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Sensory Preconditioning in the Terrestrial Mollusk, *Limax flavus*

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ABSTRACT—Sensory preconditioning (SPC) in the terrestrial slug, *Limax flavus*, was studied with carrot odor and cucumber odor as the conditioned stimuli (CSs) and quinidine sulfate solution as the unconditioned stimulus (US). When slugs experienced CS1-CS2 and CS2-US training pairs, their odor preference for CS1 was reduced as well as that for CS2. The reduction in CS1 odor preference was observable only when slugs experienced both training pairs. In the second experiment, in order to study the stimulus-stimulus associations after SPC, the conditioned slugs were cooled immediately after presentation of CS1 or CS2. As a result, both odor preferences increased after both CS1+cooling and CS2+cooling treatments, which suggested that CS1-CS2 and CS2-US associations were formed after SPC. From these results, it is concluded that *Limax* shows sensory preconditioning and that its stimulus-stimulus associations are similar to those of mammals.

INTRODUCTION

Mollusks are being intensively studied in order to clarify the cellular and molecular mechanisms of associative learning because of the relative simplicity of their nervous systems [2, 3]. Against the background of the excitement generated by these studies, the question of whether one can generalize the emerging principles to vertebrates arises. Comparative studies at the behavioral level might provide an insight into the relationship between vertebrate and invertebrate learning processes and partly answer this question. Gelperin and his colleagues demonstrated that the terrestrial slug, *Limax maximus,* showed some logical operations similar to those known in vertebrates, such as first- and second-order conditioning and blocking [4, 13]. They also showed that stimulus-stimulus associations formed during two types of second-order conditioning in the slug were parallel to those in vertebrates [14]. However, there have been no reports that show sensory preconditioning in mollusks.

Sensory preconditioning (SPC) is a kind of classical conditioning where two conditioned stimuli (CSs) are associated without an unconditioned stimulus (US). The SPC procedure consists of two phases. In phase 1, conditioned stimulus 1 (CS1) is associated with conditioned stimulus 2 (CS2), and in phase 2, CS2 is associated with US. As a result of SPC, CS1, which is not associated directly with US, becomes able to evoke the conditioned response (CR). Sensory preconditioning was first studied by Brogden [1], but has sometimes been dismissed on the grounds that the level of conditioning observed to CS1 is too slight to warrant serious theoretical attention [17]. Later the phenomenon was proved to be a real one [6], and several well-controlled experiments have shown reliable levels of response to CS1 in such diverse situations as conditioned suppression in rats [9, 12], heart-rate conditioning in rabbits [9], and flavor-aversion conditioning in rats [5, 11].

Rizley and Rescorla [12] studied stimulus-stimulus associations after sensory preconditioning using a "postconditioning treatment strategy", in which, after conditioning, the ability of CS2 to evoke CR was reduced by repeated presentation of CS2 to extinguish the CS2-US association. As a result, not only the ability of CS2 to evoke CR but also that of CS1 was dismissed, which indicates that CS1-CS2 and CS2-US associations were formed during sensory preconditioning. Thus, sensory preconditioning is one piece of evidence against strict stimulus-response or S-R theory [7], and is a good example of the ability of animals to integrate several associations into one [8].

In the present study, we demonstrated that the terrestrial slug, *Limax flavus,* showed sensory preconditioning. In addition, the stimulus-stimulus associations formed after the conditioning were studied using cooling-induced retrograde amnesia [15]. It was shown that the associations in *Limax* were parallel to those in vertebrates.

MATERIALS AND METHODS

Animals

Specimens of *Limax flavus* were maintained in laboratory culture on frog chow (Oriental Yeast Co. Ltd.) with a 14 hr/10 hr light-dark cycle at 19°C. Two or three days before the start of training, 3 to 6 month old animals (1.5–2.0 g) were placed into individual plastic containers (113×105×28 mm) lined with moistened filter paper and then starved until the start of the experiments.

Materials used for stimulation

Carrot juice was made in the laboratory. Several carrots were ground in a blender and centrifuged for 30 min at 7,000×*g*. The supernatant was used as carrot juice, which was kept at −20°C until use. Cucumber juice was made in exactly the same way. During

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training, the carrot (Ca) or cucumber juice (Cu) was applied to filter paper and used as the conditioned stimulus (CS). A saturated solution of quinidine sulfate (Q: 1 g/90 ml pure water) was applied to filter paper and used as a bitter-taste unconditioned stimulus (US).

Sensory preconditioning (SPC)

The conditioning procedure consisted of two phases. The slugs were divided into three groups, PP [Paired (phase 1)-Paired (phase 2)], PU (Paired-Unpaired) and UP (Unpaired-Paired). During phase 1 of training, slugs in groups PP and PU were transferred with tweezers to a plastic container lined with filter paper moistened with CS1. After 2 min exposure, the slugs were directly transferred to another plastic container lined with filter paper moistened with CS2, and after 2 min exposure were returned to their individual containers. This paired presentation of CS1 and CS2 was repeated three times with a 2 hr intertrial interval (ITI). Slugs in group UP received the same number of CS1 and CS2 presentations as those in groups PP and PU, but the CS1-CS2 interstimulus interval (ISI) was 30 min. On the next day, in phase 2, slugs in groups PP and UP were exposed to CS2 for 2 min in the same way as in phase 1 training, then transferred to another plastic container lined with filter paper immersed with quinidine sulfate solution. After 1 min exposure, they were rinsed with saline for 5 sec and returned to their individual containers. The paired CS2-US treatment was repeated three times with a 2 hr ITI. Slugs in group PU received three unpaired (ISI=30 min) presentations of CS2 and US with a 2 hr ITI. On the third day, odor preferences for Ca and Cu were tested.

As was described previously [18], although the slugs were able to sense both the odor and taste of the CSs, they did not show taste-taste conditioning by the conditioning procedure. Thus, the taste of the CSs has no influence on the conditioning.

Cooling-induced retrograde amnesia

According to Sekiguchi *et al.* [15], cooling-induced retrograde amnesia reflects Pavlovian conditioning associations. Thus, amnesia was induced to study the associations of sensory preconditioning. The conditioned slugs were tested for their odor preferences and divided into two groups, CS1F and CS2F ("F" represented cooling treatment). They were then exposed to CS1 (group CS1F) or CS2 (group CS2F) for 2 min, transferred to their individual containers, and placed in the freezer compartment of a refrigerator for 5 min. Within 3 min, the body temperature fell to about 1°C as determined by a thermocouple. On the next day, the slugs were again tested for their odor preferences.

Testing and the measure of conditioning

The testing apparatus has been described previously [16]. Briefly, it consisted of three chambers, two of which were for carrot/cucumber juice and frog chow as odor sources. These odor sources were placed on the floor of each side-chamber. The slug was placed in the center chamber, the wall of which was perforated. A line divided the center chamber into a "carrot/cucumber" and a "chow" side.

The testing procedure was exactly the same as that also described previously [16]. For each test, a slug was placed in the center chamber with its body aligned along the center line and observed until it crossed the center line. During the next 120 sec, the time spent by the slug's head on the carrot/cucumber side was recorded. Each slug underwent three trials of carrot odor versus frog chow odor and another three trials of cucumber odor versus frog chow odor, with a 2-hr interval. The observer did not know which treatments had been experienced by the slug being tested.

The measure of conditioning used, "odor preference", was the percentage of the total time the slug spent on the carrot/cucumber side in the carrot/cucumber odor versus frog chow odor trials. This was obtained by dividing the total time each slug's head spent on the carrot/ cucumber side by the total measured time (120 sec×3 trials=360 sec). It has been confirmed that the testing procedure itself does not influence the odor preference [16].

Statistical analysis

Analysis of variance (ANOVA) was used to compare the odor choices of the groups. Newman-Keuls post hoc tests were used for further analysis of the data when the F ratio reached significance. We also used the paired *t*-test when analyzing the pre- and post-amnesia differences for individual slugs.

RESULTS

Sensory preconditioning in Limax flavus

Forty-one slugs were divided into three groups, PP (n=17), PU (n=12) and UP (n=12). In the first experiment, cucumber juice (Cu) and carrot juice (Ca) were used as CS1 and CS2, respectively. Thus, slugs in groups PP and PU were treated with three CuCa conditioning pairs on the first day of training, and slugs in groups PP and UP were treated with three CaQ conditioning pairs on the second day.

The result is shown in Fig. 1a. It is evident that the PP and UP slugs showed much less Ca odor preference than the PU slugs (solid columns). Analysis of variance (ANOVA) showed differences among the groups, $F(2,38)=16.51$, $P<0.001$. Post hoc individual comparisons (Newman-Keuls test) indicated that group PU was significantly different from groups PP and UP ($P<0.05$). On the other hand, the PP slugs showed much less Cu odor preference than PU and UP slugs (clear columns). ANOVA again showed differences among the groups, $F(2,38)=12.93$, $P<0.001$. Individual comparisons revealed that group PP was significantly different from groups PU and UP ($P<0.05$). These results suggest that the reduced preference for Cu odor in group PP slugs is due to SPC.

In the next experiment, to examine the possibility that the observed changes in odor preferences were odor-specific, the CSs were exchanged (CS1: carrot, CS2: cucumber). Twenty-four further slugs were divided into three groups, PP (n=8), PU (n=8) and UP (n=8). Slugs in groups PP and PU experienced three CaCu training pairs, and slugs in groups PP and UP experienced three CuQ training pairs. As shown in Fig. 1b, the results were parallel to those of the previous experiment. ANOVA showed differences among the groups in both Cu and Ca odors (cucumber, $F(2,21)=4.64$, $P<0.025$; carrot, $F(2,21)=17.98$, $P<0.001$). Individual comparison revealed that group PU was significantly different from groups PP and UP in Cu odor preference ($P<0.05$) and that group PP was significantly different from groups PU and UP in Ca odor preference ($P<0.05$). Figure 1c represents combined data from Fig. 1a and 1b, in which the Cu odor preferences from Fig. 1a and the Ca odor

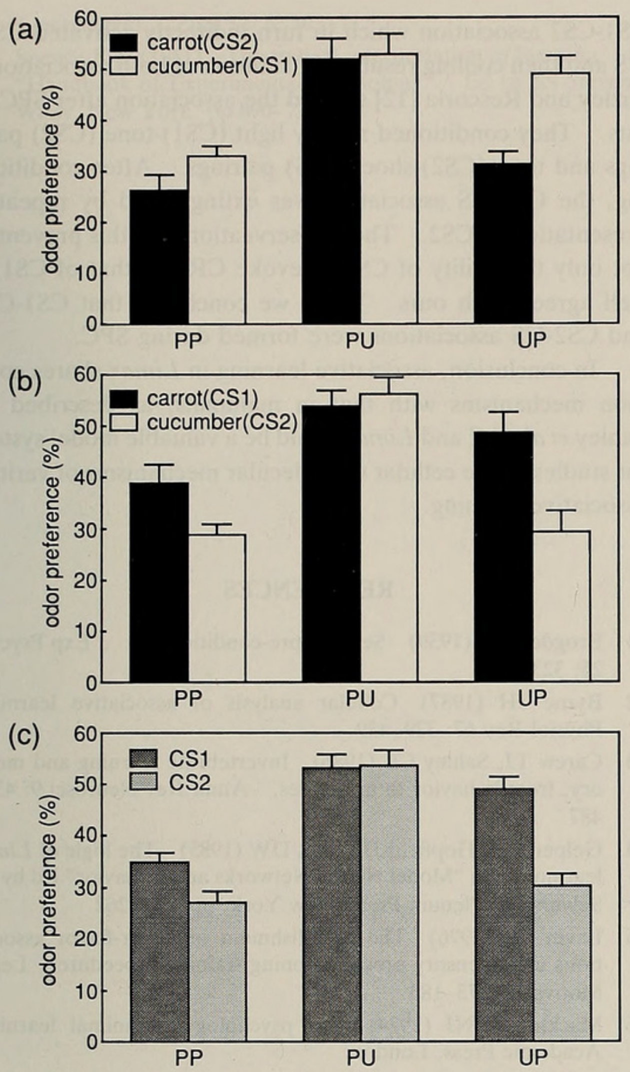

FIG. 1. Odor preferences of slugs that experienced both CS1-CS2 and CS2-US pairs (PP), only CS1-CS2 pairs (PU) and only CS2-US pairs (UP). (a) CS1: cucumber, CS2: carrot. (b) CS1: carrot, CS2: cucumber. (c) combined (a) and (b) data. US is quinidine sulfate solution. Odor preferences for carrot and cucumber are represented by solid and clear columns, respectively. Bars: standard errors of means.

preferences from Fig. 1b are combined to show CS1 odor preferences, and the Ca odor preferences from Fig. 1a and the Cu odor preferences from Fig. 1b are combined to show CS2 odor preferences. These combined results indicate that the slugs showed SPC.

Cooling-induced retrograde amnesia in the conditioned slugs

When slugs conditioned to avoid food odor were cooled immediately after presentation of the conditioned odor, retrograde amnesia was induced. Yamada *et al.* [18] reported that in *Limax flavus* CS presentation reactivates the memory involved with the conditioning and memory reactivation prior to cooling is necessary for amnesia induction. In addition, Sekiguchi *et al.* [15] indicated that cooling-induced retrograde amnesia reflects stimulus-stimulus associations formed after a variety of associative conditionings. Thus, the association after SPC was studied using amnesia.

Nineteen slugs were divided into two groups, CuF (n=9) and CaF (n=10). Slugs in groups CuF and CaF were conditioned to avoid Ca and Cu odors in the same way as those in group PP of Fig. 1a, in which Cu and Ca were used as CS1 and CS2 respectively. On the third day, after testing odor preferences, slugs in groups CuF and CaF were exposed to Cu and Ca respectively, immediately followed by cooling.

As shown in Fig. 2a, when conditioned slugs were treated with CuF (group CuF), both Ca and Cu odor preferences increased [cucumber, $t(8)=3.039$, $P<0.02$; carrot, $t(8)=2.695$, $P<0.05$, paired *t*-test]. A similar increase in both odor preferences was observed in the slugs treated with CaF [cucumber, $t(9)=3.197$, $P<0.02$; carrot, $t(9)=4.482$, $P<0.002$, paired *t*-test]. Thus, both CuF and CaF treatments resulted in an increase in both odor preferences.

In order to counterbalance the two CSs, twenty slugs were conditioned identically except that CS1 and CS2 were Ca and Cu respectively (Fig. 2b). They were treated with CaF (CS1+cooling, n=10) or CuF (CS2+cooling, n=10). After CaF treatment, both Ca (CS1) and Cu (CS2) odor preferences increased [carrot, $t(9)=5.708$, $P<0.001$; cucumber, $t(9)=5.820$, $P<0.001$], and similar results were

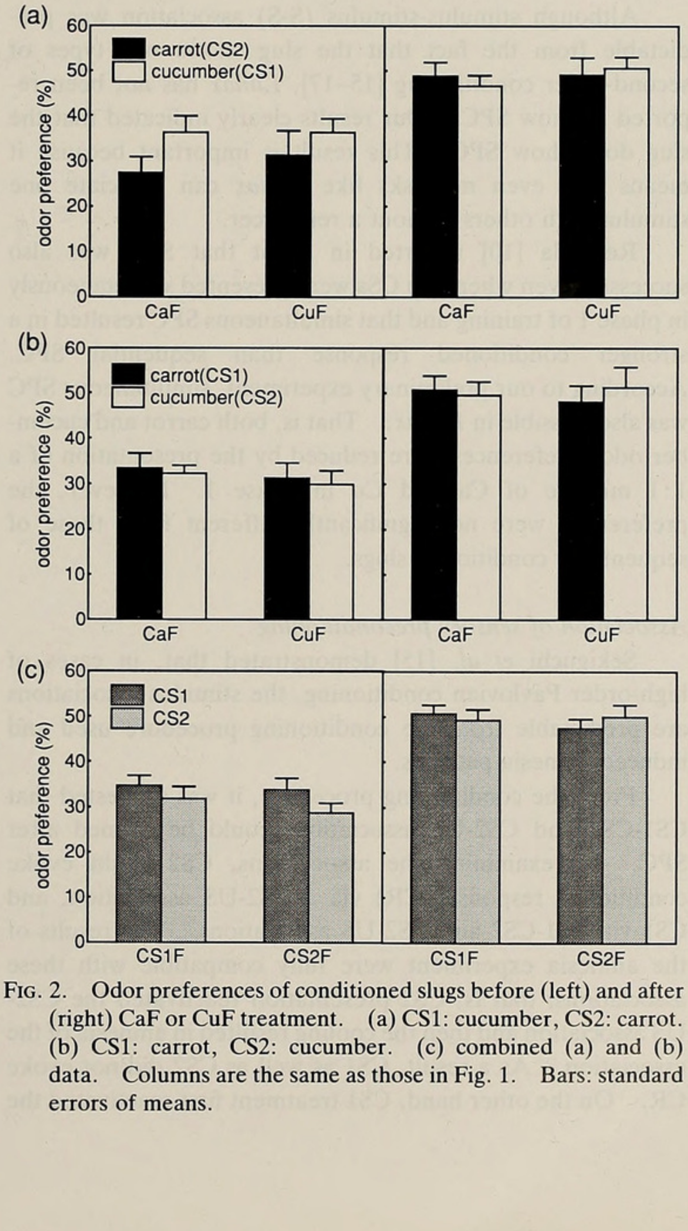

FIG. 2. Odor preferences of conditioned slugs before (left) and after (right) CaF or CuF treatment. (a) CS1: cucumber, CS2: carrot. (b) CS1: carrot, CS2: cucumber. (c) combined (a) and (b) data. Columns are the same as those in Fig. 1. Bars: standard errors of means.

observed after CuF treatment [carrot, t(9)=3.812, $P<0.005$; cucumber, t(9)=3.732, $P<0.005$]. Fig. 2c represents combined data from Fig. 2a and 2b, in which the CuF group data from Fig. 2a and the CaF data from Fig. 2b are combined to show the CS1F group results and the CaF group data from Fig. 2a and the CuF group data from Fig. 2b are combined to show the CS2F group results. These combined results indicate that in slugs conditioned by SPC, both CS1F and CS2F treatments resulted in amnesia with respect to both CS1 and CS2.

DISCUSSION

Sensory preconditioning in the terrestrial mollusk, *Limax flavus,* was studied. The first experiment indicated that the slug shows sensory preconditioning. In the second experiment, in order to study the stimulus-stimulus associations after SPC, retrograde amnesia was induced by cooling the conditioned slugs. As a result, an increase in both odor preferences was observed in response to CaF and CuF treatments.

Sensory preconditioning in Limax flavus

Although stimulus-stimulus (S-S) association was predictable from the fact that the slug shows two types of second-order conditioning [15–17], *Limax* has not been reported to show SPC. Our results clearly indicated that the slug does show SPC. This result is important because it means that even mollusks like *Limax* can associate one stimulus with others without a reinforcer.

Rescorla [10] reported in a rat that SPC was also successful even when two CSs were presented simultaneously in phase 1 of training and that simultaneous SPC resulted in a stronger conditioned response than sequential SPC. According to our preliminary experiment, simultaneous SPC was also possible in *Limax*. That is, both carrot and cucumber odor preferences were reduced by the presentation of a 1:1 mixture of Ca and Cu in phase 1. However, the preferences were not significantly different from those of sequentially conditioned slugs.

Association of sensory preconditioning

Sekiguchi *et al.* [15] demonstrated that, in cases of high-order Pavlovian conditioning, the stimulus associations are predictable from the conditioning procedure used and induced amnesia patterns.

From the conditioning procedure, it was suggested that CS1-CS2 and CS2-US associations could be formed after SPC. On examining the associations, CS2 could evoke conditioned response (CR) via a CS2-US association, and CS1 via CS1-CS2 and CS2-US associations. The results of the amnesia experiment were fully compatible with these associations; that is, CS2 presentation reactivated the CS2-US association and then the cooling resulted in amnesia of the association. As a result, CS1 as well as CS2 did not evoke CR. On the other hand, CS1 treatment first reactivated the CS1-CS2 association which in turn indirectly activated CS2-US and then cooling resulted in amnesia of both associations. Rizley and Rescorla [12] studied the association after SPC in rats. They conditioned rats by light (CS1)-tone (CS2) pairings and tone (CS2)-shock (US) pairings. After conditioning, the CS2-US association was extinguished by repeated presentation of CS2. Their observeation that this prevented not only the ability of CS2 to evoke CR but that of CS1 as well agreed with ours. Thus, we concluded that CS1-CS2 and CS2-US associations were formed during SPC.

In conclusion, associative learning in *Limax* shares common mechanisms with that in mammals, as described by Sahley *et al.* [14] and *Limax* could be a valuable model system for studies of the cellular or molecular mechanisms of various associative learning.

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