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Time Course of Protein Synthesis-Dependent Phase of Olfactory Memory in the Cricket *Gryllus bimaculatus*

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ABSTRACT—The cricket *Gryllus bimaculatus* forms a stable olfactory memory that lasts for practically a lifetime. As a first step to elucidate the cellular mechanisms of olfactory learning and memory retention in crickets, we studied the dependency of memory retention on the *de novo* brain protein synthesis by injecting the protein synthesis inhibitor cycloheximide (CHX) into the head capsule. Injection of CHX inhibited ^{3}H-leucine incorporation into brain proteins by > 90% for 3 hr. Crickets were trained to associate peppermint odor with water (reward) and vanilla odor with saline solution (non-reward) and were injected with CHX before or at different times after training. Their odor preferences were tested at 2 hr, 1 day and 4 days after training. Memory retention at 2 hr after training was unaffected by CHX injection. However, the level of retention at 1 day and 4 days after training was lowered when CHX was injected 1 hour before training or at 1 hr or 6 hr after training. To study the time course of the development of CHX-sensitive memory phase, crickets that had been injected with CHX at 1 hr after training were tested at different times from 2 to 12 hr after training. The level of retention was unaffected up to 4 hr after training but significantly lowered at 5 hr after training, and the CHX-sensitive memory phase developed gradually during the next several hours. CHX dissociates two phases of olfactory memory in crickets: earlier protein synthesis-independent phase (< 4 hr) and later (> 5 hr) protein synthesis-dependent phase.

Key words: learning, memory, olfaction, cricket, cycloheximide

INTRODUCTION

Behavioral and pharmacological studies in a wide variety of species, ranging from insects to mammals, have shown that long-lasting form of memory is dependent upon protein synthesis (reviewed by Davis and Squire, 1984). In vertebrates, different inhibitors of protein synthesis such as cycloheximide (CHX), puromycin or anisomycin distort the formation of long-lasting memory, but not that of short-term memory, if they are applied shortly before or after training (Squire and Barondes, 1974; Flood *et al*., 1975a,b). In the molluscan *Aplysia*, long-term facilitation of synaptic transmission from sensory neurons to motor neurons induced by the application of serotonin requires protein synthesis (Montarolo *et al*., 1986). In the fruit-fly *Drosophila*, olfactory memory formed by multiple spaced training (repeated training sessions with a rest interval between each) contains two memory components; one lasts for at least 7 days and is sensitive to CHX, and the other decays away within 3–4 days and is insensitive to CHX (Tully *et al*., 1994; DeZazzo and Tully, 1995). The formation of the former protein synthesis-dependent memory component is also critically dependent upon the expression of cAMP-responsive element-binding protein (CREB) gene (Yin *et al*., 1995).

In honey bees, protein synthesis-dependent memory phase develops only very slowly after olfactory conditioning: blockage of either transcription (actinomycin D) or translation (anisomycin) had no effect on memory retention in tests performed at 1 day and 2 days after training, and the impairment of memory retention was observed only after 4 days of training (Wüstenberg *et al*., 1998). In the fruit-fly *Drosophila*, however, CHX-sensitive memory phase was observed in tests performed at 1 day after training (Tully *et al*., 1994), but the time course of the development of this memory phase has not been studied. Thus, the extent by which the time course of the development of protein synthesis-dependent memory phase varies among different insect species remains unclear.

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Recently, we found that the cricket *Gryllus bimaculatus* has a highly developed olfactory learning capability, characterized by fast acquisition, long-term retention and easy rewriting of memory (Matsumoto and Mizunami, 2000). Olfactory memory of crickets is extremely long-lasting: memory established in the early nymphal stage can last for practically a lifetime (Matsumoto and Mizunami, 2002b). Since crickets allow for detailed electrophysiological studies of brain neurons (Böhm and Schildberger, 1992; Staudacher and Schildberger, 1998), crickets may provide a useful model for studying cellular processes underlying the formation of long-lasting memory. As a first step to clarify the cellular mechanisms of olfactory long-term memory retention in crickets, we carried out experiments to determine the effect of the CHX on olfactory memory retention in crickets. The results were compared to those reported for honey bees.

MATERIALS AND METHODS

Insects

Adult male crickets, *Gryllus bimaculatus*, reared in a 12-hr light-dark cycle (photophase: 8 am–8 pm) at 27±2°C were fed a diet of insect pellets and water *ad libitum.* All experiments were carried out 1–2 weeks after the imaginal molt.

Effects of CHX on protein synthesis

The level of protein synthesis inhibition in the brain was measured by the incorporation of ^{3}H-leucine into trichloroacetic acid (TCA)-precipitable polypeptides after injection of cycloheximide (CHX), an antibiotic that blocks translation of RNA. CHX (Sigma Co.) was dissolved in cricket physiological saline (NaCl 150 mM, KCl 9 mM, $CaCl_2$-H_2O 5 mM, $NaHCO_3$ 2 mM, glucose 40 mM, adjusted to pH 7.2 with NaOH) to a concentration of 20 mM. This concentration corresponds to 16.8 μg CHX per injection volume of 3 μl and amounts to a dosage of 24.0 mg CHX/kg body weight, since a cricket weighs about 0.7 g. L-4,5-^{3}H-leucine (NEN^{TM} Life Science Products Inc., U.S.A.; specific activity, 1.63 TBq/mmol) was diluted in cricket saline to a final concentration of radioactivity of 37 MBq/ml. Each cricket was injected with CHX manually by a 10 μl microsyringe through a hole pricked into the median ocellus. At 30 min after CHX injection, 1 μl of ^{3}H-leucine (1.0 μCi) was injected manually by a 10 μl microsyringe through the same hole. After 2.5 hr of tracer incorporation, the crickets were immobilized with ice-cold water for 30 sec. The brains were freed of adhering tracheae and, after several washes with cricket saline, were dissected from the head capsules. The brains were immediately placed into a 1 ml tissue grinder containing 40 μl of ice-cold phosphate-buffered saline (PBS; 0.04 M, pH 6.7). Each brain was manually homogenized and was transferred to a centrifuge tube. The homogenizer was rinsed twice with 40 μl of PBS. The tubes were spun at 10,000 × g for 10 min.

To determine the amount of ^{3}H-leucine incorporation into proteins, 50 μl of supernatant was applied to a filter paper (15×15 mm) and air-dried. Proteins were precipitated on the filter paper with ice-cold TCA (10%, w/v) for 10 min. After that, the filter paper was washed twice with 10% TCA and twice with ethanol, and then the precipitated proteins were dissolved with an ACS-II (Aqueous Counting Scintillant) and bound label was quantified by liquid scintillation counting (LSC). Tracer incorporation was expressed as dpm/brain and adjusted for background, nonspecific binding of ^{3}H-leucine to the filter paper. Counting efficiency was determined by analysis of the spectral quench parameters of the isotope.

Behavioral experiments

Four days before the start of the experiment, a group of 30 crickets was placed in a container and they were fed a diet of insect pellets *ad libitum* but deprived of drinking water to enhance their motivation to search for water. On the day of the experiment, they were individually placed in 100-ml glass beakers.

Classical conditioning training and operant test

We used classical conditioning and operant testing procedures (Matsumoto and Mizunami, 2002a). Individual crickets were given differential conditioning trials, an appetitive conditioning trial being followed by an aversive conditioning trial. In the appetitive conditioning trial, peppermint odor was associated with water reward (P^+ conditioning trial). In the aversive conditioning trial, vanilla odor was associated with saline solution (non-reward) (V^- conditioning trial). One-ml hypodermic syringes were used for conditioning. A small filter paper (3×3 mm) was attached to the needle of the syringe at 10 mm from its tip. The syringe used for the P^+ conditioning trial was filled with water, and the filter paper attached to the needle was soaked with peppermint essence. The syringe used for the V^- conditioning trial was filled with 20% NaCl solution, and the filter paper was soaked with vanilla essence. For odor presentation, the filter paper was placed within 1 cm of the cricket's head. At 2 sec after the onset of odor presentation, a drop of water or saline solution was given to the mouth of the cricket for 2 sec. Then, the air in the beaker was ventilated for 2 sec using a hand-held vacuum cleaner. Two sessions of differential conditioning trials were performed, with the sequence of P^+, V^-, P^+ and then V^- conditioning trials. The inter-trial interval (ITI) was 5 min.

The apparatus used for the odor preference test was slightly modified from that described previously (Matsumoto and Mizunami, 2002a). In short, the apparatus consisted of three chambers, a 'test

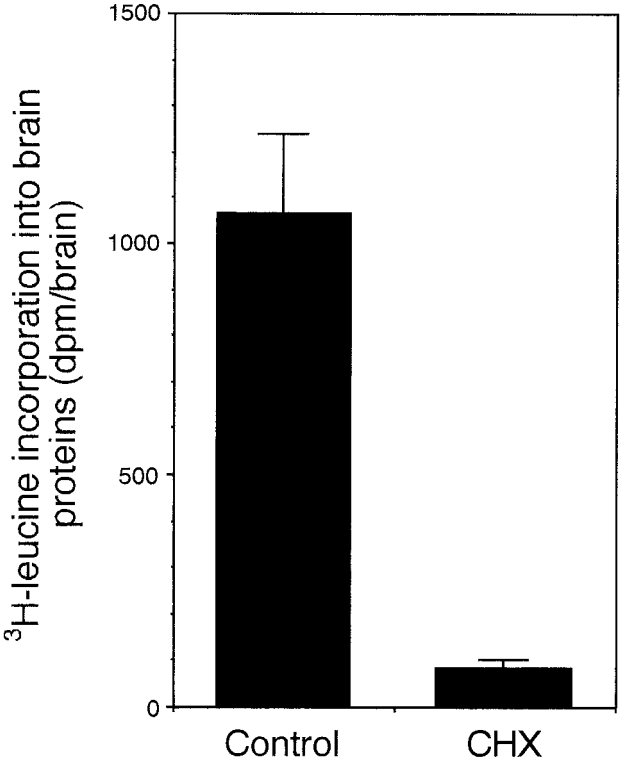

Fig. 1. Tracer incorporation into proteins in the brains of crickets. Crickets were injected with 3 μl of cricket saline (Control; *N*=12) or saline containing 16.8 μg cycloheximide (CHX; *N*=8). After 30 min of drug exposure, 1 μl of ^{3}H-leucine (1.0 μCi) was injected. Tracer incorporation was terminated after 2.5 hr.

chamber' and two removable 'waiting chambers', one of which was placed at the 'waiting position' and the other at the 'entrance position'. There was a sliding door between the waiting chamber at the entrance position and the test chamber. On the floor of the test chamber, there were two circular holes that connected the chamber with two of three sources of odor. Each odor source consisted of a cylindrical plastic container covered with a fine gauze net. The three containers were mounted on a rotatable holder. Two odor sources could be located simultaneously just below the holes at the 'offer position' by rotating the holder.

Before the preference test, a cricket was transferred from the beaker to the waiting chamber at the waiting position and left for approximately 4 min to become accustomed to the surroundings. The waiting chamber was then slid into the entrance position, and the door to the test chamber was opened. When the cricket entered the test chamber, the door was closed and the test started. Two minutes later, the relative positions of the vanilla and peppermint sources were changed by rotating the container holder. An odor source was considered to have been visited when the cricket probed the top net with its mouth. The time spent for visiting each odor source was measured cumulatively. The preference test lasted for 4 min. At the end of training, the sliding door was opened and the cricket was gently pushed into the waiting chamber, and then the cricket was transferred to a beaker. Daily testing and training began at 11 a.m. with a maximum duration of 4 hr. After completing the test session, crickets were fed a diet of insect pellets *ad libitum* until the next retention test. In experiments in which crickets were subjected to retention tests at 1 day and 4 days after training, a few drops of water were given after the 1-day retention test and also on the subsequent day, but not thereafter. Each cricket was used only once in an experiment and not reused in another experiment.

Experimental procedures

1) Memory retention after classical conditioning

Untreated crickets that had undergone two sets of differential conditioning trials were given odor preference tests just before training (PT-0) and at 2 hr (PT-1), 1 day (PT-2) and 4 days (PT-3) after training (Fig. 2); thus, each cricket underwent three retention tests. The extinction effects of retention tests are negligible (Matsumoto

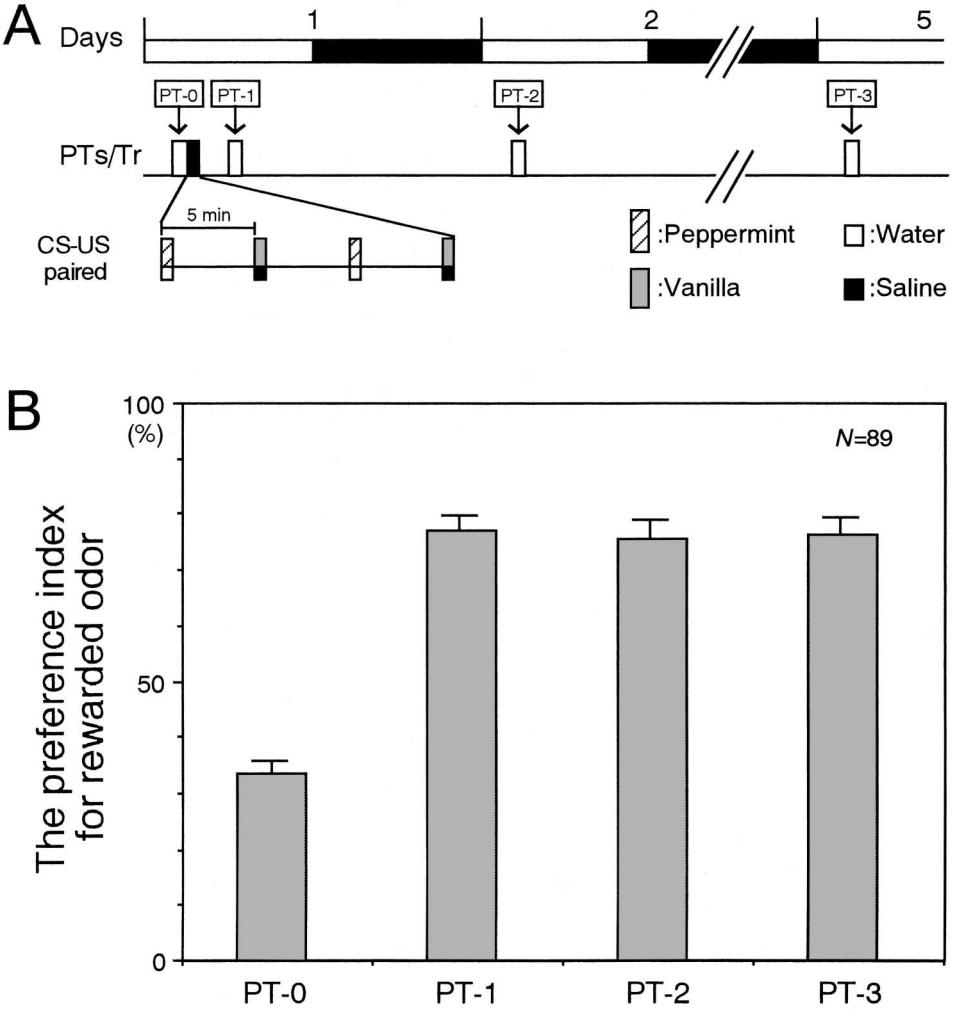

Fig. 2. Memory retention after classical conditioning in untreated crickets. **A,** Time schedule for training and testing, where preference tests (PTs, white bars) were performed before (PT-0) and at 2 hr (PT-1), 1 day (PT-2) and 4 days (PT-3) after training (Tr, black bar). The white and black parts of the time bar indicate photophase (12 hr) and scotophase (12 hr), respectively. A stimulus schedule for training is illustrated at the bottom, in which the hatched and shaded bars on the line indicate the presentations of peppermint and vanilla odor, respectively (as conditioned stimulus), and the white and black squares below the line indicate the presentation of water and saline solution, respectively (as unconditioned stimulus). **B,** Preference indexes for rewarded odor (peppermint) before training and at 2 hr, 1 day and 4 days after training are shown as means ± S. E. *N* is the number of crickets tested.

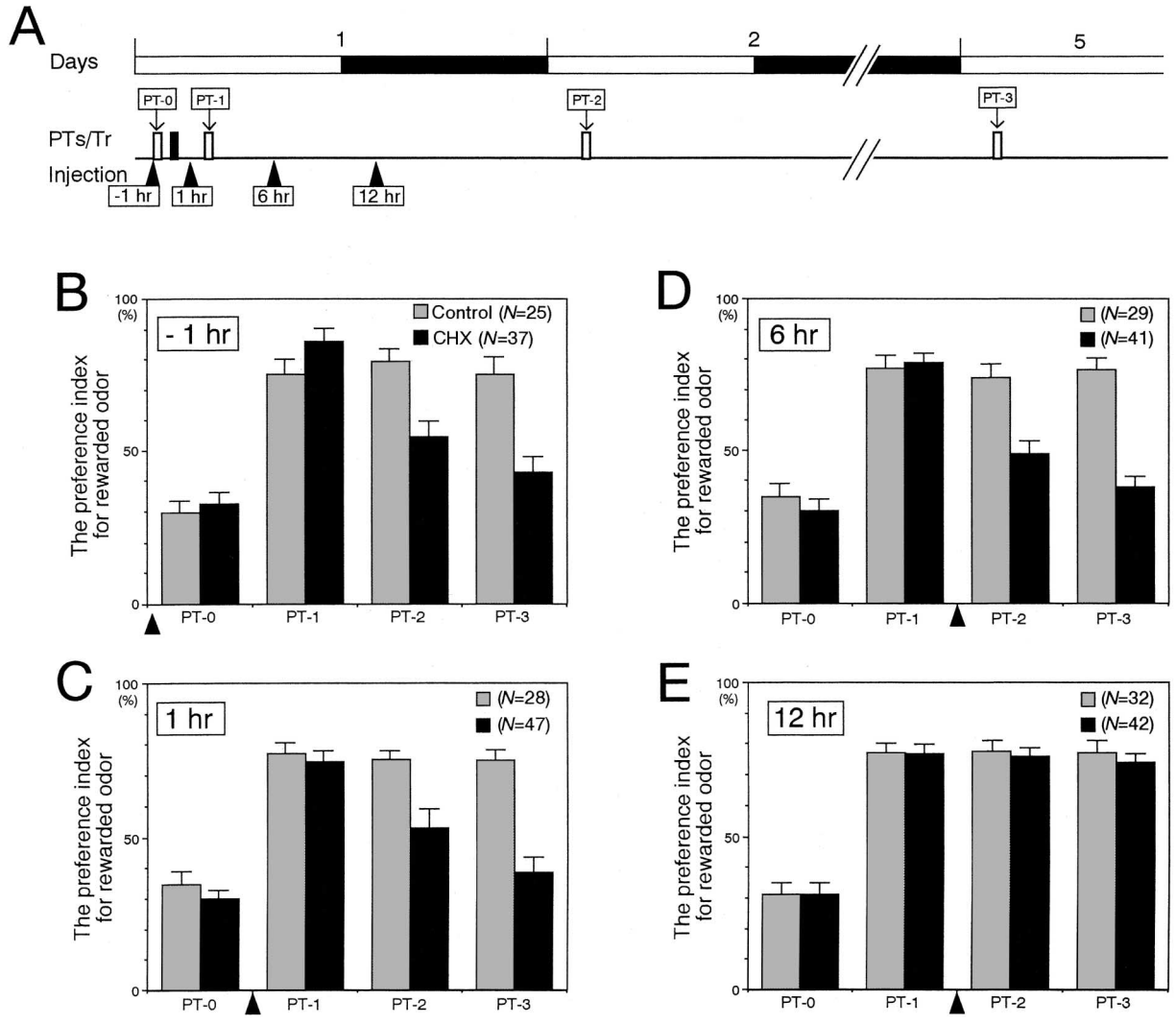

Fig. 3. Effects of CHX injection. **A,** Time schedule for training (Tr, black bar), testing (PTs, white bars) and CHX or saline injection (black triangles). **B–E,** Preference indexes for rewarded odor (peppermint) before training (PT-0) and at 2 hr (PT-1), 1 day (PT-2) and 4 days (PT-3) after training in the control group (gray bar) and CHX-injected group (black bar) are shown as means ± S. E. CHX or saline (Control) was injected either 1 hr before (**B**) or 1 (**C**), 6 (**D**) or 12 hr (**E**) after training. Black triangles indicate the timing of injection of CHX or saline. *N* is the number of crickets tested.

and Mizunami, 2000a).

2) Effects of CHX injection on learning and memory

To study the effects of CHX on learning and memory retention, 3 μl of 20 mM CHX in cricket saline or cricket saline alone (control) was injected 1 hr before training or 1, 6 or 12 hr after training (Fig. 3). Odor preferences were tested before training (PT-0) and at 2 hr (PT-1), 1 day (PT-2) and 4 days (PT-3) after training.

3) Time course of the development of CHX-sensitive memory phases

To study the time course of the development of CHX-sensitive memory phases (Fig. 4), eight groups of crickets were injected with CHX at 1 hr after training and were tested at various times from 2 to 12 hr after training. As a control, one group of crickets was injected with cricket saline at 1 hr after training and was tested at 2 hr and 12 hr after training.

Data analysis

Relative odor preference of individual crickets was measured using the preference index (PI) for rewarded odor (peppermint) (%), defined as $t_p/(t_p+t_v) \times 100$, where t_p is the time spent exploring the peppermint source and t_v is the time spent exploring the vanilla source. The χ^2 test was used to evaluate initial preference. Wilcoxon's test (WCX) was used to compare the preferences in different tests of a given cricket group, and the Mann-Whitney *U*-test (M-W) was used to compare the preferences of different groups. The Kruskal-Wallis test (K-W) was used to compare the preferences in three or more tests of different groups.

RESULTS

Protein synthesis

Tracer incorporation in the brain in the CHX-injected group and that in the saline-injected group were 1067±170 dpm and 82±16 dpm (±SE), respectively (Fig. 1), indicating that injection of 16.8 μg CHX (24 mg/kg body weight) led to 92.3% inhibition of synthesis of water-soluble, TCA-precipitable proteins in the cricket brain for at least 3 hr after CHX injection.

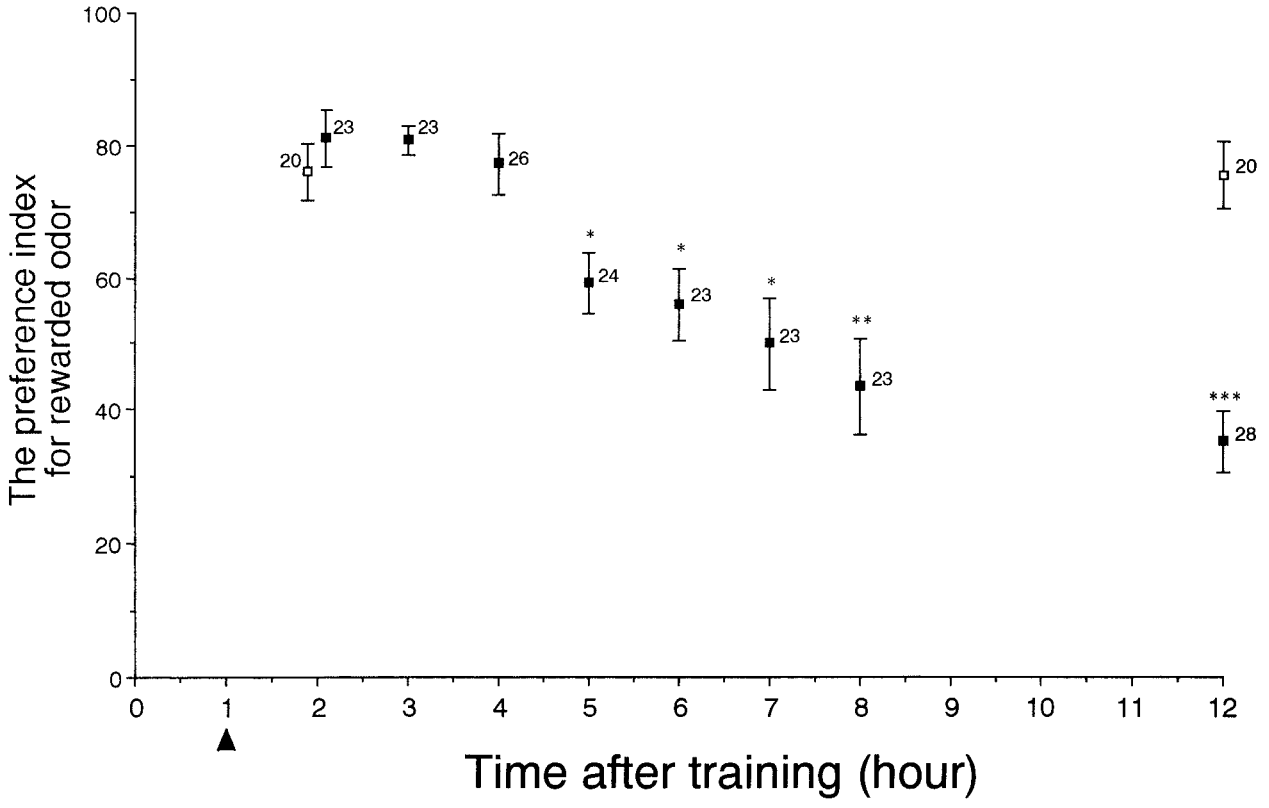

Fig. 4. Time course of the development of CHX-sensitive memory phase. Preference indexes for rewarded odor (peppermint) of eight CHX-injected groups (black squares) and a control group (white square) are shown as means ± S. E. (%) A black triangle indicates that CHX or saline was injected at 1 hr after training. The number of crickets tested are shown near each data point. Asterisks indicate significant differences compared to the preferences of the control group at 2 hr and 12 hr after injection (*, p<0.05; **, p<0.005; ***, p<0.0001 Kruskal-Wallis test).

Behavioral experiments

Initial preference

In the initial preference test, 713 of 800 crickets used in this study spent more time exploring the vanilla source than they did exploring the peppermint source, and the χ^2 test showed that the number of crickets that preferred vanilla over peppermint was significantly (p<0.0001) greater than the number of crickets that preferred peppermint, in agreement with our previous results (Matsumoto and Mizunami, 2000). No significantly different initial preferences for rewarded odor (peppermint) were found among the nineteen groups of crickets used in this study (p>0.5, df=18, *H*=6.858, K-W).

Memory retention after classical conditioning

At 2 hr after training (PT-1), crickets that had undergone differential conditioning trials exhibited significant levels of conditioning: The preference for rewarded odor (peppermint) at 2 hr after training was significantly greater than that before training (p<0.0001, df=1, *T*=47, WCX). No significant decay of memory retention was found from 2 hr to 1 day or to 4 days: The preference for rewarded odor at 1 day (PT-2) or 4 days (PT-3) did not significantly differ from that at 2 hr after training (p>0.3, df=1, *T*=1278 for PT-2; p>0.5, df=1, *T*=1502.5 for PT-3, WCX). The results indicate that our conditioning procedure led to a high level of olfactory conditioning and that the memory formed was retained for at least 4 days without significant decay. All subsequent experiments were performed using this conditioning procedure.

Effects of CHX injection

The mortality rate of CHX-injected crickets did not differ from that of saline-injected (control) crickets when compared at 4 days after injection.

In the experiment for which the results are shown in Fig. 3, CHX was injected at various times before or after training. Fig. 3B shows the results from crickets that had been injected with CHX or saline at 1 hr before training. The saline-injected (control) group exhibited a significant level of conditioning at 2 hr after training and exhibited no significant decay of memory retention from 2 hr to 4 days: The preference for rewarded odor at 2 hr after training was significantly greater than that before training (p<0.0001, df=1, *T*=12, WCX) and did not significantly differ from that at 1 day (PT-2) or 4 days (PT-3) after training (p>0.5, df=1, *T*=107.5 for PI-2; p>0.5, df=1, *T*=114.5 for PI-3, WCX). The preferences of saline-injected crickets for rewarded odor before and after training did not significantly differ from those of untreated crickets (p>0.3, df=1, *U*=1004.0 in PT-0; p>0.5, df=1, *U*=1080.0 in PT-1; p>0.1, df=1, *U*=954.0 in PT-2; p>0.5, df=1, *U*=1023.5 in PT-3, M-W). The preferences of CHX-injected crickets for rewarded odor before training and at 2

hr after training did not significantly differ from those of the control crickets (p>0.5, df=1, *U*=423.5 in PT-0; p>0.05, df=1, *U*=299.0 in PT-1, M-W). At 1 day (PT-2) and 4 days (PT-3) after training, however, the preferences of CHX-injected crickets for rewarded odor were significantly less than those of the control crickets (p<0.005, df=1, *U*=241.0 in PT-2; p<0.001, df=1, *U*=202.5 in PT-3, M-W): memory after 1 day of training was affected by CHX injection. The CHX-injected group exhibited a significant level of retention at 1 day after training but not at 4 days after training: The preference of CHX-injected crickets for rewarded odor at 1 day after training (PT-2) was significantly greater than that before training (PT-0) (p<0.01, df=1, *T*=126.0, WCX), but that at 4 days after training (PT-3) was not significantly greater than that before training (p>0.1, df=1, *T*=235.0, WCX).

Fig. 3C and 3D show the effects of CHX injection at 1 and 6 hr after training, respectively. The effects of CHX injection at 1 hr and 6 hr after training were similar to those of CHX injection at 1 hr before training (Fig. 3B): the preferences of CHX-injected crickets for rewarded odor before training (PT-0) and at 2 hr after training (PT-1) did not significantly differ from those of the control crickets (Fig. 3C: p>0.3, df=1, *U*=578.0 in PT-0; p>0.5, df=1, *U*=648.0 in PT-1; Fig. 3D: p>0.3, df=1, *U*=516.5 in PT-0; p>0.5, df=1, *U*=585.5 in PT-1, M-W), but the preferences of CHX-injected crickets for rewarded odor at 1 day (PT-2) and 4 days (PT-3) after training were significantly less than those of the control crickets (Fig. 3C: p<0.001, df=1, *U*=314.0 in PT-2; p<0.0001, df=1, *U*=218.0 in PT-3; Fig. 3D: p<0.001, df=1, *U*=286.5 in PT-2; p<0.0001, df=1, *U*=111.5 in PT-3, M-W). CHX-injected groups exhibited significant levels of retention at 1 day after training but not at 4 days after training: The preference for rewarded odor at 1 day after training (PT-2) was significantly greater than that before training (PT-0) (Fig. 3C: p<0.0001, df=1, *T*=179.0; Fig. 3D: p<0.01, df=1, *T*=180.0, WCX), but that at 4 days after training (PT-3) was not significantly greater than that before training (Fig. 3C: p>0.1, df=1, *T*=394.0; Fig. 3D: p>0.05, df=1, *T*=257.5, WCX).

Fig. 3E shows the effects of CHX-injection at 12 hr after training. The preferences of CHX-injected crickets for rewarded odor at 1 day and 4 days after training did not significantly differ from those of the control crickets (p>0.5, df=1, *U*=624.0 in PT-2; p>0.3, df=1, *U*=583.5 in PT-3, M-W).

In summary, CHX impairs 1-day and 4-day memory retention, but not 2-hr memory retention when it is injected 1 hr before or at 1 or 6 hr after training.

Time course of the development of CHX-sensitive memory phase

To examine the time course of the development of CHX-sensitive memory phase, eight different groups of crickets were injected with CHX at 1 hr after training and were tested at various times from 2 to 12 hr after training (Fig. 4). In the saline-injected (control) group, no significant decay of retention was found from 2 to 12 hr after training: the preference for rewarded odor at 2 hr after training did not significantly differ from that at 12 hr after training (p>0.5, df=1, *U*=198.5, M-W). In the CHX-injected groups, the preferences for rewarded odor from 2 to 4 hr after training were not significantly different from those of the control crickets at 2 hours and 12 hr after training (p>0.5, df=2, K-W), but the CHX-sensitive memory phase was detected 5 hr after training: the level of memory retention at 5 hr after training was significantly less than the levels in the control crickets at 2 and 12 hr after training (p<0.05, df=2, K-W). The CHX-sensitive memory phase developed gradually during the next several hr. We conclude that the CHX-sensitive memory phase emerges after 5 hr of training.

DISCUSSION

We found that the protein synthesis inhibitor CHX impairs olfactory memory retention after 5 hr of training in crickets. Injection of 16.8 μg CHX into the head capsule led to over 90% of protein synthesis inhibition for at least 3 hr in the cricket brain (Fig. 1). This was comparable to the results in honey bees, where over 90% of protein synthesis inhibition was maintained for at least four hr after injection of 4.2 μg CHX (Wittstock *et al*., 1993). In the crickets, the mortality rate of CHX-injected crickets did not differ from that of saline-injected controls when compared at 4 days after injection. Thus, it is likely that the impairment of long-term memory retention is due to the inhibition of protein synthesis, not due to the toxic side effect of CHX.

It could be argued that CHX might act not on memory formation but on retrieval. If this is the case, the effect of CHX should be a monotonous function of the time from CHX injection to the time of the retention test. However, no significant effect of CHX injection was found in a test performed 84 hr after injection when CHX was injected 12 hr after training, but significant effects on retention were found in tests performed earlier (at 18 and 23 hr after injection) and later (at 90 and 95 hr after injection) when CHX was injected 1 or 6 hr after training (Fig. 3). Therefore, the impairment of retention is likely to be due to an impairment of memory formation rather than that of retrieval.

In many systems of learning in both vertebrates and invertebrates, protein synthesis necessary for long-term memory has been reported to be restricted to one or two hr after training (Davis and Squire, 1984). We found, however, an impairment of retention when CHX was injected not only 1 hr before or after training but also 6 hr after training (Fig. 3B-D). Similar results have been obtained in honey bees, in which olfactory memory retention at 4 days after conditioning was impaired when actinomycin D was injected 1 or 6 hours after training (Wüstenberg *et al*., 1998). Thus, a relatively long time-window of susceptibility to protein synthesis inhibition may be a characteristic feature of olfactory memory in insects. Studies in chicks (Rose, 1995a, b) and rats (Grecksch and Matthies, 1980) suggest two windows of susceptibility to protein synthesis inhibition: one immediately after training and another beginning several hours after

training. It would be interesting to investigate whether the long time-window of susceptibility to interference with protein production in cricket olfactory memory represents a one-window or a two-window organization of protein synthesis.

In honey bees, protein synthesis-dependent olfactory memory phase emerges only after 3–4 days after training, and no significant effect of protein-synthesis inhibition was found in tests performed at 1 or 2 days after training (Menzel *et al*., 1993; Wittstock *et al*., 1993; Wittstock and Menzel, 1994; Wüstenberg *et al*., 1998; Grünbaum and Müller, 1998). We found that protein synthesis-dependent memory phase emerges much earlier in crickets. CHX-sensitive memory phase was observed at 5 hr after training, and this memory phase developed gradually during the next several hr. It would be interesting to examine the functional significance and neural basis of different time course of the formation of protein synthesis-dependent memory phase between crickets and honey bees. The difference in the time course of the formation of long-term olfactory memory between crickets and honey bees may reflect the difference in the variability of their food sources: In honey bees, flowers as food sources are unreliable are changeable with days, while in crickets food items may be less changeable. Therefore, it may be beneficial for crickets to establish long-term olfactory memory about food sources more quickly than honey bees do in order to achieve effective foraging. In the fruit-fly *Drosophila*, CHX-sensitive memory phase was observed in tests performed 1 day after training (Tully *et al*., 1994), but the time course of the development of this memory phase has not been studied.

Insects have been shown to be pertinent models for studying the neural basis of olfactory memory formation. In *Drosophila,* the mushroom body is thought to be involved in the retrieval of olfactory memory (Dubnau *et al*., 2001; McGuire *et al*., 2001). In honey bees, both the mushroom body and the antennal lobe are thought to be involved in olfactory memory processing (Erber *et al*., 1980; Menzel and Müller, 1996; Menzel, 1999), and an identified mushroom body extrinsic neuron, the PE1 neuron, has been suggested to be involved in the processing of nonassociative and associative short-term memory (Mauelshagen, 1993). However, the mechanisms underlying memory formation and retention at the levels of neurons and neural networks remain unclear. Crickets may emerge as a useful model for electrophysiological study of the neural processes underlying long-term memory formation, because we have established a method using wire electrodes to chronically record the activities of brain neurons for more than 6 hr in unrestrained crickets (Y. Matsumoto and M. Mizunami, unpublished results) by modifying methods used to record the activities of extrinsic neurons of the mushroom body of the cockroach (Mizunami *et al*., 1998; Okada *et al*., 1999). The next step for us is to study changes in the activity of antennal lobe and mushroom body neurons associated with the formation of protein synthesis-dependent long-term memory.

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