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Higher-order conditioning of taste-odor learning in rats: Evidence for the association between emotional aspects of gustatory information and olfactory information

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HIGHLIGHTS

- Associative structure of taste-odor learning was examined in rats.
- Second-order conditioning of taste-odor learning was acquired.
- Alternatively, sensory preconditioning of the learning was not.
- Odors may be mainly associated with the emotion evoked by tastes.

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ABSTRACT

Previous studies have shown that rats prefer an odor paired with saccharin solution to an odor paired with quinine solution (taste-odor learning). However, it remains unclear whether the odors are associated with the emotional (i.e., positive and/or negative hedonics) or qualitative (i.e., sweetness and/or bitterness) aspects of gustatory information. This study aimed to examine this question using higher-order conditioning paradigms: second-order conditioning (SOC) and sensory preconditioning (SPC). Adult Wistar rats were divided into SOC and SPC groups. Food flavors, purchased from a Japanese market, such as melon (0.05%), lemon (0.1%), vanilla (0.1%), and almond (0.1%), were randomly used as odors A, B, C, and D for each rat. The SOC group was exposed to 0.005 M saccharin solutions with odor A and 0.02 M quinine solutions with odor C in the first 5 days of learning. Additionally, they were exposed to water with a mixture of odors A and B, and water with a mixture of odors C and D in the next 5 days of learning. The order of these two learning sessions was reversed in the SPC group. We hypothesized that if odor was associated with the emotional, or qualitative, aspects of gustatory information, the SOC, or SPC groups, respectively, would prefer odor B to odor D. Our results showed that the SOC group preferred odor B to odor D, whereas the SPC group did not show any such preference. This suggests that odors may be primarily associated with emotion evoked by gustation in taste-odor learning.

1. Introduction

We often perceive an odor, such as vanilla or lemon, as ‘sweet’ or ‘sour’ smelling, respectively. Nevertheless, gustation and olfaction are discrete perceptual systems. Taste-odor synesthesia—perception of an odor as having some taste-like property—is thought to be acquired and modulated by daily food experience, and consequently has been described as “learned synesthesia” [1,2]. For instance, Stevenson, Prescott, and Boakes [3] showed that after repeated pairing of the sweet taste of sucrose with unfamiliar odors such as lychee or water chestnut, these odors were judged as smelling sweeter. This perceptual change of odor is thought to be based on classical conditioning [2]. First, when an unfamiliar odor (e.g., lychee)—a conditioned stimulus (CS)—is paired with a taste (e.g., sucrose)—an unconditioned stimulus (US)—association between these two stimuli (CS-US) is acquired. Once this association has been acquired, the odor (CS) always activates the representation of the taste (US), and thus, the lychee odor is

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perceived as smelling sweet. Similar results were obtained in subsequent studies using human participants [4], with taste-odor associations thought to be more robust than others, such as taste-color associations [5,6].

Although researchers carefully select odor stimuli, which are unfamiliar to most of their participants, humans have such diverse histories with their own food experiences that the effects of these variables in the experiments cannot be controlled. Therefore, animal studies are needed to better elucidate and understand the mechanism of taste-odor learning. For instance, Fanselow and Birk [7] showed that rats acquired a preference for an odor that had been paired with saccharin solution, and an avoidance of another odor that had been paired with quinine solution. This result appeared to suggest that the rats acquired an association between the odor (CS) and sweetness (or bitterness) of the taste (US). However, it is possible that positive or negative hedonics, and not just sweet or bitter taste quality, could elicit the same preference or avoidance behavior [2,8]. To elucidate this point, taste-odor learning was tested with brain-lesioned rats [9]. The results showed that rats with lesions in the amygdala, a region involved in processing emotional aspects of gustatory information [10], showed rapid extinction of the preference for the saccharin-associated odor. On the other hand, rats with lesions in the insular cortex, a region involved in processing qualitative aspects of gustatory information [11,12], showed normal acquisition of the preference. This result suggested that the rats mainly acquired an association between olfactory information and the emotional aspects (i.e., positive and/or negative hedonics) but not the qualitative aspects (i.e., sweetness and/or bitterness) of gustatory information.

To further elucidate this finding, we introduce higher-order conditioning paradigms as non-invasive tools that investigate processes involved in taste-odor associative learning and memory [13]. Higher-order conditioning paradigms consist of second-order conditioning (SOC) and sensory preconditioning (SPC), whereby a CS (CS2) acquires the ability to elicit a conditioned response (CR) by being paired with another CS (CS1), rather than being directly paired with a US (Table 1). Pairing of CS1 and the US is followed by pairing of CS2 and CS1 in SOC, whereas the order of pairing is reversed in SPC. In any case, CS2 acquires the ability to elicit a CR even though it is never directly paired with the US.

It is suggested that there are critical differences between the SOC and SPC paradigms. The most important difference for the present study is that in the SOC paradigm, CS1 is thought to be associated mainly with the emotional and motivational states evoked by the US. Conversely, in the SPC paradigm, CS2 is thought to be associated mainly with the representation of CS1, and thus the US [13]. In the SPC paradigm, CS2 and CS1 are paired before CS1 is paired with the US. Therefore, the association between the representations of CS2 and CS1, and the association between the representations of CS1 and the US, are acquired. Rizley and Rescorla [14] confirmed this assumption: repeated non-reinforcement of CS1 (CS1 was presented without the US) caused extinction of the CR, not only to CS1, but also to CS2 in the SPC paradigm. These results indicate that CS2 is associated with the representation (i.e., perceptual, qualitative information) of CS1 and the US in the SPC paradigm. Alternatively, in the SOC paradigm, CS2 and CS1 are paired after development of the strong association between the US and CS1. Rizley and Rescorla [14] showed that repeated non-reinforcement of CS1 did not cause extinction of the CR to CS2 in the SOC paradigm. Therefore, the associations acquired in the SOC paradigm seem to be different from those acquired in the SPC paradigm.

Furthermore, Holland and Rescorla [15] showed that devaluation of the US (e.g., making animals sated in food appetitive conditioning) reduced conditioned response to CS1, but did not reduce them to CS2 in SOC. Holland [16] also showed that light (CS1) paired with food (US) elicited a rearing response, whereas tone (CS2) paired with light (CS1) elicited a startle-like response. Therefore, it has been suggested that CS2 is associated with emotional and motivational states elicited by the US or CS1 in SOC [13,16].

Taken together, these findings suggest that CS2 acquires the ability to elicit a CR through the development of an association with the emotional states elicited by the US in SOC, whereas in SPC, this occurs through an association with the representations of CS1 and US. In other words, CS2 seems to be mainly associated with the emotional information of the US in SOC, whereas CS2 is associated with the qualitative information of the US in SPC. If learning is based primarily on the development of the associations between representations of CSs and emotional aspects of the US, SOC would be applicable to the learning. However, if learning is based mostly on the association between the representations of CSs and the US, SPC would be more applicable to learning. Herein, by examining whether SOC or SPC paradigms are acquired successfully, we can determine which aspects (emotional or qualitative) of the US are associated with those of the CSs in taste-odor learning.

The present study consisted of three behavioral experiments. In Experiment 1, we aimed to replicate previous taste-odor learning findings from studies that used first-order conditioning [7,9], and to select and validate our odor stimuli. In Experiments 2 and 3, we aimed to examine whether odors were associated with the emotional or qualitative aspects of gustatory information using higher-order conditioning of taste-odor learning.

2. Experiment 1

2.1. Materials and methods

2.1.1. Subjects

Twelve adult Wistar male rats (300–360 g body weight) were used. The rats were housed in individual home cages in a temperature-controlled (23 ± 2 °C) and humidity-controlled (50 ± 5%) room on a 12:12 light/dark cycle, where they had free access to food (dry pellets, Oriental Yeast Co., Ltd., Japan) and deionized water, except when deprived for training, learning, and testing as described below. This study was reviewed and approved by the ethics committee of the Center for Laboratory Animal Research, Tohoku University.

2.1.2. Stimuli

Sodium saccharin (0.005 M) and quinine hydrochloride (0.02 M) were used as taste stimuli. The odor stimuli were food flavors (Narizuka Corporation, Japan) and consisted of melon (0.05%), lemon (0.1%), vanilla (0.1%), and almond (0.1%). Our pilot research revealed that odor stimuli in these concentrations were estimated to be of the same intensity by a panel of human judges. Two of these odor stimuli were presented as odor A and odor B, differently in each rat (counterbalanced). Stimuli were presented in the manner shown in Table 2.

Table 1
Procedural difference between first-order conditioning and higher-order conditioning (second-order conditioning and sensory preconditioning) (revised from Gewirtz and Davis [13]).

	Phase 1	Phase 2	Test
Classical conditioning (first-order conditioning)	CS-US		CS ?
Higher-order conditioning			
Second-order conditioning (SOC)	CS1-US	CS2-CS1	CS2 ?
Sensory preconditioning (SPC)	CS2-CS1	CS1-US	CS2 ?

Table 2
Flow chart of the sessions and stimuli presented in Experiment 1.

Training	Learning (Days 1–5)	Test (Days 6–10)
w	As vs. Bq	A vs. B

w: deionized water, As: saccharin solution with odor A, Bq: quinine solution with odor B, A: deionized water with odor A, B: deionized water with odor B.

2.1.3. Training session

The rats were deprived of water for 17 h and were allowed to drink the deionized water freely in an open-field apparatus with eight glass dishes (2 cm in diameter) on its circular (63.5 cm in diameter) floor for 5 min in the morning. Two milliliters of deionized water was put in each dish. Additional water was supplied for 3 h in their home cage via a drinking bottle after the training. When the volume of water was stable in the apparatus, the rats were entered into the learning session.

2.1.4. Learning session

After the training session, the learning session began. In this session, saccharin solution flavored with odor A was put into four of the eight glass dishes instead of water, and quinine solution flavored with odor B was put into the other four dishes (2 ml each). The rats were exposed to these flavored solutions for 5 min in the morning in the open-field apparatus. The dishes were randomly placed in the eight positions on the apparatus. The session was conducted once in the morning every day, and the rats were allowed to drink additional water in their home cages for 3 h after the session. This session was repeated for 5 days (see Table 2).

2.1.5. Test session

After the learning session, the test session began. We aimed to test the rats' preference for odor A. Therefore, water flavored with odor A was put into four of the eight glass dishes, and water flavored with odor B was put into the other four dishes (2 ml each). All of the other procedures were same as those in the learning session. This session was also repeated for 5 days (see Table 2).

2.1.6. Data analysis

The dishes were weighed before and after each session to measure intake volume (g). The preference ratio (PR) for each rat was calculated every day on the basis of the intake volume: PR = (intake of stimulus/total intake in 8 dishes) × 100. If the ratio for a stimulus was greater than the chance level (i.e., 50%, in the case of paired comparison of two different stimuli), the rats were considered to prefer that particular stimulus to others. The PR was analyzed by one-tailed z-test (vs. 50%). Probability values less than 0.05 ($p < 0.05$) were considered as statistically significant.

2.2. Results

PRs for the saccharin solution flavored with odor A in the learning session, and for water flavored with odor A in the test session, were calculated (Table 3). If the ratio was 50%, the rats did not show any preference. As shown in Table 3, on day 1, the rats' intake of the saccharin solution was relatively higher than that of the quinine solution. On day 2, the rats drank the saccharin solution intensively, and from day 3 onwards, they drank the saccharin solution almost exclusively, without drinking the quinine solution. One-tailed z-tests showed that the PR for the saccharin solution flavored with odor A was significantly greater than the chance level (50%) on days 2, 3, and 5 ($ps < 0.001$). On day 4, the mean PR was 100%, and the standard deviation was 0. Hence, the statistical test was not applied. At the beginning of the learning session, there were slight differences in behavior based on the odor stimuli; rats exposed to the saccharin solution flavored with almond did not drink any solutions on day 1. However, from day 2 onwards, all rats drank the saccharin solution intensively, regardless of which flavor was added to it.

During the test session, from days 6 to 10, the rats drank the water flavored with odor A almost exclusively, but not the water flavored with odor B. One-tailed z-tests showed that the PR for the water flavored with odor A was significantly greater than the chance level on all test days ($ps < 0.001$). These behaviors were not flavor-dependent; all the rats drank almost only the water flavored with the odor that had been paired with the saccharin solution, regardless of which flavor was presented in that manner.

2.3. Discussion

In this experiment, we aimed to replicate the findings of previous studies [7,9]. Our results showed that the rats preferred the odor that had been paired with the saccharin solution compared to the odor that had been paired with the quinine solution, which is in line with the results of previous studies [7,9]. The present results suggest that after repeated pairing of odor (CS) with taste (US) (i.e., first-order conditioning of taste-odor learning), the odor (CS) alone activates the representation of the taste (US). These results also suggest that the association was easily acquired and robust to extinction; only 5 pairings led to retention for at least 5 days. However, it is still unclear whether odor (CS) was associated with the emotional aspects of taste (US) or with the qualitative aspects of it. Experiment 2 aimed to address this question. In addition, we confirmed that the acquisition and retention of learning was not flavor-dependent. Therefore, we decided to keep using these four flavors (i.e., melon, lemon, vanilla, and almond) as the odor stimuli in Experiment 2.

Table 3
Preference ratios for the saccharin solution flavored with odor A in the learning session and for the water flavored with odor A in the test session.

			Learning: As vs. Bq					Test: A vs. B				
Subject #	Odor A	Odor B	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	VA	AL	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
2	ME	VA	100.0	100.0	100.0	100.0	100.0	100.0	97.7	84.5	100.0	88.7
3	AL	LE	0.0	100.0	100.0	100.0	97.9	100.0	96.5	100.0	100.0	100.0
4	LE	ME	90.0	100.0	98.4	100.0	100.0	100.0	100.0	100.0	100.0	100.0
5	ME	AL	–	100.0	98.4	100.0	100.0	98.4	100.0	100.0	98.6	100.0
6	LE	AL	100.0	98.0	98.0	100.0	100.0	95.7	100.0	100.0	98.1	100.0
7	VA	ME	–	98.5	100.0	100.0	96.5	100.0	97.9	100.0	100.0	100.0
8	LE	VA	50.0	0.0	100.0	100.0	100.0	96.8	98.4	98.5	100.0	100.0
9	VA	LE	50.0	100.0	98.5	100.0	100.0	94.3	100.0	100.0	100.0	100.0
10	ME	LE	75.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
11	AL	VA	–	100.0	100.0	100.0	100.0	98.2	98.0	100.0	100.0	100.0
12	AL	ME	–	97.5	98.2	100.0	100.0	100.0	94.3	100.0	98.1	97.1
		M	70.6*	91.2*	99.3*	100.0	99.5*	98.6*	98.6*	98.6*	99.6*	98.8*
		SD	35.5	8.3	0.3	0.0	0.3	0.6	0.5	1.3	0.2	1.0

ME: melon flavor (0.05%), LE: lemon flavor (0.1%), VA: vanilla flavor (0.1%), AL: almond flavor (0.1%), As: saccharin solution with odor A, Bq: quinine solution with odor B, A: deionized water with odor A, B: deionized water with odor B. – indicates the rat did not drink any solutions in the trial.

* Indicates that the ratio was significantly ($p < 0.001$) greater than chance level (50%).

3. Experiment 2

3.1. Materials and methods

3.1.1. Subjects

Twenty-four adult Wistar male rats (170–210 g body weight) were used. The environment and living conditions were the same as those described in Experiment 1. The rats were randomly divided into two experimental groups, SOC ($n = 12$) and SPC ($n = 12$).

3.1.2. Stimuli

The taste and odor stimuli were the same as those in Experiment 1. The odor stimuli were presented as odors A, B, C, and D, differently in each rat (counter-balanced). The stimuli were presented in the manner shown in Table 4.

3.1.3. Training session

The apparatus and the procedure were the same as those in Experiment 1. In this session, there were no procedural differences between the SOC and the SPC groups.

3.1.4. Learning sessions

After the training session, phase 1 of the learning session began. For the SOC group, saccharin solution flavored with odor A was put into four of the eight glass dishes instead of water, and quinine solution flavored with odor C was put into the remaining four dishes (2 ml each). For the SPC group, deionized water flavored with a mixture of odors A and B (odor AB mixture) was put into four of the eight glass dishes. Deionized water flavored with a mixture of odors C and D (odor CD mixture) was put into the remaining four dishes (2 ml each).

All of the rats were exposed to these flavored solutions for 5 min in the morning in the open-field apparatus. The dishes were randomly placed in the eight positions on the apparatus. The session was conducted once in the morning every day, and the rats were allowed to drink additional water in their home cages for 3 h after the session. Phase 1 was conducted for 5 days.

After phase 1, phase 2 of the learning session began. For the SOC group, deionized water flavored with odor AB mixture was put into four of the eight glass dishes. Deionized water flavored with odor CD mixture was put into the remaining four dishes (2 ml each). For the SPC group, saccharin solution flavored with odor A was put into four of the eight glass dishes, and quinine solution flavored with odor C was put into the remaining dishes (2 ml each). All other procedures were the same as those in phase 1. Phase 2 was also conducted for 5 days (Table 4).

3.1.5. Test sessions

After phase 2 of the learning session, testing began and the rats' preferences for each odor were evaluated. In test 1, we aimed to examine whether higher-order conditioning of taste-odor learning was acquired or not; all rats were exposed to deionized water flavored with odor B (four dishes; 2 ml each) and deionized water flavored with odor D (another four dishes; 2 ml each). In test 2, we aimed to examine whether generalization from a single conditioned odor (i.e., odor A and/or odor C) to a binary odor mixture containing the conditioned odors (i.e., odor AB mixture and/or odor CD mixture) would occur; all rats were exposed to deionized water flavored with odor AB mixture (four dishes; 2 ml each) and deionized water flavored with odor CD mixture (another four dishes; 2 ml each). In test 3, we aimed to confirm whether first-order conditioning of taste-odor learning was acquired or not; all rats were exposed to deionized water flavored with odor A (four dishes; 2 ml each) and deionized water flavored with odor C (four dishes; 2 ml each). In test 4, we aimed to compare the preference for an odor with that for another; all rats were exposed to deionized water flavored with one of the odors A, B, AB mixture, C, D, or CD mixture (one 2-ml dish per odor, for a total of 6 dishes for each rat), and deionized water without any odor (one 2-ml dish). The dishes were randomly placed in the eight positions for tests 1–3 and in the seven positions for test 4. All rats were allowed to drink these flavored solutions for 5 min in the morning in the open-field apparatus. Each test was conducted once in the morning every day, and the rats were allowed to drink water in their home cages for 3 h after the session. All tests were sequentially repeated once again after test 4 was completed (Table 4).

3.1.6. Data analysis

The PR was calculated in the manner described in Experiment 1. The PR was analyzed by one-tailed z-test and ANOVA, followed by a post hoc analysis (Shaffer's method). Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

3.2. Results

3.2.1. Phase 1 of the learning session

Mean PRs for saccharin solution flavored with odor A in the SOC group and for water flavored with odor AB mixture in the SPC group were calculated (Fig. 1A). If the ratio was 50%, the rats did not show any preference. In the SOC group, one-tailed z-tests showed that the PRs for saccharin solution flavored with odor A were significantly greater than the chance level from day 2 to day 5 ($ps < 0.001$). In the SPC group, on the other hand, one-tailed z-tests showed no significant differences between the PRs for water flavored with odor AB mixture and the chance level on all 5 days ($ps > 0.05$). This result suggests that these odor mixtures were hedonically neutral in themselves.

3.2.2. Phase 2 of the learning session

Mean PRs for water flavored with odor AB mixture in the SOC group and for saccharin solution flavored with odor A in the SPC group were calculated (Fig. 1B). In the SOC group, one-tailed z-tests showed that the PRs for water flavored with odor AB mixture were significantly greater than the chance level on all 5 days ($ps < 0.001$). In the SPC group, the PRs for saccharin solution flavored with odor A were significantly greater than the chance level from day 7 to day 10 ($ps < 0.001$).

3.2.3. Test 1 session

To examine whether higher-order conditioning of taste-odor learning had been acquired, all rats were exposed to water flavored with odor B and water flavored with odor D in test 1. If learning had been acquired, the rats would show a preference for water flavored with odor B over that flavored with odor D, just as they showed a preference for saccharin solution flavored with odor A over quinine solution flavored with odor C in the learning session. Mean PRs for water flavored with odor B were calculated for each group on each test day. A 2 (group: SOC vs.

Table 4
Flow chart of the sessions and presented stimuli in Experiment 2.

	Training	Phase 1 (Days 1–5)	Phase 2 (Days 6–10)	Test 1 (Days 11, 15)	Test 2 (Days 12, 16)	Test 3 (Days 13, 17)	Test 4 (Days 14, 18)
SOC	w	As vs. Cq	AB vs. CD	B vs. D	AB vs. CD	A vs. C	A vs. B vs. AB vs. C vs. D vs. CD vs. w
SPC		AB vs. CD	As vs. Cq				

w: deionized water, As: saccharin solution with odor A, Cq: quinine solution with odor C, AB: deionized water with a mixture of odors A and B, CD: deionized water with a mixture of odors C and D, A–D: deionized water with one of the odors, A, B, C, or D.

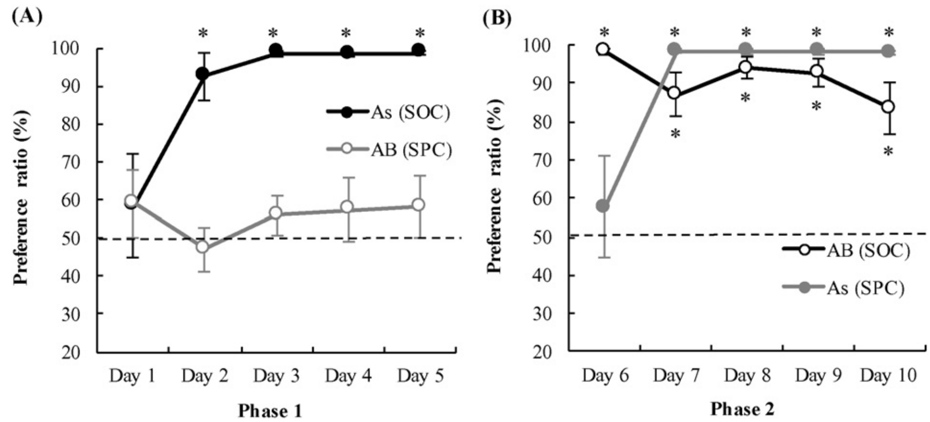

Fig. 1. (A) Mean preference ratios for saccharin solution flavored with odor A (As) in SOC group and for water flavored with odor AB mixture (AB) in SPC group in phase 1. (B) Mean preference ratios for AB in SOC group and for As in SPC group in phase 2. * indicates that the ratio was significantly ($p < 0.001$) greater than the chance level (50%, shown as a dashed line). The vertical lines indicate standard error.

SPC) × 2 (test day: first vs. second) ANOVA revealed that there was a significant main effect of group [$F(1, 22) = 6.339$, $p < 0.05$]; the PR in the SOC group ($M = 72.0\%$) was significantly greater than that in the SPC group ($M = 42.2\%$). However, there was no significant main effect of test day [$F(1, 22) = 0.014$, $p = 0.908$], nor an interaction [$F(1, 22) = 0.116$, $p = 0.737$]. Therefore, the data from the two test days were averaged in each group and used in the following analyses (Fig. 2A). One-tailed z-tests showed that the PR in the SOC group was significantly greater than the chance level ($p < 0.01$), but the PR in the SPC group was not ($p = 0.802$). These results showed that the SOC group preferred water flavored with odor B over that flavored with odor D, but the SPC group did not.

3.2.4. Test 2 session

To examine whether generalization from a single conditioned odor (i.e., odor A and/or odor C) to a binary odor mixture containing the conditioned odors occurred, all rats were exposed to water flavored with odor AB mixture and water flavored with odor CD mixture in test 2. Mean PRs for water flavored with odor AB mixture were calculated for each group on each test day. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) ANOVA revealed that there was a significant interaction between group and test day [$F(1, 22) = 5.383$, $p < 0.05$]. Post hoc analysis revealed a significant simple effect of group on the first test day [$F(1, 22) = 4.905$, $p < 0.05$]; on the first test day the PR in the SOC group ($M = 90.0\%$) was significantly greater than that in the SPC group ($M = 68.7\%$). However, there was no significant difference between the PRs on the first and the second test days in both groups. Therefore, the data from the two test days were averaged in each group and used in the following analyses (Fig. 2B). One-tailed z-tests showed that the PRs were significantly ($ps < 0.001$) greater than the chance level in both the SOC ($M = 86.1\%$) and the SPC groups ($M = 77.9\%$). These results showed that the rats drank the water flavored with odor AB mixture more than the water flavored with odor CD mixture.

3.2.5. Test 3 session

To confirm whether first-order conditioning of taste-odor learning had been acquired, all rats were exposed to water flavored with odor A and water flavored with odor C in test 3. Mean PRs for the water flavored with odor A were calculated for each group on each test day. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) ANOVA revealed that there were no significant main effects of group [$F(1, 22) = 1.297$, $p = 0.267$] or test day [$F(1, 22) = 0.894$, $p = 0.355$], nor an interaction [$F(1, 22) = 0.115$, $p = 0.737$]. Therefore, the data from the two test days were averaged (Fig. 2C), and one-tailed z-tests showed that the PRs were significantly ($ps < 0.001$) greater than the chance level in both the SOC ($M = 88.4\%$) and the SPC groups ($M = 95.1\%$). These results showed that rats drank the water flavored with odor A, which had been paired with saccharin, more than the water flavored with odor C, which had been paired with quinine.

3.2.6. Test 4 session

For tests 1 through 3, we used a paired comparison paradigm, whereby it is unclear whether the rats acquired a preference for

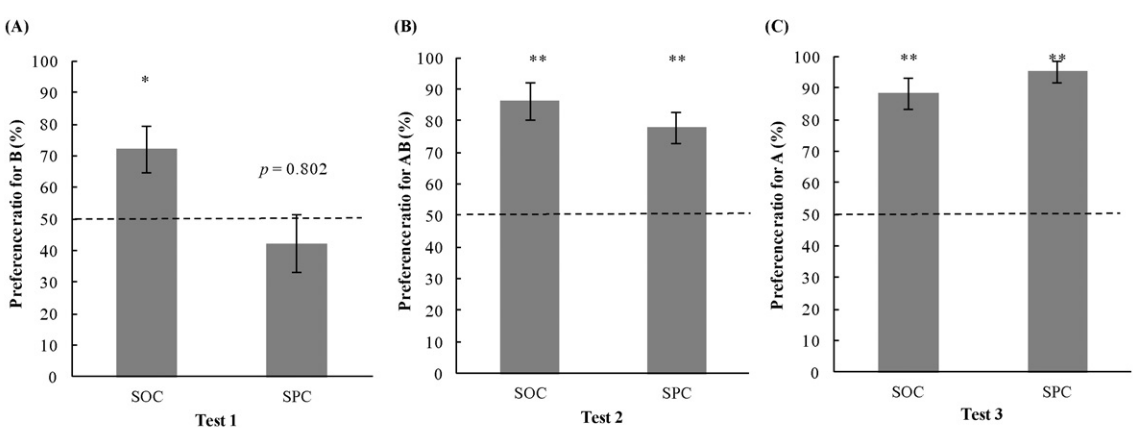

Fig. 2. Mean preference ratios for (A) water flavored with odor B in test 1, (B) water flavored with odor AB mixture in test 2, and (C) water flavored with odor A in test 3. * or ** indicates that the ratio was significantly (*$p < 0.01$, **$p < 0.001$) greater than the chance level (50%, shown as a dashed line). The vertical lines indicate standard error.

saccharin-associated odors and their mixture (i.e., odor A, odor B, and AB mixture) or the avoidance of quinine-associated odors and their mixture (i.e., odor C, odor D, and CD mixture), or both. To examine this point, all rats were exposed to water flavored separately with each of the odors A, B, AB mixture, C, D, and odor CD mixture, as well as odorless water in test 4. The mean PR for each stimulus was calculated for each group (Fig. 3). If preference or avoidance were acquired for a stimulus, the PR for the stimulus would be greater or less, respectively, than that for odorless water. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) × 7 (odor: A vs. B vs. AB vs. C vs. D vs. CD vs. odorless) ANOVA revealed that there were no significant main effects of group or test day, nor interactions ($ps > 0.05$). However, there was a significant main effect of odor [$F(6, 132) = 18.28$, $p < 0.001$]. Post hoc analysis showed that the PRs for water flavored with odor C and odor CD mixture were significantly lower than those for odorless water ($ps < 0.001$). Alternately, the PRs for water flavored with odor A and odor AB mixture were not greater than those for odorless water ($ps > 0.05$). These results showed that the rats avoided water flavored with odor C and odor CD mixture compared to odorless water.

3.3. Discussion

This experiment aimed to examine whether the odors were associated with the emotional aspects of gustatory information or with the qualitative aspects of it in taste-odor learning, using higher-order conditioning paradigms (SOC and SPC). In SOC, CS2 and CS1 are paired after development of the strong association between the US and CS1. In SPC, on the other hand, CS2 and CS1 are paired before pairing with the US. Because of this difference in the paradigms, it was suggested that CS2 might be primarily associated with the emotional information of the US in SOC, whereas CS2 might be associated with the qualitative information of CS1 and the US in SPC [13–16]. Therefore, in this experiment, we hypothesized that if the odors (CSs) were mainly associated with the emotional, or qualitative, aspects of taste (US) in taste-odor learning, the SOC (or SPC) group would prefer water flavored with odor B to that flavored with odor D (i.e., response to CS2), as they would prefer water flavored with odor A to that flavored with odor C (i.e., response to CS1). Test 1 aimed to examine this main hypothesis. The results showed that the SOC group had a preference for water

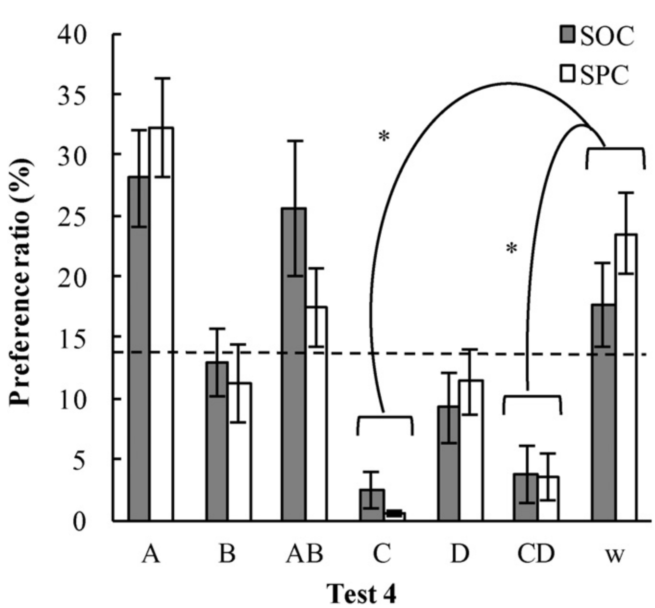

Fig. 3. Mean preference ratios for water flavored with odors A, B, AB mixture, C, D, or CD mixture, and for odorless water (described as w) in test 4. * indicates significant difference compared to odorless water ($p < 0.001$). The dashed line indicates chance level (14.3%). The vertical lines indicate standard error.

flavored with odor B over that flavored with odor D, whereas the SPC group did not, which indicated that higher-order conditioning of taste-odor learning was acquired in the SOC group, but not in the SPC group. Therefore, it seems that the odors were mainly associated with the emotional aspects of gustatory information. These results are consistent with those of Sakai and Yamamoto [9], who found that rats with lesions in the amygdala showed rapid extinction of the learned odor preference. It appears that the qualitative aspects of gustatory information might play relatively weak roles in taste-odor learning compared to the emotional aspects, at least in the present study and that of Sakai and Yamamoto [9].

Test 3 aimed to confirm whether first-order conditioning of taste-odor learning (i.e., response to CS1) had been acquired or not. The results showed that both the SOC and SPC groups showed a preference for water flavored with odor A over that flavored with odor C. Furthermore, the results indicated that all rats preferred the odor paired with saccharin to the odor paired with quinine. Hence, it can be concluded that not only the SOC, but also the SPC group, had acquired first-order conditioning of taste-odor learning, as shown in previous studies [7,9] and Experiment 1 of the present study. This finding suggests that the failure of higher-order conditioning in the SPC group was not due to the failure of acquisition of first-order conditioning of taste-odor learning.

In this study, we used a paired comparison paradigm (e.g., odor A vs. odor C), and thus, it is unclear whether the rats acquired a preference for odor A or the avoidance of odor C, or both. Consequently, we introduced test 4, in which the PR for each stimulus was compared to that of odorless water. These results showed that all rats preferred odorless water to water flavored with odor C, whereas they did not show a preference for water flavored with odor A over odorless water. Therefore, at least in the present study, the results suggest that rats only acquired avoidance of the quinine-associated odor (i.e., odor C) by first-order conditioning of taste-odor learning.

In this experiment, odors (CSs) were mixed by pairing two odors in compound (e.g., pairing odor A with odor B within odor AB mixture). We employed this procedure based on evidence from two previous studies. First, rats can acquire higher-order conditioning even when CSs (e.g., sucrose and hydrochloric acid) are paired in compound [17–19]. Second, rats can generalize a response to an odor (e.g., odor A) to a binary odor mixture containing the same odor (e.g., odor AB mixture) and vice versa [20,21]. These studies suggest that rats can elementally encode a binary odor mixture and acquire the association between odors, which is why we employed this procedure. If this suggestion were true in the current study, rats that acquired avoidance of odor C would also show avoidance of the odor CD mixture. Test 2 aimed to examine this hypothesis. Our results showed that all rats preferred water flavored with odor AB mixture to that flavored with odor CD mixture. In addition, the results of test 4 showed that all rats preferred odorless water to water flavored with odor CD mixture. Since the rats showed avoidance of odor CD mixture and odor C that had been paired with quinine, it appears that the rats perceived the conditioned odor in the binary odor mixture, as suggested by previous studies [20,21].

The PR for water flavored with odor B in the SOC group in test 1 ($M = 72.0\%$) was lower compared to that for water flavored with odor A in test 3 ($M = 88.4\%$). This result indicates that preference acquired in higher-order conditioning is weaker than that in first-order conditioning. This result seems to be based on the nature of higher-order conditioning; higher-order conditioning is intrinsically weaker than first-order conditioning because higher-order conditioning requires more complex processes for animals than first-order conditioning does [13]. In accordance with this, in test 4 the SOC rats drank odorless water more than water flavored with odors C, D, and odor CD mixture, but there was no significant difference between the PRs of odorless water and that of water flavored with odor D. Due to the intrinsic weakness of higher-order conditioning and some procedural limitations in test 4, such as the small volume of the presented stimuli (2 ml each), avoidance of odor D in the SOC group was not detected.

Furthermore, in test 4, both the SOC and SPC groups showed weak (not significant) avoidance of water flavored with odor D, but also water flavored with odor B. This result may be based on neophobic responses to odors B and D. In tests 2 and 4, the rats showed avoidance of odor CD mixture, as well as that of odor C. Although these results indicate that the rats perceived the elements in the binary odor mixture, odors B and D were not individually presented, and thus, these odors might be less familiar than odors A, C, and odor AB and CD mixtures. For these reasons, not only odor D, but also odor B, might be weakly avoided in test 4, in which all stimuli were presented at once.

Classically, in higher-order conditioning paradigms, the acquisition of higher-order conditioning (i.e., the response to CS2) is always tested first in order to avoid any alterations of the response that could occur by initially testing with first-order conditioning (i.e., response to CS1). Experiment 2 followed this traditional procedure. To replicate and validate our findings further, we conducted Experiment 3. In Experiment 3, the acquisition of higher-order conditioning was tested after the first-order conditioning was done (i.e., non-reinforcement of CS1). Rizley and Rescorla [14] showed that non-reinforcement of CS1 resulted in extinction of the CR to CS2 in SPC, but did not in SOC. Therefore, we hypothesized that significant preference for odor B over odor D would also be found in the SOC group in Experiment 3.

4. Experiment 3

4.1. Materials and methods

4.1.1. Subjects

Fifty-two adult Wistar male rats (170–210 g body weight) were used. The environment and living conditions were the same as those described in Experiment 2. The rats were randomly divided into SOC ($n = 26$) and SPC ($n = 26$) groups.

4.1.2. Stimuli

The taste and odor stimuli were the same as those in Experiment 2. The odor stimuli were presented as odors A, B, C, and D, differently in each rat (counter-balanced). The stimuli were presented in the manner shown in Table 5.

4.1.3. Training and learning sessions

The apparatus and the procedure of the training and learning sessions were the same as those in Experiment 2.

4.1.4. Test sessions

After phase 2 of the learning session, testing began and the rats' preferences for each odor were evaluated. The apparatus and procedure of the test sessions were almost the same as those in Experiment 2. An important difference was in the order of the tests. In this experiment, we examined the acquisition of higher-order conditioning of taste-odor learning in test 3, compared to test 1 in Experiment 2, and we examined the acquisition of first-order conditioning in test 1, compared to test 3 in Experiment 2.

4.1.5. Data analysis

The PRs were calculated and analyzed by one-tailed z-test and ANOVA, followed by a post hoc analysis (Shaffer's method). Probability values <0.05 ($p < 0.05$) were considered statistically significant.

4.2. Results

4.2.1. Phase 1 of the learning session

Mean PRs for saccharin solution flavored with odor A in the SOC group and for water flavored with odor AB mixture in the SPC group were calculated (Fig. 4A). If the ratio was 50%, the rats did not show any preference. In the SOC group, one-tailed z-tests showed that the PRs for saccharin solution flavored with odor A were significantly greater than the chance level on all 5 days ($ps < 0.001$). Alternately, in the SPC group, one-tailed z-tests showed no significant differences between the PRs for water flavored with odor AB mixture and the chance level on all 5 days ($ps > 0.05$). This result suggests that these odor mixtures were hedonically neutral in themselves.

4.2.2. Phase 2 of the learning session

Mean PRs for water flavored with odor AB mixture in the SOC group and for saccharin solution flavored with odor A in the SPC group were calculated (Fig. 4B). In the SOC group, one-tailed z-tests showed that the PRs for water flavored with odor AB mixture were significantly greater than the chance level on all 5 days ($ps < 0.001$). In the SPC group also, the PRs for saccharin solution flavored with odor A were significantly greater than the chance level on all 5 days ($ps < 0.001$).

4.2.3. Test 1 session

To confirm whether first-order conditioning of taste-odor learning had been acquired, all rats were exposed to water flavored with odor A and water flavored with odor C in test 1. Mean PRs for water flavored with odor A were calculated for each group on each test day. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) ANOVA revealed that there was a significant main effect of group [$F(1, 50) = 7.42, p < 0.01$]; the PR in the SPC group ($M = 96.8\%$) was significantly greater than that in the SOC group ($M = 89.3\%$). However, there was no significant main effect of test day [$F(1, 50) = 3.15, p = 0.082$], nor an interaction [$F(1, 103) = 1.28, p = 0.263$]. Therefore, the data from the two test days were averaged and used in the following analyses (Fig. 5A). One-tailed z-tests showed that the PRs were significantly ($ps < 0.001$) greater than the chance level in both the SOC and the SPC groups. These results showed that rats drank the water flavored with odor A, which had been paired with saccharin, more than the water flavored with odor C, which had been paired with quinine.

4.2.4. Test 2 session

To examine whether generalization from a single conditioned odor (i.e., odor A and/or odor C) to a binary odor mixture occurred, all rats were exposed to water flavored with odor AB mixture and water flavored with odor CD mixture in test 2. Mean PRs for water flavored with odor AB mixture were calculated for each group on each test day. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) ANOVA revealed that there were no significant main effects of group [$F(1, 50) = 0.216, p = 0.644$], test day [$F(1, 50) = 0.014, p = 0.908$], or an interaction [$F(1, 103) = 1.52, p = 0.224$]. Therefore, the data from the two test

Table 5
Flow chart of the sessions and presented stimuli in Experiment 3.

	Training	Phase 1 (Days 1–5)	Phase 2 (Days 6–10)	Test 1 (Days 11, 15)	Test 2 (Days 12, 16)	Test 3 (Days 13, 17)	Test 4 (Days 14, 18)
SOC	w	As vs. Cq	AB vs. CD	A vs. C	AB vs. CD	B vs. D	A vs. B vs. AB vs. C vs. D vs. CD vs. w
SPC		AB vs. CD	As vs. Cq				

w: deionized water, As: saccharin solution with odor A, Cq: quinine solution with odor C, AB: deionized water with a mixture of odors A and B, CD: deionized water with a mixture of odors C and D, A–D: deionized water with one of the odors, A, B, C, or D.

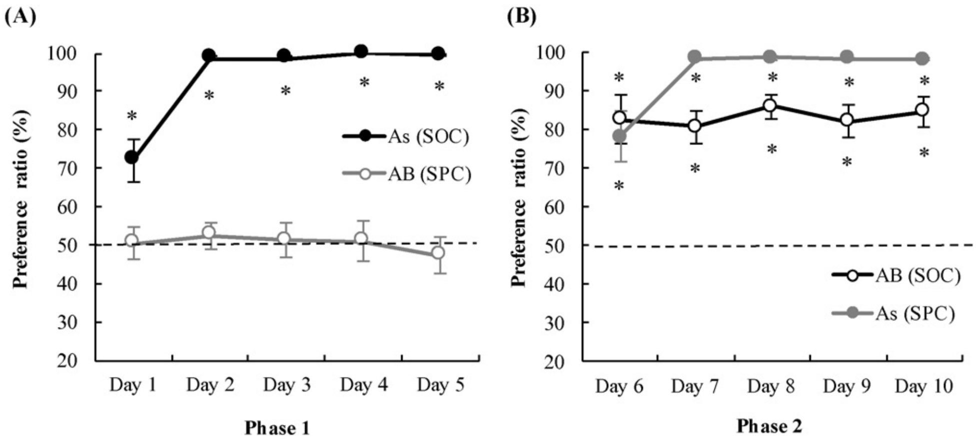

Fig. 4. (A) Mean preference ratios for saccharin solution flavored with odor A (As) in the SOC group and for water flavored with odor AB mixture (AB) in the SPC group in phase 1. (B) Mean preference ratios for AB in the SOC group and for As in the SPC group in phase 2. * indicates that the ratio was significantly ($p < 0.001$) greater than the chance level (50%, shown as a dashed line). The vertical lines indicate standard error.

days were averaged (Fig. 5B), and one-tailed z-tests showed that the PRs were significantly ($ps < 0.001$) greater than the chance level in both the SOC ($M = 82.9\%$) and the SPC groups ($M = 80.1\%$). These results showed that the rats drank the water flavored with odor AB mixture more than the water flavored with odor CD mixture.

4.2.5. Test 3 session

To examine whether higher-order conditioning of taste-odor learning had been acquired, all rats were exposed to water flavored with odor B and water flavored with odor D in test 3. If learning had been acquired, the rats would show a preference for water flavored with odor B over that flavored with odor D, as they showed a preference for water flavored with odor A over that flavored with odor C in test 1. Mean PRs for water flavored with odor B were calculated for each group on each test day. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) ANOVA revealed that there were no significant main effects of group [$F(1, 50) = 3.99$, $p = 0.051$], test day [$F(1, 50) = 0.265$, $p = 0.609$], nor an interaction [$F(1, 103) = 1.37$, $p = 0.247$]. Therefore, the data from the two test days were averaged (Fig. 5C), and one-tailed z-tests showed that the PR in the SOC group ($M = 57.3\%$) was significantly greater than the chance level ($p < 0.05$), but that in the SPC group ($M = 44.3\%$) was not ($p = 0.842$). These results showed that the SOC group showed a preference for water flavored with odor B over that flavored with odor D, but the SPC group did not.

4.2.6. Test 4 session

The mean PR for each stimulus was calculated for each group on each test day (Fig. 6). If preference or avoidance was acquired for a stimulus, the PR for the stimulus would be greater or less, respectively, than that for odorless water. A 2 (group: SOC vs. SPC) × 2 (test day: first vs. second) × 7 (odor: A vs. B vs. AB vs. C vs. D vs. CD vs. odorless) ANOVA revealed that there were no significant main effects of group, test day, nor any interactions ($ps > 0.05$). However, there was a significant main effect of odor [$F(6, 300) = 35.39$, $p < 0.001$]. Post hoc analysis showed that the PRs for water flavored with odors B, C, D, and odor CD mixture were significantly lower than those for odorless water ($ps < 0.001$). Alternately, the PRs for water flavored with odors A and AB mixture were not greater than those for odorless water ($ps > 0.05$). These results showed that the rats avoided water flavored with odors B, C, D, and CD mixture compared to odorless water.

4.3. Discussion

This experiment aimed to replicate and validate the results in Experiment 2, with a different test order; response to CS2 was always tested after the test of CS1 (i.e., non-reinforcement). Rizley and Rescorla [14] showed that non-reinforcement of CS1 did not result in extinction of the CR to CS2 in SOC because CS2 seemed to not be associated with the representation of CS1 and that of the US in SOC. Therefore, we

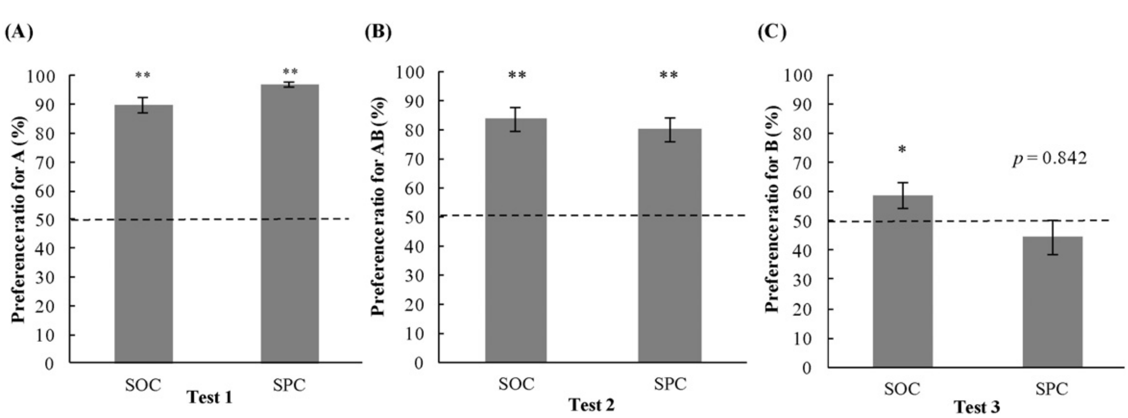

Fig. 5. Mean preference ratios for (A) water flavored with odor A in test 1, (B) water flavored with odor AB mixture in test 2, and (C) water flavored with odor B in test 3. * or ** indicates that the ratio was significantly (*$p < 0.05$, **$p < 0.001$) greater than the chance level (50%, shown as a dashed line). The vertical lines indicate standard error.

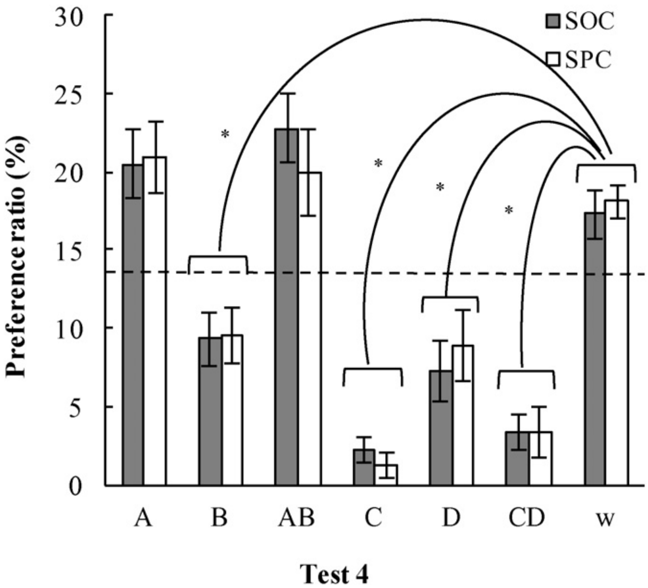

Fig. 6. Mean preference ratios for water flavored with odors A, B, AB mixture, C, D, or CD mixture, as well as for odorless water (described as w) in test 4. * indicates significant difference compared to odorless water ($p < 0.001$). The dashed line indicates chance level (14.3%). The vertical lines indicate standard error.

hypothesized that significant preference for odor B over odor D would be found in the SOC group. The results of test 3 showed that the SOC group had a significant preference for water flavored with odor B over that flavored with odor D, whereas the SPC group did not. These results again demonstrated that higher-order conditioning of taste-odor learning was acquired only in the SOC group, as shown in Experiment 2, and that it was not extinguished by the non-reinforcement of CS1. The results of tests 1 and 2 confirmed that both the SOC and SPC groups successfully acquired first-order conditioning, and rats perceived the conditioned CS1 odor in the binary odor mixture. There was only a significant difference between the SOC and SPC groups in the acquisition of higher-order conditioning. Therefore, these results suggest that the odors were mainly associated with the emotional aspects of gustatory information.

The PR for water flavored with odor B in the SOC group ($M = 57.3\%$) was lower compared to that for water flavored with odor A in test 1 ($M = 89.3\%$). This result indicates that preference acquired in higher-order conditioning is weaker than that in first-order conditioning, as shown in Experiment 2. Furthermore, the rats equally showed avoidance of water flavored with odor B and that with odor D in test 4. We interpreted this result based on the intrinsic weakness of higher-order conditioning and the neophobic responses to odors B and D. In tests 2 and 4, the rats showed avoidance of odor CD mixture, as well as that of odor C. Although these results indicate that the rats perceived the elements in the binary odor mixture, odors B and D were not separately presented, and thus, these odors might be less familiar than odors A and C, and odor AB and CD mixtures. For these reasons, odor D, as well as odor B, may have been avoided in test 4.

5. General discussion

In the literature, it has been suggested that after repeated pairing of an odor with a taste, the odor alone activates the representation of the taste [1,2]. However, the odor could be associated with both positive or negative hedonics, and sweet or bitter taste quality of the gustatory information, respectively, and both associations could lead to the same approach or avoidance behavior in animals [2,8]. The present study aimed to clarify this point.

Experiment 1 aimed to replicate previous findings [7,9]. These results showed that rats preferred the odor that had been paired with saccharin solution to the odor that had been paired with quinine solution (i.e., first-order conditioning of taste-odor learning), as shown in previous studies [7,9]. These results also showed that the acquisition and retention of learning was not dependent on which flavor was presented as the odor stimulus.

Experiments 2 and 3 aimed to examine our main hypothesis, using the higher-order conditioning paradigm (see Gewirtz & Davis [13]). The higher-order conditioning paradigm consists of two different procedures: second-order conditioning (SOC) and sensory preconditioning (SPC). In these paradigms a CS (CS2), which is not paired directly with the US, acquires the ability to elicit the CR by being paired with another CS (CS1) that is paired with the US. Based on previous findings of the associative structure of higher-order conditioning [13–16], it has been suggested that CS2 acquires the ability to elicit the CR through the development of an association with the emotional states elicited by the US in SOC, whereas in SPC, this occurs through an association with the representation of CS1 and the US. Hence, we hypothesized that if the odors were associated mainly with the emotional, or qualitative, aspects of gustatory information by taste-odor learning, SOC (or SPC) would be successfully acquired in rats. The results of Experiment 2, in which the response to CS2 was tested first, showed that higher-order conditioning of taste-odor learning was acquired in the SOC group, but not in the SPC group. Even in Experiment 3, in which the response to CS2 was tested after the test of CS1 (i.e., non-reinforcement of CS1), a significant response to CS2 was found in the SOC group. This result was consistent with that of Rizley and Rescorla [14], who showed that non-reinforcement of CS1 did not result in extinction of the CR to CS2 in SOC. Therefore, these results suggest that the odors were mainly associated with the emotional aspects of gustatory information; the odor alone activated the emotional state elicited by the taste. Furthermore, these results were consistent with the findings of Sakai and Yamamoto [9], who demonstrated that brain lesions in the amygdala interrupt learning.

Alternatively, many studies have shown that odors are associated with the qualitative, perceptual aspects of gustatory information in human psychophysics [3–6]. These studies suggest that human taste-odor learning is the basis of the qualitative taste-odor interaction, such as odor-induced taste enhancement [22–27]. Rats can also acquire the association between the salty taste quality of NaCl and an odor, as shown by Sakai and Imada [8]. Therefore, odors may also be associated with the qualitative and perceptual aspects of gustatory information, in addition to the emotional aspects, in parallel. However, the function of the latter seems to be stronger than the former, at least in the present study and in that of Sakai and Yamamoto [9]. Currently, odor-induced taste enhancement is thought to be a useful strategy to prevent salt overconsumption, which has been linked to the development of health problems (e.g., hypertension and cardiovascular disease), without reducing food acceptance [23,24]. Therefore, understanding in which conditions odors are associated with qualitative aspects of gustatory information is theoretically and practically important. Further research should elucidate this point in future.

6. Conclusions

The present study showed that higher-order conditioning of taste-odor learning was acquired only in the SOC group, but not in the SPC group. Our results suggest that in the case of sweet/bitter taste stimuli, odors were mainly associated with the emotional aspects of gustatory information, possibly based on the function of the neural substrates involved in emotional processing, such as the amygdala. The present study contributes to our understanding of flavor perception, such as taste-odor interaction, which has consequences on ingestive behavior. Additionally, this study emphasizes the significance of higher-order conditioning paradigms, which enable us to elucidate the associative neural mechanisms of learning simply by observing animal behavior.

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