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Aversive Conditioning to a Compound Odor Stimulus and Its Components in a Terrestrial Mollusc

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ABSTRACT—To understand the perception of an odor mixture by the slug *Limax marginatus*, a mixture of two odors (carrot and cucumber) was used to condition the slugs, and internal representation of the odor mixture and its components was determined by cooling-induced retrograde amnesia. Slugs conditioned with the odor mixture showed aversive behavior, not only towards the mixture, but also towards the individual odor components. When the conditioned slugs were cooled after presentation of one of the odor components, odor preferences for both components recovered, suggesting that the slugs perceived the odor mixture as an entity. However, when the slugs were exposed to the components of the odor mixture after conditioning with the mixture, cooling treatment resulted in amnesia, which was specific towards the odor presented before the cooling treatment. This suggests that slugs exposed to odor components after conditioning were able to recognize the odor components individually. Thus, slugs learn a binary mixture as an entity as long as they have no experience about the individual components of the mixture. These results are discussed in relation to other conditioning strategies, such as second-order conditioning or blocking, where a mixture of cues is used.

INTRODUCTION

Environmental stimuli consist of a large number of single chemical or physical components, and animals perceive these stimuli simultaneously by different sensory modalities. In the case of olfaction and taste, a single odor or taste usually consists of a mixture of chemical substances. Thus, it is important to study how animals perceive and learn to recognize a mixture of stimuli in order to respond to their environment.

Perception of a mixture of stimuli may occur in one of two ways: (i) the mixture is perceived as a combination of its components, or (ii) it is perceived as a unique entity. Evidence for both methods of perception have been reported. Rescorla and colleagues (Rescorla, 1972, 1973; Rescorla *et al.*, 1985) and Pearce (1987) proposed that, when two stimuli are presented simultaneously, recognition of both components occurs and the joint activation of this recognition represents a mixture-unique cue. This phenomenon has been observed in honeybees (Smith and Cobey, 1994). Conversely, it has also been reported that a configural cue can dominate elemental cues (Rudy and Sutherland, 1992). For example, under certain conditions animals can be conditioned to respond to stimuli A or B when each stimulus is presented alone, but not to respond to the mixed stimulus AB (Rescorla, 1972; Kehoe and Graham, 1988). Recently, Livermore *et al.* (1997) proposed that, at least in olfactory processing, an animal changes its strategy between elemental perception and configural perception depending on the requirements of a specific task.

In this study, we have investigated memory association after conditioning of the terrestrial slug *Limax marginatus* with an odor mixture, using cooling-induced retrograde amnesia. *Limax* is a highly olfactory animal and has been used for behavioral or physiological studies of olfactory learning and memory. It is well known that conditioning in *Limax* species can occur in a variety of ways, including second-order conditioning, blocking (Sahley *et al.*, 1981; Gelperin *et al.*, 1985) or sensory pre-conditioning (Suzuki *et al.*, 1994). In addition, Hopfield and Gelperin (1989) demonstrated that *Limax maximus* could acquire an aversion to a mixed odor AB while odors A and B remain attractive when presented individually. Thus, the slug seems to adapt a configural perception strategy when it is conditioned to avoid a mixture of odors.

Cooling-induced retrograde amnesia is a useful tool to study memory association after conditioning (Sekiguchi *et al.*, 1994, 1997). This is because amnesia can only be induced for reactivated memories (Sekiguchi *et al.*, 1991; Yamada *et al.*, 1992). Thus, one can estimate the memory association by observing how the amnesia occurred. An example of this is sequential second-order conditioning, in which two conditioning stimuli (CS1, CS2) are associated with an unconditioning stimulus (US) as CS1+US and CS2+CS1. After conditioning, slugs are exposed to CS1 immediately followed by cooling,

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which results in amnesia of CS1 but not CS2 memory. When CS2 presentation is followed by cooling, on the other hand, both CS1 and CS2 memories are lost. Thus, one can predict that three associations, CS1→US, CS2→CS1 and CS2→US, are formed after sequential second-order conditioning (Sekiguchi *et al.*, 1994). The predicted associations agree well with those of Rizley and Rescorla (1972) and Sahley *et al.* (1984) where a specific memory association is extinguished by repeated presentation of CS1 and CS2 without US. Similar experiments have revealed the memory association of two-independent first-order conditioning, simultaneous second-order conditioning (Sekiguchi *et al.*, 1994) and sensory pre-conditioning (Suzuki *et al.*, 1994). Thus, by applying cooling-induced retrograde amnesia to slugs that have been conditioned with an odor mixture, one can clarify the kind of memories and associations that are formed after conditioning.

In this study, the perception of an odor mixture or the internal representation of stimuli was examined in slugs by cooling-induced retrograde amnesia. Slugs were conditioned with a mixture of two odors, exposed to each of the odor components, and then cooled. The results suggest that slugs learn a binary mixture as a configuration as long as they have no experience of each component individually.

MATERIALS AND METHODS

Animals

The terrestrial mollusc, *L. marginatus*, was cultivated in the laboratory on frog chow (Oriental Yeast Co. Ltd.,) with a 14 h/10 hr light-dark cycle at 19°C. Three days before starting training, 2- to 4-month-old animals (1.0–1.5 g) were placed individually into a plastic container (113 mm×105 mm×28 mm) lined with moistened filter paper and then starved until the start of the experiments.

Materials used for stimulation

Carrot juice (CA) was prepared in the laboratory by homogenizing several carrots in a blender (100 g carrot/100 ml saline). The homogenate was centrifuged for 30 min at 7 000 g and the supernatant was stored at –20°C until use. Cucumber juice (CU) was prepared (100 g cucumber/100 ml saline) and stored in exactly the same manner. For training, a mixture (1:1) of CA and CU (Mix) was used as the CS. A saturated solution (1 g/90 ml saline) of quinidine sulfate (Q) was applied to the moistened filter paper and used as a bitter-taste US. The saline consisted of 52.9 mM NaCl, 4.0 mM KCl, 7.0 mM $CaCl_2$, 4.6 mM $MgCl_2$, 0.2 mM KH_2PO_4, 2.5 mM $NaHCO_3$ and 5.0 mM dextrose (pH 7.6).

Conditioning procedures

The slugs in the conditioned (paired) groups were transferred with tweezers to individual plastic containers lined with filter paper moistened with Mix. After 2 min exposure the slugs were transferred to another plastic container lined with filter paper thoroughly moistened with Q (US), and after 1 min they were washed with saline for 5 sec and returned to their original containers. This paired presentation of CS and US was repeated three times with a 2-hr inter-trial interval. The slugs in the control (unpaired) group received the same number of CS and US presentations as those in the conditioned group, but each CS-US inter-stimulus interval was 30 min.

In the above conditioning procedure, the slugs were able to sense both odor and taste of the CS. However, as described in Yamada *et al.* (1992), taste-taste conditioning does not contribute significantly to the degree of odor-taste first-order conditioning.

Cooling of the slugs

Cooling-induced retrograde amnesia was used to study memory associations after aversive conditioning to the odor mixture (Sekiguchi *et al.*, 1994). In order to induce retrograde amnesia, each conditioned slug was transferred with tweezers to another plastic container lined with filter paper moistened with CA or CU, exposed for 2 min, returned to its original container and cooled immediately to about 1°C for 5 min in the freezer compartment of a refrigerator. This cooling procedure was termed “F”.

Testing and response measurement

The testing apparatus has been described elsewhere (Yamada *et al.*, 1992). Briefly, it consisted of two side chambers for the odor sources (CA/CU/Mix and frog chow) which were placed on the floor of the chambers, and a central chamber with a perforated wall. A line bisected the central chamber into “stimulus” and “chow” sides.

For each test, a slug was placed in the central chamber with its body aligned along the center line and observed until it crossed this line. The total time spent by the slug’s head on the stimulus side during the next 2 min was recorded. Each slug was subjected to three trials of CA odor versus frog chow odor and three trials of CU odor versus frog chow odor with a 2-hr interval. The order of the CA or CU odor tests was random. A test was also performed of Mix versus frog chow odor with the same interval and random test order. The experimenter was unaware of the conditioning treatment experienced by the slug under test.

The conditioning measure used (odor preference) was defined as the percentage of time the slug spent on the stimulus side in the CA/CU/Mix versus frog chow odor trials. This was obtained by dividing the total amount of time each slug’s head spent on the stimulus side by the total experiment time (2 min×3 trials=6 min). The odor preference test was carried out on the day following the conditioning or cooling treatment. The equivalence in the odor preference between CA and CU has already been verified (Sekiguchi *et al.*, 1994).

The student’s *t*-test was used to compare the odor preferences within each group.

RESULTS

Conditioning to an odor mixture

Slugs in the conditioned (n=16) and control groups (n=15)

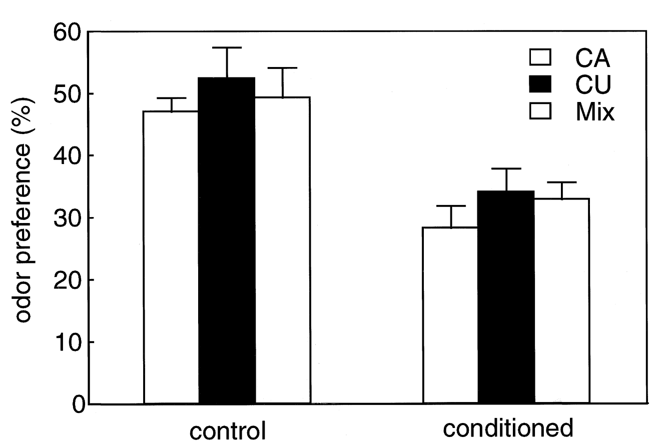

Fig. 1. Odor preferences of slugs that were conditioned with the binary odor mixture (CA+CU) and Q. Clear, solid and gray columns represent odor preferences for CA, CU and Mix, respectively. Bars: standard error of the mean.

received three paired and three unpaired presentations of Mix and Q, respectively. On the following day, all slugs were tested for their odor preferences to CA, CU and Mix. Slugs in the conditioned group showed much less odor preference, not only to Mix, but also to CA and CU, than those in the control group (CA, t (29)=4.75, P<0.001; CU, t (29)=3.84, P<0.001; Mix, t (29)=3.08, P<0.005; t-test) (Fig. 1). Thus, the slugs conditioned to avoid the odor mixture showed an aversive response to each individual odor.

Cooling-induced retrograde amnesia

Two hypotheses can be proposed from the above result: (i) the aversive conditioning to Mix resulted in representation not only of Mix but also of CA and CU individually; and (ii) conditioning to Mix resulted in representation of Mix only, and the presentation of CA or CU activated the representation of Mix, thereby evoking aversive behavior. Slugs were, therefore, conditioned with Mix+Q and received CA+F or CU+F, because cooling-induced retrograde amnesia can only be induced for reactivated memories (Yamada *et al.*, 1992). If the former hypothesis is the case, CA presentation would reactivate the representation of CA and Mix and the amnesia would occur for CA and Mix memories. Similarly, CU presentation would reactivate CU and Mix representation and CU and Mix memories would be lost. Thus the recovery of odor preference would be expected to occur selectively to the odor presented before cooling treatment. In the latter case, both CA and CU presentation would reactivate the representation of Mix and amnesia would be expected to occur for Mix. Thus both CA and CU odor preferences would recover.

Forty-two slugs were divided into three groups, conditioned (n=14), CA+F (n=14) and CU+F (n=14). The slugs in all groups were subjected to three paired presentations of Mix+Q. The following day, the slugs in the CA+F or CU+F groups received CA+F or CU+F, respectively. All slugs were tested for their odor preferences to CA and CU the following day. As shown in Fig. 2, both the CA and CU odor preferences increased in the slugs that had received CA+F, (CA, t (26)=5.59, P<0.001; CU, t (26)=3.13, P<0.005; t-test). Similarly, CU+F resulted in an increase in both CA and CU odor preferences (CA, t (26)=4.34, P<0.001; CU, t (26)=3.58, P<0.005; t-test). Thus, both CA+F and CU+F recovered both the CA and CU odor preferences of the conditioned slugs and the second hypothesis was supported.

Effect of post-conditioning exposure to odor components on amnesia

To examine above two hypotheses further, CA and CU were presented to the slugs after conditioning with Mix+Q, and the slugs were then cooled. Post-conditioning exposure to the odor components would form internal representation of these odors in addition to Mix, or it would reorganize the representation of Mix into two (CA and CU) memories. In any case, it is certain that two representation, CA and CU would be formed by the post-conditioning exposure.

Thirty-five slugs were conditioned with three Mix+Q pairs, and then divided into three groups, conditioned (n=15), CA+F (n=9) and CU+F (n=11). On the following day, slugs in the CA+F and CU+F groups received 1-min exposures to both CA and CU, and then received CA+F and CU+F, respectively, while slugs in the conditioned group did not receive any treatment. On the third day, all slugs were tested for their odor preference to CA and CU. The results are shown in Fig. 3. The slugs in the CA+F group showed an increased odor preference to CA but not to CU compared to those in the conditioned group (CA, t (22)=2.91, P<0.01; CU, t (22)=0.101, P>0.5; t-test). Slugs in the CU+F group (n=11), on the other hand, showed increased odor preference to CU but not to CA

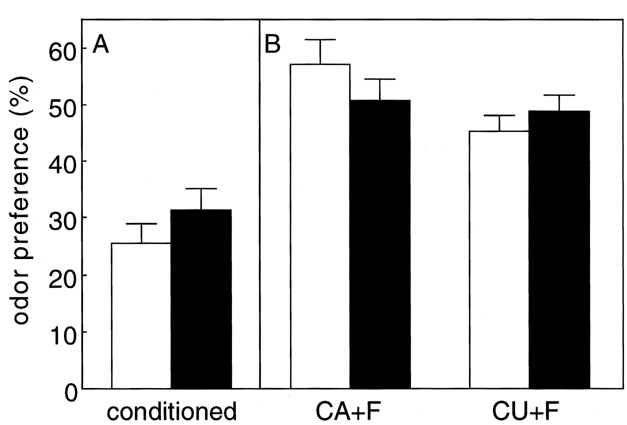

Fig. 2. Effect of cooling-induced retrograde amnesia on slugs conditioned with Mix+Q pairs. **A**. Odor preferences of slugs after Mix+Q conditioning. **B**. Odor preferences of slugs after Mix+Q conditioning, followed by F. Slugs in the CA+F and CU+F groups were conditioned with three Mix+Q pairs and received CA+F or CU+F, respectively. Columns are the same as those in Fig. 1. Bars: standard error of the mean.

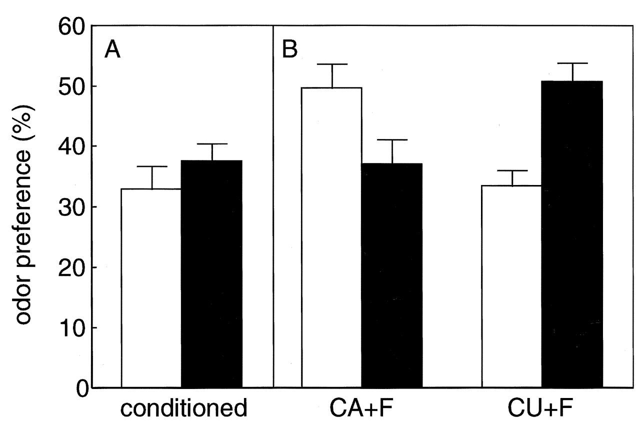

Fig. 3. Effect of post-conditioning treatment with the odor components after conditioning with Mix. **A**. Odor preferences of slugs after Mix+Q conditioning. **B**. Odor preferences of slugs which were presented with the individual odor components between Mix+Q conditioning and F. Slugs in the CA+F and CU+F groups were conditioned with three Mix+Q pairs, exposed to CA and CU odors, and then received CA+F or CU+F. Columns are the same as those in Fig. 1. Bars: standard error of the mean.

(CA, *t*(24)=0.121, P>5; CU, *t*(24)=3.11, P<0.01; *t*-test). Thus, when the slugs were exposed to the individual odor components after Mix+Q conditioning, amnesia occurred selectively on the odor presented before cooling.

DISCUSSION

The results of this study suggest that slugs conditioned with Mix are able to recognize Mix only and are not able to identify the individual components of Mix, as long as they are not exposed to them individually.

A previous study by Hopfield and Gelperin (1989) demonstrated that when a binary odor mixture AB were presented to slugs separately, followed by Q, *L. maximus* showed aversive behavior towards the odor mixture but not towards odors A or B alone. Our results on the other hand (Fig. 1) demonstrate that when *L. marginatus* was exposed to the odor mixture, followed by Q, the slugs showed aversive behavior not only towards Mix but also towards its individual components. One possible explanation is the difference in the conditioning procedure used in our study. The slugs in the study of Hopfield and Gelperin (1989) were conditioned only with the odor, whereas the slugs in our study were conditioned with a mixture of CA and CU, which permitted them to ingest the CS. However this was not the case because Yamada *et al.* (1992) suggested that taste-taste conditioning is very weak and does not influence odor-taste conditioning. Another explanation is the nature of the CS. Hopfield and Gelperin (1989) used pure chemicals that mimicked potato and mushroom odors as CS, whereas we used food odors. The mixture of food odors contain a variety of chemicals which would activate a variety of sensory neurons compared to the binary mixture of pure chemicals. Thus, it is possible that the neural representation of CA and CU odors which evoked by stimulation overlaps with the internal representation of Mix formed after conditioning with Mix + Q, to the extent that stimulation with CA or CU would be expected to activate the representation of Mix.

As the olfactory center of *Limax* species, procerebrum, is described as a nonlinear oscillator network with dense interaction among the oscillators (Gelperin and Tank, 1990; Kleinfeld *et al.*, 1994) and the olfactory information is processed by the oscillatory activity (Kimura *et al.*, 1998c), such an activation is possible. In general, nonlinear oscillators show phase-locked oscillation depending on the interaction among the oscillators. Under some conditions, changes in such as amplitudes or frequencies in a part of the oscillators result in the changes in behavior of whole oscillator network. In addition, it was reported that a conditioned odor activated a group of neurons in the procerebrum (Kimura *et al.*, 1998a, b). Thus, if the group of oscillators which is activated by CA and CU odors overlaps to some extent with that of Mix, CA or CU odors could evoke the oscillatory activity which represents Mix. In the study of Hopfield and Gelperin (1989), the overlap would be little, as the chemicals cannot activate internal representation of the mixture. Further studies are required to clarify these discrepancies.

The result that slugs showed aversive behavior to CA and CU after conditioning with Mix+Q suggests that internal representations of CA and CU are formed in addition to that of Mix. However, cooling-induced retrograde amnesia studies (Figs. 2 and 3) did not support this hypothesis. When the slugs conditioned with Mix+Q had been exposed to the individual components of Mix (Fig. 3), it is certain that internal representations of CA and CU were formed. In these slugs, amnesia occurred in a different manner from slugs that had not been exposed to the individual components (Fig. 2). This means that internal representations of CA and CU are not formed after conditioning with Mix+Q. Thus, *Limax* adapts a configural perception as long as it does not experience the components of an odor mixture.

It is very important to understand how animals perceive a mixture of stimuli. Even *Limax* species show blocking of conditioning when the slugs are conditioned to avoid an odor, and then a mixture of the CS and a second odor are paired with an US (CS1+US; Mix (CS1, CS2)+US). This results in conditioning to CS1 but not CS2 (Sahley *et al.*, 1981). Conversely, in the case of simultaneous second-order conditioning (CS1+US; Mix (CS1, CS2)), conditioning occurs to both CS1 and CS2 (Sahley *et al.*, 1984; Sekiguchi *et al.*, 1994). In both cases, slugs are first exposed to an odor and then to the odor mixture containing the CS. In the former case, in which Mix is followed by US, slugs ignore the presence of CS2, while in the latter case CS2 is segregated from the odor mixture and conditioning occurs to it. In addition, slugs also show simultaneous sensory pre-conditioning when they are first exposed to a binary odor mixture and then one of the odor components is paired with a US (Mix (CS1, CS2); CS1+US). By this conditioning procedure, the slugs show conditioned behavior to both CS1 and CS2 (Suzuki *et al.*, 1994). In this case, slugs segregate not only CS1 (the presented odor) but also CS2 from the odor mixture as observed in Fig. 3. Thus, slugs change the perception of an odor mixture depending on the conditioning procedure used, or their experiences.

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