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Authors: Nishinokubi, Izumi, and Tomioka, Kenji

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Analysis of the Mechanism underlying the Rhythm Reversal from Diurnal to Nocturnal in the Cricket *Gryllus bimaculatus*, with Special Reference to the Role of Serotonin

Izumi Nishinokubi[1†] and Kenji Tomioka[1,2*]

[1]*Department of Physics, Biology and Informatics, Faculty of Science and*
[2]*Research Institute for Time Studies, Yamaguchi University,*
Yamaguchi 753-8512, Japan

ABSTRACT—The cricket, *Gryllus bimaculatus*, shows a rhythm reversal from diurnal to nocturnal in about a week after the imaginal molt. In the present study, we investigated the role of serotonin (5-HT) in the rhythm reversal. The 5-HT content in the brain measured by HPLC equipped with an electrochemical detector gradually increased after the imaginal molt, and in fully nocturnal adults it was about 2 times of nymphal level. We then examined the effects of 5,7-dihydroxytryptamine (5,7-DHT), a selective neurotoxine to serotonergic neurons, on the locomotor rhythm. In most animals with 5,7-DHT (25 μM or 250 μM, 32.2 nl) injected into the brain, daytime activity significantly increased even after the rhythm reversal, while nighttime activity was not significantly affected, forming rather diurnal pattern. The serotonin content in the brain of animals injected with 250 μM 5,7-DHT was reduced by about 30%. On the basis of these results, possible involvement of 5-HT in the neural mechanism controlling the locomotor rhythm is discussed.

INTRODUCTION

Circadian rhythms are essential functions that are commonly seen in a wide variety of organisms. The rhythms are controlled by an endogenous physiological mechanism called circadian pacemaker. The pacemaker has been localized in restricted tissues in the central nervous system, including the optic lobe in the hemimetabolous insects such as crickets and cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Tomioka and Chiba, 1992), the pineal gland of birds and reptiles (Takahashi *et al.*, 1980; Toshini and Menaker, 1998), and the suprachiasmatic nucleus in mammals (Inouye and Kawamura, 1979).

Physiological approaches to the regulatory mechanism for the overt locomotor activity have so far revealed that the relationship between the waveforms of the pacemaker and the overt behavioral rhythms is often not consistent. For example, adult crickets (*Gryllus bimaculatus*) and cockroaches (*Leucophaea maderae*) are both nocturnally active, but the output of the pacemaker is antiphase to each other (Colwell and Page, 1990; Tomioka and Chiba, 1992). In mammalian suprachiasmatic nucleus, the electrical firing activity is reportedly always diurnally increasing irrespective of diurnal, nocturnal or arrhythmic in animal's overt activity (Inouye and Kawamura, 1979; Sato and Kawamura, 1984). The cricket, *G. bimaculatus* seems especially suitable for the research on the neural mechanism downstream from the pacemaker to the overt activity rhythm, since it shows ontogenetic change in its locomotor rhythm from diurnal to nocturnal in several days after the imaginal molt (Tomioka and Chiba, 1982).

Neural basis of the regulatory system has been also examined in view of neurotransmitters or neuroactive substances. Serotonin (5-HT) is a biogenic amine well studied in respect of its relationship to the circadian system. 5-HT is widely distributed in the central nervous system in insects(Nässel, 1987), and has been suggested to be involved in the circadian system. For example, it shifts the phase of circadian pacemaker in cockroaches and crickets (Page, 1987; Tomioka, 1999), regulates circadian rhythms of flight activity in moths (Hinks, 1967), visual systems in flies and crickets (Pyza and Meinertzhagen, 1996; Tomioka, 1993) and locomotor activity in crickets (Cymborowski and Muszynska, 1974; Germ and Tomioka, 1998).

In the present study, we investigated the possible role of 5-HT in the circadian rhythm reversal in the cricket *Gryllus bimaculatus*. The results show that there is a positive relationship between the cerebral 5-HT level and the phase of the locomotor rhythm, suggesting possible involvement of 5-HT in the regulation of the cricket's locomotor rhythm.

* Corresponding author: Tel. +81-83-933-5714;
FAX. +81-83-933-5714.
E-mail: tomioka@po.cc.yamaguchi-u.ac.jp
† Present address: National Institute of Bioscience and Human Technology, AIST, MITI, Higashi 1-1, Tsukuba, Ibaraki 305-8566, Japan

MATERIALS AND METHODS

Animals

Adults and nymphs of the cricket *Gryllus bimaculatus* were used. They were obtained from a laboratory colony maintained at 25°C in a 12 hr light and 12 hr dark cycle (LD12:12, L: 0600-1800 Japanese Standard Time). They were fed laboratory chow and water via damped absorbent cotton.

Activity recording and analysis

Animals were kept individually in an activity chamber of a transparent plastic box (180 x 90 x 50 mm) with a rocking substratum. Before housing the animal into the chamber, either of the forewings was removed to prevent any sound communication between individuals. The rocking movement of the substratum caused by a moving insect was sensed by a magnetic reed switch placed on the bottom of the box. A signal from the switch was collected by a computer (NEC, PC-9801NS/A), which summed the signal every 6 min. The total count of every 6 min was stored on a floppy diskette. The activity chambers were placed in an environment controlled room in which the temperature was kept constant at 25±1°C. Light and dark cycles were given by a 20W cool white fluorescent lamp controlled by an electric timer. The light intensity within actographs were about 400 lux at the animal's level.

At the end of an experiment, the raw data from individual cricket were double plotted in conventional manner to facilitate the visual inspection of the rhythmicity. Daily activity profiles were obtained by averaging the activity for 7 to 20 days. Data for the first 5 days after the imaginal molt were included for nymphal activity since the nymphal activity pattern persisted until this period.

Measurements of biogenic amines

The concentrations of 5-HT and its derivatives in the brain were measured by means of high pressure liquid chromatography (HPLC; Eicom, EP-10) with an electrochemical detector (ECD; Eicom, ECD300). The brains were dissected quickly and placed in 50 µl of ice-cold 0.1M perchloric acid containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The sample was homogenized and centrifuged at 12,000 g for 30 min at 4°C. 10µl of supernatant was directly injected to the HPLC column using the autosampler (Toso, AS-8021). The mobile phase was composed of 0.18 M monochloroacetic acid, 0.16 M NaOH, 50 µM ethylenediaminetetraacetic acid (EDTA) disodium, with 1.85 mM sodium-1-octanesulfonic acid (SOS) as the ion pair reagent and 8.5% (v/v) acetonitrile as the organic modifier. The pH was adjusted to 3.6 by the addition of NaOH. The HPLC system consisted of a solvent delivery pump, an injection valve (Rheodyne), a C18 reversed-phase column (150 mm × 4.6 mm) placed in a column oven (Sugai, U-620). An electrochemical detector with a carbon graphite electrode (Eicom, WE-3G) was used. The detector potential was set at 0.7V versus an Ag/AgCl reference electrode. Signals from the detector were recorded and integrated by computer (Waters, Data Station 805). Quantifications, which were based on the peak area of the chromatograms, were obtained by calculating the ratio of the peak area of the substances to the peak area of the internal standard. Concentrations were obtained by a comparison of ratios between the sample and standard chromatograms. The results were calculated in pmol/mg of brain wet weight.

Injection

Animals were fixed on a platform and anesthetized by a continuous flow of CO_2. The brain was exposed by cutting a small square piece of head capsule with a razor knife. 32.2 nl of 25 µM or 250 µM 5,7-DHT (Sigma) was injected into near center of the brain with a glass micropipette using an injector (WPI, A203XVY) mounted on a micromanipulator (Narishige, M-3333). 5,7-DHT was dissolved in insect Ringer's solution (Fielden, 1960) containing 1% ascorbic acid. The Ringer's solution was injected as a control.

RESULTS

Rhythm reversal from diurnal to nocturnal

The locomotor activity was assayed from the last (8th) instar to adult in 14 male crickets. A representative record is shown in Figure 1. Under LD12:12, the nymphal cricket was

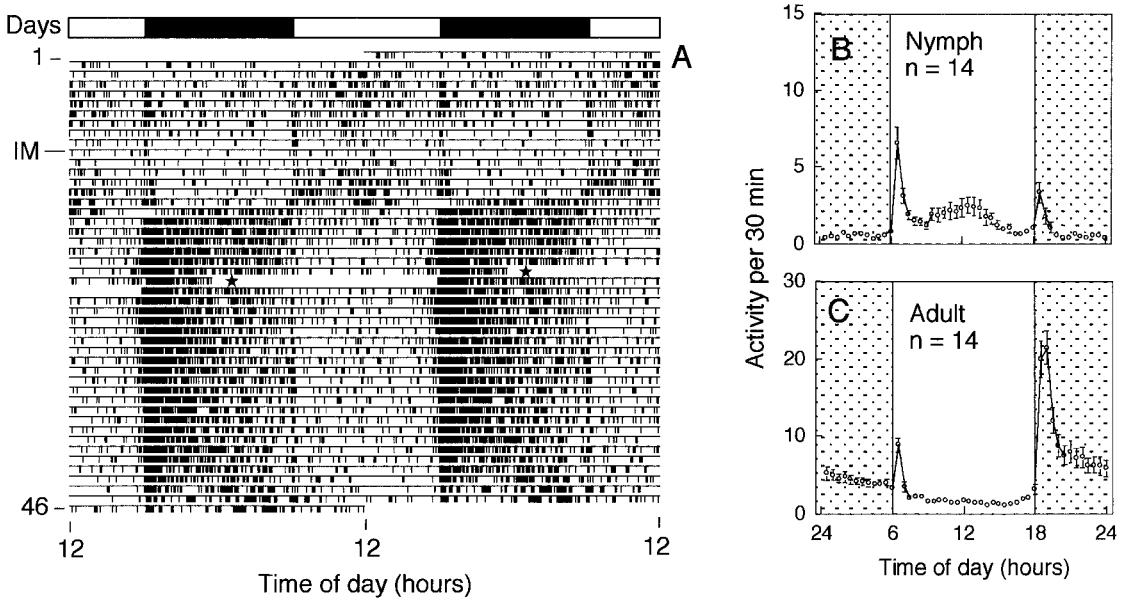

Fig. 1. A: A representative double plotted actogram of a male cricket *G. bimaculatus* under a 12 hr light and 12 hr dark cycle. The cricket was diurnally active in nymphal stage and became nocturnal within 7 days after the imaginal molt. White and black bars indicate light (white) and dark (black) cycle. IM, imaginal molt. During the blank area indicated by a star, data were lost by a failure of recording system. B, C: Average daily activity profiles of locomotor activity in 14 male crickets. Plotted are means and SEM. Dotted areas indicate the dark phase.

diurnally active with a peak in the middle of day. In this particular animal, 7 days after the imaginal molt, an activity bout suddenly appeared in the night, quickly advanced to synchronize to the lights-off in a few days. In contrast, the diurnal component gradually decreased; a nocturnal pattern was thus established, as previously reported (Tomioka and Chiba, 1982). Once the nocturnal pattern was established, it persisted throughout the adulthood. The rhythm reversal from diurnal to nocturnal was observed in all the crickets, with the establishment of nocturnality in 6.0±0.9 days after the imaginal molt (n=14).

Change in 5-HT level during the late post-embryonic development

5-HT levels in the brain during the rhythm reversal were examined in a total of 72 animals. Sampling was performed with 6th to 8th (last) instar nymphs, adults of day 0-9 and completely nocturnal adults that had undergone more than 10 days after the imaginal molt. Sampling was carried out at the middle of the light phase (12:00) and at least 5 animals were used for each point.

Figure 2 shows the concentration of 5-HT in the brain. 5-HT level in the brain was rather constant throughout the late nymphal stages and the mean value was 6.2 pmol/mg (WW). After the imaginal molt, however, it increased gradually until day 5 and then rapidly increased at day 6. Statistically signifi-

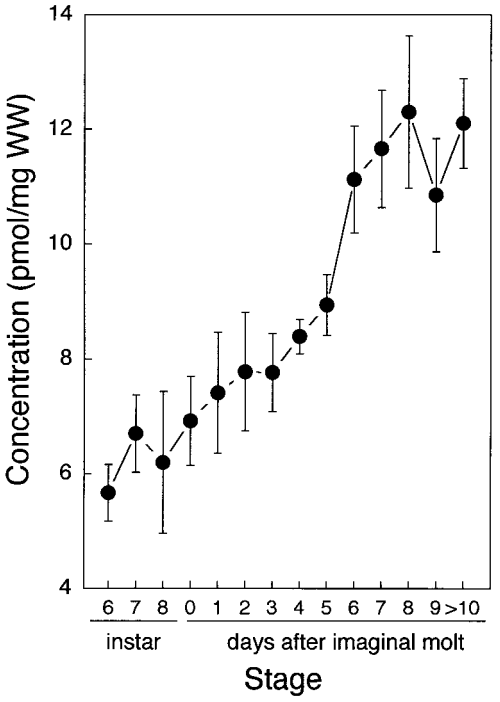

Fig. 2. The change in 5-HT concentration in the cerebral lobe of the cricket *G. bimaculatus* during the late post-embryonic development. Samples were collected at 12:00 JST. Data from 5–6 animals were pooled. Vertical bars indicate standard deviation.

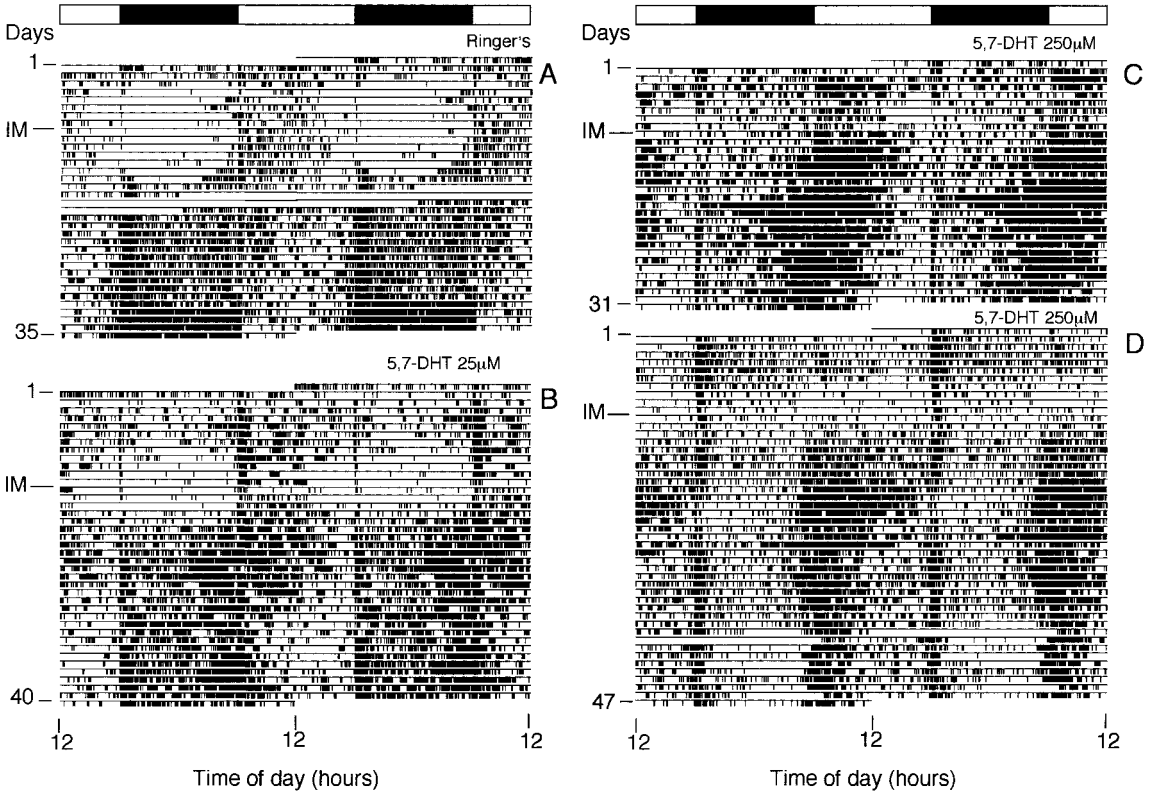

Fig. 3. Four double plotted actograms of crickets injected with either 32.2 nl of Ringer's solution (A), 25 μM (B) or 250 μM 5,7-DHT (C, D). In A, data were missed during days 17–19 because of the failure of recording system. Note that heavy activity from late night to early day occurred in the 5,7-DHT injected animals. For further explanations see text and Fig.1.

cant increase was observed when compared values of day 0 with day 5 (t-test, $p<0.05$). In mature crickets, the concentration reached over 12.0 pmol/mg (WW). It was about 2 times that in nymphal brain and very close to the value previously reported by Nagao and Tanimura (1988).

Effect of 5,7-DHT on the locomotor rhythm

We then examined the effects of 5-HT reduction on the rhythm reversal by injecting 5,7-DHT, a neurotoxine specific to the serotonergic neurons, into the brain. The chemical was dissolved in the Ringer's solution at a concentration of 25 μM or 250 μM. Four and 6 crickets at early 8th instar nymphal stage were injected with 32.2 nl 5,7-DHT of 25 μM and 250 μM, respectively. Same amount of Ringer's solution was injected in 5 crickets as control. Figure 3 shows representative activity records after the injection. All animals injected with Ringer's solution exhibited the normal rhythm reversal as exemplified in Figure 3A. The average daily activity pattern was very similar to that of intact animals both in nymphal and adult stages (Fig. 4A, B). In 5,7-DHT injected animals, however, considerable abnormality in their locomotor activity pattern was observed (Figs. 3B-D, 4C-F). In nymphs injected with 25 μM 5,7-DHT (Figs. 3B, 4C), slight but significant increase was induced in nighttime activity (t-test, $P<0.01$). Daytime activity often increased with a sharp lights-on peak, but the increase was not statistically significant (t-test, $P>0.3$). After the imaginal molt, the dense activity occurred in the latter half of the dark phase to early light phase with the development of nighttime activity (example: Fig. 3B), resulting in significant increase of the daytime activity compared with Ringer's solution injected controls (Fig. 4D, t-test, $P<0.05$). The daytime activity tended to decrease after two weeks of imaginal molt, whereas the heavy activity persisted throughout the adult life. Animals injected with 250 μM of 5,7-DHT reproduced these tendencies more clearly (example: Fig. 3C). The nocturnal activity, which started with an intense light-off peak, significantly increased even in the nymphal stage compared with Ringer's solution injected control animals (t-test, $P<0.05$, Fig. 4E), forming, in some cases, a rather nocturnal pattern (Fig. 3D). At lights-on a sharp peak occurred (Fig. 4E). In adults, activity level during the daytime was again significantly increased (t-test, $P<0.05$) because of the occurrence of the dense activity from the late night to early day (Figs. 3C, D and 4F). It appeared that lights-on peak still existed but was obscured by the gradual increase

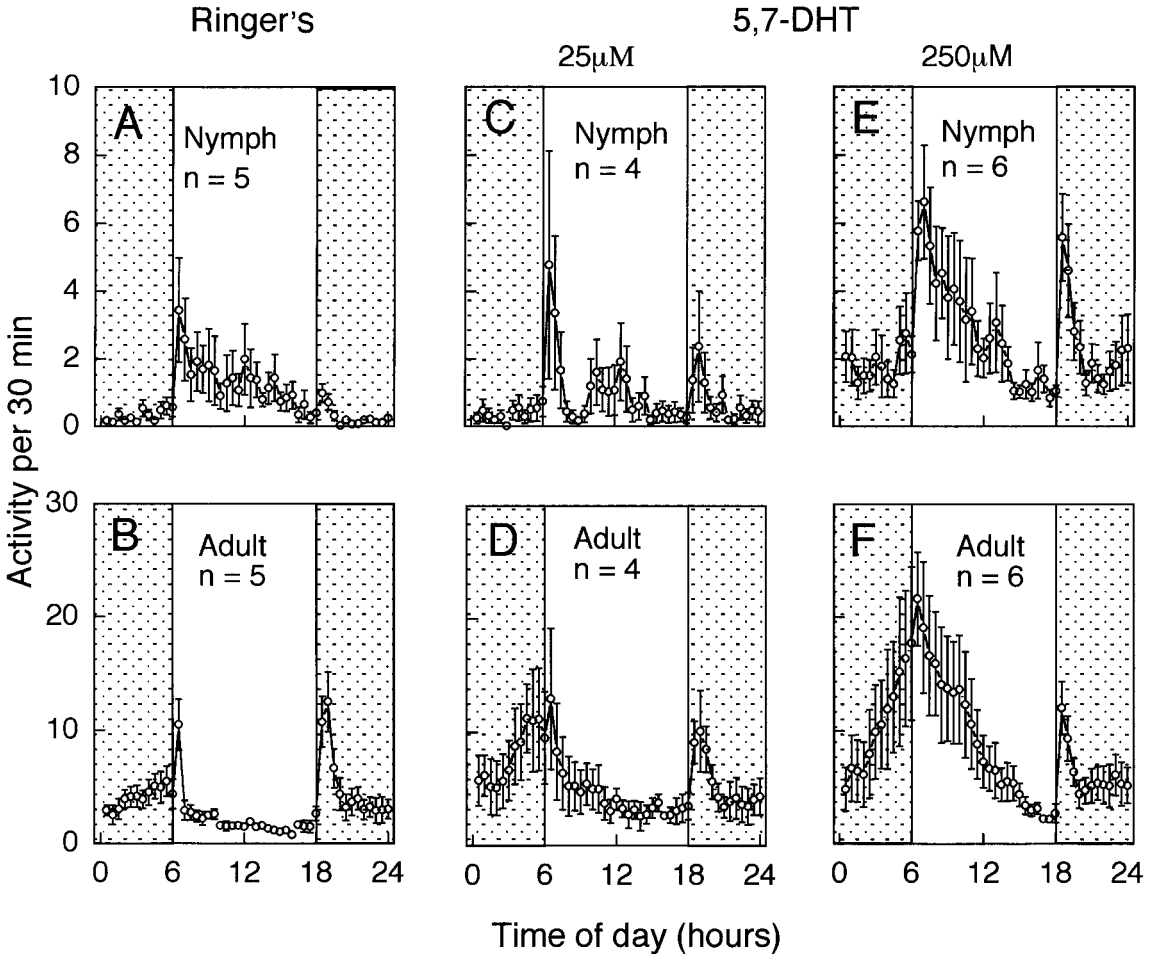

Fig. 4. Average daily activity profiles of Ringer's solution injected and 5,7-DHT injected nymphal and adult crickets. Dotted areas indicate the dark phase. Vertical bars indicate SEM. For further explanations see text.

of the activity in the late night (Fig. 4F). The activity in the nighttime was not significantly affected (t-test, P>0.1). The maximal activity at lights-off occurred earlier in the 250 μM 5,7-DHT injected adults than those injected with Ringer's solution and with 25 μM 5,7-DHT. The 5-HT level in the brain injected with 250 μM 5,7-DHT was 8.5±1.4 (SD) pmol/mg (n=5), being significantly lower than the control value of 12.1±1.0 (SD) pmol/mg (n=5, t-test, p<0.001).

DISCUSSION

The present study revealed that an increase in the cerebral 5-HT level occurred almost simultaneously to the rhythm reversal from diurnal to nocturnal, suggesting that 5-HT plays a role in the post-embryonic change in the locomotor rhythm. The involvement of 5-HT is further suggested by the results of treatment with 5,7-DHT. The chemical treatment enhanced the daytime activity both in nymphs and adults (Figs. 3 and 4), although in nymphs the enhancement was not statistically significant. The simplest explanation for this increased daytime activity level would be that 5-HT may suppress the daytime activity to facilitate the nocturnal rhythmicity in adults and this inhibitory system may also work in nymphal stage. The injection of 5,7-DHT resulted in an increase of nocturnal activity only in nymphal stage, suggesting that the nocturnal activity may be also suppressed by serotonergic system in nymphs.

It is more likely, however, that treatments with 5,7-DHT affected the coupling between the optic lobe pacemaker and the secondary oscillatory system in the brain. The previous study showed that the nymphal diurnal and the adult nocturnal rhythms coexisted during the early adult stage and that the two rhythms freeran with different freerunning period in constant darkness, suggesting that the two rhythms are controlled by two separate oscillatory centers in *G. bimaculatus* (Tomioka and Chiba, 1982). The circadian pacemaker in the optic lobe consistently shows the same circadian oscillation in its electrical activity peaking in the night (Tomioka and Chiba, 1992). It is thus suggested that the post-embryonic change from the diurnal to the nocturnal rhythm is attributable to the developmental change in the coupling between the pacemaker and the oscillatory centers possibly located in the brain (Tomioka and Chiba, 1989). 5-HT may modulate this coupling by reducing and enhancing the coupling strength of the pacemaker to the diurnal and the nocturnal oscillatory centers, respectively, rather than directly stimulates the locomotor activity center. In fact, the endogenous control of the diurnal activity in 5,7-DHT injected adults is evident by its onset preceding the lights-on by several hours. The hypothesis is further supported by the fact that adult crickets showed a clear diurnal rhythm similar to that of nymphs when ambient temperature was lowered to 20°C (Ikeda and Tomioka, 1993) where the 5-HT content in the brain was lowered (Nishinokubi and Tomioka, unpublished data). To examine the validity of this hypothesis, measurement of freerunning locomotor rhythm in constant darkness after the 5,7-DHT treatment should be carried out in the future study.

The rising phase of diurnal activity of adults injected with 5,7-DHT occurred significantly earlier than that of not only intact and Ringer's injected nymphs but also 5,7-DHT injected nymphs. This advance in phase may be caused by a slight shift in the coupling phase between the optic lobe pacemaker and the cerebral diurnal oscillatory center. Since the gradual advance of the onset of diurnal activity was often observed in the early adult stage (Fig. 3B-D), it may be dependent on some developmental change in the nervous system.

The result that sharp activity peaks occurred at lights-on when 5-HT was reduced by the chemical treatment (Fig. 4) is consistent with our previous report that injection with 5,7-DHT into the optic lobe resulted in the enhanced masking activity at lights-on that occurred after 6 hr phase advance (Germ and Tomioka, 1998). The sharp peaks may reflect hyperactivity of the photic information pathway for the locomotor center, since there are lines of evidence showing that 5-HT is a neuromodulator involved in the visual pathways (Nässel, 1987). For example, the administration of serotonin results in reduction of the sensitivity of visual interneurons in the crickets (Tomioka *et al*., 1993) and the honey bees (Kloppenburg and Erber, 1995).

It has been shown that 5-HT is involved in the control of locomotor and flight activity in some insects. In nocturnally active moth *Noctua pronuba*, it was shown that normal night flight was abolished, when cerebral median A cells containing a large amount of tryptophan, the precursor of 5-HT, were removed (Hinks, 1967). When injected with 5-HT, they showed enhanced duration and amplitude of the night flight (Hinks, 1967). In the cricket, *Acheta domesticus*, an injection of p-CPA (para-chlorophenylanalnin), an inhibitor of serotonin biosynthesis, resulted in arrhythmicity followed by a reversed phase activity rhythm (Renucci *et al*., 1989). Similar pattern changes in *A. domesticus* were also reported when treated with reserpine which also reduces the serotonin content (Cymborowski and Muszynska, 1974). Taken together with the results presented here, 5-HT seems to be a common neuroactive substance among insects, which determines the daily activity pattern.

The circadian pacemakers have been localized in specific tissues in various animals and their physiology has been extensively studied (Jacklet, 1969; Inouye and Kawamura, 1982; Colwell and Page, 1990; Tomioka and Chiba, 1992). However, data are scarce for the downstream regulation from the pacemaker toward the overt activity rhythms. In this study, we have suggested that serotonin has some role in the rhythm reversal from diurnal to nocturnal in the cricket *G. bimaculatus* by showing the positive correlation between its content in the brain and the behavior. This rhythm reversal could be a good model to understand the neural mechanism underlying the nocturnality and diurnality.

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