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Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*

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Summary

Accumulating evidence suggests that the accessory medulla is the location of the circadian pacemaker in the fruit fly *Drosophila melanogaster* and the cockroach *Leucophaea maderae*. γ-Aminobutyric acid (GABA) and Mas-allatotropin are two putative neurotransmitters in the accessory medulla in the cockroach *Leucophaea maderae*. Neurons immunoreactive to the neuropeptide Mas-allatotropin are local neurons with arborizations in the noduli of the accessory medulla, while GABA-immunoreactive neurons connect the noduli of the accessory medulla to the medulla and to the lamina *via* processes in the distal tract. Injections of GABA and Mas-allatotropin into the vicinity of the accessory medulla resulted in stable phase-dependent resetting of the circadian locomotor activity of the cockroach. The resulting phase response curves closely matched light-dependent phase response curves, suggesting that both substances play a role in circuits relaying photic information from circadian photoreceptors to the central pacemaker.

Key words: circadian rhythm, locomotor activity, light entrainment, cockroach, insect, *Leucophaea maderae*, Mas-allatotropin, γ-aminobutyric acid, GABA, neuropeptide.

Introduction

Lesion studies in the cockroach *Leucophaea maderae* have localized circadian pacemakers in the optic lobes, ventrally between the medulla and the lobula (Nishiitsutsuji-Uwo and Pittendrigh, 1968a,b; Roberts, 1974; Sokolove, 1975; Wills et al., 1985; for reviews, see Page, 1985; Chiba and Tomioka, 1987; Helfrich-Förster et al., 1998). These mutually coupled pacemakers control the phase and the period of circadian locomotor activity as well as circadian changes in the sensitivity of the compound eyes (Page et al., 1977; Page, 1978, 1982, 1983a; Wills et al., 1985; Colwell and Page, 1989, 1990). Both pacemakers can be entrained by photoreceptors in or near the compound eyes (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968a; Page et al., 1977; Page, 1983a,b) and receive coupling input from the contralateral pacemaker (Page, 1981). From these studies, however, the neuronal substrate of the circadian pacemaker and its input and output pathways remained elusive.

More recent evidence suggests that the accessory medulla, a small neuropil in the region where the lesion studies had located the circadian pacemaker, is the actual site of the internal clock in the cockroach and in other insects (Homberg et al., 1991; Stengl and Homberg, 1994; Helfrich-Förster, 1995; Frisch et al., 1996; Petri et al., 1997; Petri and Stengl, 1997; Kaneko et al., 1997; Reischig and Stengl, 1998; Helfrich-Förster et al., 1998). The organization of the accessory medulla of *L. maderae* into noduli, an internodular region and loose neuropil around the accessory medulla has been studied in detail (Petri et al., 1995; Reischig and Stengl, 1996). Also, neuronal systems appropriate to form input and output pathways of the circadian system have been identified (Stengl and Homberg, 1994; Petri et al., 1995; Reischig and Stengl, 1996; Petri and Stengl, 1997). One of these pathways, the distal tract, is currently the best candidate for a photic entrainment pathway because it appears to connect the distal medulla and/or the lamina to the noduli of the accessory medulla (Reischig and Stengl, 1996).

The prominent arborizations of the distal tract in the noduli of the accessory medulla suggest that photic information might be processed in the noduli. Thus, in immunocytochemical studies, we searched for a neurotransmitter candidate in the distal tract (Petri et al., 1995). In addition, we searched for neurotransmitter/neuropeptide candidates of neurons with dense arborizations in the noduli of the accessory medulla, the presumptive photic processing area. In a previously published report, we showed that Mas-allatotropin-immunoreactive neurons densely innervate the noduli (Petri et al., 1995) and, therefore, are candidates for processing photic information received from the distal tract. In contrast, injection experiments and computer modelling studies show that pigment-dispersing-hormone-immunoreactive neurons, which arborize in the internodular neuropil, are involved in non-photic entrainment of the clock (Petri and Stengl, 1997, 2001).

In the present account, we show that antibodies against GABA recognize the distal tract and that immunostaining is particularly prominent in the noduli of the accessory medulla. Immunostained axons of the distal tract connect the lamina and medulla to the accessory medulla. In addition, we show that injections of GABA and Mas-allatotropin into the vicinity of the accessory medulla lead to biphasic phase shifts in circadian wheel-running activity similar to those induced by light. This suggests that both transmitters are involved in circuits relaying photic information to the clock. Preliminary results of this study have appeared previously as abstracts (Petri et al., 1997, 1999; Petri and Stengl, 1998).

Materials and methods

Behavioural assays and data analysis

Leucophaea maderae (Fabricius) were reared in laboratory colonies at 30 °C, 30 % humidity and with a 12 h:12 h light:dark photoperiod (lights on from 06:00 h to 18:00 h). Only males were chosen for the pharmacological experiments (N=172) because they expressed a more robust circadian locomotor activity rhythm than females. Behavioural analysis was performed in constant darkness (DD) and at constant temperature (28±0.5 °C) and constant humidity (70 %). Experimental animals were provided with food (dry rat pellets) and water *ad libitum*. Locomotor activity was recorded in running wheels (modified from running wheels provided by Dr Wolfgang Engelmann, Tübingen, Germany, described by Wiedenmann, 1977a) equipped with a magnetic switch. One revolution of the running wheel resulted in one impulse. Impulses were continuously counted by a microcomputer over 1 min intervals and condensed and processed by custom-designed PC-compatible software (developed by H. Fink, University of Konstanz, in collaboration with Dr M. Stengl and Dr F. Wollnik). The data were plotted in double-plot activity histograms. The heights of the bars represent the number of revolutions per 5 min; they were cut off at 30 revs min^{-1}.

The free-running period τ and the induced phase shifts were estimated by converting the raw data into ASCI format. They were then merged into 20 min intervals and analyzed with Chrono II software (generously provided by Dr Till Roenneberg; see Roenneberg and Morse, 1993) on a Macintosh computer. Data were evaluated from 128 of the 172 animals used. The remaining 44 animals were excluded from further analysis because they showed little activity after the injection or died within a week following the operation.

The free-running periods (τ) before and after the injection (see below) were calculated by linear regression through daily activity onsets and by χ^2 periodogram analysis (Sokolove and Bushell, 1978; Enright, 1965). Changes in τ ($\Delta\tau=\tau_{after}-\tau_{before}$) were calculated, with periods estimated by regression through activity onsets. After the injections, activity onsets varied transiently until the animals resumed stable activity rhythms. These transients were excluded from calculations of phase and period. Phase shifts ($\Delta\varphi$) were determined as time differences between the regression lines before and after the injection extrapolated to the day of treatment. The phase shifts were then normalized with respect to τ before the treatment. Phase delays were plotted as negative values and phase advances as positive values. Time on the x-axis of the resulting phase response curve is shown as circadian time (CT), with CT12:00 h=activity onset=beginning of the subjective night. Daily activity onsets were determined using Chrono II (Roenneberg and Morse, 1993).

The microinjection data were merged into 2 h time intervals, and the mean, the standard error of the mean (S.E.M.) and the standard deviation (S.D.) were calculated. Changes in phase and period in a given time interval were considered to be significantly different from zero when the calculated 95 % confidence interval (95 % CI, Table 1, values superscripted b) of the respective time interval did not contain the value zero. The phase response curves were analyzed by one-way analysis of variance (ANOVA) with a Scheffé multiple-range test. Significance in all cases was taken as P<0.05. Statistical analyses were performed with SPSS (Superior Performing Software Systems; SPSS Inc.) on a personal computer. To analyze the statistical significance of the phase-dependence (Table 1, values superscripted c, e, g) and to test whether transmitter-dependent phase shifts were significantly different from control values (Table 1, values superscripted a, see asterisks in Fig. 5), we used one-way ANOVA in combination with a Scheffé multiple-range test. Smoothed phase response curves were produced with Excel.

Operation and injection

All manipulations were performed in dim red light at 25 °C with a microinjector (Microinjector 5242, Eppendorf, Hamburg, Germany). Experimental animals were removed from the running wheels and mounted in metal tubes at different times during the circadian cycle. After anaesthetization with CO_2, a small window was cut in the head capsule of the cockroach, and the optic lobe was revealed by moving the trachea, ocellus and fat body carefully to one side. The neurolemma of the optic lobe was penetrated with a borosilicate glass capillary whose tip was broken to give a tip diameter of 2–3 μm (Clark, Pangbourne Reading, UK). Great care was taken to pressure-inject, under visual control, Mas-allatotropin or GABA very close to the accessory medulla into one medulla. After injection, the removed piece of cuticle was waxed back and the animal was returned to the running wheel. A treatment typically lasted 6–12 min. Only one optic lobe was injected; the other one was left intact. All experimental animals were kept under constant conditions (DD) before, during (red light=DD) and after (DD) the injection.

Injections (N=128) consisted of 10^{-4} to 10^{-2} mol l^{-1} GABA (Sigma) and 10^{-4} mol l^{-1} synthetic Mas-allatotropin (Sigma). The injected volume ranged from 0.5 to 2 nl (1.5±0.6 nl; mean ± S.D.). Thus, injected doses ranged from 0.15 to 15 pmol. These doses were chosen because similar doses were effective in previous experiments (Petri and Stengl, 1997). The solutions contained 10 % aqueous blue food dye (McCormic, Baltimore, USA) to visualize the exact site and spread of the injection

without the need for further neuroanatomical processing of the brain. Each micropipette was calibrated by measuring the injected volume. Before and after the injection, a test pulse was injected into mineral oil to control for changes in tip diameter during penetration of the neurolemma. Control injections consisted of 10 % aqueous blue food dye without neuroactive substances.

Immunocytochemistry

Immunocytochemistry of GABA was performed on free-floating Vibratome sections and on paraffin sections by means of the indirect peroxidase/antiperoxidase (PAP) technique (Sternberger, 1979). Brains were dissected out of the head capsule under glutaraldehyde fixative (Boer et al., 1979), fixed for another 2 h and rinsed in phosphate buffer. For immunostaining of paraffin sections, brains were subsequently dehydrated through a graded series of aqueous ethanol solutions and toluene and embedded in Paraplast Plus (Monoject Scientific, St Louis, MO, USA). Sections at 8 µm were cut with a rotary microtome and processed for GABA immunoreactivity as described previously (Homberg et al., 1987). For immunostaining of Vibratome sections, brains were fixed as described above and embedded in gelatin/albumin. Sections of 25 µm thickness were made with a Vibratome (Technical Products, St Louis, MO, USA).

The immunocytochemical staining protocol was performed as described by Homberg (1991). Secondary antiserum (goat-anti rabbit; Sigma, Deisenhofen, Germany) was used at a dilution of 1:40 and rabbit PAP (Dako, Hamburg, Germany) at a dilution of 1:300. The anti-GABA antiserum was provided by Dr T. G. Kingan (University of Arizona, Tuscon, AZ, USA). The antiserum was raised in rabbit against a conjugate of GABA–glutaraldehyde–keyhole limpet haemocyanin. Its specificity has been characterized by Hoskins et al. (1986). The antiserum was used at a dilution of 1:40 000 on Vibratome sections and at 1:1500 on paraffin sections.

Fibre tracts and neuropil structures immunostained for GABA were reconstructed from serially stained sections with the aid of a *camera lucida* attachment on a Leica compound microscope. Photographic images were captured using a Polaroid DMC digital camera linked to a Pentium II computer. Images were processed using Adobe Photoshop and Corel Draw software. Fig. 1A,B are overlaid images from adjacent areas of the same section (Fig. 1A) and from the same area of two consecutive sections (Fig. 1B). Images were printed with an Epson Stylus Photo ink jet printer.

Results

In immunocytochemical and microinjection studies combined with behavioural analysis, we examined how photic *Zeitgeber* information might reach the noduli of the presumptive circadian clock in the accessory medulla. Stimulated by a report from Füller et al. (1989) on dense GABA immunostaining in the optic lobe of the cockroach *Periplaneta americana*, we investigated whether GABA is the likely neurotransmitter candidate of the distal tract (Reischig and Stengl, 1996), the currently most likely photic entrainment pathway to the noduli of the circadian clock. In addition, we tested whether Mas-allatotropin and GABA produce phase-dependent phase shifts similar to those produced by light when injected into the vicinity of the accessory medulla.

GABA immunostaining

GABA immunostaining was detected in a large number of neurons in the optic lobe of *L. maderae*, and immunostained somata were distributed throughout the cortex of the optic lobe (Fig. 1). All three optic neuropils were innervated by GABA-immunoreactive (GABA-ir) processes. A median layer of the medulla exhibited particularly intense immunostaining (Fig. 1A,C). Approximately 25 small immunostained cell bodies in the vicinity of the accessory medulla sent primary neurites into the accessory medulla (Figs 1B,C, 2A). The nodular neuropil of the accessory medulla showed strong granular immunostaining. Two bundles composed of at least 20 densely packed GABA-ir axons left the accessory medulla, joined and entered the distal tract along the distal surface of the medulla towards the lamina (Figs 1B,C, 2A). Along the surface of the medulla, GABA-ir processes gradually left the distal tract in several fascicles that entered the medulla neuropil at right angles (Fig. 1C). Because of the large number of GABA-ir neurons in the optic lobe, the branching pattern of these processes in the medulla could not be elucidated.

In addition to small cell bodies, a large GABA-ir neuron in the accessory medulla could be reconstructed individually (Fig. 2B). From a cell body adjacent to the accessory medulla, its primary neurite projected through the accessory medulla into the medulla, but not *via* the distal tract. One set of branches innervated the medulla, another set of six neuronal fibres projected over the frontal surface of the medulla and entered the posterior face of the lamina. Varicose processes throughout the lamina also extended to and branched within small neuropil structures adjacent to the posterior lamina termed accessory laminae (Loesel and Homberg, 2001). A side branch of the neuron also invaded the accessory medulla, but the full extension of these ramifications could not be distinguished from other GABA-ir processes.

Effects of GABA and Mas-allatotropin injections on the phase of the circadian locomotor activity rhythm

To investigate whether GABA and Mas-allatotropin are input signals to the circadian clock, we examined whether they phase-shift the rhythm of locomotor acitivity of the cockroach. Both substances were injected into the vicinity of the accessory medulla, and the locomotor activity of the animals was recorded before and after the injections. Control injections of 10 % aqueous blue food dye (N=43) did not cause significant phase changes in circadian locomotor rhythms irrespective of the circadian time of the injections (P=0.69, one-way ANOVA) (Table 1). Injections of GABA (N=35) and synthetic Mas-allatotropin (N=36) into the medulla resulted in time-dependent phase shifts in the

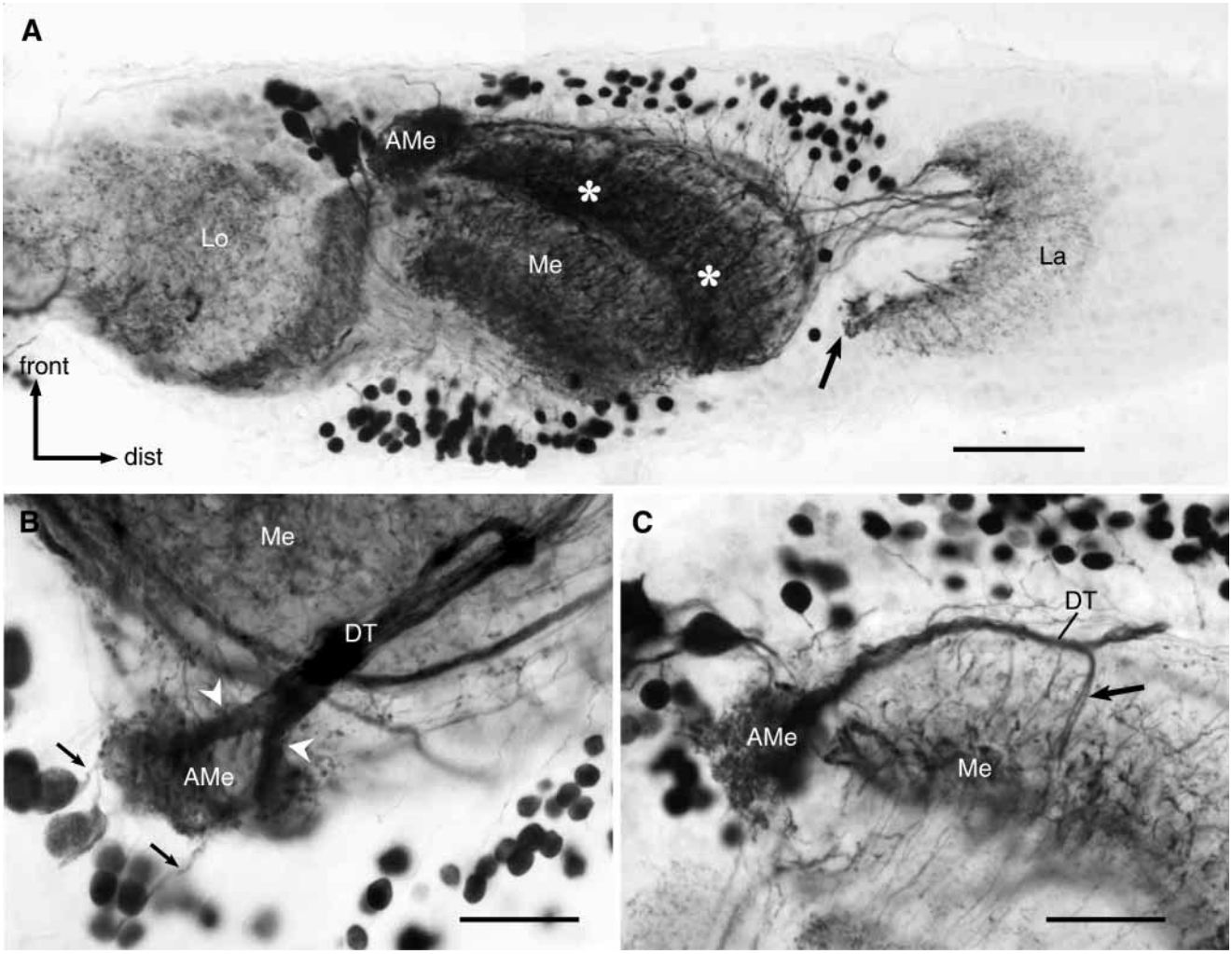

Fig. 1. γ-Aminobutyric acid (GABA) immunostaining in the optic lobe of *Leucophaea maderae*. (A) Horizontal section through the optic lobe, showing immunostaining in the lamina (La), medulla (Me), accessory medulla (AMe) and lobula (Lo). The arrow points to immunostained accessory lamina at the posterior proximal edge of the lamina. Asterisks indicate a strongly stained layer in the medulla. dist, distal; front, frontal. Scale bar, 100 μm. (B) Superimposed images from two frontal sections through the accessory medulla (AMe) and ventro-median aspects of the medulla (Me). GABA-immunoreactive processes in the distal tract (DT) separate into two fibre bundles (arrowheads) and innervate the noduli of the AMe. The thin black arrows mark primary neurites of GABA-immunoreactive somata. Scale bar, 50 μm. (C) Horizontal view showing the frontal part of the medulla (Me), the accessory medulla (AMe) and the distal tract (DT). A fascicle of immunostained axons leaves the distal tract and enters a strongly immunopositive layer of the medulla (arrow). Scale bar, 50 μm.

circadian activity rhythm of *L. maderae* (Figs 3–6; Table 1). Maximal phase delays occurred when GABA (–4.2 h) and Mas-allatotropin (Mas-At –4.9 h) were applied early in the subjective night (GABA, CT14:50 h; Mas-At, CT14:05 h), and maximal phase advances (GABA, 3.05 h; Mas-At, 3.2 h) occurred in response to injections in the middle of the subjective night (GABA, CT16:50 h; Mas-At, CT17:09 h). Examination of the 95 % confidence interval (CI, see Materials and methods) for the phase shifts in 2 h bins indicates that significant GABA- and Mas-allatotropin-dependent phase delays occurred at CT08:00–16:00 h, while significant phase advances occured at CT18:00–22:00 h (values labelled b in Table 1; asterisks in Fig. 5). No statistically significant phase shifts occurred during the rest of the cycle. The phase-dependence was statistically significant (P<0.00005, one-way ANOVA) since GABA- and Mas-allatotropin-dependent phase delays at CT12:00–14:00 h (c in Table 1) and CT14:00–16:00 h (e in Table 1) as well as phase advances at CT18:00–20:00 h (g in Table 1) were significantly different from GABA- and Mas-allatotropin-dependent phase shifts during other times of the circadian cycle (d, f, h, respectively in Table 1). In addition, phase shifts induced by GABA at CT12:00–16:00 h and CT18:00–20:00 h and Mas-allatotropin injections at CT14:00–16:00 h and CT18:00–20:00 h were significantly different from control phase shifts (Table 1). Effects of control injections at different times were in no case significantly different from those at 0 h (Table 1). In nearly

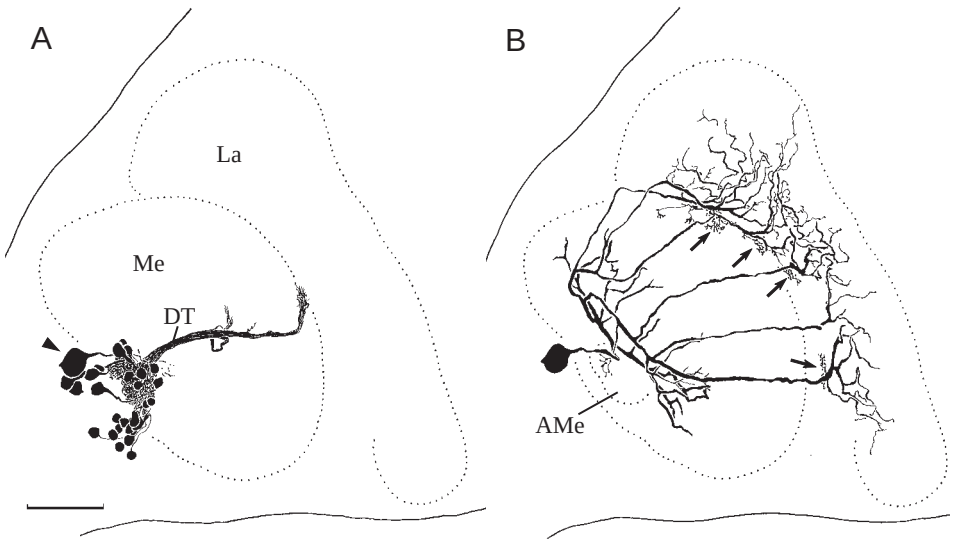

Fig. 2. Frontal reconstructions of γ-aminobutyric acid (GABA)-immunoreactive neurons innervating the accessory medulla (AMe). (A) Approximately 25 neurons send primary neurites into the AMe. From the AMe, immunostained processes continue in the distal tract (DT) along the anterior surface of the medulla (Me) and enter the medulla at several sites. The arrowhead points to the cell body of a neuron with a unique morphology shown in B. (B) The arborizations of a large immunostained neuron could be reconstructed individually. This neuron innervates the AMe and has tangential arborizations in the medulla. Six neuronal processes give rise to extensive ramifications in the lamina (La) and in small accessory laminae at the posterior inner edge of the lamina (arrows). Scale bar (applies to both), 100 μm.

Table 1. *Phase shifts (in circadian hours) resulting from control (saline) injections and injections of 15 pmol of γ-aminobutyric acid (GABA) and 0.15 pmol of Mas-allatotropin (Mas-At) at different times of the circadian cycle (circadian time)*

Circadian time (h)	Phase shift (mean ± S.E.M.)			95 % CI (lower limit to upper limit)			*N*		
	GABA	Mas-At	Saline	GABA	Mas-At	Saline	GABA	Mas-At	Saline
00:00–02:00	–0.64	–0.29	0.01±0.29	–	–	–0.89 to 0.67	1	1	5
02:00–04:00	–0.86±0.13[h]	0.10	–	–2.51 to 0.79	–	–	2	1	0
04:00–06:00	–0.22±0.09	–0.5±0.81	–0.11±0.34	–1.41 to 0.97	–10.75 to 9.77	–0.8 to 0.46	2	2	3
06:00–08:00	–0.71	–0.82±0.11	–0.55±0.49	–	–2.18 to 0.55	–1.53 to 0	1	2	3
08:00–10:00	–	–0.83±0.15[h]	–0.01±0.24	–	–1.32 to –0.35[b]	–0.52 to 0.22	0	4	3
10:00–12:00	–1.15±0.01	–1.97	–0.3±0.01	–1.3 to –1[b]	–	–0.53 to –0.07	2	1	5
12:00–14:00	–2.71±0.33[a,c,h]	–2.14±0.15[c,h]	–0.32±0.52	–3.62 to –1.79[b]	–4.07 to –0.22[b]	–1.36 to 0.25	5	2	3
14:00–16:00	–2.11±0.52[a,e,h]	–3±0.39[a,e,h]	–0.57±0.24	–3.45 to –0.77[b]	–3.93 to –2[b]	–1.51 to 0.12	6	7	6
16:00–18:00	1.01±0.76[d,f]	0.38±0.72[d]	–0.5±0.38	–1.05 to 3.2	–1.38 to 2.14	–2.35 to 0.84	4	7	7
18:00–20:00	2.24±0.34[a,d,f,g]	2.44±0.4[a,d,f,g]	–0.12±0.47	1.15 to 3.33[b]	0.71 to 4.17[b]	–0.78 to 0.79	4	3	3
20:00–22:00	0.43±0.71[d,f]	1.53±0.28[d,f]	0.36±0.24	–8.56 to 9.41	0.64 to 2.42[b]	–0.26 to 0.74	2	4	4
22:00–24:00	0.5±0.23[d]	–0.25±0.05	0.19	–0.18 to 1.17	–0.92 to 0.42	–	6	2	1
00:00–24:00	–	–	–0.24±0.11	–	–	–	35	36	43

[a]Phase shifts significantly different from control injections (P<0.05 Scheffé multiple-range test).
[b]Significant phase shifts as judged by the 95 % confidence interval (CI) (see Materials and methods).
[c]Phase shifts significantly different from transmitter-dependent phase shifts at other circadian times ([d]) (P<0.05 Scheffé multiple-range test).
[e]Phase shifts significantly different from transmitter-dependent phase shifts at other circadian times ([f]) (P<0.05 Scheffé multiple-range test).
[g]Phase shifts significantly different from transmitter-dependent phase shifts at other circadian times ([h]) (P<0.05 Scheffé multiple-range test).

all experiments, the new phase relationship was reached after 14 days of transients with variable periods of apparent unstable activity onset (Fig. 3A,C), and no differences in transition times were observed for phase delays and phase advances.

Effects of GABA and Mas-allatotropin injections on the period of the circadian locomotor activity rhythm

After the injection of saline, GABA or Mas-allatotropin, the free-running period of individual cockroaches did not

Table 2. *Changes in the free-running period (Δτ) after injections of saline, γ-aminobutyric acid (GABA) and Mas-allatotropin*

	Saline	GABA	Mas-allatotropin
One-way ANOVA	P=0.43	P=0.19	P=0.25
Δτ (h); mean ± S.D.	0±0.15	0±0.13	0±0.15
95 % CI (h)	–0.01 to 0.01	–0.01 to 0.01	–0.01–0.01
N	43	35	36

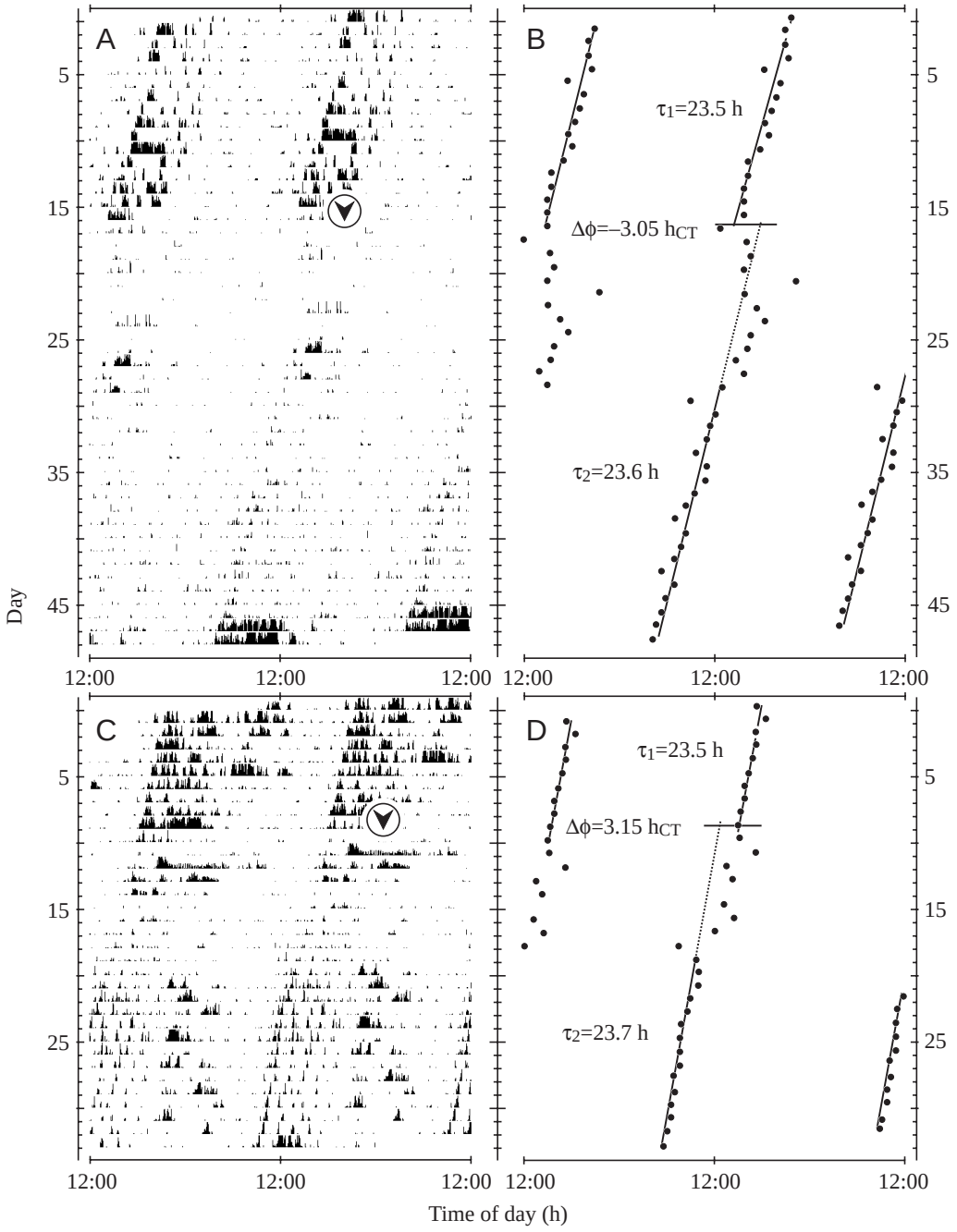

Fig. 3. Recordings of circadian wheel-running activity and plots of activity onsets from cockroaches kept in constant darkness. (A,B) After injection of 200 fmol of Mas-allatotropin in 2 nl of saline at CT15:30 h of day 16 (arrowhead), regression analysis through consecutive activity onsets (B) revealed a phase delay $\Delta\phi$ of 3.05 circadian hours (h_{CT}) after the injection. (C,D) Injection of 50 pmol of GABA in 0.5 nl of saline with blue food dye at CT17:30 h of day 9 (arrowhead) induced a phase advance of 3.15 circadian hours. Neither experiment had any effect on the period τ of the activity rhythm, after a new stable state, through several transient states (days 17–28 in A; days 10–18 in C), had been established. CT, circadian time.

change significantly (Fig. 3; Table 2). Observed changes in period were always small, included both lengthening (maximally –0.41 h) and shortening (maximally 0.48 h) of the period, and were independent of the time of injection in the circadian cycle. Thus, the mean period of 23.46±0.18 h (mean ± S.D.; N=124) was not altered (Table 2) by any treatment.

Dose-dependency of GABA-dependent phase shifts

GABA-induced phase delays in circadian wheel-running activity at CT12:00–16:00 h were positively correlated with the dose injected. Injections of 0.15 pmol of GABA caused phase delays of –1.5±0.21 h (95 % CI=–2.00 to –1.11 h, N=10), while injections of 15 pmol of GABA resulted in phase delays of –2.4±0.33 h (95 % CI=–3.10 to –1.67 h,

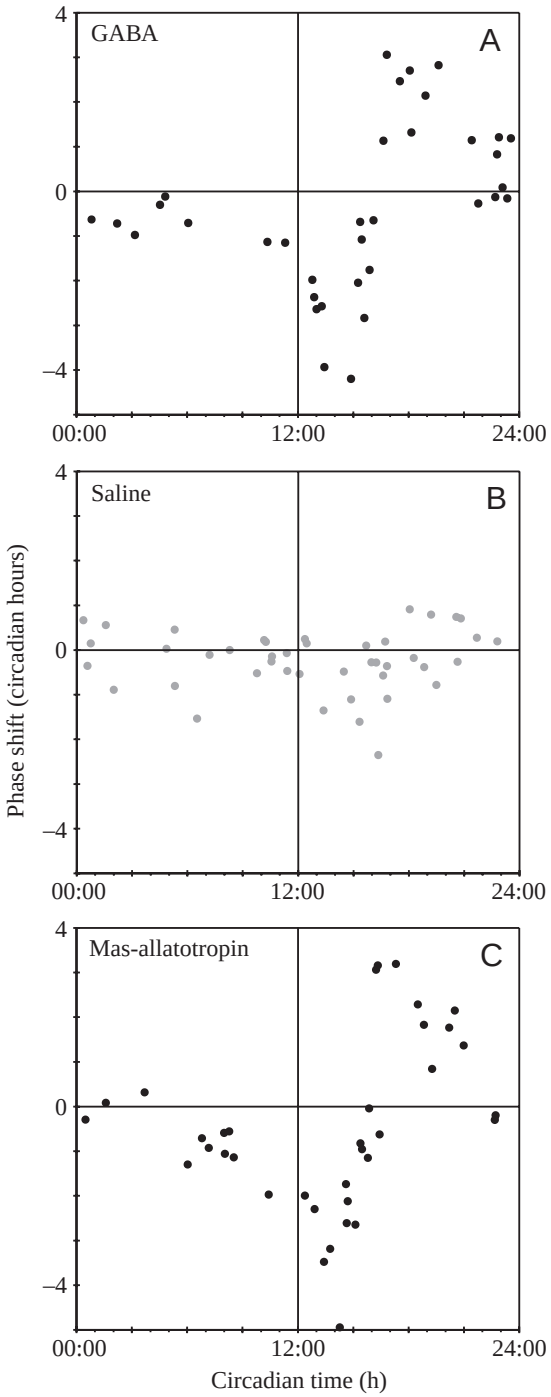

Fig. 4. Scatterplots of γ-aminobutyric acid (GABA)- and Mas-allatotropin-dependent phase shifts at different times in the circadian cycle. (A) GABA injections (15±6 pmol in 1.5±0.6 nl of saline with blue food dye, mean ± S.D.; *N*=35) cause maximal phase delays during the early subjective night (–4.2 h at CT14:50 h) and maximal phase advances during the middle of the subjective night (3.05 h at CT16:50 h). (B) Irrespective of the time of day, control injections (0.5–2 nl of saline with blue food dye; *N*=43) caused only small statistically non-significant phase delays and phase advances. (C) Injections of Mas-allatotropin (150±60 fmol in 1.5±0.6 nl of saline with blue food dye, mean ± S.D., *N*=36) cause maximal phase delays during the early subjective night (–4.9 h at CT14:05 h) and maximal phase advances during the middle of the subjective night (3.2 h at CT17:09 h). Each point represents the phase shift (in circadian hours) resulting from a single injection. Phase advances are shown as positive values and phase delays as negative values.

N=11). Only phase delays induced by 15 pmol of GABA had a significantly different effect ($P<0.05$, one-way ANOVA) from control injections (–0.49±0.22 h, 95 % CI=–0.99 to 0.01 h, *N*=9) at the same circadian time ($P=0.85$, one-way ANOVA, Scheffé multiple-range test). Both 15 pmol of GABA and 150 fmol of Mas-allatotropin caused phase delays that were significantly different from control injections at the same circadian time ($P<0.05$, one-way ANOVA, Scheffé multiple-range test). A 100-fold higher dose of GABA compared with Mas-allatotropin was required to induce the same phase-shift.

Discussion

To search for the photic entrainment pathway of the circadian clock of the cockroach *L. maderae*, we performed anti-GABA immunocytochemistry and GABA and Mas-allatotropin microinjection assays. GABA is a likely neurotransmitter candidate of the distal tract which connects the noduli of the accessory medulla, the presumptive circadian clock, to the medulla and lamina. Injections of neuroactive substances of nodular neurons (GABA and the neuropeptide Mas-allatotropin) into the vicinity of the accessory medulla resulted in phase-dependent phase shifts in circadian wheel-running activity. Therefore, GABA and a substance related to Mas-allatotropin are part of the circadian clock, or modulate an input to the circadian clock, of the cockroach. The phase response curves for both substances closely match the phase response curve obtained previously for light pulses (Fig. 6) (Page and Barret, 1989) and, therefore, suggest an involvement of both substances in circuits relaying photic information to the circadian pacemaker. Because neurons immunoreactive to Mas-allatotropin (Petri et al., 1995) and GABA innervate the noduli of the accessory medulla, we assume that light-dependent information is processed in the noduli.

Specificity of Mas-allatotropin and GABA-dependent phase shifts

The phase shifts of locomotor activity rhythm in the subjective night are specifically dependent on GABA and Mas-allatotropin because they are dose-dependent and significantly different from phase shifts in response to control injections. In addition, the phase response curves obtained differ from curves produced by pigment-dispersing hormone (PDH) or serotonin injections (Page, 1987; Petri and Stengl, 1997). Thus, the type of phase response curve obtained depends on the neuroactive substance injected. The 100-fold difference in sensitivity of the circadian system for Mas-allatotropin and PDH injections *versus* GABA injections might be a consequence of the longer half-life of neuropeptides compared with GABA. Alternatively, the difference in sensitivity might reflect

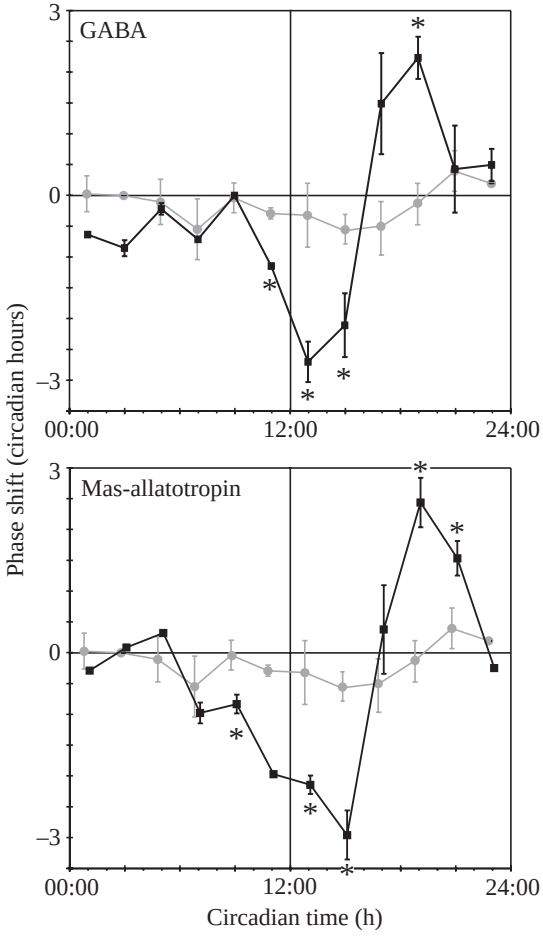

Fig. 5. Phase response curves obtained in response to 15 pmol of γ-aminobutyric acid (GABA), 0.15 pmol of Mas-allatotropin and control injections. Data were merged into 2 h bins. Mean ± S.E.M. transmitter-dependent phase shifts (black squares) and phase shifts following control injections (grey circles) are plotted (see Table 1 for values of *N*) in the middle of each 2 h bin. Asterisks indicate significant transmitter-dependent phase shifts (see values marked a in Table 1).

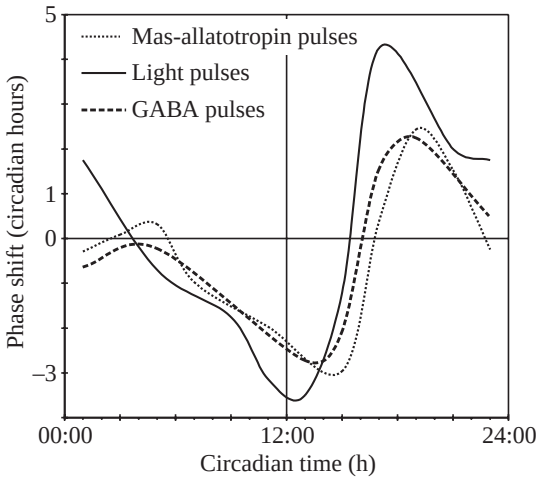

Fig. 6. Smoothed phase response curves for injections of 15 pmol of γ-aminobutyric acid (GABA) and 0.15 pmol of Mas-allatotropin compared with the phase response curve for 6 h light pulses (Page and Barret, 1989).

differences in affinity between GABA and neuropeptide receptors for their ligands.

Mas-allatotropin has been sequenced from the sphinx moth *Manduca sexta* (Kataoka et al., 1989), and a related peptide, termed Lom-AG-myotropin-I, has been identified in the locust *Locusta migratoria* (Paemen et al., 1991) and in the beetle *Leptinotarsa decemlineata* (Spittaels et al., 1996). These peptides are probably members of a larger insect family of allatotropin-related peptides, and their wide distribution throughout the nervous systems of different insect species, including *L. maderae*, has been suggested by immunocytochemistry (Paemen et al., 1992; Žitňan et al., 1993; Veenstra and Hagedorn, 1993; Würden and Homberg, 1995; Petri et al., 1995). The phase response curve obtained with Mas-allatotropin in *L. maderae* strongly suggests that a related peptide is released by neurons in the circadian system of this insect. Because the only Mas-allatotropin-ir neurons that innervate the accessory medulla are local neurons with arborizations restricted to the noduli of the accessory medulla, we hypothesize that these neurons release a Mas-allatotropin-related peptide in response to light. Thus, we assume that light input reaches the accessory medulla *via* pathways that arborize in the noduli.

Neuronal pathways for light entrainment of the circadian pacemaker of the cockroach

Evidence is increasing that the accessory medulla with associated PDH-immunoreactive (PDH-ir) neurons is the site of the circadian clock in the cockroach as well as in *Drosophila melanogaster* (Homberg et al., 1991; Stengl and Homberg, 1994; Helfrich-Förster, 1995; Frisch et al., 1996; Petri et al., 1997; Petri and Stengl, 1997; Kaneko et al., 1997; Reischig and Stengl, 1998; Helfrich-Förster et al., 1998). In *D. melanogaster*, PDH-ir neurons contain the clock molecules PERIOD and TIMELESS and are circadian pacemaker candidates (Helfrich-Förster, 1995; Kaneko and Hall, 2000; Kaneko et al., 1997). In the cockroach *L. maderae*, we also found PERIOD-immunoreactivity in cells next to the accessory medulla, at the location of PDH-ir neurons (Stengl et al., 2001). However, it is not known how light entrainment information reaches the PDH-ir pacemaker candidates in the cockroach, while several parallel pathways are known in the fruit fly (Helfrich-Förster et al., 2001). Photoreceptors of the circadian system of *L. maderae* occur in or near the compound eyes, but the light entrainment pathway to the pacemaker has not been identified (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Wiedenmann, 1977b; Page, 1978). Page (1978, 1983b) proposed inputs into the pacemaker by ipsi- and contralateral light-entrainment pathways as well as input of phase information by the contralateral pacemaker *via* a coupling pathway. Recent backfill experiments strongly suggest that some of the PDH-ir neurons form a direct circadian coupling

pathway (Reischig and Stengl, 2001). Because of this apparently direct coupling pathway in the cockroach, a single injection of neurotransmitter and neuropeptide into one pacemaker region may well cause transient changes in activity onsets over several days before a stable, new phase relationship between both pacemaker centres is reached.

In addition to the circadian coupling pathways relaying phase information from the contralateral pacemaker, cells projecting *via* the posterior optic tract with projections in the ipsi- and contralateral accessory medulla and ipsi- and contralateral medulla might bring contralateral light input into the clock (Reischig and Stengl, 2001; Loesel and Homberg, 2001). A recent study on histamine immunostaining by Loesel and Homberg (1999) demonstrated that photoreceptor axons of the compound eye do not enter the accessory medulla of *L. maderae* directly, but terminate in the lamina and in distal layers of the medulla. This indicates that there is no direct photoreceptor input to the clock. Our data presented here suggest that GABAergic neurons in the distal tract might be the missing link between compound eye photoreceptors and the accessory medulla neurons. Although this hypothesis is the most straightforward interpretation for both the immunocytochemical and injection data, we cannot, at present, rule out the additional possibility that GABA, like Mas-allatotropin, acts in local circuits, which would affect an unknown photic input to the clock. A second photic input pathway to the clock, distinct from the distal tract, could be provided by the single large-field GABA-ir neuron (Fig. 2B). This neuron appears to connect the medulla to the accessory medulla, the lamina and the accessory laminae. Intracellular recordings showed that neurons with close similarity to this cell respond strongly to light, as would be expected for neurons involved in photic entrainment of the clock (Loesel and Homberg, 2001).

In contrast to GABA, Mas-allatotropin immunoreactivity was found in 20–30 intrinsic neurons of the accessory medulla (Petri et al., 1995). These local neurons extend a dense network of varicose terminals throughout the noduli of the accessory medulla, which overlaps with GABA immunostaining (Petri et al., 1995). We cannot at present rule out the possibility that GABA and Mas-allatotropin are co-localized in some local neurons. However, striking differences in the morphological appearance of the two staining patterns, in particular the large number of GABA-ir axons in the distal tract and the fasciculated (Mas-allatotropin) *versus* non-fasciculated (GABA) entry of primary neurites into the accessory medulla, make it unlikely that the GABA- and Mas-allatotropin-immunoreactive neurons are identical. Thus, only immuno-electronmicroscopic studies will allow us to distinguish whether most of the GABA-ir neurons that form an input into the accessory medulla originate from the distal tract or from local GABA-ir neurons. Finally, Fleissner et al. (2001) recently described two putative extraocular photoreceptor organs in the cockroach optic lobe, the lamina and lobula organs, and proposed a role for these organs in light entrainment of the circadian clock. At present, however, physiological evidence for a photoreceptor role for these organs and for neuronal connections to the clock have not been demonstrated.

Since injections of low doses of GABA and Mas-allatotropin were directed towards the vicinity of the accessory medulla, it is likely that the resulting phase shifts were caused by neurons postsynaptic to the GABA- and Mas-allatotropin-immunoreactive networks in the noduli of the accessory medulla. But it cannot be excluded that, in addition to neurons innervating the noduli of the accessory medulla, other unknown neurons elsewhere in the optic lobes with connections to the accessory medulla might contribute to the phase shifts observed.

The similarity of the phase response curves obtained for light pulses and for GABA and Mas-allatotropin injections (Fig. 6) suggests that both substances are released in response to light (Petri and Stengl, 2001), possibly within the noduli of the accessory medulla. In contrast, injections of PDH, a presumptive circadian coupling signal, revealed a monophasic non-photic phase response curve (Petri and Stengl, 1997). Computer modelling of a molecular oscillator shows that biphasic and monophasic phase response curves result from disturbance to different, specific variables in the model molecular clock (Petri and Stengl, 2001). Our oscillator model confirms that substances that produce light-like phase response curves are likely to be involved in circuits relaying photic information to the clock (Petri and Stengl, 2001). Therefore, our complementary analysis of GABA and Mas-allatotropin immunostaining associated with the presumptive circadian pacemaker centre provides important information about the neuronal pathways through which light acts to reset the circadian clock in the cockroach. It still remains to be shown which neurons convey light information from the noduli of the accessory medulla to the internodular neuropil of the accessory medulla, where PDH-ir neurons, the presumptive circadian pacemaker candidates and circadian outputs arborize.

This study suggests that the light entrainment pathway of *L. maderae* might be as complex as that for light entrainment in mammals (Ralph and Menaker, 1985, 1986, 1989; Hastings et al., 1991; Aronson et al., 1993; Ding et al., 1994; Gillespie et al., 1997; Wagner et al., 1997; van Esseveldt et al., 2000). As in the mammalian system, light entrainment involves more than one neurotransmitter and consists of more than direct, unmodulated transmission of photic information from photoreceptors to the pacemaker in the accessory medulla. Future studies will examine the nature of circadian photoreceptors, parallel entrainment pathways and the types of receptor involved in the transmission of photic information to the circadian pacemaker in the cockroach. In addition, we have begun an immuno-electronmicroscopic analysis of the neuronal network of the accessory medulla.

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