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Serotonin precursor (5-hydroxytryptophan) has a profound effect on the post-copulatory time-fixed sexually refractory stage in the male cricket, *Gryllus bimaculatus* DeGeer

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Abstract This study addresses a potentially general basis of measuring time in a biological timer. Here, we examined the effects of biogenic amines on the time-fixed post-copulatory sexually refractory stage (ca. 1 h) which is defined as the time interval between spermatophore protrusion and the onset of a calling or a mating response in the reproductive cycle of the male cricket. For subcuticular injection of amines (0.15 ml, 10^{-2} mol l^{-1}), the interval of the refractory stage was shortened by octopamine, serotonin, 5-hydoxytryptophan and *N*-acetyl-serotonin but was unchanged by tryptophan, melatonin or 5-hydroxyindol-3-acetic acid. The effect of 5-hydoxytryptophan was most potent (maximum shortening, 38%) and long lasting (ca. 4.5 h) while other amines effected only the injected cycle. Injection of 5-hydoxytryptophan (180 nl, 10^{-2} mol l^{-1}) into the terminal abdominal ganglion also decreased the interval to a similar extent. Simultaneous injection of 5-hydoxytryptophan with the inhibitor of the serotonin synthesis enzyme reduced the 5-hydoxytryptophan effect suggesting that this effect results from synthesis of serotonin from 5-hydoxytryptophan. The protein synthesis inhibitor cycloheximide had no effect on the interval. These results suggest that the reproductive timer is regulated by serotonergic neurons in the terminal abdominal ganglion without protein synthesis during the interval of the time-fixed sexually refractory stage.

Keywords 5-Hydroxytryptophan · Male cricket · Serotonin · Sexual refractoriness · Timer

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Abbreviations *5-HT* serotonin · *5-HTP* 5-hydroxytryptophan · *CS* calling song · *MR* mating response *RS1* refractory stage 1 · *RS2* refractory stage 2 · *SE* spermatophore extrusion · *SP* spermatophore protrusion · *SPCS* interval between spermatophore protrusion and calling song · *SPMR* interval between spermatophore protrusion and mating response

Introduction

Animal behavior is often regulated in a time-dependent fashion. The general activity of animals in the day-night cycle is mostly under the control of circadian clocks (Edmunds 1988), and occasionally under the control of an hourglass timer triggered by an external stimulus (Lees 1973; Skopik and Bowen 1976; Arai 1977; Veerman 2001). In mammalian reproductive behavior, ovulation in many females occurs periodically under the automatic control of neurohormonal mechanisms (Freeman 1994), while in males ejaculation following repetitive intromission recurs with some interval (Sachs and Barfield 1974; Meisel and Sachs 1994) which is several minutes in the rat (Dewsbury 1967; Kurtz and Adler 1973; Horio et al. 1986) and about 30 min in the monkey (Oomura et al. 1983). It has been assumed that such a post-copulatory sexually refractory state is due to a central inhibitory mechanism rather than to a simple recovery process from fatigue (Kurtz and Adler 1973; Sachs and Barfield 1974). However, it seems unnecessary to postulate a "timer" for the sexually refractory state because it is variable, shortened by electrical shock, and it gradually increases as ejaculation is repeated (Kurtz and Adler 1973; Barfield and Geyer 1975).

In invertebrate reproductive behavior, some female insects, such as the fruit fly (Pyle and Gromko 1978), butterfly (Sims 1979) and grasshopper (Loher and Huber 1966), show mating refusal after copulation and their sexual receptivity is gradually resumed after days or weeks (Thornhill and Alcock 1983). This long and

loose period of the sexually refractory state is known to be caused by the spermatophore, or its contents, transferred from the male during copulation (Sugawara 1979, 1981; Obara 1982; Chen et al. 1988; Wolfner 1997; Hartmann and Loher 1999). Thus, again it is unnecessary to assume an innate time-keeping mechanism in such female insects.

One of the best examples of an innate "timer" concerns the post-copulatory sexual refractoriness in male crickets (Khalifa 1950; Huber 1955; von Hörmann-Heck 1957; Alexander 1961; Beck 1974; Loher and Rence 1978; Adamo and Hoy 1994; Zuk and Simmons 1997). In the cricket *Gryllus bimaculatus* the male becomes sexually inactive immediately after it extrudes the spermatophore (spermatophore extrusion) in the last stage of copulation. A few minutes later, it protrudes spermatophore material onto the ventral lobes of the external genitalia (spermatophore protrusion) to prepare for the next copulation and shows an aggressive attitude toward the female. The male remains sexually inactive for a rather fixed period after spermatophore protrusion, and then recommences courtship to the female and will even show a mating response to an artificially applied contact stimulus (Huber 1955; Sakai and Ootsubo 1988; Sakai et al. 1995; Matsumoto and Sakai 2000a, 2000b). Thus, the male has a reproductive cycle consisting of three main events: spermatophore extrusion, spermatophore protrusion and courtship (or mating response).

Within this cycle, the interval (RS1, refractory stage 1) between spermatophore extrusion and spermatophore protrusion is lengthened by stress or by the absence of a female (Nagao and Shimozawa 1987; Ootsubo and Sakai 1992), but the interval (RS2, refractory stage 2) between spermatophore protrusion and the calling song or mating response is unchanged by internal stress or external disturbances (Nagao and Shimozawa 1987; Sakai et al. 1995). Although RS2 varies among individuals, it is fairly constant within individuals, and it is thus called the time-fixed sexually refractory stage (Ureshi and Sakai 2001). Moreover, RS2 is neither controlled by afferent feedback arising from the presence of the maturing spermatophore (Loher and Rence 1978; Nagao and Shimozawa 1987) nor from the genitalia itself (Sakai et al. 1991).

Recently, we have used local cooling of the central nervous system (CNS) to show that the timer for RS2 is located within the terminal abdominal ganglion (Ureshi and Sakai 2001). The timer is temperature dependent; its cycle time decreases linearly at higher temperatures and increases at lower temperatures. It is stopped below 10°C and re-started without reset when returned to normal temperature. These facts suggested that some metabolic processes may underlie the time-keeping mechanism of the reproductive timer.

Previously, Nagao and Shimozawa (1987) hypothesized that the biogenic amines, octopamine and serotonin (5-HT) in the hemolymph may be the substrates for the timer: both amine concentrations in the hemolymph change gradually after spermatophore protrusion and attain a certain ratio after the pre-set time. However, we disagreed with this hemolymph amine-hypothesis because perfusion of the hemocoel with saline during RS2 did not change the interval of RS2 (Ureshi and Sakai 2001). This fact does not mean, however, that biogenic amines are not related to the reproductive timer system. Rather, in some way they may be involved in regulating the timer within the ganglia since there are a number of reports that serotonin modifies the time-keeping system in circadian rhythms (Eskin et al. 1982; Page 1987; Proccer et al. 1993; Tomioka 1999; Chen et al. 1999; Saifullah and Tomioka 2002).

Thus, we re-examined the effects of octopamine and serotonin on the time-fixed sexually refractory stage (RS2) and then we investigated the effects of other amines by subcuticular and intraganglionic injection. As a result, we found that the precursor of 5-HT, 5-hydroxytryptophan (5-HTP) was strikingly potent in shortening RS2. A preliminary report appeared elsewhere (Ureshi and Sakai 2000).

Materials and methods

As the experimental procedures have already described in detail in a previous paper (Ureshi and Sakai 2001), only brief descriptions are given here.

Animals

Crickets, *Gryllus bimaculatus* DeGeer, were reared under a constant light-dark cycle (L:D = 12:12) at 27 ± 2°C. They were fed with insect diet and water ad libitum. Male and female crickets were used 10–15 days after the final molt. Males were separated from females at least 24 h before use.

Responses to a model stimulus

Males in the pre-copulatory mating stage normally exhibit courtship, including a calling song and a courtship song to the female, and show a mating response consisting of cercal vibration, backward walking and hooking onto a model stimulus mimicking the female (Matsumoto and Sakai 2000a). In contrast, males in RS2 show an evasion response including body withdrawal, kicking or forward walking to the same stimulus.

Test procedures

The mating response test commenced after a male had copulated with a female (in a 100-ml beaker) and spermatophore protrusion had occurred in readiness for subsequent mating. The male was stimulated with a model female stimulus every minute. Males continuously respond to this stimulus by evasion for about the first 60 min, and then they begin to show the mating response and calling song (Ureshi and Sakai 2001).

Definitions of intervals

The time interval from spermatophore extrusion (SE) to the mating response (MR) or to the calling song (CS) is called here the sexually refractory stage, and the time interval from the CS to SE is called the mating stage (Fig. 1). The former is divided into the interval (RS1) between SE and spermatophore protrusion (SP), and the interval (RS2) between SP and the recommencement of the MR or

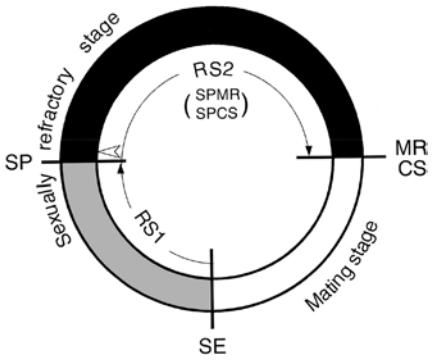

Fig. 1 The reproductive cycle of the male cricket. *CS* calling song; *MR* mating response; *RS1* sexually refractory stage 1; *RS2* sexually refractory stage 2; *SE* spermatophore extrusion; *SP* spermatophore protrusion. *SPCS* the interval between SP and CS; *SPMR* the interval between SP and MR. The *white arrowhead* by SP indicates the timing of drug injection. See Materials and methods for details

the re-emission of the CS. RS2 measured by the MR is abbreviated as SPMR, and RS2 measured by the CS, SPCS.

Drugs

Drugs were dissolved in saline [(in mmol l^{-1}): NaCl 150; KCl 9.0; $CaCl_2/H_2O$ 5.0; $NaHCO_3$ 2.0; glucose 40 in distilled water adjusted to pH 7.2 with NaOH] to different concentrations (10^{-2} to 10^{-4} mol l^{-1}) just before use. Only melatonin was dissolved in 2% methanol and diluted 1:10 (maximum concentration was thus 10^{-3} mol l^{-1}). Amines used were as follows: DL-octopamine (OA, Sigma), L-tryptophan (Trp, Wako), 5-hydroxytryptophan (5-HTP, Sigma), 5-hydroxytryptamine (5-HT, Sigma), *N*-acetyl-5-hydroxytryptamine (NA-5-HT, Sigma), melatonin (Met, Nakarai), 5-hydroxyindole-3-acetic acid (5-HIAA, Sigma). To inhibit the 5-HT synthesis enzyme (nonspecific aromatic L-amino acid decarboxylase, AADC), *m*-hydroxybenzylhydrazine (NSD-1015, Sigma) was used. Cytosolic protein synthesis at the translation level was inhibited by cycloheximide (CHX, Sigma)

Drug injection

Drugs were administered to males as soon as they exhibited SP (Fig. 1, open arrowhead).

Subcuticular injection

An amine solution (0.15 ml) was injected into the hemocoel with a 1-ml syringe through the cuticle at the base of a hindleg. The volume injected was approximately 1.5 times as much as the typical volume of the cricket hemolymph (0.1 ml). To check the distribution of the injected solution in the hemocoel, methylene blue solution was injected. The entire central and peripheral nervous system, including the terminal abdominal ganglion, was stained uniformly blue. The amines and NSD-1015 were injected alone or in combination with 5-HTP.

Intraganglionic injection

Males were anesthetized by immersing the head in water/ice for 1 min. A U-shaped window was then cut in the 6–8th abdominal sternites and the terminal abdominal ganglion was slightly pulled up with a stainless holder. The ganglion was then penetrated with the tip (about 30 μm diameter) of a 1-mm-diameter glass pipette. Amine solution (180 nl, a similar volume of the terminal abdominal ganglion) was injected with a pressure microinjection device (Narishige, IM-30) for 2 s. Success in injecting a solution into the ganglion was confirmed by swelling of the ganglion. For controls, only saline was injected, and for a sham-control the ganglion was penetrated with only the tip of a glass pipette. The cut cuticle was then closed. Injection into the metathoracic ganglion was performed similarly after opening the metathoracic sternite. To facilitate quick recovery from the cold anesthesia the head of the cricket was immersed in warm water (35°C). It took about 8 min for the operation and injection, and 2 min for the recovery. After this treatment, the male was separated from the female for at least 5 min to avoid biting of the cut area by the female.

Hemocoel washing

To wash out the injected drug from the hemocoel, saline (0.2 ml) was slowly injected into the hemocoel through the dorsal region of the thorax with a syringe. When the abdomen was expanded, two holes were made on the cuticle near the cerci with an insect pin to drain the hemolymph. Washing was facilitated by rubbing the abdomen gently several times with the experimenter's fingers. This treatment (about 1 min, each) was repeated twice with saline.

Data presentation and analysis

The RS2 (SPMR and SPCS) time intervals are presented as medians and 95% confidence intervals (CI) (Tables 1, 2). To show the effect of drugs on RS2, the change ratio was calculated by dividing a treatment RS2 by the RS2 in the non-injected cycle. Some of the data are shown in histograms to indicate the variation in RS2 between individuals. For statistical analysis, the Mann-Whitney *U*-test was used, with the significance level set at $P = 0.05$.

Results

Subcuticular injection of biogenic amines

Saline controls

In order to examine the effect of volume of the injected solution, only saline (0.15 ml) was injected into the hemocoel. The SPMR interval and the SPCS interval were both 60 min (CI, 58–62, $n = 34$). These values were nearly the same as 59 min (CI, 57–61) and 60 min (CI, 58–62, $n = 34$) respectively in males with no injection. Males exhibited the normal mating response (MR) and calling song (CS), except one male which showed auto-spermatophore extrusion (auto-SE) (see Ureshi and Sakai 2001): it ejected the spermatophore without any prior MR or courtship. Since neither RS2 interval was affected by saline injection, RS2 values in males with amines injected were compared with RS2 values in males with no injection in the following experiments.

Effects of amines

OA, 5-HT, NA-5-HT, or 5-HTP was injected at different concentrations. OA significantly shortened both SPMR and SPCS at 10^{-2} mol l^{-1} but not at lesser concentrations (Table 1). The change ratio was 1.00 for SPMR and 1.00 for SPCS at 10^{-3} mol l^{-1}, and 0.88 and 0.83 at

Table 1 Effects of subcuticular injection of biogenic amines on refractory stage (RS). The values of RS2 are presented as medians with confidence intervals in brackets. *n* number of males showing calling song (CS) and mating response (MR). (*5-HIAA* 5-hydroxyindole-3-acetic acid; *5-HT* serotonin; *5-HTP* 5-hydroxtryptophan; *aSE* auto-spermatophore extrusion; *Met* melatonin; *NA-5-HT* *N*-acetylserotonin; *OA* octopamine; *SPCS* the interval between spermatophore protrusion (SP) and CS; *SPMR* the interval between SP and MR; *Trp* L-tryptophan)

*Significant difference at $P < 0.05$;
**$P < 0.01$

	Concentration (mol l^{-1})	RS2 (min)				*n*	Abnormal males
		Before injection		After injection			aSE
		SPMR	SPCS	SPMR	SPCS		(% of males tested)
Saline	/	59 [57–61]	60 [58–62]	60 [58–62]	60 [58–62]	34	1 (2.9)
OA	10^{-2}	64 [61–67]	65 [62–68]	56 [53–59]**	54 [51–57]**	20	1 (5.0)
	10^{-3}	64 [61–67]	66 [63–69]	64 [61–67]	66 [63–69]	22	0
	10^{-4}	59 [57–61]	65 [63–68]	65 [62–68]	68 [65–71]	26	1 (3.8)
5-HT	10^{-2}	61 [58–64]	64 [61–67]	41 [38–44]**	51 [46–56]**	32	6 (18.8)
	10^{-3}	60 [57–63]	62 [60–64]	54 [51–57]	56 [53–59]	25	4 (16.0)
	10^{-4}	61 [57–64]	62 [59–65]	61 [57–64]	62 [59–65]	26	0
NA-5-HT	10^{-2}	60 [57–63]	61 [58–64]	45 [42–48]**	48 [46–51]**	30	4 (13.3)
	10^{-3}	60 [57–63]	61 [57–63]	61 [57–63]	63 [60–66]	22	2 (9.1)
	10^{-4}	62 [60–64]	62 [60–64]	60 [57–63]	63 [61–65]	34	0
5-HTP	10^{-2}	64 [61–67]	67 [64–70]	43 [41–45]**	53 [50–56]**	35	0
	10^{-3}	61 [59–63]	62 [59–65]	54 [52–56]*	56 [54–58]*	21	0
	10^{-4}	59 [57–61]	62 [60–64]	58 [56–60]	62 [60–64]	27	1 (3.7)
Trp	10^{-2}	62 [59–65]	63 [60–66]	64 [61–67]	64 [61–67]	24	3 (12.5)
5-HIAA	10^{-2}	65 [62–68]	65 [61–69]	66 [62–70]	68 [65–71]	22	1 (4.8)
Met	10^{-3}	62 [59–65]	66 [63–69]	63 [60–66]	67 [65–69]	25	0

Table 2 Effects of biologenic amines injected into the terminal abdominal ganglion (TAG) or metathoracic ganglion (MtG) on RS2. RS2 is given as median with confidence intervals in brackets. *n* number of males showing CS and MR; *nRA* (no reproductive action) – males not exhibiting MR, CS or aSE

	Ganglion	RS2 (min)				*n*	Abnormal males	
		Before injection		After injection			aSE	nRA
		SPMR	SPCS	SPMR	SPCS		(% of males tested)	
Sham	TAG	64 [61–67]	66 [63–69]	65 [61–69]	63 [54–72]	22	5 (22.7)	4 (18.2)
Saline	TAG	62 [60–64]	64 [62–66]	68 [62–74]	74 [69–79]**	33	5 (15.2)	12 (36.4)
5-HT	TAG	61 [56–66]	62 [57–67]	48 [36–60]**	58 [47–69]	24	6 (25)	6 (25)
5-HTP	TAG	64 [61–67]	65 [62–68]	35 [30–40]**	39 [35–43]**	28	1 (3.6)	11 (39.3)
	MtG	64 [59–68]	65 [60–69]	66 [63–68]	68 [65–71]	15	1 (6.7)	0

10^{-2} mol l^{-1}, respectively (Fig. 2a). Auto-SE rate was 5.0%. Males injected with OA tended to be more aggressive to females or to the model stimulus, compared with saline-injected males.

5-HT shortened both SPMR and SPCS (Table 1). The change ratio was 0.90 for SPMR and 0.90 for SPCS at 10^{-3} mol l^{-1}, and 0.67 and 0.80 at 10^{-2} mol l^{-1}, respectively (Fig. 2b). A significant decrease in RS2 was observed only at 10^{-2} mol l^{-1}. The degree of RS2 shortening (20 min for SPMR and 13 min for SPCS) was significantly greater ($P < 0.01$) than that (8 min and 11 min, respectively) in males injected with OA. The auto-SE rate (18.8%) was more than that in males injected with saline or OA. Males often excreted feces, rubbed their abdominal tips against the substrate and cleaned the cerci with the hindlegs. They occasionally rubbed out the spermatophore. But this SE occurred passively because all the males re-commenced the MR and CS but not spermatophore protrusion (SP) thereafter.

NA-5-HT significantly shortened both SPMR (0.75) and SPCS (0.79) at 10^{-2} mol l^{-1} (Table 1, Fig. 2c). These values were not significantly different from those in males injected with 5-HT. The auto-SE rate (13.3%) was similar to that in males injected with 5-HT. Abnormal behavior, such as frequent feces excretion, was not observed.

5-HTP significantly shortened both SPMR (0.67) and SPCS (0.79) at 10^{-2} mol l^{-1} (Table 1, Fig. 2d). The degree of RS2 shortening was greater ($P < 0.01$) than that in males injected with OA, but comparable to that in males injected with 5-HT and NA-5-HT. However, 5-HTP significantly ($P < 0.05$) shortened RS2 even at a concentration of 10^{-3} mol l^{-1}. In contrast to males injected with 5-HT, neither auto-SE nor feces excretion were observed. Males tended to be more sensitive to stimulation than males injected with saline: they responded vigorously to the contact stimulation by evasion or by a copulation response. Some males frequently raised the abdomen upward for some time after injection (behavioral meaning is unknown).

Duration of amine effects

To examine how long an injected amine remains effective, RS2 was measured at least two cycles after injection at a concentration of 10^{-2} mol l^{-1} (except for Met at 10^{-3} mol l^{-1}; Fig. 3). For the effect of OA, the change ratios of SPMR were 0.88 ($n = 19$) in the injected cycle and 0.98 in one cycle after injection, and those of SPCS were 0.83 and 1.01, respectively (Fig. 3a). For 5-HT, the change ratios were 0.69 and 0.95 ($n = 26$) for SPMR, and

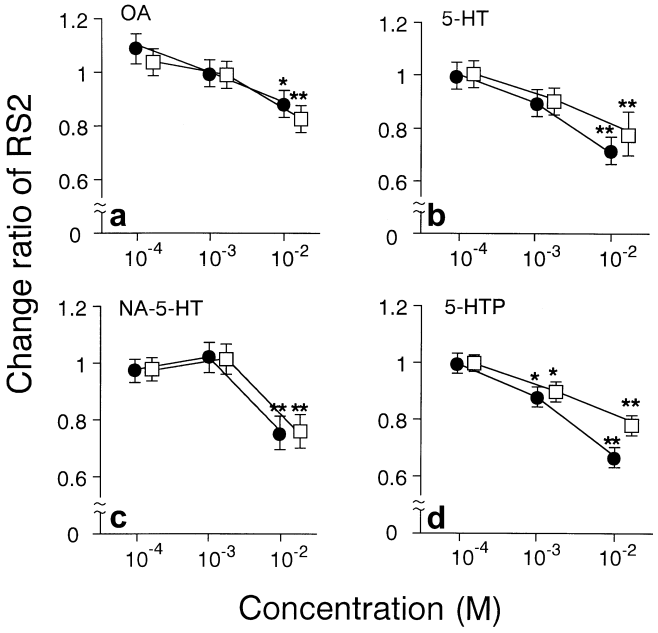

Fig. 2a–d Effects of amines at different concentrations on RS2. *Abscissa*: amine concentration; *ordinate*: the change ratio of RS2. **a** Octopamine (OA); **b** serotonin (5-HT); **c** *N*-acetylserotonin (NA-5-HT); **d** 5-hydroxytryptophan (5-HTP). *Dark circle* shows SPMR and *white square* shows SPCS. *Vertical bars*: 95% confidence interval. Significance level is indicated by *asterisks*: $*P<0.05$); $**P<0.01$. These conventions are also used in subsequent figures

0.80 and 0.94 for SPCS, respectively (Fig. 3c). No difference was present in the change ratio of SPMR or SPCS between the first cycle (non injected) and the third cycle, indicating that the effects of OA and 5-HT did not continue for more than 1 h. NA-5-HT had a similar effect to that of 5-HT (Fig. 3d). In contrast, Trp, 5-HIAA or Met had no effect on RS2 even in the injected cycle (Table 1, Fig. 3b, e, f).

In contrast to the six amines just described, 5-HTP caused a pronounced shortening of both SPMR and SPCS over at least five cycles after injection. The change ratios of SPMR ($n=8$) were 1.00 (first), 0.67 (second), 0.38 (third), 0.46 (fourth), 0.62 (fifth), 0.80(sixth), and 0.97 (seventh) (Fig. 4a). The medians of the second to sixth cycles were significantly shorter than 61 min (CI 56–66) in the non-injected cycle (first). It should be stressed that SPMRs in the third and fourth cycles were significantly shorter than that even in the injected cycle (second). The shortest SPMR (38%) was 23 min (CI, 16–30) in the third cycle. On the other hand, the change ratios of SPCS ($n=8$) were 1.00 (first), 0.76 (second), 0.67 (third), 0.63 (fourth), 0.76 (fifth), 0.79 (sixth) and 0.98 (seventh), respectively. The shortest SPCS (64%) occurred two cycles (4th) after injection which was 40 min (CI 33–47) compared with 63 min (CI, 58–68) in the first cycle.

To examine whether the prolonged effect was due to 5-HTP remaining in the hemolymph, the hemocoel was washed out with saline immediately after the male exhibited SP in the third cycle. The change ratios of SPMR ($n=6$) were 1.00 (first), 0.62 (second, injected), 0.59 (third, washed), 0.73 (fourth), 0.92 (fifth) and 1.01 (sixth). Those of the SPCS ($n=6$) were 1.00 (first), 0.71 (second, injected), 0.74 (third, washed), 0.74 (fourth), 0.90 (fifth) and 0.97 (sixth). In spite of eliminating injected 5-HTP by washing, significant shortening still occurred over four cycles after injection (Fig. 4b).

In addition, the recommencement of the MR in Fig. 4a far preceded that of the CS in males injected with 5-HTP. This is seen in the differences between SPMR (Fig. 5a–c, black bars to the left in each pair of graphs)

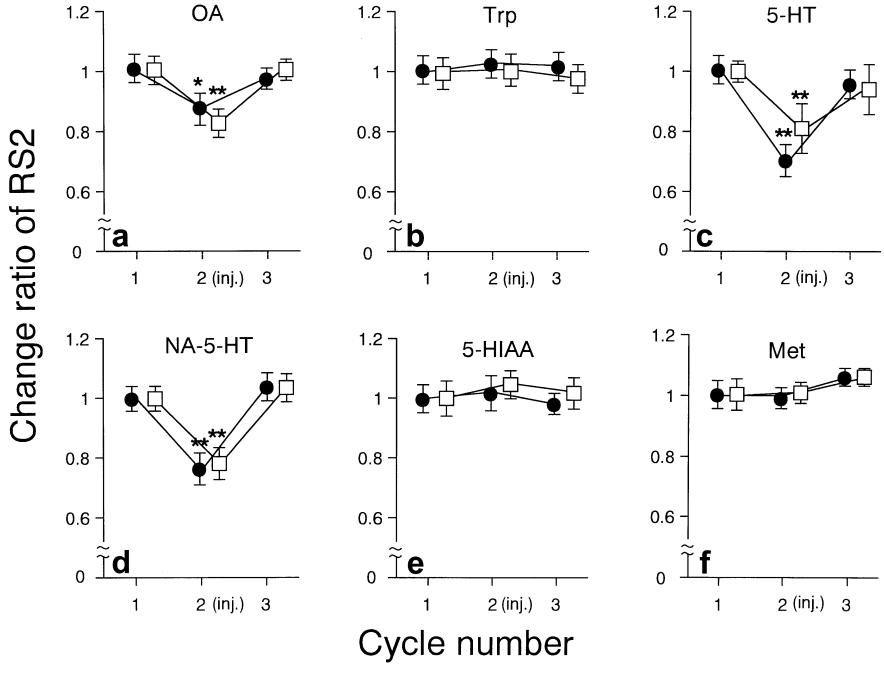

Fig. 3a–f Duration of the effects of six amines on RS2. *Abscissa* shows three successive reproductive cycles: *1* one cycle before injection (first cycle); *2 (inj.)* injected cycle (second cycle); and *3* one cycle after injection (third cycle). *Ordinate*: change ratio of RS2 in relation to the first cycle. **a** OA; **b** tryptophan (Trp); **c** 5-HT; **d** NA-5-HT; **e** 5-hydroxyindole-3-aceticacid (5-HIAA); **f** melatonin (Met). Drug concentration was 10^{-2} mol l^{-1} except for Met (10^{-3} mol l^{-1})

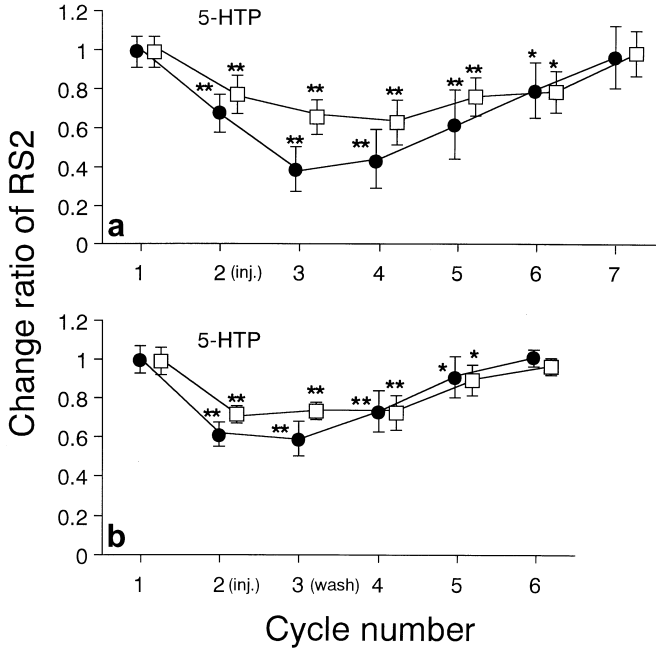

Fig. 4a, b Duration of the effect of 5-HTP on RS2. **a** Subcuticular injection of 5-HTP (10^{-2} mol l^{-1}) soon after spermatophore protrusion in the second cycle. **b** The same as **a** but the hemocoel was washed out with saline soon after spermatophore protrusion in the third cycle

and SPCS (Fig. 5a–c, black and/or white bars) in the injected males. The median difference increased from 4 min (CI, 2–6, $n=8$) in the first cycle (Fig. 5a) to 8 min (CI, 1–15) in the second cycle (Fig. 5b) and to 21 min (CI 11–31) in the third cycle (Fig. 5c). The SPCS-SPMR difference was not significant between the first and second cycles but it was significant ($P<0.05$) between the first and third cycles, due primarily to a decrease in SPMR (Fig. 5c).

Time-course to renew the reproductive cycle in males injected with 5-HTP

Because 5-HTP-injected males showed a considerable shortening of RS2 (Fig. 4), it was possible that the MR and/or the CS occurred independently of the timer (by definition, the sexually refractory stage should have ended at the recommencement of the MR or CS). If this were the case, subsequently occurring reproductive behaviors may have taken unusual time-courses. To examine this, the time interval from the re-emission of the CS to SP via copulation was analyzed in males injected with 5-HTP (from data in Fig. 4a). All the males, which exhibited the CS and MR, soon copulated with the female and showed SP in the normal time-course. The median time from the CS to SP via copulation (gray bars to the right in each pair of graphs in Fig. 5) was 8 min (CI, 5–11, $n=8$) in the first cycle (Fig. 5a), 10 min (CI, 3–17) in the second (injected) cycle (Fig. 5b), and 11 min (CI, 8–14) in the 3rd cycle (Fig. 5c). No significant difference was present among these intervals, indicating that males showing shorter RS2 had the normal time courses following the recommencement of the MR and CS.

5-HT synthesis enzyme inhibitor NSD-1015

To examine whether RS2 shortening was due to the effect of 5-HTP itself or to its metabolite 5-HT, the central L-aromatic amino acid decarboxylase inhibitor NSD-1015 (2×10^{-2} mol l^{-1}), was injected into the hemocoel in combination with 5-HTP (10^{-2} mol l^{-1}). The change ratios of the SPMR were 1.00 [first, 66 min (CI 59–73, $n=7$)], 1.00 (second, injected), 0.79 (third) and 0.68 (fourth), while they were 1.00 [first, 58 min (CI 55–61),

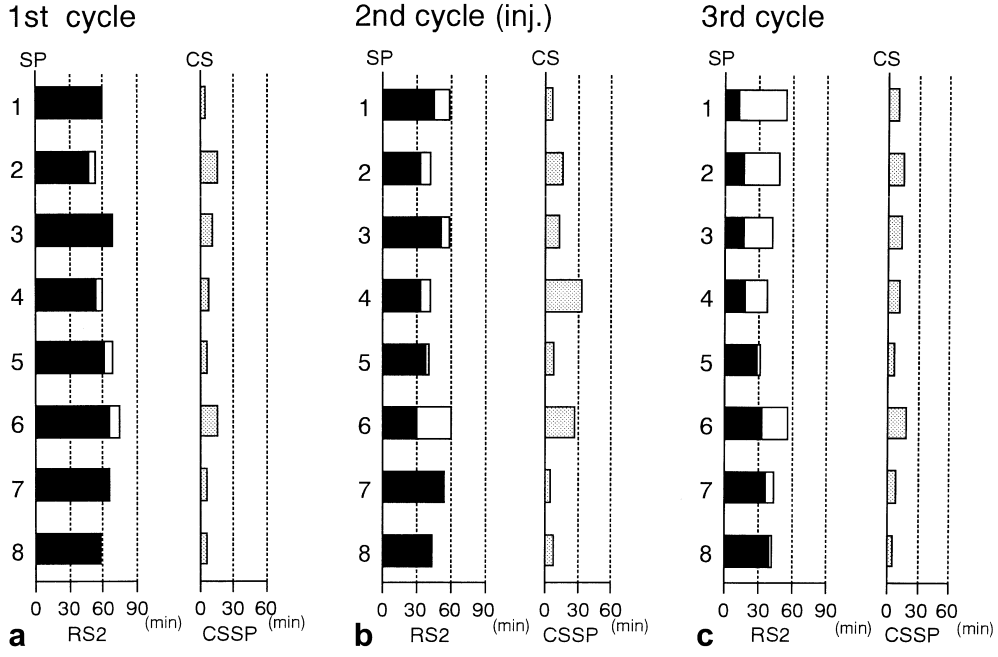

Fig. 5a–c The interval from the onset of the CS to SP via copulation in eight males injected with 5-HTP. In each pair of graphs, RS2 is shown to the left [SPMR, *black bar*; SPCS, *white bar* (shorter intervals are not seen)]. To the *right*, the interval (CSSP) from the onset of the CS to SP via copulation is shown. **a** One cycle before injection (*1st cycle*); **b** injected cycle (*2nd cycle*); **c** one cycle after injection (*3rd cycle*). Data for RS2 were the same as those in the first–third cycles of Fig. 4a. Note that the shorter the SPMR, the longer the CSSP (**b**, **c**). No significant difference is present in the average of CSSP between the three cycles (**a**–**c**)

$n = 11$], 0.69 (second), 0.53 (third) and 0.67 (fourth) with 5-HTP alone (Fig. 6a). The differences in RS2 between males with and without NSD-1015 injected were significant in the second ($P < 0.01$) and third ($P < 0.01$) cycle. However, the difference disappeared in the fourth cycle indicating that the nullifying effect of NSD-1015 was shorter than the shortening effect of 5-HTP. On the other hand, the change ratios of the SPCS were 1.00 [first, 70 min (CI 64–76), $n = 7$], 1.04 (second), 0.79 (third,) and 0.74 (fourth) with 5-HTP and NSD-1015, while they were 1.00 [first, 61 min (CI 58–64), $n = 11$], 0.84 (second), 0.69 (third) and 0.77 (fourth) with 5-HTP alone (Fig. 6b). The differences in RS2 between males with and without injected NSD-1015 were also significant in the second ($P < 0.01$) and third ($P < 0.01$) cycles. These results indicate that NSD-1015 significantly reduced the effect of 5-HTP on the RS2 shortening.

Intraganglionic injection of 5-HT and 5-HTP

To determine the target of injected amines in the CNS, intraganglionic injection of 5-HT and 5-HTP at 10^{-2} mol l^{-1} was carried out. The sham controls (the penetration of the terminal abdominal ganglion with a glass micropipette) showed no significant change in RS2: the change ratios were 1.02 for SPMR and 0.96 for SPCS compared to 1.00 in intact males (Fig. 7a). Twenty-two percent of the males exhibited auto-SE and 18.2% did not show courtship or copulatory behavior in the following 3-h observation periods (Table 2).

For saline injection, the change ratios were 1.10 for SPMR and 1.16 for SPCS. No significant difference was observed in SPMR before and after injection, although the SPCS slightly increased after injection (Fig. 7b). In contrast to males in the sham control, no males succeeded in genitalia coupling following the success in hooking during copulation with the female. Accordingly, normal SE did not follow. This deficiency was also present in males injected with 5-HT and 5-HTP, which prevented data collection for the third cycle.

5-HT injection caused shortening of RS2 but only in SPMR (Fig. 7c). The change ratios were 0.79 for SPMR and 0.94 for SPCS. These values were greater than those for SPMR (0.67) and for SPCS (0.80) in the subcuticular injection, though not statistically significant. Auto-SE rate was 25%. A further 25% of the males showed no reproductive behavior (Table 2). However, the excretion of feces was not observed, which contrasted the result of subcuticular injection. This indicated that feces excretion was due to the direct action of 5-HT on the intestines.

5-HTP injection into the terminal abdominal ganglion had a remarkable effect on shortening RS2. The change ratios were 0.55 for SPMR and 0.60 for SPCS, both of which were significantly smaller than 1.00 before

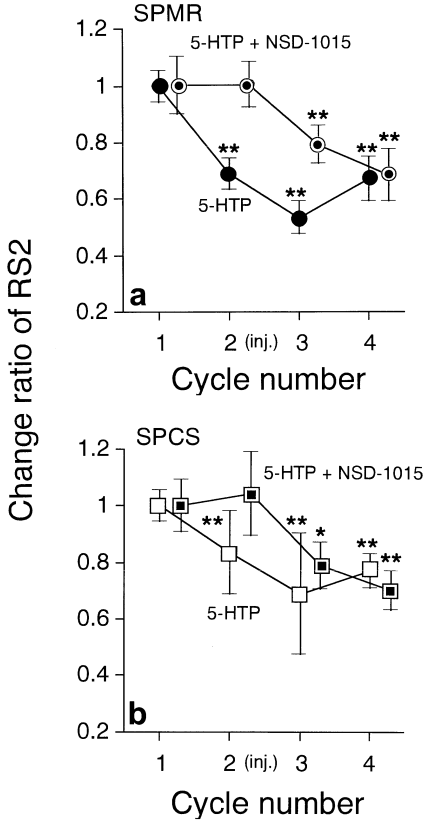

Fig. 6a, b The effect of 5-HT synthesis enzyme inhibitor NSD-1015 on RS2 shortening by 5-HTP. **a** RS2 (SPMR) measured by MR. *Filled circles*: 5-HTP alone; *double circles*: 5-HTP with NSD-1015. **b** RS2 (SPCS) measured by CS. *Open squares*: 5-HTP alone; *double squares*: 5-HTP with NSD-1015. Note that NSD-1015 almost nullified the shortening effect of 5-HTP on RS2 in the second cycle (**a**, **b**)

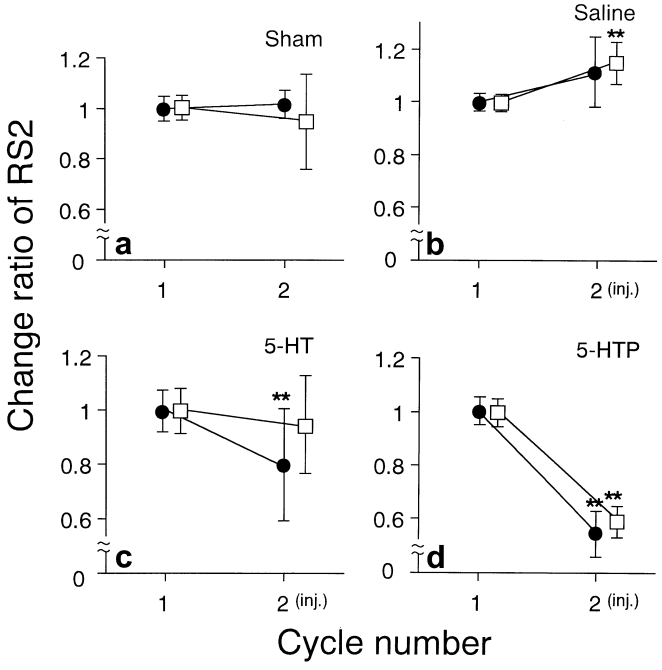

Fig. 7a–d Effects of intraganglionic injection of 5-HT and 5-HTP. **a** Sham control; **b** saline injection; **c** 5-HT injection; **d** 5-HTP injection. Amine (10^{-2} mol l^{-1}, 180 nl) was injected into the terminal abdominal ganglion

injection (Fig. 7d). When RS2 was represented in actual time values, SPMR was shortened from 64 min to 35 min, and SPCS from 65 min to 39 min (Fig. 8a). The amounts of RS2 shortening by intraganglionic injection were significantly less ($P < 0.05$ for SPMR and $P < 0.01$ for SPCS) than in subcuticular injection. Auto-SE rate was 3.6% and 39.3% of the males showed no reproductive behavior.

In contrast, the change ratios in males with 5-HTP injected into the metathoracic ganglion were 1.03 for SPMR and 1.05 for SPCS (Fig. 8b). No significant differences were present in the SPMR or SPCS between before and after injection, indicating that intraganglionic injection of 5-HTP into the metathoracic ganglion had no effect on shortening RS2.

Effects of injection of protein synthesis inhibitor

The shortening of RS2 could result from protein synthesis initiated during RS2 by 5-HT or 5-HTP. To examine this, a protein synthesis inhibitor, cycloheximide (CHX), was injected. For subcuticular injection (10^{-2} mol l^{-1}), the change ratios of the second cycle (injected) to the first cycle were 0.95 for SPMR [61 min (CI 58–64, $n = 28$) to 64.5 min (CI 62–67)] and 0.99 for SPCS [65 min (CI 63–67) to 66 min (CI 64–68)] (Fig. 9a). The values in the second cycle were not significantly different from those in the first cycle. For intraganglionic injection (10^{-2} mol l^{-1}), the change ratios in the second cycle were 0.98 for SPMR [63 min (CI 59–67, $n = 20$) to 64 min (CI 60–68)] and 1.07 for SPCS [70 min (CI 67–73) to 65.5 min (CI 63–68)](Fig. 9b). There was no significant difference between the first cycle and the second cycles. These results indicated that protein synthesis is not required for the reproductive timer to keep time.

Discussion

OA and 5-HT in invertebrate behavior

It has been established that biogenic amines play crucial roles in the initiation, execution and modification of invertebrate behavior (Orchard 1982; Orchard et al. 1993; Kravitz 1988; Roeder 1999). OA induces defensive posture (Livingstone et al. 1980; Hoyle and Field 1983; Harris-Warrick and Kraviz 1984), flight (Sombati and Hoyle 1984; Claassen and Kammer 1986; Stevenson and Kutsch 1987; Weisel-Eichler and Libersat 1996; Duch and Pflüger 1999), swimming (Mulloney et al. 1987) and age-related division of labor (Wagener-Hulme et al. 1999). More recently, we found that OA facilitated copulatory motor actions in the male cricket (Matsumoto and Sakai 2001). On the other hand, 5-HT mediates aggressive behavior (Edwards and Kraviz 1997; Kostowski and Tarchalska 1972; Antoson and Paul

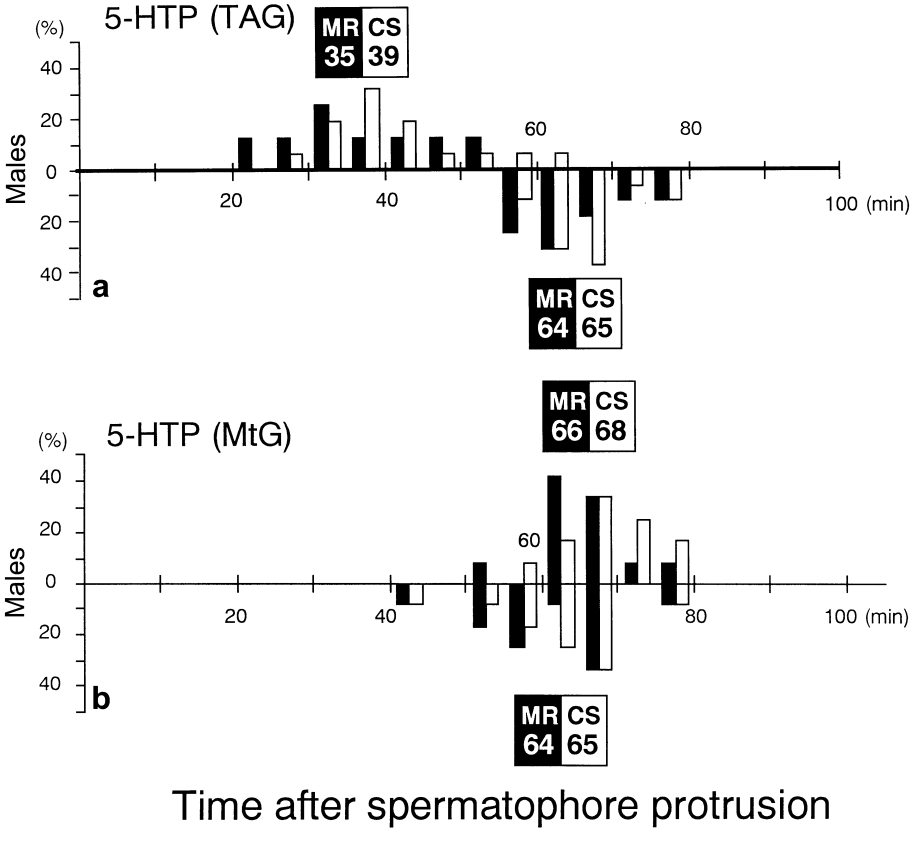

Fig. 8a, b The difference in the effect of intraganglionic injection of 5-HTP between the terminal abdominal ganglion (*TAG*) and the metathoracic ganglion (*MtG*). **a** Injection of 5-HTP into the TAG. **b** Injection of 5-HTP into the MtG. *Ordinate*: percentage of males that showed an MR (*black bar*) or a CS (*white bar*). *Black box*: the median interval (SPMR) between SP and MR; *white box*: the median interval (SPCS) between SP and CS. **a** and **b** were constructed using data from the third cycle in Fig. 4a and those from the second cycle in Fig. 7d, respectively

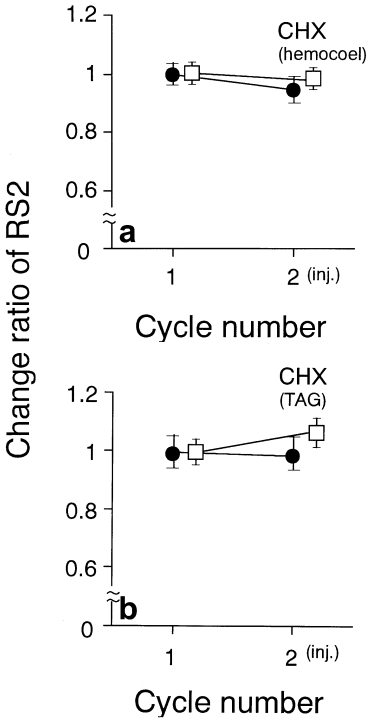

Fig. 9a, b The effect of cycloheximide (CHX) on RS2. **a** Subcuticular injection of CHX (10^{-2} mol l^{-1}). **b** Intraganglionic (TAG) injection of CHX (10^{-2} mol l^{-1}). Note that neither SPMR nor SPCS significantly changed with CHX injection

1997), feeding (Rosen et al. 1983; Lent and Dickinson 1984; Yeoman et al. 1994; Kabotyanski et al. 2000), and escape reactions (Glanzman and Krasne 1983). More generally, 5-HT is involved in arousal (Kupfermann and Weiss 1982), memory formation (Kandel and Schwarz 1982; Bicker and Menzel 1989) and circadian rhythm control (Eskin et al. 1982; Nässel et al. 1985; Page 1987; Proccer et al. 1993; Tomioka et al. 1993; Tomioka 1999; Chen et al. 1999).

In the insect CNS, octopaminergic and serotonergic neurons are commonly found (Nässel 1988; Homberg 1991; Spörhase-Eichmann et al. 1992; Stevenson and Spörhase-Eichmann 1995; Roeder 1999). Although their numbers in each ganglion are relatively small, their axonal arborizations are widespread. Except for the brain, OA is found in dorsal unpaired median neurons with large somata, paired ventral neurons with intermediate somata and paired lateral neurons with small somata in each ganglion (Evans and O'Shea 1978; Dymond and Evans 1979; Konings et al. 1988; Lee and Wyse 1991; Eckert et al. 1992; Spörhase-Eichmann et al. 1992; Bräunig et al. 1994; Schneider et al. 1993; Stevenson and Spörhase-Eichmann 1995; Bräunig and Pflüger 2001). On the other hand, 5-HT is found in bilaterally paired neurons with intermediate somata dorsally or ventrally in the lateral and posterior regions of each ganglion (Bishop and O'Shea 1983; Tyrer et al. 1984; Hustert and Topel 1986; Longley and Longley 1986; Valles and White 1988). Serotonergic neurons in the terminal abdominal ganglion in the male cricket *Acheta domesticus* (Hustert and Topel 1986; Klemm et al. 1986) are of special interest because the reproductive timer in question is plausibly located in that ganglion (Ureshi and Sakai 2001).

Effects of OA and 5-HT on RS2

Previously, Nagao et al. (1991) reported that subcuticular injection of amine solutions (0.01 ml at 10^{-6} to 10^{-3} mol l^{-1}) changed the time interval (SPCS) between SP and the onset of the CS. OA shortened SPCS at 10^{-3} mol l^{-1} and produced change ratios of ca. 1.0–0.8 (from Fig. 5C, D in Nagao et al. 1991), while 5-HT lengthened SPCS at higher concentrations (change ratios ca.1.3 at 10^{-4} mol l^{-1} and 1.1 at 10^{-3} mol l^{-1}), but it shortened SPCS at lower concentrations (change ratios ca. 0.8 at 10^{-5} mol l^{-1} and 0.9 at 10^{-6} mol l^{-1}). Although the effect of OA seemed reliable, that of 5-HT was uncertain because there was no consistency for its effect on SPCS. In contrast to these results, a pilot study indicated that OA and 5-HT did not change SPCS or SPMR even at higher concentrations up to 10^{-2} mol l^{-1}. However, both amines became effective when the volume of injected solution was increased up to 0.15 ml (15 times larger than before). Although such a large dose at a high concentration may be problematic, the effects on RS2 were different for different amines: 5-HTP was highly effective, 5-HT had a moderate effect, and Trp or Met were not effective at all. Furthermore, the effects of amines were stage-specific in the reproductive cycle, i.e., they shortened RS2 but not RS1. In addition, amine injection at high doses did not apparently cause any progressive physical deterioration. These observations suggest that the amines used here, though given in large doses, probably acted at physiological concentrations on neurons in the CNS. The large dose at the high concentration may have been necessary for the amines to permeate the ganglion through the sheath and to reach particular sites of the limited number of neurons. At present, the cause of the difference between the results of Nagao et al. (1991) and ours is unknown.

Effect of 5-HTP on RS2

Our main finding is that 5-HTP, the precursor of 5-HT, is strikingly potent in shortening RS2. The shortest RS2 (SPMR) induced by 5-HTP was 38% of the control (100%) and that by 5-HT was 67%. The effect of subcuticular injection of 5-HTP (10^{-2} mol l^{-1}) continued over 5 reproductive cycles (nearly 4.5 h) while OA, 5-HT, and NA-5-HT shortened RS2 in only the injected cycle. It should be noted that the maximum effect of 5-HTP on SPMR was delayed by one cycle after the injected cycle, while that on SPCS was delayed by two cycles. Washing the hemocoel at about 1 h after injection reduced the effect of 5-HTP only slightly. These

results indicate that the effect of 5-HTP was not only the strongest among the amines used but also persistent.

Also, 5-HTP was effective when injected into the terminal abdominal ganglion, but not effective when injected into the metathoracic ganglion, indicating that 5-HTP targeted neurons in the terminal abdominal ganglion. This is in accord with our previous finding that the timer for the post-copulatory time-fixed sexually refractory stage is located in the terminal abdominal ganglion (Ureshi and Sakai 2001). Presumably the shortening of RS2 was the result of acceleration of the timer in the terminal abdominal ganglion. However, another possibility is that the earlier occurrences of the MR and CS after injection of 5-HTP was due to the removal of inhibition onto pattern generators in the thoracic and abdominal ganglia (Sakai et al. 1995; Matsumoto and Sakai 2000b). Although it cannot be eliminated, if this were the case, the disinhibitory effect of 5-HTP should have appeared sooner after injection, and subsequently occurring SE in copulation and SP should not have followed in the normal time-courses.

Is the effect of 5-HTP caused by synthesized 5-HT from 5-HTP?

5-HTP is synthesized in insect nervous tissues from Trp by a ring hydroxylation of the enzyme tryptophan hydroxylase (5-OHase). It is decarboxylated to 5-HT by nonspecific AADC (Evans 1980). 5-HT is metabolized through two different routes: one is to 5-hydroxyindolacetaldehyde (5-HIAAD) by monoamine oxidase (MAO) and then to 5-hydroxyindolacetic acid (5-HIAA) by aldehyde dehydroxylase (ADH); in the other 5-HT is converted to NA-5-HT by *N*-acetyltransferase (NAT) and then to melatonin by 5-hydroxyindol-*o*-methyltransferase (HIOMT). Among these substances, 5-HT, its precursor 5-HTP and its metabolite NA-5-HT were effective in shortening RS2 while the others were ineffective. So far, specific receptors for 5-HTP have not been identified (Evans 1980). In our experiments, 5-HT synthesis inhibitor NSD-1015 reduced the effect of 5-HTP. Thus, it appears that 5-HTP is taken up by serotonergic neurons, much as 5-HT is taken up by transporters and diffusion (Scott et al. 1985; Bermudez and Beadle 1989), decarboxylated to 5-HT, stored in vesicles, and released from the active sites of membranes to act on 5-HT receptors in target neurons. However, for RS2 in the amine-injected cycle, 5-HT was effective as much as 5-HTP. Why was 5-HT much less potent than 5-HTP? At present, we have no solid answer to this question. Extrinsic 5-HT was as effective in shortening RS2 as 5-HTP, but the former may be quickly metabolized in the tissues. However, we are cautious about hypothesizing the role of 5-HTP in accelerating the timer. There might be receptors specific for 5-HTP in neurons of the terminal abdominal ganglion, similar to a recently identified receptor for tyramine, the precursor of OA (Arakawa et al. 1990; Saudou et al. 1990; Kutsukake et al. 2000). There is a report that 5-HTP was more potent than 5-HT in producing locomotor arousal, and that it quickly activated an isolated single serotonergic neuron in snails, suggesting that 5-HTP influences the membrane excitability. It is still unknown whether the effect is direct or mediated by a raise of intracellular 5-HT (Sakharov et al. 1998). The implication is that 5-HT release may be increased not only through synthesis of 5-HT from 5-HTP, but also through the increase in excitation of 5-HT neurons by 5-HTP.

Comparison of sexual refractoriness between invertebrates and mammals

So far as we know, 5-HT has not been shown to control mating behavior in invertebrates. In our previous experiments, OA facilitated copulatory motor actions in the male cricket but other amines including 5-HT, dopamine or noradrenalin did not (Matsumoto and Sakai 2001). In mammals, it is known that dopamine is excitatory to male sexual behavior (Argiolas and Melis 1995). Copulation is correlated with an increase in dopamine in the medial preoptic area (Mas et al. 1987; Hull et al. 1995) and the nucleus accumbens (Pleim et al. 1990) in rats. Microinjection of the dopamine antagonist into the medial preoptic area impaired sexual motivation, copulation and penile erection (Warner et al. 1991). On the other hand, 5-HT is generally inhibitory to male sexual behavior (Argiolas and Melis 1995). In particular, post-copulatory sexual refractoriness is correlated with an increase in extracellular 5-HT in the lateral hypothalamus (Lorrain et al. 1997) or in the medial preoptic area (Hoffman et al. 1987), suggesting that ascending serotonergic neurons from the midbrain play an inhibitory role in initiating copulation. This is seemingly in contrast to the shortening effect of 5-HT on RS2 in crickets. However, the effect of 5-HT on the post-copulatory sexually refractoriness cannot be dealt with at the same level in crickets and rats because their inhibitory systems are different: crickets have the timer but rats have not. 5-HT effected time-keeping but not inhibition itself (see Sakai et al. 1995; Matsumoto and Sakai 2000a, 2000b). Further study is necessary to understand the mechanisms of the post-copulatory sexually refractory stage from the comparative viewpoint.

5-HT and the reproductive timer

It is not plausible that 5-HT mediates time-keeping itself as if it were sand in an hourglass. If so, RS2 should have been terminated as soon as massive 5-HT or 5-HTP was injected, and prolonged far longer by simultaneous injection of NSD-1015 which stops the conversion of 5-HTP to 5-HT. Rather, we assume that 5-HT may affect some metabolic processes responsible for time-keeping. 5-HT, synthesized from extrinsic 5-HTP, may be excessively released at synaptic sites to abnormally stimulate

5-HT receptors of the timer neuron(s). As a result, the metabolic processes, which normally progress at constant speeds, would be highly activated, as reported for the sensitization mechanism by 5-HT in *Aplysia* (Kandel and Schwartz 1982), and the resultant products would accelerate the time-keeping. By analogy, 5-HT widens the neck of the hourglass. According to recent studies on circadian rhythm control systems, it is assumed that 5-HT is involved in the regulation of time-keeping in terms of phase shifting (Page 1987; Tomioka 1999). However, we still do not know how 5-HT is associated with the normal time-keeping underlying the sexually refractory stage.

Finally, our results with the protein synthesis inhibitor CHX indicated that time-keeping does not need newly synthesized proteins during RS2. This suggests that the mechanism of time-keeping in the reproductive timer is essentially different from that of circadian oscillators which require protein synthesis (Rothman and Strumwasser 1976; Nakashima et al. 1981; Eskin et al. 1984; Khalsa et al. 1992; Koumenis and Eskin 1992; Hall 1995; Allada et al. 1998; Rutila et al. 1998; Dunlap 1999; Tomioka 2000). Studies with 5-HT agonists and antagonists, and enzyme activators and inhibitors for second messengers are now urgently needed.

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