Letter

Evaluation of the ability of mice to detect VOCs, using a positive operant reinforcement procedure

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(Received December 5, 2014; Accepted April 15, 2015)

ABSTRACT — To examine the validity of a newly established “three-odor detection (TOD)” procedure using one of volatile organic compounds (VOCs), limonene, food-restricted male mice were used. Five animals each were assigned to either TOD or single-odor detection (SOD). TOD was composed of two trainings and one test (TEST) session. Mice were trained to discriminate an odor of coffee from no odor and odors of coffee and cheese from no odor in trainings 1 and 2, respectively. In TEST, mice were required to discriminate odors of coffee, cheese, and limonene from no odor. In SOD, mice were required to discriminate an odor of limonene from no odor. Each training or test was conducted once a day until animals achieved a learning criterion (75% correct response rate for 2 consecutive days), or until a maximum number of sessions (20 sessions) was completed. The number of sessions for reaching the learning criterion of animals in TEST (8.2 ± 0.8) was smaller than that of animals in SOD (19.2 ± 0.8). Results indicated that mice in TOD detected low levels of VOCs more rapidly than animals in SOD. I concluded that TOD is a useful procedure for detecting low levels of VOCs.

Key words: Mouse, Three-odor detection (TOD), Operant behavior

INTRODUCTION

As many volatile organic compounds (VOCs) have typical smells, sometimes workers have reported that odors of VOCs induce adverse health problems or/and deprave symptoms (Berger-Sweeney *et al.*, 1998) even if those concentrations were under the acceptable levels. As there are few studies focusing on the effects of the odor of VOCs per se on workers' health, causal relationships between health effects and the odors remain to be seen. To investigate the health effects of odors by animal experiments, it is essential to use test procedures that are able to estimate behavioral changes induced by odors. Many studies have demonstrated that mice readily learn tasks cued by odors, and they improve rapidly over successive trials (Singer, 2006) and show remarkable ability to detect odors (Wolkoff *et al.*, 2006). For example, in the Go/No-go operant paradigm, mice have been found to reliably achieve high levels of detection accuracy across a variety of concentrations between 40–60 trials (Wolkoff *et al.*, 2006) or within 100 trials (Zagreda *et al.*, 1999). However, the focus in this research field was to explore the mechanism of the olfactory system in the animal, not

to measure the odor threshold or odor detection ability in mice. In particular, there are a few problems with examining the odor detection ability of animals using VOCs: sometimes animals avoid sniffing VOCs because of the unfavorable smell; habituation or addictive effects of VOCs can also be problems for animals to examine the ability of odor detection or to measure the threshold of odors of VOCs. Aim of the present study was to develop an efficient odor detection procedure using animals without degrading the precision. A new procedure made it possible for mice to detect limonene, which is a VOC, within a small number of trials. Usually many trials were required to understand the relationship between odor discrimination and responding under odor discrimination experiments. Therefore it was difficult to use odor stimuli that have adverse effects for health, such as organic solvents. If the relationship is learned by a schedule controlled operant behavior (SCOB) using harmless odors in advance, learning to discriminate novel odors might be established with a smaller number of trials, even if they are noxious. In the present study, I established a new experimental procedure using odors, named three-odor detection (TOD). In the procedure, animals were trained

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to discriminate between odor stimulus and 'no odor' by pressing levers. Then the relationship was generalized to a target (VOC) odor.

MATERIALS AND METHODS

C3H male mice (Japan Charles River, Tokyo, Japan) were housed individually under the following conditions: a 12-hr light/12-hr dark cycle (lights on at 7:00) at 21°C with free access to water. From 6 weeks of age, food was restricted to 2.5–4 g per day and maintained the mice at 80–85% of their free-feeding weights. Experiments were conducted in accordance with the *Guidelines for the Care and Use of Animals* of the National Institute for Environmental Studies (Tsukuba, Japan) and National Institute of Occupational Safety and Health (Kawasaki, Japan). All experiments were carried out using standard operant chambers for mice (370W × 280D × 280 mmH, Muromachi Kikai, Tokyo, Japan), equipped with two levers, two cue lights, a house light, a speaker for providing a tone, and a feeder. The chamber was individually located in a sound-attenuating box (600W × 500D × 500 mmH, Muromachi Kikai). Each stimulus was a tube containing the prepared odor, placed on a tray located under the floor of the chamber. Coffee and cheese, to which mice showed preference during a pilot study, were used as training odors in the TOD. Then limonene was selected as a test odor because mice strongly evaded it during the pilot study. As mice avoided the odor in the preliminary observation, it was thought that limonene was a good sample to examine the effect of our new odor detection procedure. Coffee, which was a commercial instant powder (Japan Nestle, Hyogo, Japan), was diluted with pure water to 1.0% concentration, which is available for drink. The cheese was a commercial cheddar paste (Kraft, Northfield, IL, USA) diluted with corn oil (Sigma-Aldrich, St Louis, MO, USA) to a concentration of 0.1 g/mL. Limonene (Sigma-Aldrich, Tokyo, Japan), diluted with pure water to a concentration of 1 µL/mL, was used in TOD and SOD. These concentrations of coffee and cheese were selected because they could be spread in equal proportions on a cotton ball. Each odor stimulus, 300 µL of volume, was absorbed into the cotton ball being put in the beneath of a 1.5 mL tube. The tube was covered with another cotton ball and then capped. Tubes were wrapped with white paper to eliminate visual cues. The tube was uncapped to release the odor immediately before putting the tube in a small dish, and then presented on a tray located under the grid floor of the chamber. Two weeks after the start of deprivation, 10 mice were engaged in the magazine training. At 10 weeks of age, 5 animals each were assigned to

the TOD and the SOD. The TOD was comprised of three parts: trainings 1 (T1), 2 (T2), and a test (TEST). In T1, animals were required to press one of two levers, which corresponded to coffee odor or no odor. In T2, cheese was added to odor stimuli. In TEST, animals were required to press the odor lever when any of three odors, coffee, cheese or limonene was presented. SOD simply required animals to discriminate between limonene and no odor respectively. In T1 and T2, odor and no odor trials were shown 50 times each. TEST contained 25 odor and 25 no odor trials. The order of stimulus presentation in both TOD and SOD was randomized.

Each animal was engaged 1 session/day, 5 days/week during 20 sessions. At the beginning of a session, the house light was on and a tone was sounded for 0.5 sec before each trial. A stimulus was manually presented approximately 0.5 sec after the tone. Cue lights above the levers were on after the animal sniffed the stimulus for 2 sec, and levers were activated. When the animal selected the correct lever, a 14 mg pellet (Bio-Serv, Flemington, NJ, USA) was delivered. On the other hand, if the animal selected the wrong lever or did not respond after more than 30 sec, the trial was terminated, house and cue lights, and levers turned off. In 30 sec intervals between trials, air of the chamber was flushed by an electric fan, and the dish and the tray were wiped with a wet paper towel. Correct response rate of each session was calculated. The session was conducted until the animal reached 75% correct in 2 consecutive sessions. The number of sessions needed to reach the criteria was defined as a learning load index (LLI). The average LLI of TOD was compared with that of SOD and was analyzed by the Student's t-test. The LLIs were verified by statistically significant increases in correct response rate at final sessions compared to those at the starting sessions by a two-tailed Student's t-test. All statistical analyses were performed with SPSS statistical package 19.0 software (IBM, Chicago, IL, USA). A *P*-value of ≤ 0.05 was considered significant.

RESULTS

In SOD, the average LLI was 19.2 ± 0.8 (Table 1). The range of LLIs was 18–20, the upper limit of the range exceeded 20 because 2 mice out of 5 could not reach the criteria within 20 sessions. Even though some animals did not reach the criteria, the average correct response rate in session 20 (68.3 ± 11.6) was significantly higher than that of the first session (19.5 ± 18.1) (Fig. 1), it meant that animals achieved the task as a group. The average correct response rates of limonene (54.3 ± 12.3) and no odor (61.0 ± 14.3) were not different. In T1, all animals reached

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Table 1. Average number and range of sessions required to reach the 75% correct response rate.

		Number of sessions for reaching the learning criterion (sessions)	Learning load index (sessions)	Correct response rate of each stimulus (%)	
Procedure	SOD	19.2 ± 0.8	18 - 20	Limonene	54.3 ± 12.3
				No odor	61 ± 14.3
	T1	6.8 ± 0.2*	6 - 7*	Coffee	75.4 ± 3.5
				No odor	65.3 ± 3.5
	T2	18.2 ± 1.3	16 - 19	Coffee	75 ± 2.7 ^{a, b}
				Cheese	59.9 ± 3
				No odor	65.6 ± 3.3
				Coffee	82 ± 7.8 ^{a, c}
TOD	T3	8.2 ± 0.8*	7 - 9*	Cheese	85 ± 7.1 ^{a, c}
				Limonene	63.1 ± 8
				No odor	64.5 ± 2.5
				No odor	64.5 ± 2.5

* means < 0.05 (vs. SOD), ^a means < 0.05 (vs. no odor), ^b means < 0.05 (vs. cheese), ^c means < 0.05 (vs. limonene) in the same procedure.

Fig. 1. Correct response rate (mean ± S.E.M) of mice for limonene at SOD. (*= $p < 0.05$, vs. correct response rate in the first session).

the correct response rate within the range of 6-7 sessions (Table 1). The average number of session required a 75% correct rate (6.8 ± 0.2), which was significantly lower than that of SOD. The average correct response rate of coffee (75.4 ± 3.5) in T1 was significantly higher than that of no odor (65.3 ± 3.5) (Fig. 2A). In T2, animals took 16-19 sessions to reach a 75% correct rate. The average number of sessions (18.2 ± 1.3) was significantly higher than that of T1; however, it was not different from that of SOD (Table 1). The average correct response rate of coffee (75.0 ± 2.7) in T2 was significantly higher than those of cheese (59.9 ± 3.0) and no odor (65.6 ± 3.3) (Fig. 2B and C). These results implied that animals showed more difficulty in learning to press the same lever for coffee and cheese, even if these odors were presented differently. The correct response rates of coffee were higher in

almost all trials than those of no odor at T2, even though both stimuli were experienced in the same amounts of trials in T1 and T2. In TEST, the number of sessions (8.2 ± 0.8) required to reach a 75% correct rate was significantly lower than those of SOD and T2, but not different from that of T1. The range of sessions to reach a 75% correct rate was 7-9 (Table 1). Fig. 2D and E show the correct response rate for odor and no odor stimulus during TEST, respectively. The average correct response rate for coffee (82.0 ± 7.8) and cheese (85.0 ± 7.1) were significantly higher than those of limonene (63.1 ± 8.0) and no odor (64.5 ± 2.5) in T2. Also, correct rates of coffee and cheese in sessions 2, 4, and 5 were higher than those of no odor (69.2 ± 4.22 , 52.5 ± 3.02 , and 55.0 ± 3.12 at sessions 2, 4, and 5). The average number of correct rates of coffee (82.0 ± 7.8) and cheese (85.0 ± 7.1) were greater than those of limonene (63.1 ± 8.0) and no odor (64.5 ± 2.5). The stimulus of no odor did not reach a 75% response rate until the last session.

DISCUSSION

I examined sensitivity to odor of mice using limonene as one of VOCs and evaluated effectiveness and validity of TOD. The main finding in this study is that limonene was readily detected by mice in TOD. In the Go/No-go operant paradigm, mice have been found to reliably achieve high levels of detection accuracy across a variety of concentrations between 40-100 trials (Wolkoff *et al.*, 2006; Zagreda *et al.*, 1999). In the present study, the total number of trials of limonene detection that achieved the learning criterion in TOD was 68.3 (7-9 trials/session). The ability of odor detection in mice shown in the present study is comparable to the results of other studies that evaluated olfactory learning (Huttera *et al.*, 2006). I concluded that TOD using mice might be one of useful

Fig. 2. Group means of correct response rates of mice at training1 (A), 2 (B) and Test (C), and correct response rates of each odor and no-odor stimuli (D) in training 2 and Test (E). Correct response rates of group mean ($\circ$), coffee ($\blacksquare$), cheese ($\blacktriangle$), limonene ($\times$), and no-odor ($\blacklozenge$) are shown. All data are presented as means $\pm$ S.E.M. (* = $p < 0.05$, vs. correct response rate in the first session, $\dagger = p < 0.05$, vs. correct response rate in the first session in SOD).

methods for detecting odor of VOCs. The American Conference of Governmental Industrial Hygienists (ACGIH) indicated the threshold limit value for a variety of VOCs in the workplace. Though Threshold Limit Value (TLV) for limonene is not included in the ACGIH's guidelines, levels may easily reach several hundred parts per billion (ppb) during and immediately after product use (Walker and O'Connell, 1986; Wainman *et al.*, 2000). Seifert *et al.* (1999) indicated 0.03 mg/m³ as guideline for terpenes in the guideline values for indoor air pollutant. The volume of limonene used in the TEST of the present study was 0.84 μ g (1 μ L) in the 1.5 m³-sound attenuating box, and the concentration was 0.56 μ g/m³. This concentration could be thought very low level as compared to the guideline value (Seifert *et al.*, 1999). I concluded that TOD is useful to detect low levels of VOCs in indoor air for risk assessment. The number of sessions required to reach a correct response was set at 75% in the present study, which was lower than in other studies; for example, 90% (Wolkoff *et al.*, 2006) and 80% (Nakayama and Morimoto,

2009) were set in the Go/No go experiment paradigm used by other investigators. In all procedures of the present study, the correct response rates in the last session were higher than those in the first session. This means that I can suggest that animals learn odor detection in the process of these procedures, and a 75% correct rate was enough to reason that animals had learned the odor detection. The accuracies of odor detection in the present study were unstable, as indicated when they were decreased at sessions 5-7 in the experiment. Differences in accuracy can be due to procedural differences. For acquisition of associative learning, the salience of the stimulus cues is an important factor in determining how well the task is learned (Iversen *et al.*, 1986). If stimulus cues are salient to evoke a response, conditional discrimination develops earlier and more accurately than when the cues are less salient (Iversen *et al.*, 1986). In the present study, mice had to learn to press levers not only for odor stimuli but also for no odor. Responding to an undetectable stimulus could be difficult for mice from an associative learning

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standpoint. No odor stimulus might be difficult to evoke a response because no odor would not function as a discrimination cue. Therefore, I thought that the 75% correct rate set in the present study was not lower than the other studies.

It is assumed that the animals tried to distinguish the very low level of smells in the no odor stimulus because the rate of correct responses was lower than that of the other odor stimuli. In TOD, mice learned the relationship between stimuli (odor)-response (specific lever pressing)-reward (food pellets for correct responding) during training. However, there were some problems in TOD procedure. Animals needed a lot of trials in T2 to reach the learning criterion. Animals might need time to categorize two stimuli (coffee and cheese) as odor stimuli, at the same time they might distinguish no odor as different stimulus from odor stimuli. In addition, at the beginning of TEST, correct response rates of limonene and no odor were not stable. It was thought that animals tried to categorize three odors as odor stimulus.

In the TEST session of TOD, the number of sessions to reach the criteria was significantly smaller than that in SOD. If mice associated each odor stimulus and response one by one when the conditioning was established, the results of SOD should be similar to the results of TOD. These results support that mice applied the response-behavior relationship acquired in the training to TEST as a category and did not use each odor as a simple element. Therefore, I concluded that TOD can be applied to testing other chemicals that have adverse effects, such as toxic, hedonic, or addictive effects.

In summary, mice were able to detect low levels of VOCs when trained by positive reinforcement in an operant procedure. TOD may be applicable to testing for other harmful chemicals because it minimizes exposure time.

Conflict of interest---- The authors declare that there is no conflict of interest.

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