherally determined. If responsiveness of lobsters also increases abruptly during pre-dusk/dusk arousal, then the OLs may be strongly activated during this period. It is therefore intriguing that we consistently see the highest serotonin levels in the OLs during this pre-dusk period.

By contrast, serotonin levels in the ALs rise throughout subjective day to a peak at dusk and are sustained at a high level throughout subjective night, rather than dropping precipitously as in the OLs. In this context, it is interesting that activity patterns in the ALs will be sensitive not only to chemosensory activation but also to visual and mechanosensory stimulation. It is possible that the sustained high levels of serotonin in these regions during subjective night reflect a heightened sensitivity, and corresponding increased activity, in the variety of sensory systems involved in nocturnal behaviors. The decrease in serotonin levels during the early subjective morning hours coincides with the onset of a low activity period in lobsters (Ennis, 1983; Chabot et al., 2001). The fact that serotonin has been repeatedly associated with learning and memory mechanisms (Harvey, 2003; Meneses, 2003; Orsetti et al., 2003; Wolff et al., 2003) may also be relevant to the rhythms we measure in brain regions. Certainly, the fact that serotonin is differentially regulated in specific brain regions may reflect the potential importance of time-of-day performance of those areas.

Multiple functional, entrainable circadian rhythms have also been found in the mammalian brain. Nuclei in the olfactory bulb and the ventral hypothalamus of rats are rhythmic, with peak expression of *Per* at night, while other brain areas are only weakly rhythmic or arrhythmic (Abe et al., 2002). It is believed that cells within the mitral cell layer of the olfactory bulb are competent circadian pacemakers, regulating their own gene expression and membrane excitability (Granados-Fuentes et al., 2004). Such results indicate that multiple pacemaking tissues exist and that these function semiautonomously from each other. These data, in combination with the fact that circadian modulation of olfaction has been reported in mammals (Amir et al., 1999; Funk and Amir, 2000), suggest that the presence of an independent pacemaker in the olfactory bulb may be related to the need for local regulation of olfactory processing. However, how such rhythms in transcriptional or electrical activity in the bulb relate to olfaction is not known. Therefore, in contrast to the traditional view of a single pacemaker driving multiple rhythms, the presence of independent pacemakers in functionally distinct brain regions whose activities are coordinated appears to be the standard in many tissues.

Serotonin as a regulator of neurogenesis

In both vertebrate and invertebrate species, serotonin is a potent regulator of neurogenesis (Brezun and Daszuta, 1999, 2000; Benton and Beltz, 2001; Beltz et al., 2001; Jacobs, 2002; Radley and Jacobs, 2002; Malberg and Duman, 2003). In the brain of the American lobster, reduced serotonin levels result in a decrease in neurogenesis among the deutocerebral local and projection neurons (clusters 9 and 10; Fig. 1) (Benton and Beltz, 2001; Beltz et al., 2001), while elevated serotonin levels result in an increased rate of neurogenesis (J. L. Benton, E. M. Goergen and B. S. Beltz, unpublished results). It is also known that a bundle of fine serotonergic fibers from the DGN terminate blindly in the region where new projection neurons are born in cluster 10 (Beltz et al., 2001). Serotonin is therefore thought to be important in regulating the cell cycle period of progenitor cells that produce neurons in the lobster. Hence, it is of particular interest in the context of our current study of circadian regulation of serotonin levels that the rate of neurogenesis in the lobster follows a diurnal rhythm with the lowest rate of neurogenesis at dawn and a peak rate at dusk; this rhythm is due to a light-entrainable, endogenous circadian oscillation (Goergen et al., 2002).

Data presented here demonstrate that serotonin levels in the brain also follow an endogenous circadian rhythm that is light entrainable. Electrochemical (Fig. 3) and immunocytochemical (Table 1) analyses show that serotonin levels are at their highest in whole lobster brains prior to dusk and at their lowest in the pre-dawn period. Therefore, the peak and trough in serotonin levels in the lobster brain precede the peak and trough in the rate of neurogenesis among the projection neurons.

There are two possible interpretations of the fact that light entrains brain serotonin levels *and* the timing of neurogenesis. Either the day/night cycle influences these two processes *via* parallel regulatory mechanisms, or serotonin may be an element in the direct pathway by which light regulates neurogenesis in the lobster brain. However, as we now know that serotonin levels are regulated independently in different brain regions, it is not possible at this stage to relate endogenous fluctuations in brain serotonin levels to the rate of neurogenesis. We cannot directly measure local serotonin levels in the proliferation zone of cluster 10, because this region is very small and serotonin levels would be below the limits of detection. Nevertheless, we can test whether serotonin is directly involved in circadian regulation of neurogenesis using the knowledge that the DGN innervates the region of proliferation in cluster 10. Thus, we can manipulate levels of activity unilaterally in the DGNs and ask whether the rate of neurogenesis is altered in response to stimulation of this neuron.

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