Title

Analysis and modeling of neural processes underlying sensory preconditioning

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Abstract

Sensory preconditioning (SPC) is a procedure to demonstrate learning to associate between relatively neutral sensory stimuli in the absence of an external reinforcing stimulus, the underlying neural mechanisms of which have remained obscure. We address basic questions about neural processes underlying SPC, including whether neurons that mediate reward or punishment signals in reinforcement learning participate in association between neutral sensory stimuli. In crickets, we have suggested that octopaminergic (OA-ergic) or dopaminergic (DA-ergic) neurons participate in memory acquisition and retrieval in appetitive or aversive conditioning, respectively. Crickets that had been trained to associate an odor (CS2) with a visual pattern (CS1) (phase 1) and then to associate CS1 with water reward or quinine punishment (phase 2) exhibited a significantly increased or decreased preference for CS2 that had never been paired with the US, demonstrating successful SPC. Injection of an OA or DA receptor antagonist at different phases of the SPC training and testing showed that OA-ergic or DA-ergic neurons do not participate in learning of CS2-CS1 association in phase 1, but that OA-ergic neurons participate in learning in phase 2 and memory retrieval after appetitive SPC training. We also obtained evidence suggesting that association between CS2 and US, which should underlie conditioned response of crickets to CS2, is formed in phase 2, contrary to the standard theory of SPC assuming that it occurs in the final test. We propose models of SPC to account for these findings, by extending our model of classical conditioning.

Keywords

sensory preconditioning, classical conditioning, olfactory learning, visual learning, insects

1. Introduction

The capability of learning to associate between external sensory signals and to predict future sensory events plays critical roles in survival of animals in a changing environment. Associative learning of animals typically occurs in the presence of a biologically significant sensory stimulus that serves as a reinforcing stimulus. However, many animals including insects (Müller, Gerber, Hellstern, Hammer, & Menzel, 2000), molluscs (Kojima, Kobayashi, Yamanaka, Sadamoto, Nakamura, Fujita, Kawai, Sakakibara, & Ito, 1998) and humans are also capable of learning to associate between relatively neutral stimuli in the absence of an external reinforcing stimulus, as has been demonstrated by the capability of sensory preconditioning (SPC) (Brogden, 1939). The SPC procedure consists of two phases (Rescorla, 1980). In phase 1, the subject is presented with two neutral sensory stimuli (conditioned stimuli, CS2 and CS1), and in phase 2, one of the stimuli (CS1) is paired with a rewarding or punishing stimulus (unconditioned stimulus, US). Then response of the animals to CS2 is tested (Fig. 1A). A significant learning score for CS2 indicates successful SPC.

The SPC procedure has been frequently used for analysis of associative process underlying learning (Gewirtz & Davis, 2000; Dwyer & Killcross, 2006), but the fundamental question of whether learning of association between neutral sensory stimuli in SPC occurs by the same learning rules and neural mechanisms as those of reinforcement learning remains unclear. Notably, whether neurotransmitters that mediate reinforcing signals in appetitive or aversive learning underlie formation of associations between neutral sensory stimuli has remained unsolved. One study in rodents showed that dopamine (DA) is released in the nucleus accumbens during training in phase 1 of an aversive SPC paradigm, as it is during aversive or appetitive conditioning, and the authors suggested that accumbal DA-ergic neurons serve as reinforcing neurons not only in aversive or appetitive learning (Schultz, 2007) but also in learning to associate between neutral stimuli (Young, Ahier, Upton, Joseph, & Gray, 1998). This suggestion, however, is not consistent with the finding in another study on rats that administration of a dopamine receptor antagonist before training in phase 1 of

aversive SPC did not impair SPC (Nader & LeDoux, 1999).

In insects, evidence suggests that octopaminergic (OA-ergic) and DA-ergic neurons mediate appetitive and aversive reinforcing signals, respectively, in classical conditioning (honey bees: Hammer & Menzel, 1998; Farooqui, Robinson, Vaessin, & Smith, 2003; Vergoz et a. 2007; crickets: Unoki, Matsumoto & Mizunami, 2005, 2006; fruit-flies: Schwaerzel, Monastirioti, Scholz, Friggi-Grelin, Birman, & Heisenberg M, 2003; Schroll, Riemensperger, Buchner, Ehmer, Völler, Erbguth, Gerber, Hendel, Nagel, Buchner, & Fiala, 2006; Aso, Siwanowicz, Bräcker, Ito, Kitamoto, & Tanimoto, 2010), although in *Drosophila*, critical roles of DA-ergic neurons in mediating appetitive reinforcing signals have also be suggested (Kim, Lee, & Han, 2007; Liu, Plaçais, Yamagata, Pfeiffer, Aso, Friedrich, Siwanowicz, Rubin, Preat, & Tanimoto, 2012). In crickets, moreover, we have suggested that OA-ergic or DA-ergic neurons also participate in memory retrieval after appetitive or aversive conditioning (Mizunami, Unoki, Mori, Hirashima, Hatano, & Matsumoto, 2009; Mizunami & Matsumoto, 2010).

In this study, we first established procedures for SPC in crickets, which allow long-term (1day) memory retention after SPC training. In insects, SPC has been reported in honey bees (Müller et al., 2000; Hussaini, Komischke, Menzel, & Lachnit, 2007) and fruit-flies (Brembs & Heisenberg, 2001; Guo & Guo, 2005), but not in any other species. Moreover, the effect of SPC has been found only shortly after training (<24 min) in these studies, which has hampered deeper analysis of SPC. Then, we studied the effects of pharmacological blockade of OA-ergic or DA-ergic transmission at various stages of the SPC procedure. Moreover, we addressed another fundamental question concerning SPC (Hall, 1996) of whether association between CS2 and US, which should underlie conditioned response of crickets to CS2, is formed in phase 2 or in the final test. Finally, we propose models of SPC to account for findings in this study.

2. Materials and Methods

2.1. Insects

Adult male crickets, *Gryllus bimaculatus*, at 1 week after the imaginal molt, were used in this study. Three days before the start of the experiment, animals were placed individually in beakers and deprived of drinking water to enhance their motivation to search for water.

2.2. Procedures for SPC

SPC training consisted of two phases (Fig. 1A). In phase 1, an apple or banana odor (CS2) and a white-center and black-surround pattern (CS1) were presented at the same time to the animals. For presentation of stimuli, a visual pattern and a small piece of filter paper soaked with apple essence or banana essence were attached to the needle of a syringe (Fig. 1B), and the pattern and the paper were simultaneously presented near the head of the animal for 2 sec. This trial was repeated 4 or 8 times with an inter-trial interval (ITI) of 1 min or 5 min. The procedure of phase 2 training (Fig. 1B) was the same to that of appetitive or aversive conditioning of a visual pattern described previously (Unoki et al., 2006). A visual pattern (CS1) was presented to the animal for 2 sec and then a drop of water (appetitive US) or 10% quinine solution (aversive US) was given to the mouth. The trials were repeated 4 or 6 times with an ITI of 2.5 or 5 min. The interval between phase 1 training and phase 2 training was 5 or 60 min.

For control of the non-associative effect, one group of crickets was subjected to unpaired presentations of CS2 and CS1 in phase 1 and then subjected to paired presentations of CS1 and US in phase 2 (Unpaired/Paired or UP/P group), and another group was subjected to paired presentations of CS2 and CS1 and then unpaired presentations of CS1 and US (Paired/Unpaired or P/UP group). Unpaired presentations were performed in a pseudo-random sequence with an interval of 2.5 min, with the number of presentations of stimuli being the same as that in paired trials.

All groups of animals were subjected to odor preference tests before and after

conditioning. We used the “operant testing” procedure, which is based on a high capability of crickets to transfer memory formed in a classical conditioning situation to an operant testing situation (Matsumoto & Mizunami, 2002; Unoki et al., 2005, 2006). In short, on the floor of the test chamber of the test apparatus, there were two holes that connected the chamber with two odor sources (Fig. 1C). Each odor source consisted of a plastic container containing a filter paper soaked with 3 μl solution of apple essence or banana essence, covered with fine gauze net. Three containers were mounted on a rotative holder and two of three odor sources could be located simultaneously just below the holes of the test chamber. Before the odor preference test, a cricket was transferred to the waiting chamber at the waiting position and left for about 4 min to become accustomed to the surroundings. Then the cricket was allowed to enter the test chamber and the test started. Two min later, the relative positions of the banana and apple sources were changed by rotating the container holder. The preference test lasted for 4 min. If the total time of visits of an animal to either source was less than 10 sec, we considered that the animal was less motivated to visit odor sources, possibly due to a poor physical condition, and the data were rejected.

2.3. Procedures for aversive conditioning with quinine punishment

We newly developed a procedure for conditioning of a visual pattern with 10% quinine solution, the procedure being the same as that of aversive visual pattern conditioning with sodium chloride solution (Unoki et al., 2006). Either a white-center and black-surround pattern (white-center pattern) or black-center and white-surround pattern (black-center pattern) was used for conditioning. The procedure for the visual pattern preference test was the same as that described previously (Unoki et al., 2006). In short, two white-center patterns and one black-center pattern were presented on a grey sliding wall at the end of the test chamber, and the animal was allowed to freely choose between the two patterns during a test of 4 min in duration. If the total visiting time was less than 10 sec, we considered that the animal was less motivated to visit patterns and the data were rejected.

2.4. Pharmacology

Groups of animals were injected with 3 μl of physiological saline (Matsumoto, Noji, & Mizunami, 2003) or saline containing 1 μM epinastine (Roeder, Dagen, & Gewecke, 1998) or 200 μM cis(z)-flupenthixol (Mustard, Blenau, Hamilton, Ward, Ebert, & Mercer, 2003) into the head hemolymph. The estimated final concentrations after diffusion were 3.5 nM for epinastine and 700 nM for flupenthixol, calculated from the injected volume and the approximate body weight of 850 mg. The drugs were purchased from Sigma (Tokyo, Japan).

2.5. Data analysis

An odor (or a pattern) was considered to have been visited when the cricket probed the top net of the odor source on the floor (or the pattern on the wall in the case of visual pattern conditioning) with its mouth or pulpi. The time spent visiting each odor or pattern was measured cumulatively. In appetitive SPC, relative preference of each animal was determined using the preference index (PI) for rewarded odor, defined as $t_r/(t_r+t_{nr})x100$, where t_r was the time spent exploring the odor associated with reward and t_{nr} was the time spent exploring the odor or pattern not associated with reward. In aversive SPC, relative preference was determined using the PI for unpunished odor, defined as $t_{np}/(t_{np}+t_p)x100$, where t_{np} was the time spent exploring the odor not associated with punishment and t_p was the time spent exploring the odor associated with punishment. In aversive conditioning of a visual pattern, relative preference for the control pattern was similarly calculated. The Wilcoxon's test (WCX test) was used to compare preferences before and after training, the Mann-Whitney test (M-W test) was used to compare between groups. For multiple comparisons, Holm method was used to adjust the significance level. We found no significant differences in odor preferences or visual pattern preferences among the different groups of animals before training (Kruskal-Wallis test, $p>0.5$). Since we observed no difference in conditioning effect between groups in which banana odor was used as CS2 and apple odor was used as CS2, data

from the two groups were pooled.

3. Results

3.1. Appetitive SPC with water reward

We first attempted to establish appropriate procedures for an appetitive form of SPC in crickets. In insects, appetitive SPC has been reported in honey bees (Müller et al., 2000; Hussaini et al., 2007) but not in any other species of insects. We developed procedures for SPC training by modifying procedures for appetitive conditioning of odor or visual pattern with water reward in crickets (Matsumoto & Mizunami, 2002; Unoki et al., 2006).

One group of crickets (paired/paired or P/P group) was subjected to simultaneous presentations of a white-center and black-surround pattern (white-center pattern, CS1) and an apple or banana odor (CS2) 8 times (phase 1 training) and then subjected to pairing of a visual pattern (CS1) and water reward (US^+) 4 times (phase 2 training) (Fig. 1B; insets above A-C in Fig. 2). The inter-trial interval (ITI) was 5 min, and the interval between phase 1 training and phase 2 training was also 5 min. One control group (unpaired/paired or UP/P group) was subjected to unpaired presentations in phase 1 and paired presentations in phase 2. Another control group (P/UP group) was subjected to paired presentations in phase 1 and unpaired presentations in phase 2. In all groups, relative preferences between apple and banana odors, one of which was presented in phase 1 (CS2) and the other of which was not presented (control odor), were tested in a test apparatus (Fig. 1C) before training and at 30 min after completing phase 2 training.

The experimental (P/P) group exhibited a significantly increased preference for the odor presented in phase 1 training (CS2) after training than that before training (Fig. 2A; $P<0.05$, WCX test adjusted by Holm method). On the other hand, neither the UP/P nor P/UP group exhibited a significantly different preference for CS2 after training compared to that before training (Fig. 2B,C; $P>0.05$, WCX test adjusted by Holm method). Comparisons among groups also showed that the preference after training in the P/P group was significantly

greater than that in the P/UP or UP/P group ($P<0.05$, S-D test adjusted by Holm method). The results indicate that the observed increase in preference for CS2 in the experimental group is due not to a non-associative effect but to the effect of SPC.

Next, we explored effective stimulus parameters to achieve SPC. In appetitive SPC in honey bees, in which two odors were used as CSs and sucrose solution was used as US, it has been reported that a single trail in phase 1 is sufficient to achieve maximal level of the SPC effect (Müller et al., 2000). Three groups of crickets were each subjected to 1, 2 or 4 trials of CS2-CS1 pairing in phase 1 and 4 trials of CS1-US^+ pairing in phase 2 (Fig. 2D-G). The 1-trial group and 2-trial group did not exhibit a significantly different preference for CS2 after training compared to that before training (Fig. 2D,E; $P>0.05$, WCX test), indicating that SPC was not achieved. On the other hand, the 4-trial group exhibited a significantly increased preference for CS2 after training (Fig. 2F; $P<0.01$, WCX test). In another group with 4-trial CS2-CS1 pairing and with a 1-min ITI, successful SPC was also achieved (Fig. 2G; $P<0.01$, WCX test). We conclude that 4 or 8 trials of CS2-CS1 pairing with a 1- or 5-min ITI in phase 1 are effective for achieving appetitive cross-modal SPC in crickets. We also performed appetitive SPC experiment with an odor as CS1 and a visual pattern as CS2 but we failed to find any SPC effect.

3.2. Aversive SPC with quinine punishment

We next attempted to establish procedures for aversive SPC. In insects, aversive SPC has been reported in the fruit-fly *Drosophila* (Brembs & Heisenberg, 2001; Guo & Guo, 2005), but not in any other species of insects. We have shown that crickets can achieve aversive conditioning of olfactory or visual pattern stimuli with sodium chloride solution (Matsumoto & Mizunami 2002; Unoki et al., 2006) but our preliminary study suggested that aversive SPC with sodium chloride solution was not fully successful, yielding only a marginal effect (data not shown). We thus attempted to establish, at first, aversive conditioning with quinine solution (Fig. 3A,B). In one group, one of two visual patterns (CS) paired with quinine

solution (US) was presented for four times with a 5-min ITI. Relative preferences between two visual patterns were tested before training and at 30 min after completing the training. The group exhibited a significantly increased preference for the control pattern (thus exhibiting decreased preference for punished pattern) at 30 min after conditioning (Fig. 3A; $p<0.05$, WCX test adjusted by Holm method). Unpaired presentations of the pattern and quinine solution did not yield a conditioning effect (Fig. 3*B*; $p>0.05$, WCX test adjusted by Holm method). Comparison between groups showed that the preference of the paired group after training was significantly greater than that of the unpaired group ($p<0.05$, M-W test adjusted by Holm method). The results indicate that the changed preference in the paired group is not due to a non-associative effect.

We proceeded to a study of the effect of SPC with quinine punishment (Fig. 3C-E). One group of crickets (P/P group) was subjected to simultaneous presentations of a white-center visual pattern (CS1) and an apple or banana odor (CS2) 8 times with a 5-min ITI (phase 1). Five minutes later, the group was subjected to 4 trials of CS1 (pattern)-US^- (quinine solution) pairing with a 5-min ITI (phase 2). One control group (UP/P group) was subjected to unpaired presentations in phase 1 and paired presentations in phase 2, and another control group (P/UP group) was subjected to paired presentations in phase 1 and unpaired presentations in phase 2. Relative preferences between apple and banana odors, one of which was presented in phase 1 and (CS2) and the other of which was not presented (control odor), were tested before training and at 30 min after completing phase 2 training.

The experimental (P/P) group exhibited a significantly increased preference for the control odor (and thus exhibiting decreased preference for CS2) at 30 min after training compared to that before conditioning (Fig. 3*C*; $P<0.05$, WCX test adjusted by Holm method). In contrast, neither the UP/P control group nor the P/UP control group exhibited a significantly different preference for CS2 after training compared to that before training (Fig. 3D, E; $P>0.05$, WCX test adjusted by Holm method). Comparison of preferences after training between groups showed that the preference for CS2 in the P/P group was

significantly greater than that in the P/UP or UP/P group ($P<0.05$, M-W test adjusted by Holm method). We thus conclude that the observed change of preference in the experimental group is due to the effect of SPC.

3.3. Studies on the roles of OA-ergic or DA-ergic neurons in aversive conditioning with quinine punishment

Studies on neurotransmitters involved in SPC require basic knowledge of neurotransmitters involved in classical conditioning, on which the SPC procedure is based. We have shown that OA receptor antagonists (epinastine and mianserin), but not DA receptor antagonists (chlorpromazine, spiperone, fluphenazine and flupenthixol), impair memory acquisition and retrieval in appetitive olfactory or visual conditioning with water reward (Unoki et al., 2005, 2006; Nakatani, Matsumoto, Mori, Hirashima, Nishino, Arikawa, & Mizunami, 2009; Mizunami et al., 2009). In contrast, DA receptor antagonists, but not OA receptor antagonists, impair memory acquisition and retrieval in aversive olfactory or visual conditioning with sodium chloride solution punishment (Unoki et al., 2005, 2006; Nakatani et al., 2009; Mizunami et al., 2009). We proposed a model of classical conditioning to account for these findings (Fig. 7A, Mizunami et al., 2009).

We studied, at first, whether our findings that DA-ergic neurons but not OA-ergic neurons participate in memory acquisition and retrieval in aversive conditioning with sodium chloride punishment are applicable to those in aversive conditioning with quinine punishment. We injected 3 μl of saline or saline containing 200 μM flupenthixol (DA receptor antagonist) or 1 μM epinastine (OA receptor antagonist) at 30 min before training or the final test (Fig. 4A-C). The doses of drugs were determined on the basis of our previous study (Unoki et al., 2005, Nakatani et al., 2009). The saline-injected group or epinastine-injected group exhibited a significantly increased preference for the control pattern (thus exhibiting decreased preference for punished odor) after training compared to that before training (Fig. 4B, C, $p<0.05$, WCX test adjusted by Holm method), indicating that aversive conditioning was achieved. On the

other hand, the preference for the control pattern of flupenthixol-injected group after training did not significantly differ from that before training (Fig. 4A, $p>0.05$, WCX test adjusted by Holm method), indicating impairment of conditioning. Comparison among groups also showed that the preference after training of the flupenthixol-injected group was significantly greater than that of the saline-injected group ($p<0.05$, M-W test adjusted by Holm method) but that of the epinastine-injected group was not ($p>0.05$, M-W test adjusted by Holm method). The results indicate that DA-ergic neurons, but not OA-ergic neurons, participate in memory acquisition in visual pattern conditioning with quinine punishment.

Next, effects of flupenthixol or epinastine were tested for memory retrieval after visual pattern conditioning with quinine punishment. Three groups of animals were injected with saline or saline containing 200 μM flupenthixol or 1 μM epinastine at 30 min before the final test. In another two groups, flupenthixol was injected at 60 min or 120 min before the test, to test durability of the effect of flupenthixol (This information was needed for designing experiments on SPC.). The epinastine-injected group (Fig. 4G; $p<0.05$, WCX test adjusted by Holm method) and the saline-injected group (Fig. 4H; $p<0.05$, WCX test adjusted by Holm method) exhibited a significantly increased preference for the control visual pattern after training. Comparison between groups also showed that the preference of the epinastine-injected group after training did not significantly differ from that of the saline-injected group ($p>0.05$, M-W test adjusted by Holm method). In contrast, none of the three flupenthixol-injected groups exhibited a significantly different preference for the control pattern after training compared to that before training (Fig. 4D-F; $p>0.05$, WCX test), indicating impairment of memory retrieval. The results suggest that DA-ergic neurons, but not OA-ergic neurons, participate in memory retrieval and that the effect of flupenthixol lasts for 120 min after injection.

3.4. Pharmacological study on the roles of OA-ergic and DA-ergic neurons in learning for associating between CS1 and CS2

Next, we examined the effect of flupenthixol or epinastine on CS2-CS1 association in phase 1 of appetitive or aversive SPC. This was achieved by combining two experiments: one experiment to determine the effect of injection of flupenthixol before phase 1 of appetitive SPC with water reward (Fig. 5A) and another experiment to determine the effect of injection of epinastine before phase 1 of aversive SPC with quinine punishment (Fig. 5B). Since the test was started 115 min after injection in this experiment (see legend of Fig. 5) and since we have shown that the effect of flupenthixol (Fig. 4F) or epinastine (Unoki et al., 2005) lasts for at least 120 min, the effect of flupenthixol should be maintained in all stages of the SPC experiment, namely, phase 1, phase 2 and the final test. Therefore, if flupenthixol impaired appetitive SPC, DA-ergic neurons should participate in one or more stages of the SPC experiment, and then we would proceed to the study on which stages are responsible for the effect of flupenthixol. Alternatively, if flupenthixol did not impair appetitive SPC, DA-ergic neurons should not participate in any stage of the SPC experiment.

One group of crickets was injected with 3 μl of saline containing 200 μM flupenthixol at 30 min before 8 trials of CS2-CS1 pairing and then received 4 trials of CS1-US^+ pairing. The flupenthixol-injected group exhibited a significantly increased preference for CS2 after training compared to that before training (Fig. 5A; $p<0.05$, WCX test adjusted by Holm method). Further comparison between groups showed that the preference for CS2 of the flupenthixol-injected group after training did not significantly differ from that of the non-injected group that received the same training (P/P group in Fig. 2A; $p>0.05$, M-W test adjusted by Holm method). The results suggest that DA-ergic neurons do not participate in phase 1, phase 2 or the final test of the appetitive SPC procedure. Another group of crickets was injected with 3 μl of saline containing 1 μM epinastine at 30 min before 8 trials of CS2-CS1 pairing and 4 subsequent trials of CS1-US^- pairing. The epinastine-injected group exhibited a significantly increased preference for CS2 after training (Fig. 5B; $p<0.05$, WCX test adjusted by Holm method). Further comparison between groups showed that the preference for CS2 of the epinastine-injected group after training did not significantly differ

from that of the non-injected group that received the same training (P/P group in Fig. 3C; $p>0.05$, M-W test adjusted by Holm method). The results indicate that OA-ergic neurons do not participate in phase 1, phase 2 or the final test of the aversive SPC procedure. By combining the results of the two experiments, we conclude that neither OA-ergic neurons nor DA-ergic neurons participate in learning of the association between CS2 and CS1 in phase 1, namely, associations between neutral sensory stimuli occur without involvement of OA-ergic or DA-ergic reinforcing neurons in crickets. Our findings that DA-ergic neurons or OA-ergic neurons also do not participate in phase 2 and the final test in appetitive or aversive SPC, respectively, match our previous findings that DA-ergic neurons or OA-ergic neurons do not participate in acquisition and retrieval of memory in appetitive or aversive conditioning, respectively (Unoki et al., 2005, 2006; Mizunami et al., 2009).

3.5. Effect of interval between phase 1 and phase 2 on SPC

One of the controversial issues regarding SPC is whether formation of association between CS2 and US by integration of sensory experience in phase 1 and phase 2, which should underlie SPC, occurs in phase 2 or in the final test (Rescorla & Freberg, 1978; Rescorla, 1980; Hall, 1996). In the latter case, the memory about CS1-CS2 association and that about CS1-US association formed in phase 1 and phase 2, respectively, are maintained until the final test and are integrated during retrieval. The results of our study on the effect of interval between phase 1 training and phase 2 training, however, suggested that the memory formed in phase 1 needs not to be maintained until the final test for achieving SPC (see below), thus refuting this possibility. We developed a training protocol that lead to the memory lasted for one day (~24 hours) after the phase 2 training, so that the interval between phase 1 and phase 2 could be changed from 5 min to 60 min in two groups of crickets while the interval between phase 1 training and the final test remained practically unchanged: One group was subjected to 8 trials of CS2-CS1 pairing and 6 trials of CS1-US^{+} pairing with a 2.5-min ITI with 5-min intervals between phase 1 training and phase 2 training. Another

group was subjected to the same number of trials with 60-min intervals between phase 1 training and phase 2 training. The 5-min interval group exhibited a significantly increased preference for CS2 one day after training (Fig. 6A; $p<0.01$, WCX test), indicating successful SPC. In contrast, the 60-min interval group did not exhibit a significantly changed preference one day after training (Fig. 6B; $p>0.05$, WCX test), thus indicating no SPC effect. The finding that the 5-min interval group, but not the 60-min interval group, exhibited SPC is best accounted for if the memory about CS2-CS1 association formed in phase 1 training is maintained for only a short period of time and if this memory needs to be activated in phase 2 training, but not in the final test, for achieving SPC. We thus conclude that phase 2 training, not the final test, is critical for formation of association between CS2 and US. This finding differs from the standard theory of SPC that such association is formed in the final test (Rescorla & Freberg, 1978; Hall, 1996), and urged us to construct models of SPC to account for this finding. For aversive SPC, we were not able to perform a similar experiment because we have not established procedures to achieve long-term (one-day) memory retention, a prerequisite for such an experiment.

3.6. Roles of OA-ergic neurons in phase 2 training and in memory retrieval after SPC training

Since we have shown that OA-ergic neurons, but not DA-ergic neurons, participate in memory acquisition and retrieval in appetitive conditioning with water reward (Unoki et al., 2005; Mizunami et al., 2009), we next addressed the issue of whether OA-ergic or DA-ergic neurons participate in formation of CS2-US association in phase 2 and retrieval of memory of this association in the final test. These experiments were aimed at determining whether our model of classical conditioning (Fig. 7A) can be used as a framework for constructing models of SPC.

Two groups of crickets were injected with 3 μl of saline or saline containing 1 μM epinastine at 30 min before 8 trials of CS2-CS1 pairing and subsequent 6 trials of CS1-US^{+} pairing with a 2.5-min ITI. The final test was performed one day (~24 hours) after completing

training. The animals were under the influence of epinastine in phase 1 and in phase 2 but not in the final test because the effect of epinastine lasts for 2 hours but not for one day (Mizunami et al., 2009). The saline-injected group exhibited a significantly increased preference for CS2 one day after training (Fig. 6C; $p<0.05$, WCX test adjusted by Holm method), but the epinastine-injected group did not (Fig. 6D; $p>0.05$, WCX test adjusted by Holm method). Comparison between groups showed that the preference for CS2 of the epinastine-injected group after training was significantly greater than that of the saline-injected group (M-W test adjusted by Holm method, $p<0.05$). The results indicate that epinastine impairs memory acquisition in either of phase 1 or phase 2 or both. Since we have shown that phase 1 is insensitive to epinastine (Fig. 5), phase 2 should be sensitive to epinastine. Thus, we conclude that OA-ergic neurons participate in formation of CS2-US^+ association, which is critical for achieving SPC, in phase 2 training. Another two groups of crickets were each subjected to 8 trials of CS2-CS1 pairing and 6 trials of CS1-US^+ pairing with a 2.5-min ITI. About 24 hours later, the groups were injected with 3 μl of saline (Fig. 6E) or saline containing 1 μM epinastine (Fig. 6F) at 30 min before the final test. The saline-injected group exhibited a significantly increased preference for CS2 (Fig. 6E; $p<0.01$, WCX test adjusted by Holm method), but the epinastine-injected group did not (Fig. 6F; $p>0.05$, WCX test adjusted by Holm method). Comparison between groups showed that the preference for CS2 of the epinastine-injected group after training is significantly greater than that of the saline-injected group ($p<0.01$, M-W test adjusted by Holm method). The results indicate impairment of memory retrieval by epinastine. We conclude that OA-ergic neurons participate in memory retrieval after appetitive SPC training. Taken together, OA-ergic neurons participate in memory acquisition in phase 2 and memory retrieval in the final test in appetitive SPC, as they do in appetitive conditioning, indicating that these two forms of conditioning share common neurotransmitter mechanisms.

3.7. Proposal of models of SPC

Finally, we developed models of SPC to account for our finding that formation of CS2-US association by integration of sensory experience in phase 1 and phase 2 occurs in phase 2 (Fig. 6A,B). These models were constructed on the basis of our model of classical conditioning (Fig. 7A, Mizunami et al., 2009), which assumes that (1) efficacy of synaptic transmission from "CS" neurons that represent CS to OA-ergic or DA-ergic neurons ("OA/DA" neurons) and that of synapses from "CS" neurons to "CR" neurons, the activation of which produces conditioned response (CR), are strengthened by pairing of CS and US, (2) after conditioning, presentation of CS activates "CS" neurons and then "OA/DA" neurons and (3) coincident activation of "OA/DA" neurons and "CS" neurons activates "CR" neurons (AND gate) and produces CR.

For the nature of the association between CS1 and CS2 formed in phase 1 of SPC training, two hypothesis have been proposed: One assumes formation of a configural unit that can be activated by presentation of either CS1 or CS2 (configural unit hypothesis) and the other assumes mutual associations between neurons representing CS1 and those representing CS2 (association-chain hypothesis) (Rescorla & Freberg, 1978; Hall, 1996). Since the results of our study do not allow discrimination of these two possibilities, either of the two hypotheses has been considered for constructing models of SPC (Fig. 7B and C). In these models, we assume that (1) the efficacy of synaptic connections for forming configural neurons ("CS1:2") (Fig. 7B) or for forming mutual connections between "CS1" and "CS2" neurons (Fig. 7C) is enhanced by CS2-CS1 pairing in phase 1, (2) the efficacy of synaptic connections from "CS1:2" neurons (Fig. 7B) or "CS1" and "CS2" neurons (Fig. 7C) to "OA/DA" neurons and "CR" neurons is enhanced by CS1-US pairing in phase 2, and (3) in the final test, presentation of CS2 activates "CS1:2" neurons (Fig. 7B) or "CS2" neurons (Fig. 7C) and then "OA/DA" neurons, and coincident activation of these two types of neurons activates "CR" neurons (AND gate) and produces learned responses. This model differs from previous models of SPC proposed in mammals (Hall, 1996; Rescorla & Freberg, 1978) in that (1) it is assumed that the association between CS2 and US is formed in phase 2, not in the final test,

and (2) it is assumed that activation of reinforcing neurons (OA-ergic neurons) is needed for retrieval of memory formed by SPC training.

4. **Discussion**

4.1. Major findings

SPC is a higher-order form of learning for testing the capability of animals to associate between relatively neutral sensory stimuli (Rescorla, 1980) and is useful for analysis of the associative process in learning (Gewirtz & Davis, 2000; Blundell, Hall, & Killcross, 2003; Dwyer & Killcross, 2006), but the underlying neural and molecular mechanisms remain unclear. In this study, we established procedures for aversive and appetitive forms of SPC training in crickets. Then we studied the effect of epinastine (OA receptor antagonist) and flupenthixol (DA-receptor antagonist) on each phase of SPC training and testing, and concluded that association between neutral sensory stimuli occurs without involvement of OA-ergic neurons or DA-ergic neurons, which have been suggested to convey appetitive US or aversive US in classical conditioning in insects (See references cited in the Introduction section.). We also obtained evidence suggesting that formation of CS2-US association by integration of experiences in phase 1 and phase 2 occurs in phase 2, in contrast to the widespread assumption that it occurs in the final test (Rescorla & Freberg, 1978; Hall, 1996). In addition, we suggested that OA-ergic neurons participate in acquisition of memory about association between CS2 and US in phase 2 and retrieval of the memory in the final test in appetitive SPC, as we have suggested for memory acquisition and retrieval in appetitive conditioning (Mizunami et al., 2009). By integrating these findings, we proposed models of SPC that account for our present findings (Fig. 7B, C), based on our model of classical conditioning in crickets (Mizunami et al., 2009, Fig. 7A). These findings have extended our knowledge of the neural basis of SPC and pave the way for further analysis of the cellular and molecular mechanisms of SPC in simpler model systems in insects.

Epinastine used in this study is known as a potent antagonist of insect OA receptors

(Roeder et al., 1998; Degen, Gewecke & Roeder, 2000b), but a recent study on honey bees (Beggs, Tyndall & Mercer, 2011) reported that it antagonizes not only OA receptors but also a type of DA receptor (DOP2). Because RNAi study on honey bees suggested participation of a type of OA receptor (OA1) in appetitive conditioning (Farooqui, Robinson, Vaessin & Smith, 2003) and because a study on the mutant of the DOP2 (DAMB) gene in *Drosophila* larvae showed no impairment of appetitive conditioning (Selcho, Pauls, Han, Stocker & Thum, 2009), we suppose it is less likely that the effect of epinastine observed in this study and in our previous studies is mediated by blockade of the DOP2 receptor. RNAi technique is available for the study of learning and memory in crickets (Takahashi, Hamada, Miyawaki, Matsumoto, Mito, Noji, & Mizunami, 2009), and we are currently studying the effect of RNAi of the DOP2 gene to resolve this issue.

4.2. SPC in insects: comparison with previous reports

We established procedures for appetitive SPC that allow retention of memory for ~24 hours, which matches protein synthesis-dependent long-term memory (Matsumoto et al., 2003), for the first time in insects. In previous studies on SPC in insects (honey bees: Müller et al., 2000; Hussaini et al., 2007; fruit-flies; Brembs, & Heisenberg, 2001; Guo & Guo, 2005), the effect of SPC training has been found only shortly (<24 min) after training, the memory of which matches short-term memory. Extended durability of memory after SPC training should facilitate future behavioral and pharmacological analyses of SPC. Secondly, we were able to achieve appetitive and aversive forms of SPC in a very similar experimental setting, which facilitates comparison between the two forms of learning. In insects, previous reports on appetitive SPC have been confined to honey bees, whereas those on aversive SPC have been confined to fruit-flies (See references cited above.).

We found that 4 or 8 trials, but not 1 or 2 trails, to associate between an odor and a visual pattern in phase 1 lead to SPC with water reward in crickets. In honey bees, it has been reported that a single trial to associate between two odors in phase 1 training is sufficient to

achieve SPC with sucrose reward (Müller et al., 2000). The reasons for the differences between the two studies are unknown, but a possible reason is that crossmodal sensory association in our study in crickets is more difficult to achieve than within-modal association in the previous study in honey bees. Another possible reason is the higher salience of the odors in comparison to the salience of visual stimuli.

4.3. Learning of association between neutral sensory stimuli

We showed that neither OA nor DA, which have been suggested to convey appetitive or aversive US in insect classical conditioning, participate in association between neutral sensory stimuli (Fig. 5). Neurotransmitters involved in association between neutral stimuli have been studied in SPC of rodents, but results have been conflicting: It has been shown in rodents that midbrain DA-ergic neurons mediate reinforcing signals in appetitive conditioning and aversive conditioning (Day, Roitmn, Wightman, & Carelli, 2007; Schultz, 2007), and one study suggested possible participation of these neurons in phase 1 training of SPC (Young, et al., 1998), but another study concluded that DA-ergic transmission does not participate in phase 1 training (Nader & LeDoux, 1999). Our results suggest that, in crickets, that learning of association between neutral sensory stimuli occurs by neural systems other than OA-ergic reward system or DA-ergic punishment systems. We also suggest that such mechanisms produce memory of limited durability, probably because retention of such memory is less likely to contribute to survival of animals if it is not incorporated into the memory of biologically-significant sensory events that occur subsequently. We should continue to search for neurotransmitters involved in associations between neutral stimuli. One of possible candidates is serotonin, because in some insects, participation of serotonergic neurons in some forms of aversive learning is reported (Sitaraman, Zars, Laferriere, Chen, Sable-Smith, Kitamoto, Rottinghaus, & Zars, 2008; Wright, Mustard, Simcock, Ross-Taylor, McNicholas, Popescu, & Marion-Poll, 2010).

4.4. Models of SPC

Our finding that phase 2 training needs to immediately follow phase 1 training to achieve a long-term SPC effect (Fig. 6A,B) suggests that formation of CS2-US association by integration of experiences in phase 1 and phase 2 occurs in phase 2, not in the final test. The standard theory of SPC assumes that the formation of CS2-US association occurs in the final test (Rescorla & Freberg, 1978), but to our knowledge, there have been no studies to convincingly show whether it occurs in phase 2 or in the final test (Hall, 1996). Our finding thus urged us to construct new models of SPC (Fig. 7B and 7C). In our models, either of the two hypotheses on the nature of the association of neutral stimuli in phase 1, namely, configural unit hypothesis and association-chain hypothesis (Rescorla & Freberg, 1978; Hall, 1996; Müller et al., 2000), has been incorporated into a model of classical conditioning (Fig. 7A, Mizunami et al., 2009). Our models of SPC account for the major findings in this study, namely, (1) formation of CS2-CS1 association in phase 1 occurs independent of OA-ergic or DA-ergic neurons, (2) formation of association between CS2-US occurs in phase 2, and (3) activation of OA-ergic neurons is needed for acquisition and retrieval of memory about CS2-US association in appetitive SPC. A previous study on appetitive SPC in honey bees, in which two different odors were used as CSs, provided evidence to support the configural unit hypothesis (Müller et al., 2000), and whether this is applicable to cross-modal association in SPC in crickets needs to be studied.

Results of this study do not discriminate these two hypotheses, but our models should help designing experiments for studying which of the two hypotheses better accounts for SPC. The model shown in Fig. 7B assumes that synaptic connections that activate “CS1:2” neurons by CS1 or CS2 are short-lived after phase 1 training and needs to be converted into a long-lasting form in phase 2 training so that the “CS1:2” neurons are activated by presentation of CS2 in the final test. In the model shown in Fig. 7C, on the other hand, the enhanced synaptic connections between “CS1” and “CS” neurons in phase 1 need not be maintained until the final test, because activation of these connections is not needed for memory retrieval in the

final test. Selective extinction experiments with repeated presentation of CS1 after SPC training and subsequent testing of the capability of CS2 to produce an SPC effect may be helpful for discrimination between the different hypotheses: the model in Fig. 7B expects extinction of the SPC effect by this procedure but that in Fig. 7C does not. Another possible experiment is to utilize a devaluation procedure, with CS1 paired with quinine punishment after appetitive SPC training with water reward and subsequent testing of the capability of CS2 to produce an SPC response.

4.5. Toward elucidation of the brain mechanisms of SPC

The brain areas in which associations between olfactory and visual stimuli occur in phase 1 training should be one of major subjects of our study. An obvious candidate is the mushroom body, which plays essential roles in olfactory learning (Heisenberg, 2003; Davis, 2005; Menzel & Giurfa, 2006; Watanabe, Matsumoto, Nishino, & Mizunami, 2011) and in which integration of multisensory signals, including olfactory, visual and gustatory signals, occurs (Li & Strausfeld, 1997, 1999; Mizunami, Okada, Li, & Strausfeld, 1998a, Mizunami, Weibrecht & Strausfeld, 1998b; Okada, Ikeda, & Mizunami, 1999). Another possible site for integration of olfactory and visual stimuli is the central complex, since some studies have suggested that it participates in visual learning (Liu, Seiler, Wen, Zars, Ito, Wolf & Heisenberg, 2006) and in the processing of olfactory memory (Wu, Xia, Fu, Wang, Chen, Leong, Chiang, & Tully, 2007). In mammals, there are reports suggesting that the association between CSs occurs in a brain area distinct from that for the association of a CS with a US; namely, in classical conditioning of eye blink response in rabbits, it has been reported that the cerebellum, but not the hippocampus, participates in conditioning (Thompson, 1991), whereas SPC for this response requires an intact hippocampus (Port & Patterson, 1984). Therefore, whether the association between CSs occurs in the same brain areas as those for the association between a CS and a US should be a critical question for our future research.

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Figure captions

Fig. 1. Methods for SPC. (**A**) A table showing experimental procedures for SPC. (**B**) Training for appetitive or aversive SPC. In phase 1, an apple or banana odor (CS2) was simultaneously presented with a white-center visual pattern (CS1). In phase 2, the visual pattern (CS1) was associated with water (appetitive US) or quinine solution (aversive US). (**C**) Test apparatus. For the test of relative odor preference between apple and banana odors, a cricket was placed in the waiting chamber for acclimation and then allowed to enter the test chamber to freely visit apple and banana odor sources.

Fig. 2. Appetitive SPC. (**A**) One group of animals was subjected to simultaneous presentation of CS2 (apple or banana odor) and CS1 (white-center pattern) in phase 1. In phase 2, the group was subjected to presentation of CS1 followed by presentation of water (US+) to the mouth. (**B, C**) Another two groups were each subjected to unpaired presentation in phase 1 and paired presentation in phase 2 (UP/P groups) (**B**) or paired presentation in phase 1 and unpaired presentation in phase 2 (P/UP groups) (**C**). The inter-trial interval (ITI) was 5 min. (**D-F**) Effects of different numbers of trials in phase 1 training. Three groups of animals were subjected to 1 (**D**), 2 (**E**) or 4 (**F**) trials of CS2-CS1 pairing and then 4 trials of CS1-US+ pairing. The ITI was 5 min. (**G**) Another group was subjected to 4 trials with a 1-min ITI in phase 1 and then 4 trials with a 5-min ITI in phase 2. In all experiments, the interval between phase 1 and phase 2 was 5 min. Relative odor preference (PI) between apple and banana odors was tested before and at 30 min after training. PIs for the odor used as CS2 before (white bars) and after (grey bars) training are shown as box and whisker diagrams. The line in the box is the median and the box represents the 25-75 percentiles in this and in all following figures. Whiskers extend to extreme values as long as they are within a range of 1.5× box length. The number of animals is shown below the boxes. Wilcoxon's test was used for comparison of preference before and after conditioning and Mann-Whitney test was used to

compare between groups. For multiple comparisons, Holm method was used to adjust the significance level. The results of statistical comparison are shown as asterisks (＊＊$p<0.01$; ＊$p<0.05$; NS $p>0.05$).

Fig. 3. Aversive conditioning and aversive SPC. (**A, B**) Aversive conditioning of a visual pattern. One group of crickets was subjected to 4-trial aversive conditioning to associate a visual pattern with quinine solution (US-) with ITI of 5 min (**A**). Another group received unpaired presentations of the CS and US- with intervals of 2.5 min and with pseudo-random sequences (**B**). Relative preference between white-center pattern and black-center pattern was tested before training and at 30 min after conditioning. PIs for the control pattern not used for conditioning before (white bars) and after (gray bars) training are shown as box and whisker diagrams. (**C-E**) Aversive SPC. One group of animals was subjected to 8 trials of CS2 (odor)-CS1 (pattern) pairing and then 4 trials of CS1-US- (quinine solution) pairing (**C**). One control group was subjected to unpaired presentations in phase 1 and paired presentation in phase 2 (UP/P group) (**D**) and another control group was subjected to paired presentation in phase 1 and unpaired presentation in phase 2 (P/UP group) (**E**). The ITI was 5 min, and the interval between the first and second phases was 5 min. Relative odor preference was tested before and at 30 min after completing the training. PIs for the control odor not used as CS2 before and after conditioning are shown as box and whisker diagrams. The number of animals is shown below the boxes. Wilcoxon's test was used for comparison of preference before and after conditioning and Mann-Whitney test was used to compare between groups. For multiple comparisons, Holm method was used to adjust the significance level. The results of statistical comparison are shown as asterisks (＊$p<0.05$; NS $p>0.05$).

Fig. 4. Effects of OA or DA receptor antagonist on acquisition and retrieval of memory in aversive conditioning with quinine punishment. **(A-C**) Experiments to study the effect of flupenthixol (DA receptor antagonist) or epinastine (OA receptor antagonist) on aversive

visual pattern conditioning with quinine punishment. Three groups of animals were each injected with 3 μl of saline (**C**) or saline containing 200 μM flupenthixol (**A**) or 1 μM epinastine (**B**) at 30 min before 4-trial conditioning of a white-center or black-center pattern (CS) with quinine solution (US-). Relative preference between rewarded pattern and control pattern was tested one day (~24 hours) after training. (**D-H**) Experiments to study the effect of flupenthixol or epinastine on memory retrieval after aversive visual pattern conditioning with quinine punishment. Three groups of animals were subjected to 4-trial conditioning of a white-center or black-center pattern (CS) with quinine solution (US-). One day (~24 hours) after training, each group was injected with 3 μl of saline containing 200 μM flupenthixol at 60 min (**D**), 90 min (**E**) and 120 min (**F**) before the test. Another group was subjected to 4-trial conditioning of a visual pattern with quinine solution. One day after training, the group was injected with 3 μl of saline (**H**) or saline containing 1 μM epinastine (**G**) at 30 min before the test. PIs for the control pattern before (white bars) and after (gray bars) conditioning are shown as box and whisker diagrams. The number of animals is shown below the boxes. Wilcoxon's test was used for comparison of preference before and after conditioning and Mann-Whitney test was used to compare between different groups. For multiple comparisons, Holm method was used to adjust the significance level. The results of statistical comparison are shown as asterisks ($*$ $p<0.05$; NS $p>0.05$).

Fig. 5. Effects of injection of flupenthixol before phase 1 of appetitive SPC. (**A**) or injection of epinastine before phase 1 of aversive SPC (**B**). Two groups of crickets were each injected with 3 μl of saline containing 200 μM flupenthixol (**A**) or 1 μM epinastine (**B**) at 30 min before phase 1 of appetitive or aversive SPC, respectively. The groups each received 8 trials of CS2 (odor)-CS1 (pattern) pairing and then 4 trials of CS1-US+ (water) or CS1-US- (quinine solution) pairing. The ITI was 5 min, and the interval between phase 1 training and phase 2 training was 5 min. Relative odor preference was tested before and at 30 min after training. PIs for CS2 (**A**) or control odor (**B**) before (white bars) and after (black bars)

conditioning are shown as box and whisker diagrams. The number of animals is shown below the boxes. Wilcoxon's test was used for comparison of preference before and after conditioning and Mann-Whitney test was used to compare between groups. For multiple comparisons, Holm method was used to adjust the significance level. The results of statistical comparison are shown as asterisks (＊p<0.05).

Fig. 6. Analysis of appetitive SPC. (**A, B**) Effects of interval between phase 1 training and phase 2 training on SPC. Two groups of animals were each subjected to 8 trials of CS2 (odor)-CS1 (pattern) pairing and then 6 trials of CS1-US+ (water) pairing with an ITI of 2.5 min. The intervals between phase 1 training and phase 2 training were 5 min in one group (**A**) and 60 min in the other group (**B**). Relative odor preference was tested before and at ~24 hours after the training. (**C-F**) Four groups of animals were each subjected to 8 trials of CS2-CS1 pairing and then 6 trials of CS1-US+ pairing with an ITI of 2.5 min. The interval between phase 1 training and phase 2 training was 5 min. Relative odor preference was tested before and at ~24 hours after the training. In two groups, 3 μl of saline (**D**) or saline containing 1 μM epinastine (**C**) was injected 30 min before phase 1 training. In another two groups, 3 μl of saline (**F**) or saline containing 1 μM epinastine (**E**) was injected 30 min before the final test. PIs for CS2 are shown as box and whisker diagrams. The number of animals is shown below the boxes. Wilcoxon's test was used for comparison of preference before and after training and Mann-Whitney test was used to compare between groups. For multiple comparisons, Holm method was used to adjust the significance level. The results of statistical comparison are shown as asterisks (＊＊p<0.01; ＊p<0.05; NS p>0.05).

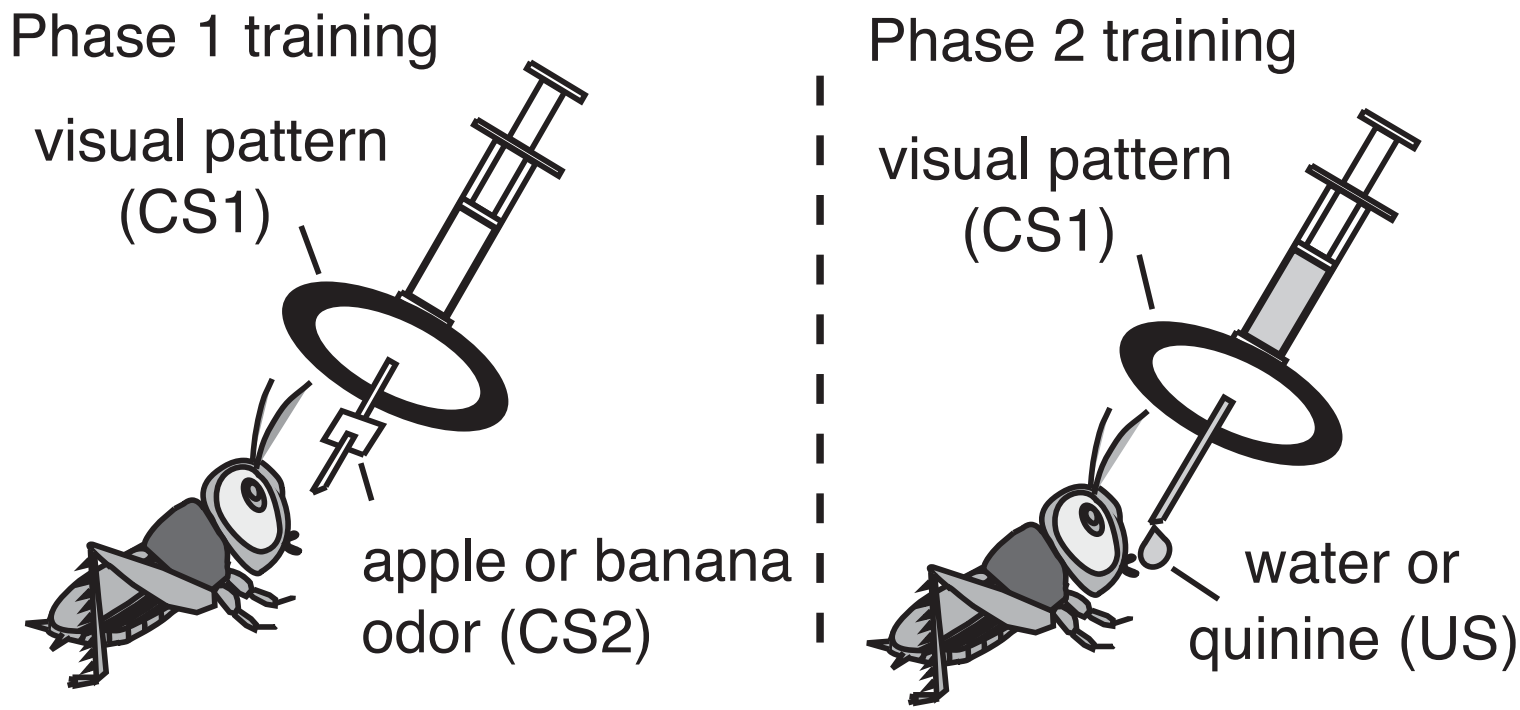
Fig. 7. Models of SPC. (**A**) A model of classical conditioning proposed to account for the finding that OA-ergic neurons or DA-ergic neurons participate in memory acquisition and retrieval in appetitive or aversive conditioning, respectively (Mizunami et al., 2009). The model assumes that (1) "CS" neurons (assuming intrinsic neurons of the mushroom body,

called Kenyon cells) that convey signals about a CS make synaptic connections with dendrites of “CR” neurons (assuming efferent (output) neurons of the mushroom body lobe), activation of which leads to a CR that mimics unconditioned response (UR), but these synaptic connections are silent or very weak before conditioning, (2) OA-ergic or DA-ergic efferent neurons projecting to the lobes (“OA/DA” neurons), which convey signals for appetitive or aversive US, respectively, make synaptic connections with axon terminals of “CS” neurons, (3) “CS” neurons also make silent synaptic connection with “OA/DA” neurons, (4) the efficacy of the synaptic transmission from “CS” neurons to “CR” neurons and to “OA/DA” neurons is strengthened by coincident activation of “CS” neurons and “OA/DA” neurons during appetitive or aversive conditioning and (5) coincident activation of “CS” neurons and “OA/DA” neurons is needed for activation of “CR” neurons (AND gate) and for production of CR in response to CS. In short, this model assumes formation of both “S-S (CS-US) connections” and “S-R (CS-CR) connections” (Holland, 1993) by conditioning and activation of both connections for producing CR (for details, see Mizunami et al., 2009). (**B, C**) Models of SPC proposed by incorporating the configural unit hypothesis (**B**) or the association-chain hypothesis (**C**) (Rescorla & Freberg, 1978; Hall, 1996) into our model of classical conditioning. In (***B)***, it is assumed that the efficacy of synaptic connections that allow CS1 or CS2 to activate configural units (input synapses to “CS1:2” neurons) is strengthened in phase 1, in accordance with the configural unit hypothesis. These connections are assumed to be relatively short-lived after phase 1 and need to be converted into long-lasting forms in phase 2, so that the connections are activated by presentation of CS2 in the final test. In (**C**), it is assumed that the efficacy of mutual synaptic transmissions between “CS1” and “CS2” neurons, which code CS1 and CS2, respectively, is strengthened in phase 1, in accordance with the association-chain hypothesis. These synaptic connections need to be maintained until phase 2 but not until the final test.

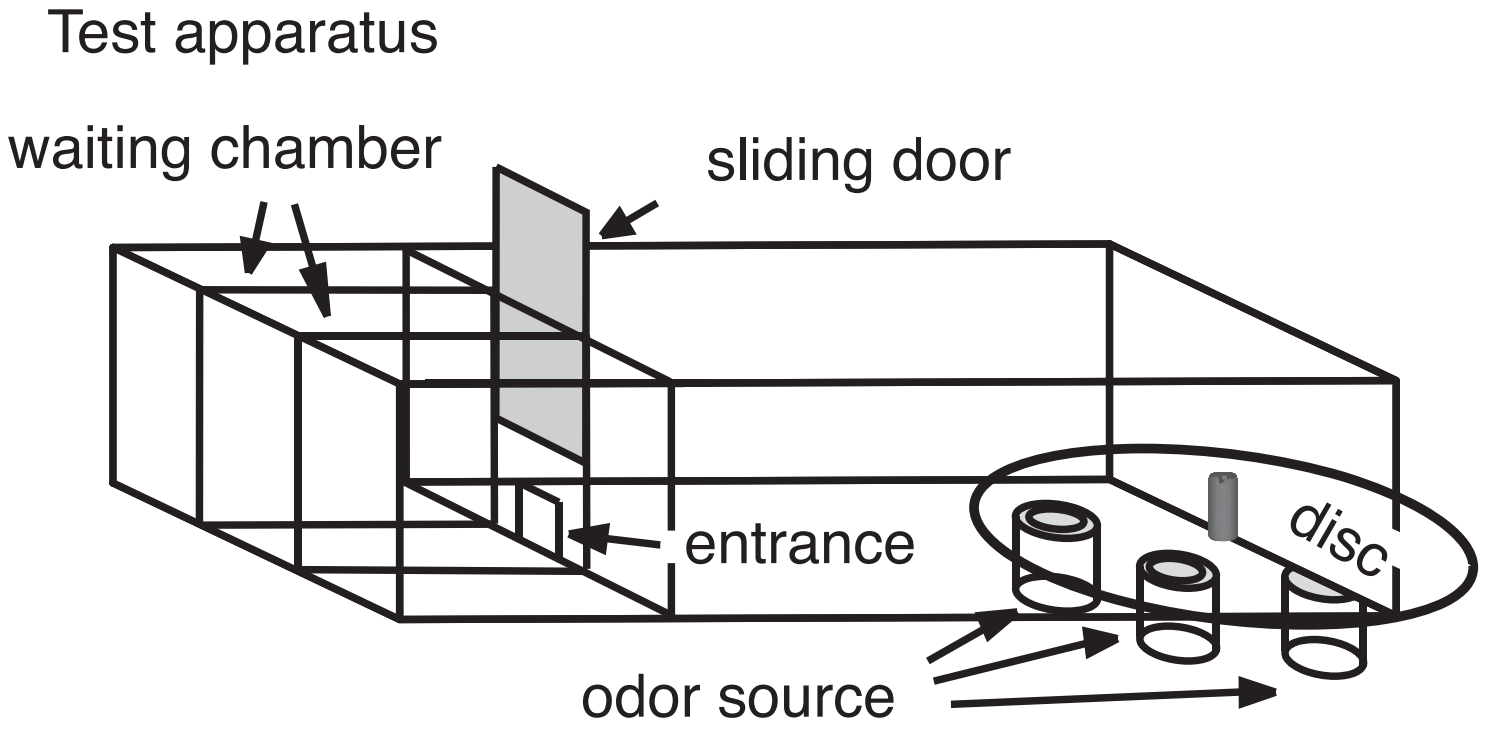
A

Phase 1	Phase 2	Test
CS2:CS1	CS1→US	CS2

B



C



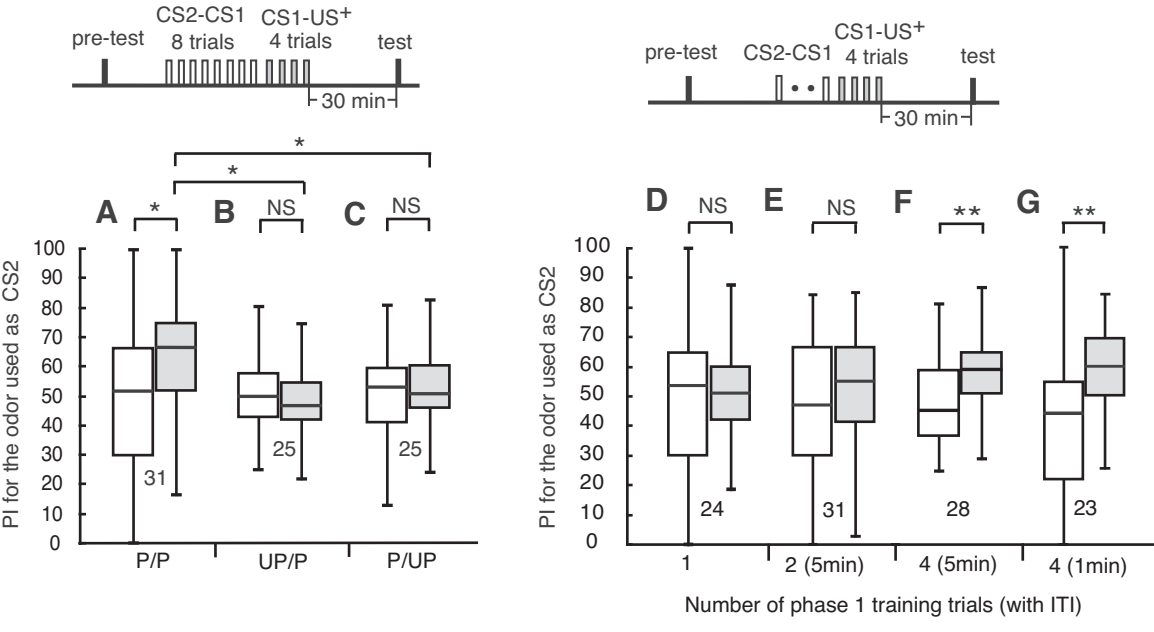

pre-test
CS2-CS1
8 trials
CS1-US+
4 trials
test
30 min
*
*
A
*
B
NS
C
NS
PI for the odor used as CS2
100
90
80
70
60
50
40
30
20
10
0
31
25
25
P/P
UP/P
P/UP
pre-test
CS2-CS1
CS1-US+
4 trials
test
30 min
D
NS
E
NS
F
**
G
**
24
31
28
23
1
2 (5min)
4 (5min)
4 (1min)
Number of phase 1 training trials (with ITI)

Fig. 2

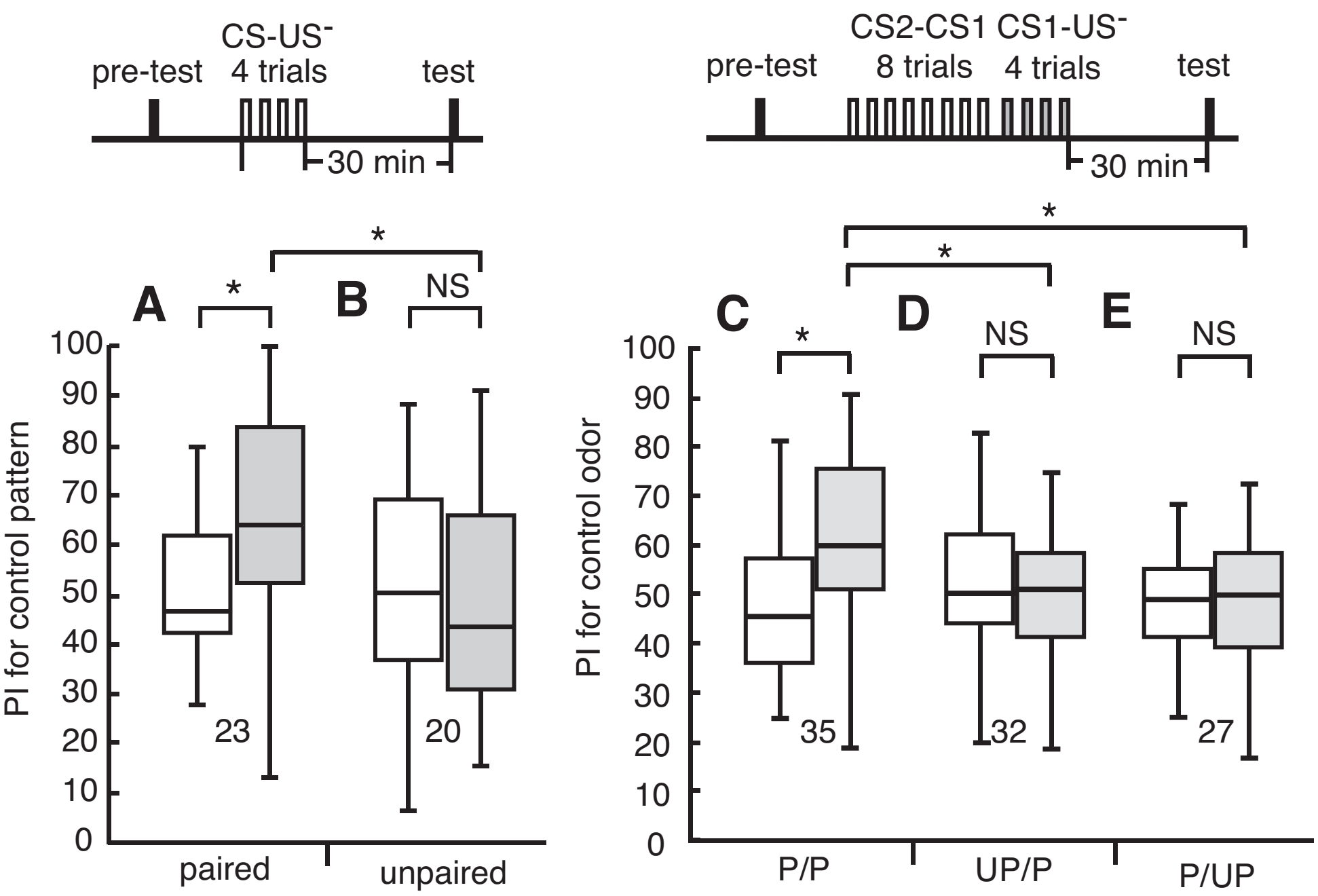

pre-test
CS-US-
4 trials
test
30 min
A
B
*
*
NS
PI for control pattern
100
90
80
70
60
50
40
30
20
10
0
23
20
paired
unpaired
pre-test
CS2-CS1
8 trials
CS1-US-
4 trials
test
30 min
C
D
E
*
*
*
NS
NS
35
32
27
P/P
UP/P
P/UP

Fig. 3

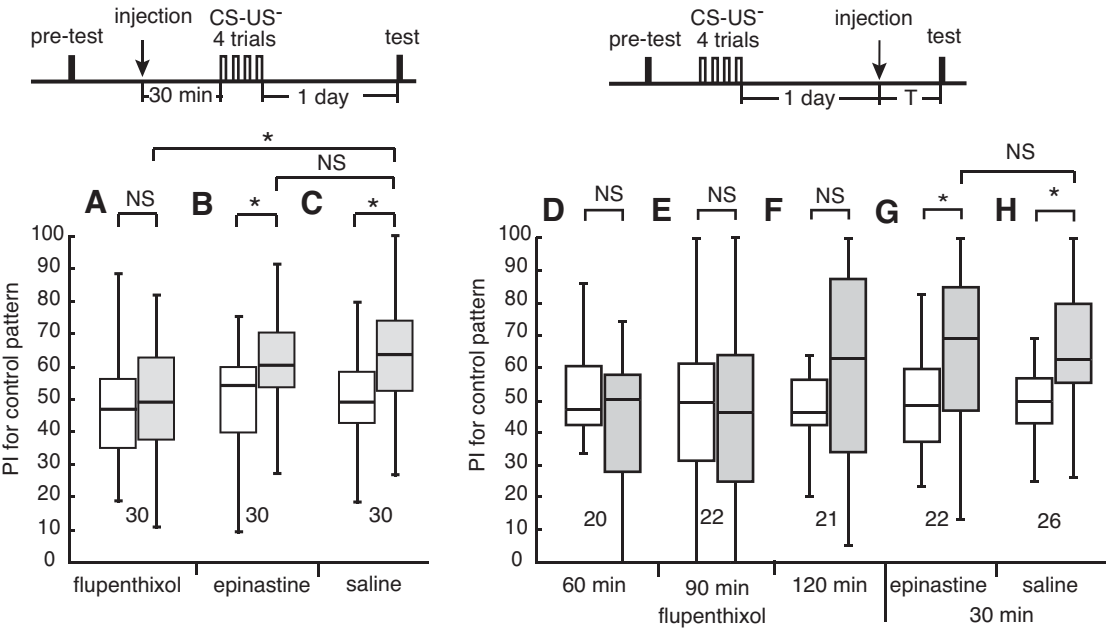

pre-test
injection
CS-US-
4 trials
test
30 min
1 day
pre-test
CS-US-
4 trials
injection
test
1 day
T
A
B
C
D
E
F
G
H
NS
*
PI for control pattern
100
90
80
70
60
50
40
30
20
10
0
30
30
30
20
22
21
22
26
flupenthixol
epinastine
saline
60 min
90 min
120 min
epinastine
saline
flupenthixol
30 min

Fig.4

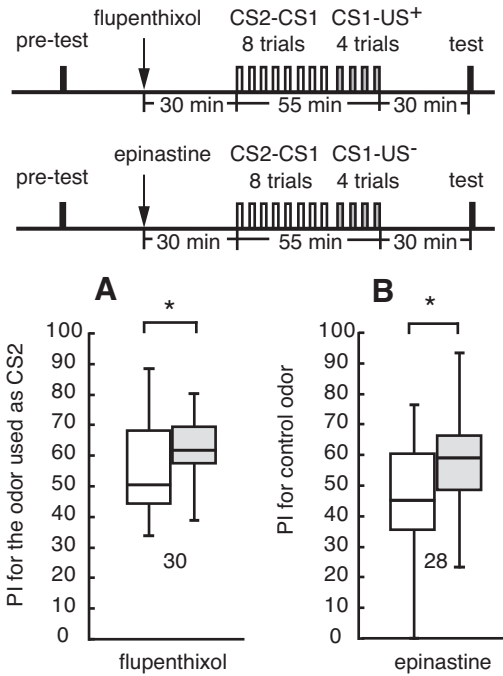

flupenthixol
CS2-CS1
8 trials
CS1-US+
4 trials
pre-test
test
30 min
55 min
30 min
epinastine
CS2-CS1
8 trials
CS1-US-
4 trials
pre-test
test
30 min
55 min
30 min
A
B
*
*
PI for the odor used as CS2
PI for control odor
100
90
80
70
60
50
40
30
20
10
0
30
28
flupenthixol
epinastine

Fig. 5

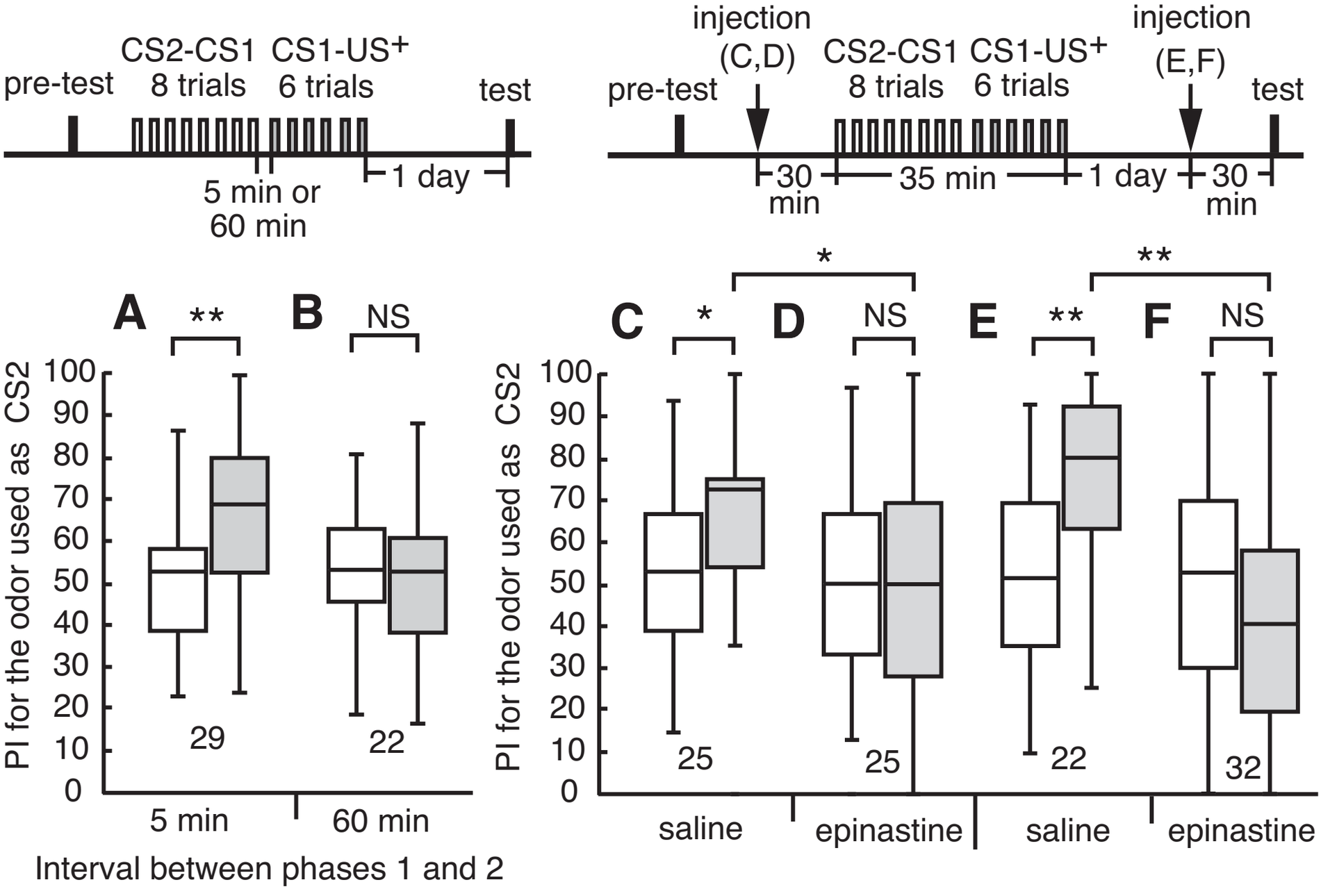
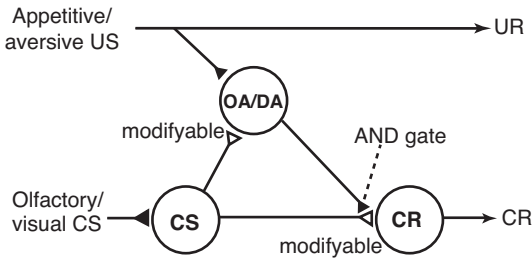

pre-test
CS2-CS1
8 trials
CS1-US+
6 trials
test
5 min or
60 min
1 day
injection
(C,D)
injection
(E,F)
30
min
35 min
1 day
30
min
A
B
C
D
E
F
**
NS
*
*
**
NS
*
**
NS
PI for the odor used as CS2
100
90
80
70
60
50
40
30
20
10
0
29
22
25
25
22
32
5 min
60 min
saline
epinastine
saline
epinastine
Interval between phases 1 and 2

Fig. 6

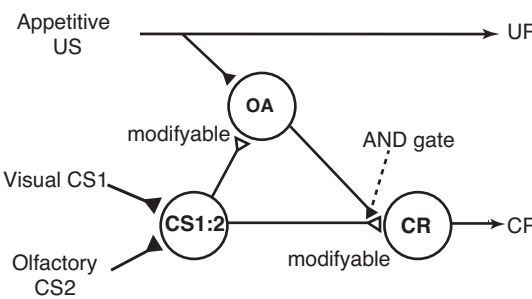
A

Training	Test
CS→US	CS



B

Phase 1	Phase 2	Test
CS2:CS1	CS1→US	CS2



C

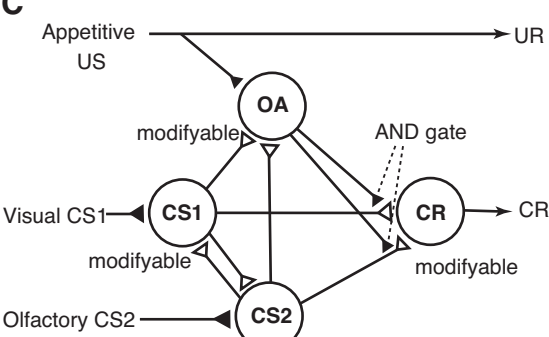


Fig.7