ZOOLOGICAL SCIENCE 15: 317–322 (1998)

Effects of 5,7-DHT Injection into the Optic Lobe on the Circadian Locomotor Rhythm in the Cricket, *Gryllus bimaculatus*

Monika Germ and Kenji Tomioka*

Department of Physics, Biology and Informatics, Faculty of Science, Yamaguchi University, Yamaguchi 753-8512, Japan

ABSTRACT—The effect of direct 5,7-dihydroxytryptamine (5,7-DHT) injection into the medulla region of the optic lobe on the locomotor activity was investigated in the adult male cricket, *Gryllus bimaculatus*. After a 6 hr phase advance of a light-dark cycle, the 5,7-DHT injected animals needed significantly longer time for resynchronization to the new cycle (6.55 ± 0.62 days) than the control, Ringer's solution injected animals (3.17 ± 0.15 days; $P < 0.001$, t-test). Light induced a bout of activity (i.e., masking effect) when light-dark cycle was phase advanced by 6 hr and the duration of the masking effect was significantly longer in 5,7-DHT injected animals. An initial bout of the nocturnal activity was significantly greater in the 5,7-DHT injected animal. Under constant darkness, the freerunning periods of both groups were not significantly different. Under constant light, a significantly higher percentage of 5,7-DHT injected animals showed arrhythmicity compared with the control group. An analysis carried by high-pressure liquid chromatography with electrochemical detection (HPLC-ECD) revealed that the serotonin content in the optic lobe was significantly reduced to less than 50% in the 5,7-DHT injected animals, even one month after the injection. These results suggest that serotonin plays important roles in the regulation of circadian locomotor rhythms of the cricket mainly by regulating the sensitivity of the photoreceptive system.

INTRODUCTION

Serotonin (5-HT) is one of the major biogenic amines distributing widely in the insect central nervous system and acting as a neuromodulator and a neurotransmitter (Evans, 1980; Nässel, 1988; Ali, 1997). Serotonin reportedly plays important roles in the circadian system. Muszynska-Pytel and Cymborowski (1978a, b) found that there is a distinct correlation between 5-HT level in the brain and intensity of locomotor activity during the course of 24 hr in the cricket, *Acheta domesticus*: The lowest level in the brain and hemolymph was found just after lights-off when the activity was highest. In cockroaches, Page (1987) showed that 5-HT treatment of the optic lobe phase shifts the circadian rhythm in a phase dependent manner. In the cricket optic lobe, the circadian rhythm of sensitivity of visual interneurons has a good correlation to the 5-HT content in the optic lobe, in that the sensitivity is highest when the 5-HT content is higher (Tomioka *et al.*, 1993). Moreover, injecting 5-HT into the fly's optic lobe partly mimics the daily change in the axonal size of the lamina monopolar cell L1 (Pyza and Meinertzhagen, 1996; Meinertzhagen and Pyza, 1996). However, it is still largely unclear how the serotonergic system is involved in the circadian system in insects.

One of the powerful strategy to approach this issue is to reduce 5-HT content by specific neurotoxins. 5,7-dihydroxytryptamine (5,7-DHT) is one of such neurotoxins, causing selective degeneration of serotonergic neurons (Sinhababu and Borchardt, 1988). In mammals this toxin has been already used to study the modification of circadian rhythms (Morin and Blanchard, 1991; Meyer-Bernstein and Morin, 1996; Ohi *et al.*, 1988; Smale *et al.*, 1990). In hamsters, it has been shown that 5,7-DHT increases the duration of the nocturnal activity phase in light to dark cycle (LD), lenghtens the freerunning period of the locomotor rhythms under constant light (LL), and magnifies the phase delay region in the phase response curve (Morin and Blanchard, 1991; Meyer-Bernstein and Morin, 1996). Serotonin immunohistochemistry showed an approximate 90% loss of cells from the dorsal rhaphe nucleus (Morin and Blanchard, 1991). These results suggest that the serotonergic system modulates the tonic and phasic actions of light on the hamster circadian system.

Recently, 5,7-DHT was found to be effective also in insects. Pyza and Meinertzhagen (1996) injected 5,7-DHT directly into the optic lobe of the blowfly and found that it reduced the mean axon size of L1 and L2 cells. The fact stimulates us to use this drug to investigate the role of serotonin in the circadian rhythm regulation in the cricket, *Gryllus bimaculatus*. We injected 5,7-DHT directly into the pacemaker locus, the medulla region of the optic lobe. Like in hamsters, in the injected animals, the nocturnal activity was longer and the light induced activity bursts were more prominent. On 6 hr advance

* Corresponding author: Tel. +81-839-33-5714; FAX. +81-839-33-5714.

of the 12 hr light to 12 hr dark cycle (LD 12:12), the 5,7-DHT injected animals needed significantly longer transients for reentrainment to the new cycle. Taken together, it is likely that, in crickets, serotonin is involved in the regulation of the sensitivity to light, in photic entrainment mechanism and in the control of the duration of the nocturnal activity as in mammals.

MATERIALS AND METHODS

Animals

All experiments were performed with intact, adult male crickets, *Gryllus bimaculatus*, obtained from our laboratory colonies, which were maintained at 25 ± 0.5°C in a 12 hr light and 12 hr dark cycle (LD12:12; light: 06:00–18:00, Japanese Standard Time, JST). To prevent any interindividual sound communication, one forewing was removed. One optic lobe of the crickets was also removed just before the activity recording was started, because, in crickets, one optic lobe is enough to create the rhythm (Okada *et al.*, 1991). The method for the surgery has been described elsewhere (Tomioka and Chiba, 1989).

Activity recording

The animals were kept individually in an activity chamber with rocking substratum whose movement caused by a moving animal was sensed by a magnetic reed-switch connected to a computer for data storage. The signals of the movement were summed every 6 min. Food and water were available *ad libitum* and the temperature was kept constant at 25 ± 0.5°C. Light dark cycles and constant light were given by a 15 W cool white fluorescent lamp controlled by an electric timer. The light intensity at the chamber was about 250 lux.

Injection

Male crickets with one optic lobe removed and with a clear nocturnal rhythm were fixed on a platform and anesthetized by a continuous flow of CO_2 to prevent high efflux of hemolymph. With a fine razor knife, a small window was cut in the head capsule to expose the optic lobe. 32.2 nl of 5,7-DHT (creatinin-sulfate salt, Sigma) solution or insect Ringer's solution (Fielden, 1960) was then injected into the medulla of the optic lobe with a glass micropipette, broken to a tip diameter of 5–10 μm, using a micromanipulator (Narishige, M-3333) and a nanoliter injector (WPI, A203XVY). The concentration of 5,7-DHT was 0.5 mM and dissolved in Ringer's solution containing 0.1% ascorbic acid. The injection was performed between 7:00 and 9:00 (JST).

Data analysis

Locomotor records of individuals were double plotted in a conventional manner by computer with a resolution of 6 min. The freerunning period of individuals was determined by the chi-square periodogram (Sokolove and Bushell, 1978). Values are expressed as mean ± SEM.

Measurements of biogenic amines

By means of high pressure liquid chromatography (HPLC; Eicom, EP-10) with electrochemical detection (ECD; Eicom, ECD 300) the levels of serotonin, dopamine and their metabolites (N-acetyldopamine, N-acetylserotonin, 5-hydroxyindole-3-acetic acid: 5-HIAA), in a single optic lobe were measured at the end of experiments. The injected optic lobes were dissected quickly and placed in 30 μl of ice-cold 0.1 M perchloric acid containing 100 ng/ml 3,4-dihydroxybenzylamide (DHBA) as an internal standard. The sample was homogenized and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was directly injected to the HPLC column or stored at –80°C until use. All chemicals were obtained from Sigma Chemical Co (St. Louis, Mo, USA). Standard solution of serotonin sulfate complex and other biogenic amines as well as the mobile phase were made as described in Nagao and Tanimura (1988). In brief, the mobile phase contained 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) CH_3CN as the organic modifier. The pH was adjusted to 3.6 by an addition of NaOH. The HPLC system consisted of a solvent delivery pump, an injection valve (Rheodyne), a C18 reversed-phase column (250 mm × 4.6 mm, 5 μm average particle size) 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 0.7 V versus an Ag/AgCl reference electrode. Signals from the electrochemical detector were recorded and integrated by computer (Waters, Data station 805). Comparing with standards the concentration of biogenic amines in the optic lobes were measured for 5,7-DHT injected animals and Ringer's-solution injected animals. The results were calculated in pmol/optic lobes (mean ± SD).

Statistical analysis

The difference between mean values was statistically analyzed with two-tailed Student's t-test or by χ^2-analysis. Significance was considered at $P < 0.05$.

RESULTS

Period under DD and LL

The locomotor activity of 12 5,7-DHT injected and 11 Ringer's injected animals was recorded. They were held in LD12:12 for the first three or four days, then transferred to constant darkness (DD) and subsequently to LL at 6:00 on day 17 or 18. Figure 1 shows representative activity records for Ringer's injected (Fig. 1A) and 5,7-DHT injected (Fig. 1B) animals. Under DD, the freerunning period of 5,7-DHT injected animals was 23.55 ± 0.11 hr, which was slightly shorter, but not significantly different from that of Ringer's-injected animals (23.79 ± 0.21 hr). Under LL, 80% of 5,7-DHT injected animals showed arrhythmicity: the ratio was significantly greater as compared with the control, Ringer's injected animals (18%, $P < 0.01$, χ^2-test). In 5,7-DHT injected animals, the duration of initial intense nocturnal activity became longer in a few days after the injection (Fig. 1B); The average duration of the first nocturnal bout was 4.05 ± 0.35 hr which was significantly longer than that of the control animals (1.63 ± 0.07 hr; $P < 0.001$, t-test).

Resychronization and masking effect

The resynchronizability to a 6 hr shifted LD was examined in 21 animals injected with 5,7-DHT and 16 animals with Ringer's-solution. Three days after the injection, the LD cycle was phase advanced by 6 hr (lights-on 0:00, lights-off 12:00), by shortening the dark phase. Figure 2 shows representative activity records for a Ringer's injected animal (Fig. 2A) and a 5,7-DHT injected animal (Fig. 2B). After the 6 hr advance shift, the Ringer's solution injected animals needed 3.17 ± 0.15 days until resynchronization to the new LD cycle. The animals injected with 5,7-DHT, however, needed 6.55 ± 0.33 days and the entrainment was not so robust in comparison with the control animals. The difference in numbers of transient cycles was statistically significant ($P < 0.001$, t-test). Two 5,7-DHT

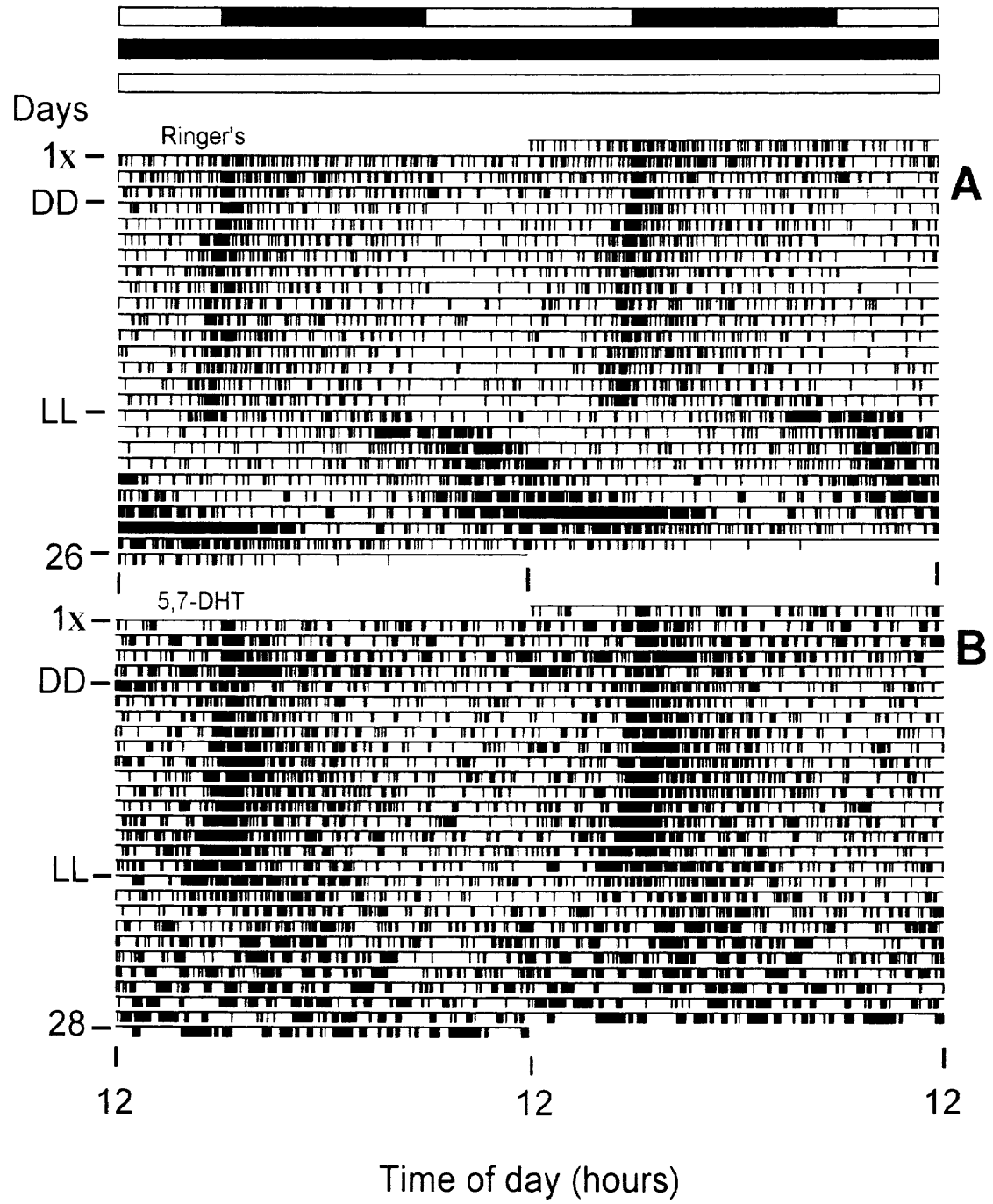

Fig. 1. Locomotor rhythms of crickets injected with Ringer's-solution (**A**) or 5,7-DHT (**B**) on the first day and transferred to constant darkness (DD) and subsequently to constant light (LL). Under DD both animals freeran with a period shorter than 24 hr. Under LL the Ringer's injected animal (**A**) freeran with a period longer than 24 hr, while the 5,7-DHT injected animal (**B**) showed no clear rhythm. Black and white bars indicate light (white) and dark (black) portions, respectively.

injected animals needed more than 12 days for the reentrainment to the new cycle.

During the transient cycles the masking effect was clearly induced at lights-on, where the late subjective night phase was exposed to light. The light induced masking effect was significantly greater in the 5,7-DHT injected animals: The duration of masking activity in the injected animals were significantly longer than that of Ringer's injected controls ($P < 0.001$, t-test) from the first day of phase-shift until the fourth day when the phase shifts almost completed in the control animals (Fig. 3). In the steady state entrainment to the shifted LD, the initial bout of nocturnal activity persisted for 1.29 ± 0.3 hr in the Ringer's solution injected animals, whereas in the 5,7-DHT injected animals it continued for 3.34 ± 0.21 hr, being significantly longer ($P < 0.01$, t-test).

Biogenic amine concentration

The biogenic amine concentration in the optic lobes of 5,7-DHT injected and Ringer's-injected animals was significantly different only for serotonin. As shown in Fig. 4, the con-

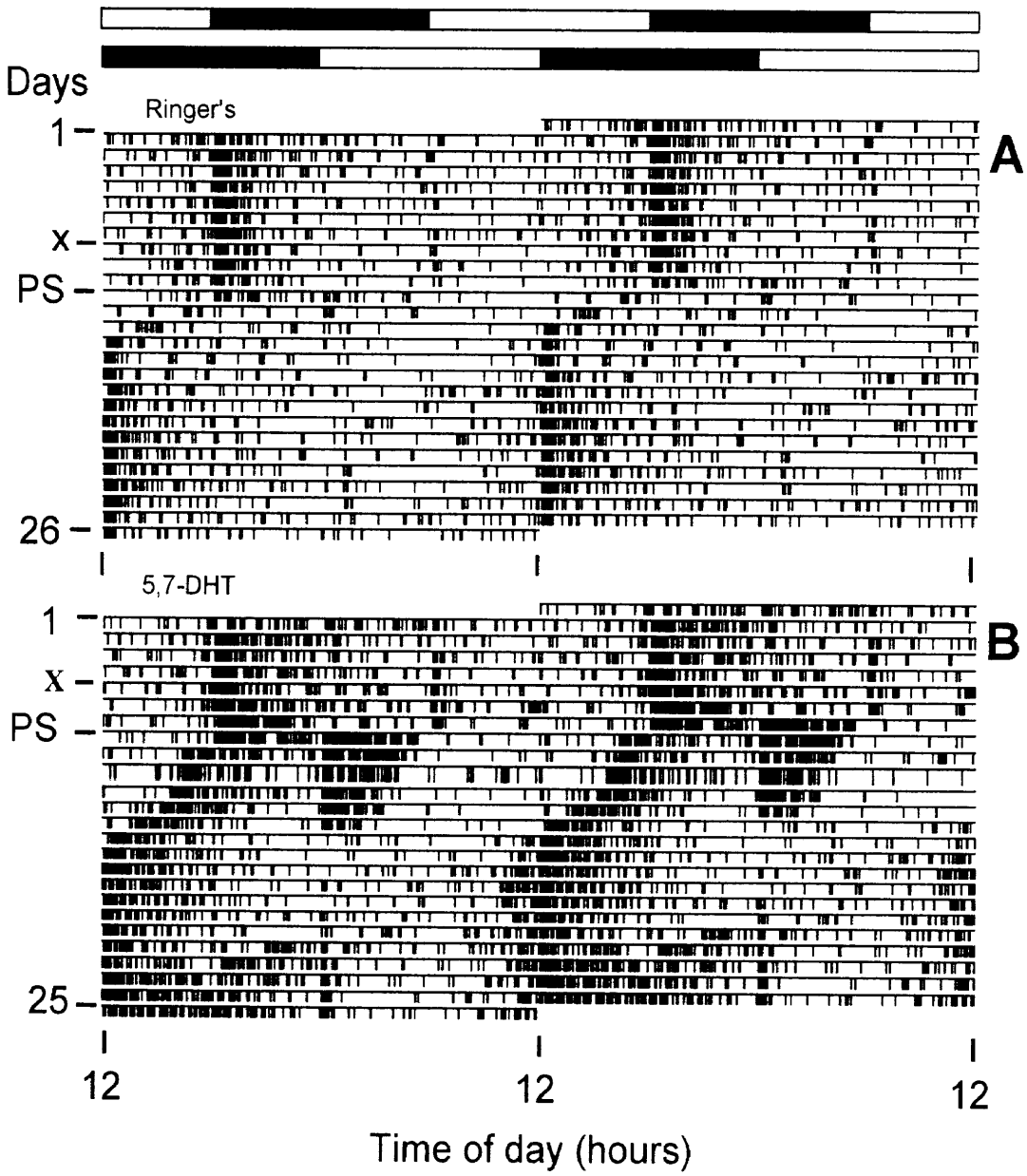

Fig. 2. Locomotor rhythms of crickets injected with Ringer's solution (**A**) or 5,7-DHT (**B**) on the day indicate by x. Three days after the injection, indicated by PS the animals were transferred to a 6 hr advanced LD cycle. The Ringer's injected animal (**A**) needed 3 days until entrained to the new cycle, while the 5,7-DHT injected animal (**B**) needed 7 days. The lights-on induced masking effect was more prominent in the 5,7-DHT injected animal. For further explanations see Fig. 1 and text.

centration of serotonin was 2.85 ± 0.88 pmol/optic lobe in the 5,7-DHT injected animals, which was significantly lower than that of Ringer's injected animals (6.43 ± 1.07 pmol/optic lobe; $P < 0.001$, t-test). The concentrations of N-acetyldopamine, dopamine, 5-HIAA, N-acetylserotonin were similar in both groups.

DISCUSSION

Selective reduction of serotonin

Animals with 5,7-DHT injected into the optic lobe showed the locomotor rhythm clearly different from that of Ringer's injected animals. Direct injection of the chemical into the medulla region of cricket's optic lobe resulted in significant reduction of serotonin level in the tissue without apparent damage to the dopaminergic system. The serotonin level was reduced to 50% of that in the Ringer's injected animals when examined 4 weeks after the injection. Preliminary results showed that it was even reduced to nearly 20% of that of the control 2 days after the injection (Germ, unpublished data). However, the content of serotonin in the brain did not change significantly after the injection of 5,7-DHT into the optic lobe (Germ, unpublished data). Since the content of dopamine as well as metabolites of dopamine and serotonin were at the similar level, the change in locomotor rhythms would be attributable to the reduced level of serotonin in the optic lobe, although we should carefully examine other biogenic amines in the future study.

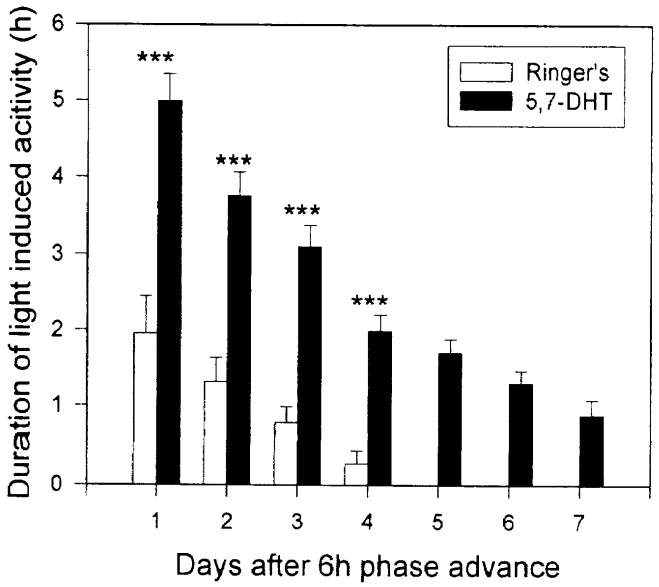

Fig. 3. The daily duration (mean ± SD) of the light induced activity (masking effect) after the 6 hr phase advance for Ringer's injected crickets (white columns) and for 5,7-DHT injected crickets (black columns). n = 16–21; ***, P < 0.001.

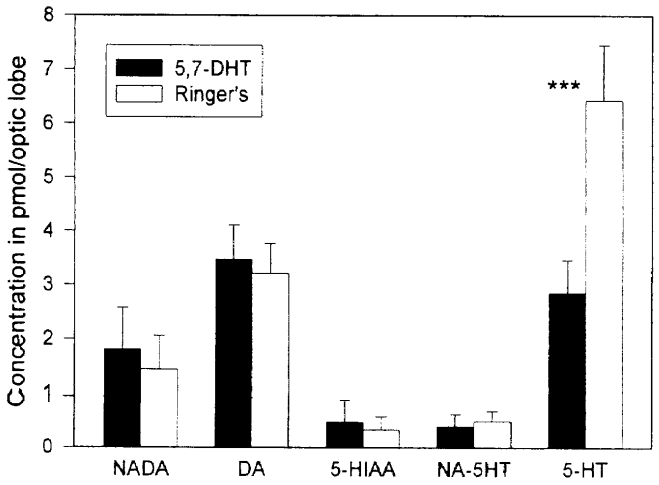

Fig. 4. The concentration (mean ± SD) of biogenic amines measured by HPLC-ECD in the optic lobes of Ringer's solution injected animals (white columns) and 5,7-DHT injected animals (black columns). NADA, N-acetyldopamine; DA, dopamine; 5-HIAA, 5-hydroxyindole-3-acetic acid; NA-5HT, N-acetylserotonin; 5-HT, 5-hydroxytryptamine, serotonin. n = 14-18; ***, P < 0.001.

Role of serotonin in the circadian system

The fact that the freerunning period under DD is not significantly different between 5,7-DHT injected animals and Ringer's injected controls suggests that serotonin is not a necessary component for the generation of circadian rhythm. However, it seems to have important roles in the circadian system. The present study revealed that reduction of serotonin in the optic lobe resulted in 4 major effects: the longer transient cycles for resynchronization, the greater masking effects of light, the longer duration of the initial bout of nocturnal activity, and frequent arrhythmicity in LL.

The 5,7-DHT injected animals showed light-induced masking effects significantly longer than the control animals. This intensified masking effect may be attributable to the reduced level of serotonin in the optic lobe. There are lines of evidence showing that serotonin is a neuromodulator in the insect visual system (Nässel, 1988). The administration of serotonin reportedly reduces the sensitivity of visual interneurons not only in the cricket (Tomioka *et al.*, 1993) but also in the honey bee (Kloppenburg and Erber, 1995). It is thus likely that the reduced level of serotonin releases the visual interneurons from serotonergic inhibition to cause a higher sensitivity to light which would eventually result in the higher light-induced masking effect. The higher percentage of arrhythmicity in 5,7-DHT injected animals under LL also suggests the increased sensitivity of the visual system, since the cricket often became arrhythmic when the illumination level of LL was increased (Tomioka and Chiba, 1982).

The 5,7-DHT injected animals showed the longer initial bout of nocturnal activity both under LD and DD (Figs. 1, 2). In the cricket, the center for generating the locomotor activity is thought to reside in the central brain and it produces the rhythm under the regulation of the optic lobe pacemaker (Tomioka and Chiba, 1989). Since the 5,7-DHT injection seems to affect the serotonin content only in the optic lobe, it is thus likely that the prolonged first nocturnal activity bout is caused by the increased output from the optic lobe pacemaker. This hypothesis is also supported by the following facts. First, when serotonin is injected into the haemoceal, the activity of the injected animal is substantially reduced (Okada and Tomioka, unpublished observation). Second, the application of exogenous serotonin to the optic lobe compound eye system *in vitro* reduces the neural activity in the optic stalk (Tomioka, 1994). Since similar relationship between the serotonin level and the locomotor activity has also been reported for 5,7-DHT injected hamsters (Morin and Blanchard, 1991), the role of serotonergic system in regulation of locomotor activity may be common among insects and vertebrates.

One of our most significant findings is that the 5,7-DHT injected animals needed significantly longer transient cycles until their locomotor rhythms were entrained to a 6 hr advanced LD. The longer transients seem to contradict to our hypothesis that the reduced level of serotonin increases the sensitivity of photoreceptive system. One possibe explanation for this is that serotonin may act more directly on the circadian pacemaker through a pathway distinct from the one for photic entrainment. There are numbers of reports showing that the serotonergic system is a phase shifting agent inducing a phase response curve different from the one for light in the marine gastropod *Aplysia californica* (Corrent and Eskin, 1982), in the cockroach *Leucophaea maderae* (Page, 1987), in the cricket *Gryllus bimaculatus* (Tomioka, 1994) and in the rat (Prosser *et al.*, 1993).

Masking activity may also contribute to the longer transient cycles. It is reported in mammals that the forced activity often causes the phase shifts in a phase dependent manner and that it is the morning phase during which the phase delay is induced (Reebs and Mrosovsky, 1989). Although there is no report available for the feedback of activity to the pace-

making system in insects, the fact that, in 5,7-DHT injected animals, the longer masking effects of lights-on always accompanied the longer transient cycles may be also interpreted by the phase-delay or deceleration of the circadian rhythm. The serotonergic system may reduces the sensitivity of the photoreceptive system to suppress the light-induced untimely activity and facilitate the reentrainment.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan. Monika Germ is a postdoctoral fellow supported by the Japan Society for the Promotion of Science.

REFERENCES

Ali DW (1997) The aminergic and peptidergic innervation of insect salivary glands. J Exp Biol 200: 1941–9

Corrent G, Eskin A (1982) Transmitterlike action of serotonin in phase shifting a rhythm from the *Aplysia* eye. Am J Physiol 242: R333–338

Evans PD (1980) Biogenic amines in the insect nervous system. Adv Insect Physiol 15: 317–473

Fielden A (1960) Transmission through the last abdominal ganglion of the dragonfly nymph. J Exp Biol 37: 832–844

Kloppenburg P, Erber J (1995) The modulatory effects of serotonin and octopamine in the visual system of the honey bee (*Apis mellifera*). II. Electrophysiological analysis of motion-sensitive neurons in the lobula. J Comp Physiol 176: 119–129

Meinertzhagen IA, Pyza E (1996) Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. TINS 19, 7: 285–291

Meyer-Bernstein EL, Morin LP (1996) Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. J Neurosci 16: 2097–2111

Morin LP, Blanchard J (1991) Depletion of brain serotonin by 5,7-DHT modifies hamster circadian rhythm response to light. Brain Res 566: 173–185

Muszynska-Pytel M, Cymborowski B (1978a) The role of serotonin in regulation of the circadian rhythms of locomotor activity in the cricket (*Acheta domesticus* L.) I. circadian variations in serotonin concentration in the brain and hemolymph. Comp Biochem Physiol 59C: 13–15

Muszynska-Pytel M, Cymborowski B (1978b) The role of serotonin in regulation of the circadian rhythms of locomotor activity in the cricket (*Acheta domesticus* L.) II. Distribution of serotonin and variations in different brain structure. Comp Biochem Physiol 59C: 17–20

Nagao T, Tanimura T (1988) Distribution of biogenic amines in the cricket central nervous system. Anal Biochem 171: 33–40

Nässel DR (1988) Serotonin and serotonin-immunoreactive neurons in the nervous system of insects. Prog Neurobiol 30: 1–85

Ohi K, Hayashi S, Takahashi K (1988) Development of circadian rhythms in rats with lesions of serotonergic system. Physiol Behav 44: 393–403

Okada Y, Tomioka K, Chiba Y (1991) Circadian phase response curves for light in nymphal and adult crickets, *Gryllus bimaculatus*. J Insect Physiol 37: 583–590

Page TL (1987) Serotonin phase-shifts the circadian rhythm of locomotor activity in the cockroach. J Biol Rhythms 2: 23–34

Prosser RA, Dean RR, Edgar DM, Heller HG, Miller JD (1993) Serotonin and the Mammalian Circadian System: I. *in vitro* phase shifts by serotonergic agonists and antagonists. J Biol Rhythms 8: 1–16

Pyza E, Meinertzhagen IA (1996) Neurotransmitters regulate rhythmic changes amongst cells in the fly's optic lobe. J Comp Physiol A 178: 33–45

Reebs SG, Mrosovsky N (1989) Effects of induced wheel running on the circadian activity rhythms of syrian hamsters: entrainment and phase response curve. J Biol Rhythms 4: 39–48

Sinhababu AK, Borchardt RT (1988) Molecular mechanism of biological action of the serotonergic neurotoxin 5,7-dihydroxytryptamine. Neurochem Int 12: 273–284

Smale L, Michels KM, Moore RY, Morin LP (1990) Destruction of the hamster serotonergic system by 5,7-DHT: effects on the circadian rhythm phase, entrainment and response to triazolam. Brain Res 515: 9–19

Sokolove PG, Bushell WN (1978) The chi square periodogram: its utility for analysis of circadian rhythms. J Theoret Biol 72: 131–160

Tomioka K (1994) Serotonin phase-shifts the cricket optic lobe circadian pacemaker *in vitro*. Zool Sci 11 (Supplement):110

Tomioka K, Chiba Y (1982) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 147: 299–304

Tomioka K, Chiba Y (1989) Photoperiodic entrainment of locomotor activity in the crickets (*Gryllus bimaculatus*) lacking the optic lobe pacemaker. J Insect Physiol 35: 827–835

Tomioka K, Ideka M, Nagao T, Tamotsu S (1993) Involvement of serotonin in the circadian rhythm of an insect visual system. Naturwissenschaften 80: 137–139

(Received February 2, 1998 / Accepted March 5, 1998)