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Serotonin sets the day state in the neurons that control coupling between the optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*

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Summary

The bilaterally paired optic lobe circadian pacemakers of the cricket *Gryllus bimaculatus* mutually exchange photic and circadian information to keep their activity synchronized. The information is mediated by a neural pathway, consisting of the so-called medulla bilateral neurons, connecting the medulla areas of the two optic lobes. We investigated the effects of serotonin on the neural activity in this coupling pathway. Spontaneous and light-induced electrical activity of the neurons in the coupling pathway showed daily variations, being more intense during the night than the day. Microinjection of serotonin or a serotonin-receptor agonist, quipazine, into the optic lobe caused a dose- and time-dependent inhibition of spontaneous and light-induced responses, mimicking the day state. The amount of suppression was greater and the recovery from the suppression occurred faster during the night. Application of metergoline, a non-selective serotonin-receptor antagonist, increased spontaneous activity and light-evoked responses during both the day and the night, with higher effect during the day. In addition, metergoline effectively attenuated the effects of serotonin. These facts suggest that in the cricket's optic lobe, serotonin is released during the daytime and sets the day state in the neurons regulating coupling between the bilaterally paired optic lobe circadian pacemakers.

Key words: cricket, *Gryllus bimaculatus*, circadian rhythm, medulla bilateral neuron, pacemaker coupling, photo-responsiveness, serotonin.

Introduction

Most organisms exhibit daily rhythms in their physiology and behavior that persist under constant conditions. These rhythms are driven by an endogenous mechanism called a biological clock or a circadian pacemaker. Hemimetabolous insects such as crickets and cockroaches have been extensively studied in order to understand the underlying circadian time-keeping mechanism (Helfrich-Förster et al., 1998; Tomioka et al., 2001). In crickets, the pacemaker that controls the behavioral and physiological rhythms has been localized: one is present in each of the paired optic lobes (Loher, 1972; Wiedenmann, 1983; Tomioka and Chiba, 1984, 1986, 1992). The paired optic lobe circadian pacemakers are mutually coupled to one another (Wiedenmann, 1983; Tomioka et al., 1991; Tomioka, 1993; Ushirogawa et al., 1997). The pacemaker is entrained not only to the light cycles perceived by the ipsilateral compound eye, but also by its contralateral partner (Tomioka et al., 1991; Tomioka, 1993). Behavioral and anatomical studies showed that in *Gryllus bimaculatus*, a group of interneurons, called medulla bilateral neurons (MBNs), which run along the ventral half of the optic stalk and directly connect the medulla areas of the two optic lobes, are the most likely candidates mediating coupling signals between the optic lobe circadian pacemakers, since severance of the neural tract that includes the MBNs decouples them (Yukizane and Tomioka, 1995).

Serotonin (5-hydroxytryptamine; 5-HT) is one of the major putative neuroactive substances in the insect optic lobe (Nässel, 1987). Lines of evidence from various experimental approaches suggest that serotonin plays important physiological roles in the circadian system of insects (Page, 1987; Pyza and Meinertzhagen, 1996; Tomioka, 1999). In crickets, serotonergic neurons are distributed almost the entire area of the optic lobe's lamina and medulla neuropiles, and the serotonin content in the optic lobe fluctuates depending on the time of day, in synchrony with circadian changes in the sensitivity of visual interneurons (Tomioka et al., 1993). Administration of exogenous serotonin shifts the phase of the cricket's optic lobe pacemaker *in vitro* in a phase-dependent manner and the phase–response curve caused by a serotonin pulse is quite similar in shape to that caused by mutual coupling between bilateral clocks (Tomioka, 1999). Taking all these facts together, serotonin seems to be involved in the cricket's circadian system. Since signals required for the mutual coupling are exchanged through a neural pathway that consists of MBNs, and since serotonin shifts the phase of the cricket's pacemaker, in this study we tried to reveal the

physiological effects of serotonin on the MBNs, using electrophysiological techniques. Our results show that serotonin reduces the MBNs' electrical activity in a dose-dependent and time-dependent manner, suggesting that serotonin not only sets the day state in the MBN but also regulates mutual coupling between the bilaterally paired pacemakers, by modulating the MBNs' electrical activity and responsiveness to light.

Materials and methods

Experimental animals

All experiments were performed with adult male crickets, *Gryllus bimaculatus* de Geer, obtained from laboratory colonies maintained at a constant temperature of 25±0.5 °C and a 12 h:12 h light:dark photoperiod (light 06:00–18:00 h; Japanese standard time) with a continuous supply of food (laboratory chow; Nihon Clea, Type CA-1) and water.

Electrophysiology

An adult male cricket was first anesthetized with CO_2. The walking legs, antenna and wings were removed to minimize body movement. In some cases, the sub-esophageal ganglion was also carefully removed with a fine pair of forceps. The head of the animal was then fixed on a specially designed plastic platform with a 1:1 mixture of beeswax and colophonium. A small square piece of cuticle around the compound eye was cut with a fine razor knife and opened to expose the optic stalk, a long nerve trunk connecting the lamina medulla complex to the lobula. The optic stalk was then cleansed from surrounding tissues and was subdivided into fine nerve filaments using a fine needle. The separated optic stalk filaments were finally severed vertically near the medulla. The cut end of a nerve filament from the ventral side of the optic stalk was sucked firmly into an electrode filled with insect Ringer's solution (Fielden, 1960). A silver reference electrode was placed near the tip of the suction electrode. The cavity of the head capsule was sealed with petroleum jelly to prevent desiccation. During the preparation, the nervous tissues were not perfused but were bathed in a small amount of Ringer's solution. A small hole was also made on the head capsule near the contralateral eye to allow injection into the contralateral optic lobe (see Fig. 1). Electrical signals from the suction electrode were amplified by an amplifier (Nihon Kohden, AVB-9) and displayed on an oscilloscope (Nihon Kohden, VC-9). Signals were then fed into a computer (IBM, 300GL) *via* an A/D converter (Cambridge Electronic Design Limited, 1401 Plus). Data were analyzed using Spike-2 software (Version 3, Cambridge Electronic Design Limited).

To examine the effects of serotonin on retinal photoreceptors, simultaneous recording of the electrical activity of the optic stalk and electroretinogram (ERG) was made. ERGs were obtained using a glass electrode filled with Ringer's solution. The active electrode was positioned just beneath the surface of the cornea and a silver wire reference electrode was placed in the hemocoel of the head capsule through a small hole made with a fine needle. To elicit the ERG, light stimuli of 500 ms duration were delivered to the compound eye through a light guide. Responses were amplified by a high-gain amplifier (Tektronix, TM502A), displayed on an oscilloscope and recorded as above. The amplitude of the ERG was measured as the excursion from the baseline to its peak. In all cases, after fixing the electrode the animal was kept in the dark for at least 30 min before photic stimulation.

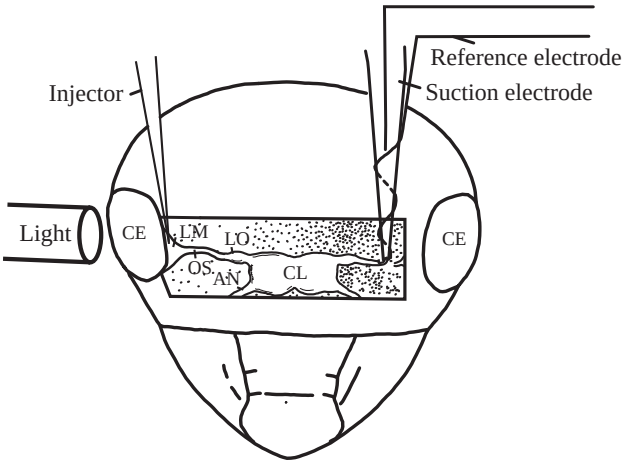

Fig. 1. The arrangement used to record neural activity from the optic stalk brain efferents. Neural activity was recorded from the separated optic stalk using a suction electrode. Light stimulation and injection of chemicals were performed on the contralateral side. AN, antennal nerve; CE, compound eye; CL, cerebral lobe; LM, lamina medulla complex; LO, lobulla; OS, optic stalk.

Photic stimulation

To examine the intensity-dependence of light-evoked responses of MBNs, 1000 ms light flashes of varying intensities were delivered to the compound eye through a 3.5 mm diameter plastic light guide. A slide projector (ELMO, CS II) equipped with a 150 W lamp (Philips, KP-8) was used as a light source. Light from the slide projector was focused on one end of the light guide and the opposite end was placed close to the compound eye. Light pulses with various intensities were regulated by an electric shutter controlled by a stimulator (Nihon Kohden, SEN-3210). Neutral density filters (Shonan Kogaku Co.) ranging from 10 to 50 % were placed between the shutter and the light source to attenuate light pulses to different intensities. The maximal light intensity ($\log I$=0) at the surface of the compound eye was 0.4 mW cm^{-2}, as measured by an optical power meter (UDT instruments, Model 371).

Microinjections

Serotonin (creatinine sulfate, Sigma), a nonspecific agonist of serotonin receptors, quipazine dimaleate (RBI), and a nonspecific antagonist of serotonin receptors, metergoline phenylmethyl ester (Tocris Cookson), were used in this experiment. Serotonin and quipazine were dissolved in Ringer's solution to the desired concentrations (0.1 mmol l^{-1},

1 mmol l^{-1} and 10 mmol l^{-1}). Metergoline was first dissolved in dimethyl sulphoxide (DMSO) (Sigma) and further diluted with Ringer's solution to make working solutions of the desired concentration. Injections were made during the day (at approximately 13:00 h) and at night (approximately 21:00 h) into the medulla area of the optic lobe using a glass micropipette equipped with a nanoliter injector (WPI, A203XVY) mounted on a micromanipulator (Narishige, M-3333). The volume of injected solution was estimated by measuring the diameter of a droplet injected into mineral oil under a microscope. The volume for single injections was 8.47–12.76 nl (mean±S.D.=10.02±1.5 nl). The micropipette tips were checked before and after injections in order to ensure that the injection was performed successfully. The same volume of Ringer's solution was injected as a control.

Data analysis

To determine the effects of injected chemicals on neural activity, the number of spikes during the light pulse was compared before and after the injection. The light-evoked response was estimated by subtracting the number of spikes occurring during the 1000 ms period just before the light pulse from that during the 1000 ms light pulse itself. Comparisons were made using the equation: $SI=100\times(B-A)/B$, where SI is the suppression index and A and B represent the total number of spikes induced by a series of light pulses with intensities of log$I$=0 to –8 before (B) and after (A) injection with chemicals. Paired or unpaired Student's t-tests were used, where applicable, to determine the statistical significance of differences. The number of animals (N) used for each recording is indicated.

Results

Day/night changes in spontaneous activity and light-induced responses of the optic stalk brain efferents

The activity of brain efferents in the cricket was recorded using extracellular suction electrodes from the cut ends of the optic stalks, which contain axons of MBNs. In the dark, the spontaneous firing rate during the night was 84±7.3 spikes s^{-1} (mean ± S.E.M., N=14); this was significantly higher than that during the day 54.3±5.8 (N=19) (t-test, $P<0.01$). When the contralateral optic lobe was removed, the number of spontaneous impulses was reduced by 38±5.7 % (N=5) and the reduction was statistically significant (t-test, $P<0.01$), suggesting that approximately one-third of the spontaneous activity is from the contralateral optic lobe or depends on the neural activity of the optic lobe.

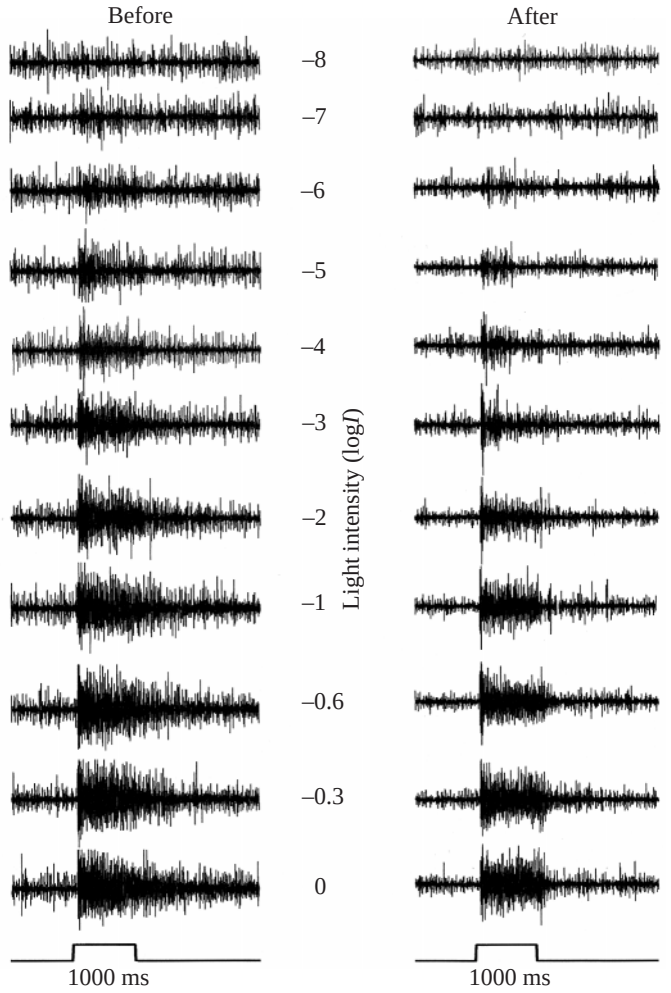

Fig. 2. Spontaneous and light-evoked activity recorded from the separated proximal optic stalk of a dark-adapted eye before and after injection of 10 pmol serotonin at daytime. Responsiveness increased with increments in light intensity. Injection of serotonin into the optic lobe reduced the spontaneous activity as well as the light-induced responses. Duration of light pulses to the contralateral eye was 1000 ms, as shown at the bottom of the waveform. The highest intensity tested was 0.4 mW cm^{-2} designated as log$I$=0.

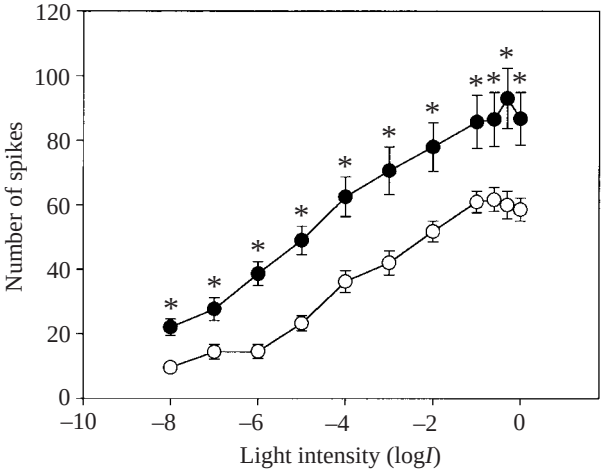

Fig. 3. Average intensity–response curves for day (open circles, N=19) and night (filled circles, N=14). The photo-responsiveness of medulla bilateral neruons (MBNs) was greater during the night than the day. During the day the response was approximately 60 % of that during the night. Vertical bars indicate ±1 S.E.M. Significant differences between day and night are indicated by asterisks ($P<0.01$, t-test).

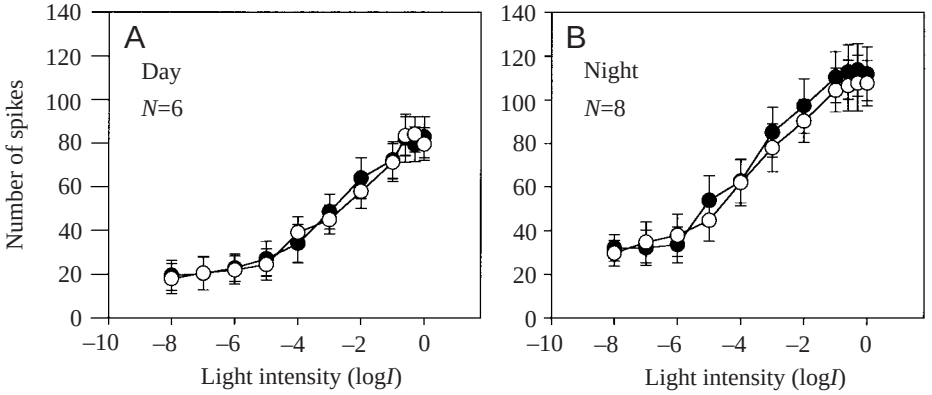

Fig. 4. Average intensity–response curves showing the effect of injection of Ringer's solution into the contralateral optic lobe on light-induced responses of medulla bilateral neurons (MBNs) during day (A) and night (B). Filled circles indicate activity before injection and open circles after injection. Vertical bars indicate ±1 S.E.M. Administration of Ringer's solution had little effect on MBN photo-responsiveness with the suppression index *SI*=1.4±2.3 (mean ± S.E.M., *N*=6) and 5.2±1.3 (*N*=8) for day and night, respectively.

A 1000 ms light pulse given to the contralateral eye induced a burst discharge in the brain efferents. Fig. 2 shows a representative result obtained during the day. The light-evoked response is practically entirely from the MBNs, since it has been shown that cutting the tract in which MBNs run resulted in a loss of light-evoked response (Tomioka et al., 1994). It gradually increased with an increase in light intensity, yielding an intensity–response curve (Fig. 3). The smallest response was detected at an intensity of log*I*=–8 and reached a maximum at log*I*=–1 (Figs 2, 3). With higher intensities, light-evoked discharges were prominent even after the light pulse (Fig. 2). There was a clear day/night difference in the magnitude of the light-evoked responses, which were significantly greater during the night than the day (*t*-test, $P<0.01$); the light intensity evoking the half-maximal response shifts from log*I*=–3.7 in the day to log*I*=–4.7 at night (Fig. 3).

Effects of serotonin on spontaneous and light-induced responses

Different doses of serotonin (1.0, 10 and 100 pmol in 10 nl) were injected into the contralateral optic lobe to reveal its effect on the brain efferents including MBNs during both day and night. In the control experiments, the same amount of Ringer's solution was injected into the optic lobe. No changes were observed in either spontaneous or light-induced responses following Ringer's solution injection (Fig. 4). The reduction of spontaneous activity in control experiments was –2.6±4.9 % (mean ± S.E.M., *N*=6) and 5.5±7.4 % (*N*=8) for day and night, respectively. The suppression index (*SI*) for the light-induced response was 1.4±2.3 (mean ± S.E.M., *N*=6) and 5.2±1.3 (*N*=8) for day and night, respectively.

Microinjection of serotonin into the optic lobe clearly suppressed the spontaneous activity of the brain efferents in

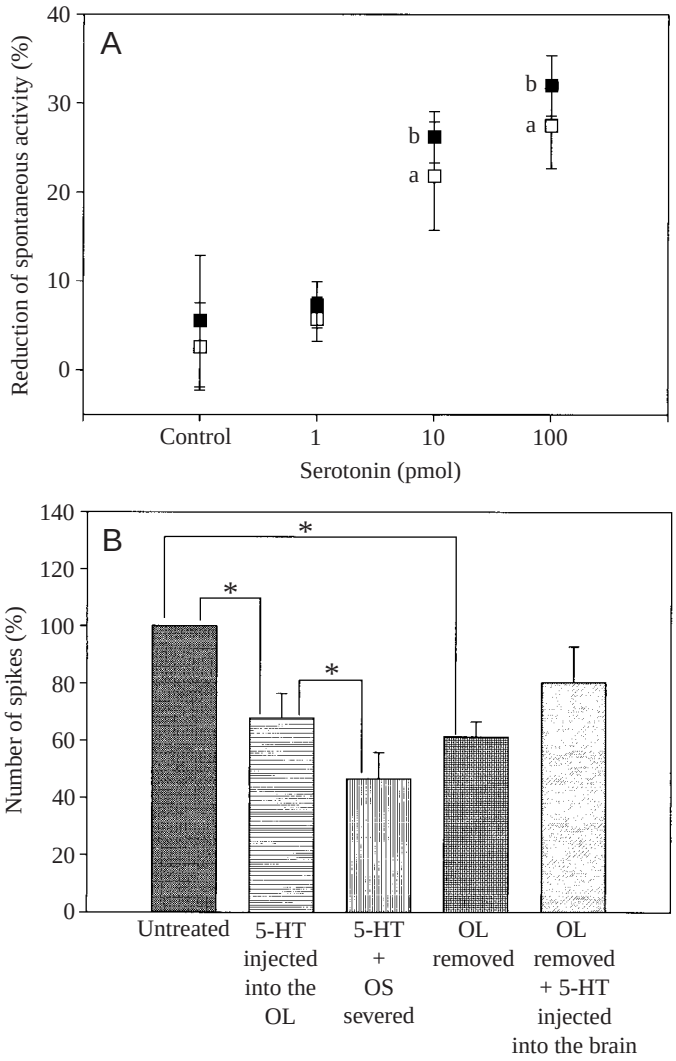

Fig. 5. Effects of serotonin on the spontaneous activity recorded from the separated proximal optic stalk. (A) Application of serotonin into the optic lobe suppressed the spontaneous activity in a dose-dependent manner during both day (open squares) and night (filled squares). a and b indicate a significant difference ($P<0.05$, *t*-test) compared with the Ringer-injected control for day and night, respectively. Values are means ± S.E.M. of 5–8 preparations. (B) Serotonin (10 pmol) injected into the optic lobe significantly suppressed the spontaneous firing rate, which was further reduced when the contralateral optic stalk was severed. When the contralateral optic lobe was removed, the spontaneous activity was significantly reduced, and this value was increased only slightly when serotonin was injected into the protocerebral lobe after the removal of the optic lobe. Values are means ± S.E.M. of 5 preparations, given as percentage of the value before treatments (untreated). *$P<0.01$, *t*-test.

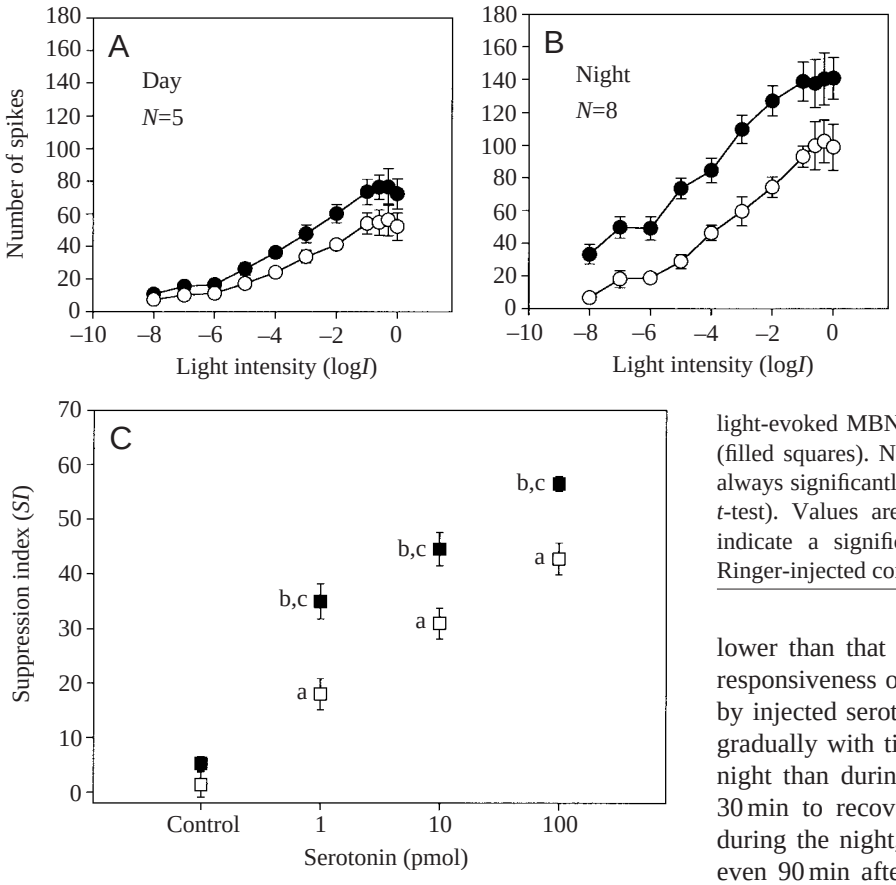

Fig. 6. Effects of serotonin on light-induced responses of medulla bilateral neurons (MBNs) recorded from the separated proximal optic stalk. (A,B) Average intensity–response curves showing the effect of serotonin (10 pmol) on MBN photo-responsiveness during the day (A) and the night (B). The number of spikes induced by a light pulse is plotted against the corresponding light intensities. Filled and open circles indicate values either before (filled circles) or after injection (open circles). The effect of serotonin was greater during the night than during the day. (C) Dose–response curve showing the ability of serotonin to suppress light-evoked MBN activity during the day (open squares) and night (filled squares). Note that the sensitivity of MBN to serotonin was always significantly greater during the night than the day (c, $P<0.01$, *t*-test). Values are means ± S.E.M. of 5–8 preparations. a and b indicate a significant difference ($P<0.01$, *t*-test) compared with Ringer-injected control values for day and night, respectively.

the optic stalk (Fig. 2). This inhibitory effect of serotonin was dose dependent during both day and night: greater suppression occurred with larger doses of serotonin. A significant reduction of spontaneous discharge rate occurred at 10 pmol both for the day and night (*t*-test, $P<0.05$, compared with that of controls) (Fig. 5A). The spontaneous activity reduced by serotonin was further reduced significantly by severing the contralateral optic stalk (*t*-test, $P<0.01$) (Fig. 5B). Removal of the contralateral optic lobe also significantly reduced the spontaneous firing rate (*t*-test, $P<0.01$) (Fig. 5B). When serotonin was injected into the brain following the removal of the optic lobe, the firing rate increased by 26 % relative to the optic-lobe-removed value but this increment was not statistically significant (*t*-test, $P>0.05$, $N=5$) (Fig. 5B).

Serotonin exerted a pronounced suppression not only on the spontaneous activity of MBNs but also on their light-evoked response (Fig. 2). Fig. 6A,B shows average intensity–response curves before and after injecting 10 pmol of serotonin. Serotonin shifts the intensity–response curve downwards. This inhibitory effect was both time and dose dependent, with greater suppression during the night and at higher dosage. A significant suppression of the light-induced response was observed at 1.0 pmol both during the day and at night (*t*-test, $P<0.01$, compared with the control). The *SI*s were significantly greater during the night by 25–40 % than those during the day for all dosages used (*t*-test, $P<0.01$) (Fig. 6C), indicating that the sensitivity of MBNs to serotonin during the light phase was lower than that during the dark phase. Although the photo-responsiveness of MBNs was almost immediately suppressed by injected serotonin during both day and night, it recovered gradually with time. The recovery occurred faster during the night than during the day: as shown in Fig. 7, it took only 30 min to recover by 100 % from serotonergic suppression during the night, while complete recovery was not observed even 90 min after the injection during the day. Spontaneous firing frequency returned to the initial level within 30 min after injection at either time, however.

Effects of serotonin on the electroretinogram

To examine whether the suppression of the light-evoked response by serotonin is attributable to reduced sensitivity of the retina, the amplitude of the electroretinogram (ERG) was compared before and after injection of serotonin into the optic lobe. The ERGs recorded were simple cornea negative waves (Fig. 8A,B), which were quite similar to those reported for *Teleogryllus commodus* (Rence et al., 1988). The waveform did not change even after cutting the optic nerve (Fig. 8A), suggesting that the ERG originated from the retinal cells. The amplitude of the ERG during the night (Fig. 8D) was approximately three times that during the day (Fig. 8C). Little change in the ERG waveform and amplitude was observed after the injection of serotonin during either the day or the night (Fig. 8B-D). Similar results were obtained when serotonin was injected directly into the retina, suggesting that the inhibitory effects of serotonin on brain efferents do not result from changes in sensitivity of the photoreceptors, but are probably attributable to changes in responsiveness of visual interneurons.

Effects of serotonin-receptor agonist and antagonist on spontaneous activity and light-induced responses

The effects of a nonspecific agonist of serotonin receptors, quipazine, were also examined. Quipazine injected into the

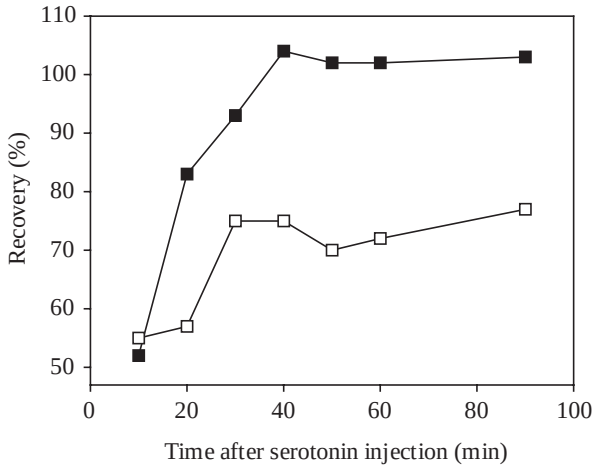

Fig. 7. An example of recovery of medulla bilateral neuron (MBN) photo-responsiveness from serotonergic suppression. Full (100 %) recovery took only 30 min during the night (filled squares), while during the day complete recovery was not achieved even after 90 min (open squares). Similar results were obtained in other preparations (day, *N*=3; night, *N*=3).

optic lobe caused a dose-dependent inhibition of both spontaneous activity and the light-induced response of the MBNs. This effect was greater during the night (Fig. 9A,B) and clearly dose dependent (Fig. 9C). The effect of 100 pmol of quipazine was approximately equivalent (*t*-test, $P>0.05$) to that of 1.0 pmol of serotonin.

The application of a nonspecific serotonin-receptor antagonist, metergoline, caused no significant alteration in MBN activity or photosensitivity at dosages of 1.0 and 10 pmol: the *SI* values for 1.0 and 10 pmol metergoline during the day were –0.5±4.5 (mean ± S.E.M., *N*=2) and –2±4.8 (*N*=4), respectively. However, at 100 pmol a substantial increase of both spontaneous and light-induced activity was observed during both the day and night. The spontaneous activity was increased by 56±7.3 % (*N*=5) during the day and by 34±6.4 % (*N*=4) during the night, while the *SI* values during the day and night were –35±6.0 (*N*=5) and –19±4.5 (*N*=6), respectively. These changes were statistically significant compared with those of controls (*t*-test, $P<0.01$). Unlike serotonin and quipazine, the effect of metergoline was greater during the day than at night (Fig. 10A,B). When injected together with serotonin, metergoline effectively antagonized the effects of serotonin on MBN photosensitivity (Fig. 10C–E).

Discussion

Daily change in spontaneous efferent activity and light-induced responses

The present study showed that spontaneous and light-evoked responses of the MBNs recorded from the proximal cut end of the optic stalk exhibited day/night changes: both are greater during the night than during the day. Daily changes in spontaneous neural activity have been reported in many insects, including crickets and cockroaches (Tyshchenko, 1973; Tomioka and Chiba, 1986; Colwell and Page, 1990). In cockroaches, the daily rhythm recorded from the cervical connective disappeared after cutting the optic tract, indicating that it is controlled by the optic lobe (Colwell and Page, 1990).

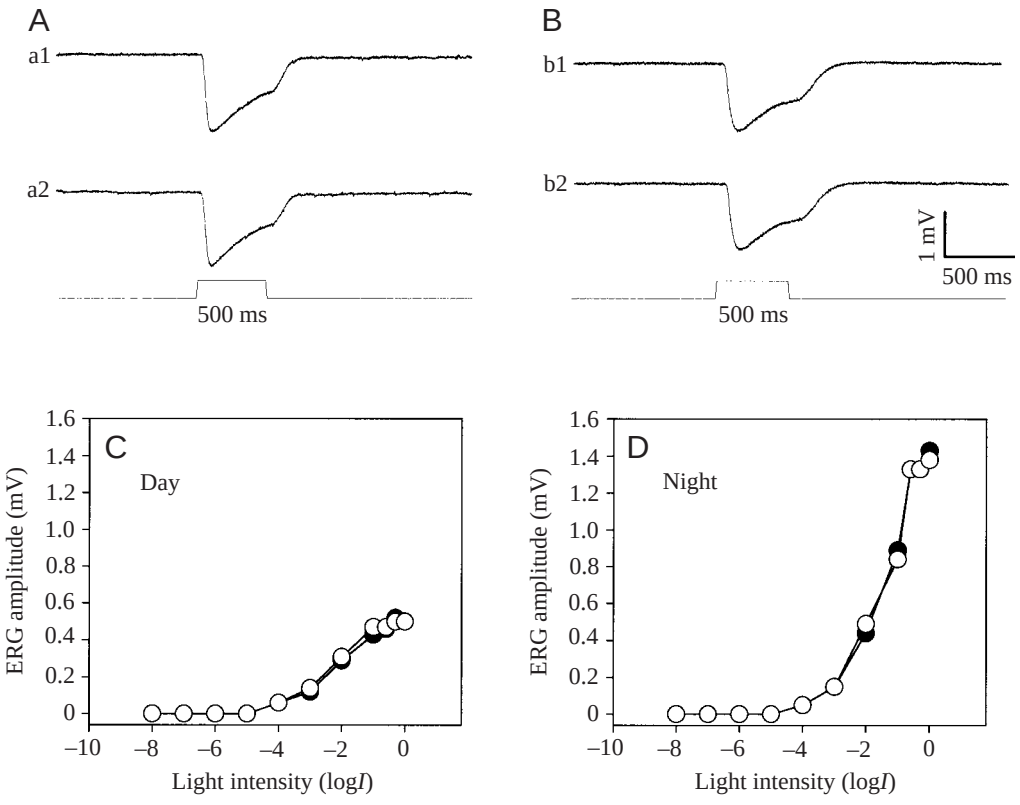

Fig. 8. (A) Examples of electroretinogram (ERG) waveform recorded from the compound eye of the cricket before (a1) and after (a2) the optic nerve was severed. The response was elicited with a 500 ms light pulse (shown beneath ERG recordings). (B) ERG waveforms before (b1) and after (b2) 10 nl of serotonin solution (10 mmol l^{-1}) was injected into the optic lobe. (C,D) Representative intensity–response curves for the on-component of the ERG obtained before (filled circles) and after (open circles) serotonin injection into the optic lobe during the day (C) and the night (D). Similar results were obtained in other preparations (day, *N*=3; night, *N*=3). Note that injection of serotonin into the optic lobe had no significant ($P>0.05$, *N*=4) effect on both waveform and amplitude of the ERG.

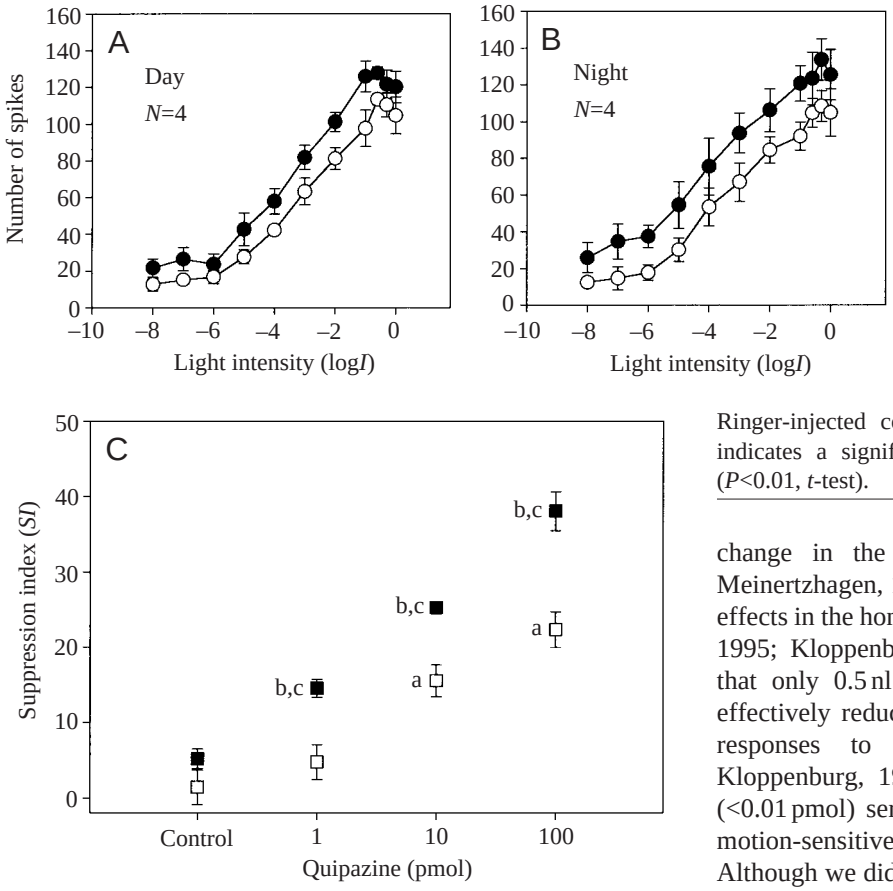

Fig. 9. (A,B) Average intensity–response curves showing that quipazine (10 pmol) suppressed medulla bilateral neuron (MBN) photo-responsiveness during both day and night, but more effectively during the night. Filled and open circles indicate results before and after quipazine injection, respectively. Vertical bars indicate ± 1 S.E.M. (B) Dose dependency of the effect of quipazine on MBN photo-responsiveness. Open and filled squares indicate results for the day and night, respectively. Values are means ± S.E.M. of 4–8 preparations. a and b indicate a significant difference (P<0.01, t-test) compared with Ringer-injected control value for day and night, respectively. c indicates a significant difference between day and night values (P<0.01, t-test).

The present study revealed that the spontaneous activity in the brain efferents towards the optic lobe also depends on the optic lobe itself: detaching the cerebral lobe from the optic lobe by transection of the contralateral optic stalk resulted in a reduction of the spontaneous activity. The spontaneous activity dependent on the contralateral optic lobe may mostly be attributable to the MBNs, since they are the only neurons that project from the contralateral optic lobe, running in the fraction of the optic stalk from which the recording was made (Tomioka et al., 1994).

Injection of serotonin into the optic lobe resulted in a reduction of the spontaneous activity in the brain efferents. When serotonin was injected into the brain after removal of the optic lobe, the spontaneous activity of the brain efferents increased. These results suggest that reduction of spontaneous impulse activity induced by the injection of serotonin occurred within the optic lobe. It is also consistent with the fact that a large number of serotonergic neurons are located in the optic lobe (Nässel, 1987; K. T. and S. Tamotsu, unpublished data).

Serotonin regulates the responsiveness of MBNs

The present study demonstrates that photo-responsiveness of the MBNs was suppressed in a dose-dependent and time-dependent manner by serotonin as well as by its non-specific serotonin-receptor agonist, quipazine, when injected into the optic lobe. The injected volume of serotonin solution in our experiment was one-tenth of that used to induce morphological change in the fly's optic lobe interneurons (Pyza and Meinertzhagen, 1995), but larger than that used to examine its effects in the honeybee visual system (Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995). It has been reported that only 0.5 nl of 10^{-5} mol l^{-1} (0.005 pmol) serotonin can effectively reduce the honeybee's direction-specific antennal responses to stripe pattern movements (Erber and Kloppenburg, 1995) and that less than 1 nl of 10^{-5} mol l^{-1} (<0.01 pmol) serotonin modulates the response properties of motion-sensitive neurons (Kloppenburg and Erber, 1995). Although we did not attempt injection of such a small amount of serotonin solution, we can estimate the minimum effective dosage by extrapolation of the serotonin dose–response curve for photo-responsiveness (Fig. 6C). The estimated minimum effective dosage at night is less than 0.01 pmol which is quite similar to the value used for honeybees.

Since serotonin injected into the optic lobe or retina had little effect on the amplitude of the ERG, it is most probable that the suppression of MBN responsiveness by serotonin occurs within the optic lobe. The fact that application of serotonin induces morphological changes in interneurons in the fly's optic lobe (Meinertzhagen and Pyza, 1996; Pyza and Meinertzhagen, 1996) is consistent with this hypothesis. Our finding that no apparent changes were observed in ERG amplitude after serotonin injection is inconsistent with the report by Chen et al. (1999) for blowflies *Calliphora erythrocephala*, in which serotonin injected into the retina reduced the amplitude of the sustained negative component of the ERG, which is derived at least partly from photoreceptor responses. There might be species specificity on the action of serotonin in the retina.

Involvement of serotonin in the visual system has been reported for a wide variety of animals including insects. Serotonin changes the sensitivity of retinal photoreceptors in *Aplysia californica* (Jacklet, 1991) and the locust (Cuttle et al., 1995). The sensitivity of visual interneurons in the optic lobe of crickets (Tomioka et al., 1993; Tomioka, 1999) and in the brain of bees *Apis mellifera* (Kloppenburg and Erber, 1995) is suppressed by serotonin. Serotonin also induces morphological

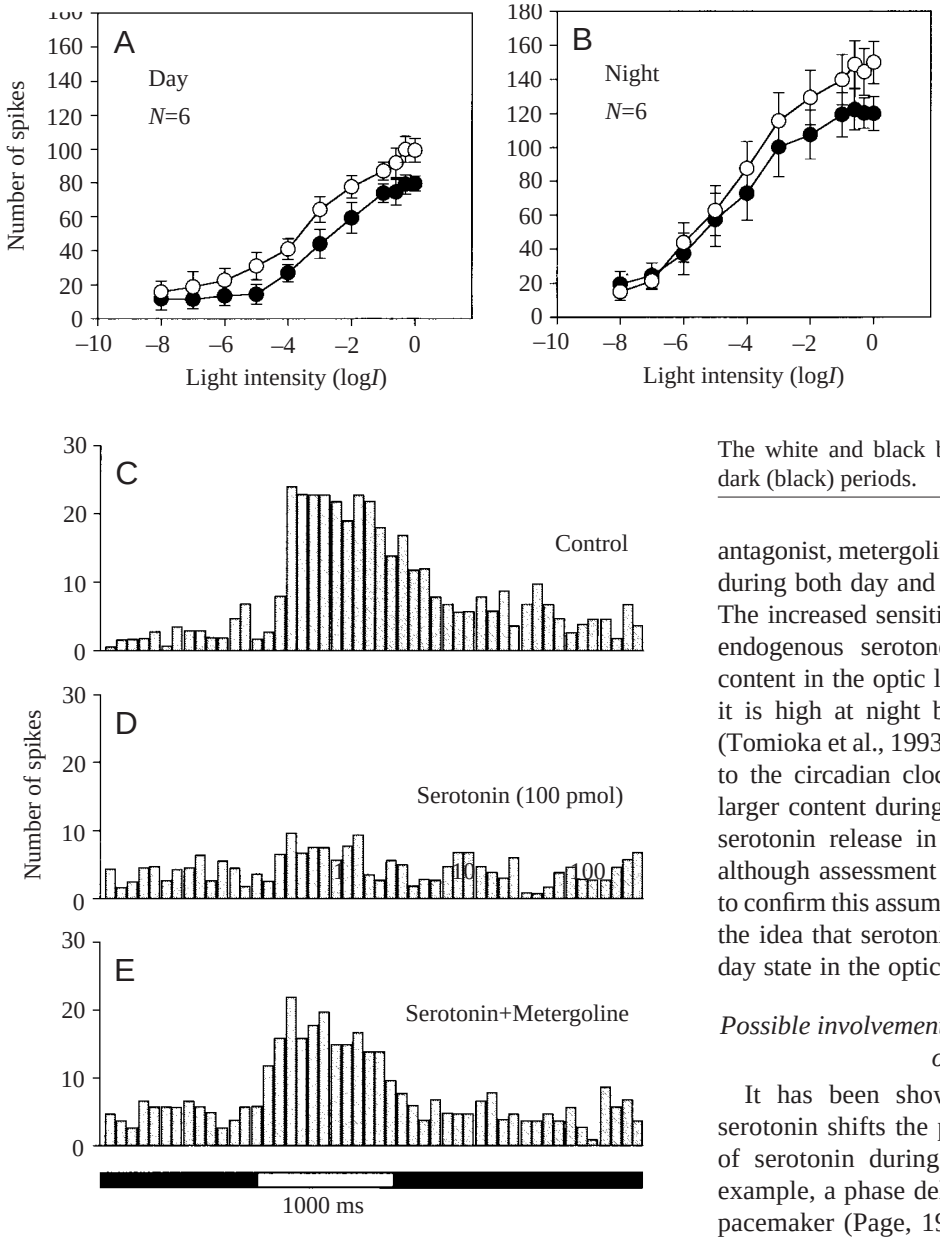

Fig. 10. Effects of metergoline (100 pmol) on medulla bilateral neuron (MBN) photo-responsiveness. (A,B) Average intensity–response curve before (filled circles) and after injection (open circles) of metergoline. Vertical bars indicate ±1 S.E.M. Metergoline increased the photo-responsiveness during both day (A) and night (B), but more effectively during the day. (C–E) Integrated rate histograms illustrating the activity per 100 ms. Serotonin suppressed the light-induced responses (D), but metergoline attenuated the suppressing effect of serotonin when administered together with serotonin (E). The white and black bars at the bottom indicate light (white) and dark (black) periods.

changes in the fly's optic lobe interneurons to mimic a day state, which may be reflected in the change in responsiveness of the interneurons (Meinertzhagen and Pyza, 1996; Chen et al., 1999). These facts suggest that serotonin is a common substance regulating the sensitivity of the visual system in various ways and in a wide variety of animals.

Time of serotonin release

The present study revealed that serotonin suppressed MBN responsiveness to light during both the day and night, but that the amount of suppression was greater at night with the same dosage. Recovery from the suppression also occurred faster during the night. The greater suppression and faster recovery during the night suggest that less endogenous serotonin is released at this time. This possibility is supported by the finding that application of a non-selective serotonin-receptor antagonist, metergoline, increased the MBNs sensitivity to light during both day and night with greater effects during the day. The increased sensitivity is consistent with the suppression of endogenous serotonergic action by metergoline. Serotonin content in the optic lobe fluctuates during the day/night cycle: it is high at night but substantially reduced during the day (Tomioka et al., 1993), suggesting that serotonin may be related to the circadian clock system in the cricket optic lobe. The larger content during the night could reflect a reduced rate of serotonin release in the optic lobe (Tomioka et al., 1993), although assessment of this release *in vivo* would be required to confirm this assumption. Nevertheless, the data so far support the idea that serotonin is released during the day and sets the day state in the optic lobe interneurons.

Possible involvement of serotonin in mutual coupling between optic lobe pacemakers

It has been shown in a wide variety of animals that serotonin shifts the phase of the circadian clock: application of serotonin during the early subjective night causes, for example, a phase delay in the cockroach optic lobe circadian pacemaker (Page, 1987), whereas in *Aplysia californica* and the cricket *G. bimaculatus*, it induces a phase delay during the subjective night, but phase advances during the subjective day (Koumenis and Eskin, 1992; Tomioka, 1999). Since the phase–response curve for serotonin is quite similar to that caused by mutual entrainment between the two optic lobe pacemakers, serotonin is thought to be a candidate molecule working in the coupling pathway in the cricket *G. bimaculatus* (Tomioka, 1999).

In addition to its phase-shifting effect, the present study demonstrated that serotonin affects coupling less directly, by suppressing the sensitivity of MBNs to light. It has been suggested that coupling signals between optic lobe pacemakers are mediated by MBNs (Yukizane and Tomioka, 1995; Tomioka and Yukizane, 1997). Immunohistochemical labeling with anti-serotonin antibodies revealed that the MBNs were not serotonergic themselves. Serotonin is somehow released by serotonergic neurons under the regulation of the pacemaker

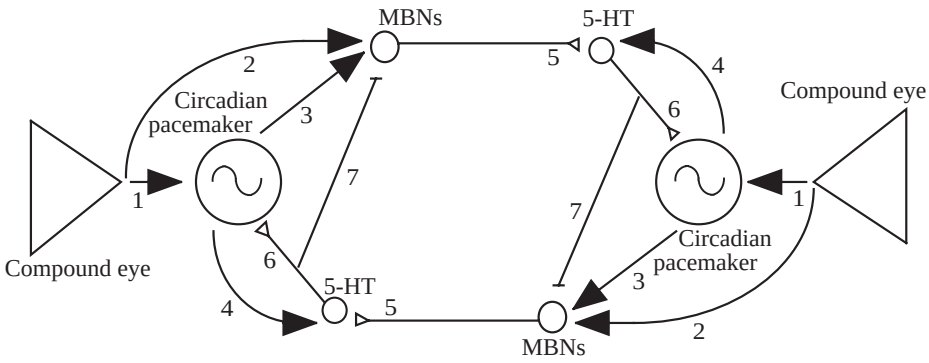

Fig. 11. Possible involvement of serotonin in the coupling mechanism between the optic lobe pacemakers. The circadian pacemakers in the optic lobe synchronize not only to the environmental light/dark cycle through photic information from the compound eye (1) but also to their contralateral partner. The medulla bilateral neurons (MBNs) are the major component of the coupling system and receive photic information from the photoreceptor (2) and circadian information from the pacemaker (3) on their own side. Serotonin (5-HT) is released from the serotonergic neurons under regulation by the circadian pacemaker (4) and the contralateral MBNs (5). The released serotonin shifts the phase of the pacemaker (6) in a phase-dependent manner, and at the same time, it reduces the coupling signals (7) by suppressing the activity of MBNs.

and MBNs. Probably, released serotonin in one optic lobe shifts the phase of the pacemaker on that side in a phase-dependent manner and, at the same time, reduces the coupling signals by suppressing the activity of MBNs, keeping, in turn, the phase of the contralateral pacemaker stable (Fig. 11).

The phase shifts caused by serotonin in a compound eye and optic lobe complex isolated and kept *in vitro* were much larger than those expected from the phase–response curve for the mutual coupling obtained from the interaction between the experimentally dissociated pacemakers (Tomioka et al., 1991; Tomioka, 1993, 1999). Although one may attribute this difference to the rather high concentration of serotonin used in the phase-shifting experiment (Tomioka, 1999), the regulation of MBN sensitivity by serotonin appears to contribute, keeping the phase shifts in a smaller range than that in which the phase difference between the bilateral pacemakers is normally kept.

The role of serotonin in the circadian system of many other animals has been discussed extensively. In mammalian species, it is suggested that serotonin is released during the night in the clock tissue, suprachiasmatic nuclei, by the increased activity of Raphé neurons which has been linked to arousal state, with increased Raphé neuron activity associated with increased arousal (Prosser et al., 1993). The activity of the serotonergic system is thought to synchronize the pacemaker to social or behavioral changes in the environment (Medanic and Gillet, 1992). In *Aplysia californica*, the ocular circadian pacemaker in the eye receives efferent innervation from the cerebral ganglion of which the putative neurotransmitter is serotonin (Takahashi et al., 1989). The serotonergic system is hypothesized to have two roles: carrying coupling information from the contralateral ocular pacemaker or from oscillators located in the central nervous system (Lickey et al., 1983); mediating the effects of an extraocular light pathway on the oscillator in the eye (Colwell, 1990). Taking these implications together with our present results, it is apparent that the serotonergic system is commonly involved in the efferent regulatory pathway of the circadian pacemaker. Our results further suggest that the serotonergic system modulates the efferent coupling pathway to set its day state.

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