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Food aversion learning based on voluntary running in non-deprived rats: a technique for establishing aversive conditioning with minimized discomfort

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Abstract: This article presents an experimental preparation for establishing conditioned food aversion (CFA) by voluntary wheel running in rats with laboratory chow and water freely available. In Experiment 1, unfamiliar food (raisins) was avoided by rats when they first encountered it. This neophobic food avoidance was habituated by repeated tests; the rats gradually increased their raisin consumption. However, the consumption remained suppressed in rats that accessed the raisins after wheel running. This finding implies that running yielded CFA, which suppressed consumption of the unfamiliar food rather than increasing it. Because running generated kaolin clay ingestion, which is a behavioral marker of nausea, it is suggested that the running-based CFA was mediated by weak gastrointestinal discomfort. Experiment 2 supported the claim that the suppressed consumption is due to running-based CFA by showing the specificity of food suppression. Demonstration of CFA based on voluntary activity in non-deprived rats will contribute to basic research on learning and memory as an alternative technique for studying aversive conditioning with minimized discomfort in animals.

Key words: conditioned taste aversion, nausea, neophobia, rat, wheel running

Introduction

Aversive conditioning studies have greatly contributed to our understanding of behavioral and neural mechanisms of learning and memory, and its clinical applications should not be overrated [3, 20, 26]. However, concerns for laboratory animal welfare [7, 17] lead us to develop and use more humane experimental techniques than the conventional ones. For example, the animal research guidelines of the American Psychological Association [1] say, "Whenever possible behavioral procedures should be used that minimize discomfort to the nonhuman animal." In light of this trend, the present article provides a new technique for establishing conditioned food aversion (CFA), in which animals develop a dislike for a particular food by Pavlovian associative processes. Because the major sensory cue in food aversion learning is gustatory for rats [49], CFA is also called conditioned taste aversion (CTA), which is one of the most adopted research paradigms for studying aversive conditioning in rats [18, 27, 61].

In the conventional CTA paradigm for laboratory rats, a target taste solution is paired with gastrointestinal discomfort induced by radiation exposure [29] or injection of emetic drugs such as lithium chloride [48] and cyclophosphamide [23]. Other effective procedures involve such things as high-speed rotation [31], high ambient room temperature [11], magnetic field [35], electric

shock [39], tumor implantation [10], thiamine deficiency [64], histidine-free amino acid load [14], and injections of a variety of drugs such as apomorphine [72], ethanol [24], methyl mercury [16], insulin and formalin [22], psychoactive drugs [9], and so on (see Riley and Freeman [63] for a database). All of these treatments are coercive, and many of them are highly aversive for rats.

A unique agent for establishing CTA in rats is voluntary wheel running [30, 32, 33, 40, 41, 51, 54, 58]. Because wheel running is a positive reinforcer for preceding instrumental responses, such as lever pressing [8, 19, 37, 38], one may regard wheel running as pleasant. A recent finding that wheel access elicits 50-kHz ultrasonic calls [34], which are thought to reflect positive affective states, supports this idea. Notably, one might argue that some addictive drugs also have CTA-inducing and response-reinforcing properties (see Hunt and Amit [36] for a review) and evoke 50-kHz ultrasonic calls (see Barker, Simmons, and West [6] for a review). However, these drugs have adverse effects on the health of rats. On the other hand, a running wheel is frequently introduced to the animal cage as a source of environmental enrichment for rodents including rats, and their salubrious effects are well reported [15, 42, 60, 71]. Despite this pleasant nature of wheel running, it functions as an agent for establishing CTA, as already mentioned.

Among the many studies on running-based CTA (see Boakes and Nakajima [13] for a review), the most relevant to the present argument concerning animal welfare are the experiments reported by Lett *et al.* [41]: they demonstrated running-based CTA in rats with food and water freely available. Their study is unique, since water- or food-deprivation is standard practice for CTA training. The primary aim of the present research was to replicate their study because of its significance from the perspective of animal welfare. Notably, their rats were water-deprived during the pretraining phase in order to make the rats readily drink from a bottle, which would provide a target taste solution in the subsequent, non-deprived, conditioning phase. The present research did not even use such a deprivation technique: the rats were under a non-deprived condition throughout the experiment.

The first experiment reported below attempted to demonstrate running-based aversive conditioning in rats under the completely non-deprived condition. For this purpose, raisins were chosen as unfamiliar, but potentially attractive, food in the present study. Notably, the term CFA, rather than CTA, is used hereafter because the critical sensory component of this aversive conditioning was experimentally unidentified in this report. Although rats are reluctant to eat unfamiliar food [4, 46], this reluctance is expected to wane after repeated experience with the same food. In other words, the rats show habituation of neophobic reactions to a novel food item [66]. Running-based CFA is expected to counteract this process, resulting in continued suppression of raisin eating. A subsidiary purpose of this experiment was to monitor the gastrointestinal discomfort induced by voluntary running in the situation prepared here. Because a large number of rat studies [44, 45, 47, 65, 69, 70, 73] have shown that various emetic treatments generate pica behavior (kaolin clay ingestion), pica is now considered as a useful behavioral marker of nausea in rats [2]. Furthermore, recent research from my laboratory has shown that pica is elicited by wheel running in rats [52, 54, 55]. In the first experiment, nausea was assessed in rats by this measure.

The second experiment was designed to ensure that the suppressed raisin consumption observed in Experiment 1 was due to running-based CFA, and this was accomplished by employing a differential conditioning procedure, in which access to one of two snacks (raisins and cheese) was followed by wheel running, whereas access to the other was never followed by wheel running. Running-based specific food suppression was expected in Experiment 2. It is notable that the raisins and cheese employed here were chosen as proper snacks through a number of pilot studies prior to this research project.

Material and Methods

The experimental procedures reported in this article were approved by the Animal Care and Use Committee of Kwansei Gakuin University (2017-01, 2018-01) and were based on Japanese law and the guidelines published by the Science Council of Japan in 2006.

Experiment 1

Animals and housing: The subjects were 16 experimentally naïve male Slc:Wistar/ST rats purchased from a commercial breeder (Japan SLC, Hamamatsu, Japan) when they were 9 weeks old. They were housed in individual hanging cages (20 cm wide, 25 cm long, 18.7 cm high) made of wire bars and metal side walls and maintained in a vivarium on a light-dark cycle of 12-12 h (lights on at 0900 h) with controlled temperature (23°C)

and humidity (55%). The animals were handled daily for a week before the experimental procedure began. The chow pellets (MF diet, Oriental Yeast Co., Ltd., Tokyo, Japan) were placed in a metal container positioned inwards with its end apertures 3.5 cm above the cage floor. The kaolin pellets were made of kaolin powder (Shin Nihon Zokei Co., Ltd., Tokyo, Japan) and gum arabic powder (Wako Pure Chemical Industries, Osaka, Japan) at a 99:1 ratio; they were mixed with distilled water to form cylindrical pellets and were completely dried before use. A hanging metal container (8 cm wide, 4.5 long, 6 cm deep) filled with 12–16 kaolin pellets was installed next to the chow container a day before the experiment. The lower end of this container was 9 cm above the floor, and rats could access the kaolin pellets not only from the end apertures but also from the top opening. Tap water was always available from a metal nozzle positioned in the opposite wall. A plastic tray with paper bedding was positioned 10 cm under each cage.

Apparatus: The rats were transferred from their home cages by a cart having individual plastic compartments (10.5 cm wide, 13 cm long, 14.2 cm high) to a conventionally illuminated experimental room, which had 16 test cages (copies of the home cages) on a table and 8 activity wheels on a wall. The travelling time from the vivarium to the experimental room was around 1 min. Each test cage had a metal bowl (8 cm in diameter, 3.5 cm deep) clipped to the cage wall at floor level with a metal hoop holder. The activity wheels were hung on a wire net arranged in a 4 × 2 fashion. The top and bottom rows were 140 and 90 cm above the room floor, respectively, and a long plastic plate was fixed under each row to catch excretions. Each wheel had an internal width of 15 cm and a diameter of 30 cm. The running surface was made of 0.2-cm metal rods spaced 1 cm apart. The two sides of the wheel were perforated metal sheets, and the rats were placed into the individual wheels via doors on the sides. The wheels could be turned in both directions.

Procedure: Laboratory assistants, who were not informed of the aim of the research, administered the experimental protocols. On each day, all rats were moved to the experimental room for a treatment session starting at 13:50 h. On the initial 3 days (adaptation phase), the rats were confined in the test cages without any food in the bowls for 60 min per day. On the next six days (conditioning phase), each rat was given access to 8–9 raisins (TON'S Brand California Raisins, Toyo Nuts, Kobe, Japan) weighing around 4 g in the bowl for 15 min. Half

of the rats (running rats) were immediately kept in the wheels for 45 min, while the others (non-running rats) were kept in the compartments of the cart for the same time period.

Measurement: The amounts of raisin ingested in the test cages (i.e., 15-min intakes) were measured by weighing the raisins before and after the eating period, using tweezers and an electric balance (HT-120, A&D Co., Tokyo, Japan) to the nearest 0.01 g. The amounts of chow and kaolin consumed in the home cages (i.e., 23-h intakes) were recorded every day in the vivarium by removing the containers immediately after the rats were moved to the experimental room. The containers were weighed with an electric balance (BJ-1500, Sartorius Japan, Tokyo) to the nearest 0.1 g, refilled, and replaced before the rats were returned to their home cages. Crushed chow and kaolin fragments in the trays under the home cages were collected, dried for a day, segregated, and weighed to obtain the correct amounts of consumption. Therefore, the effect of administration of the treatment on Day X on consumption was evaluated on Day X+2 (Fig. 1).

Each full turn of a wheel was counted automatically by a handcrafted system consisting of a small magnet on the outer rim of the wheel, a reed switch, and an electric pedometer fixed on the wire net. The body weights of the rats were measured prior to each session with an electric balance (KS-251, Dretec Co., Ltd., Koshigaya, Japan) to the nearest 1 g.

Data analysis: An analysis of variance (ANOVA) with a between-subject factor of group and a within-subject factor of day was applied to each data set of major interest to us. A one-way repeated measures ANOVA was also employed for the running data. Statistical decisions were based on an alpha error level of $P<0.05$.

Experiment 2

Animal, housing, and apparatus: The subjects were 8 experimentally naïve rats of the same sex and strain as those used in Experiment 1. They were purchased from the same breeder at the age of 8 weeks and maintained in the same manner as those in Experiment 1. Because pica behavior was not monitored in this experiment, there were no kaolin containers in the home cages, and trays were not set under the home cages. The apparatus and the experimental room were identical to those of Experiment 1, except that the number of test cages was 8 and that only 4 of the 8 wheels were employed.
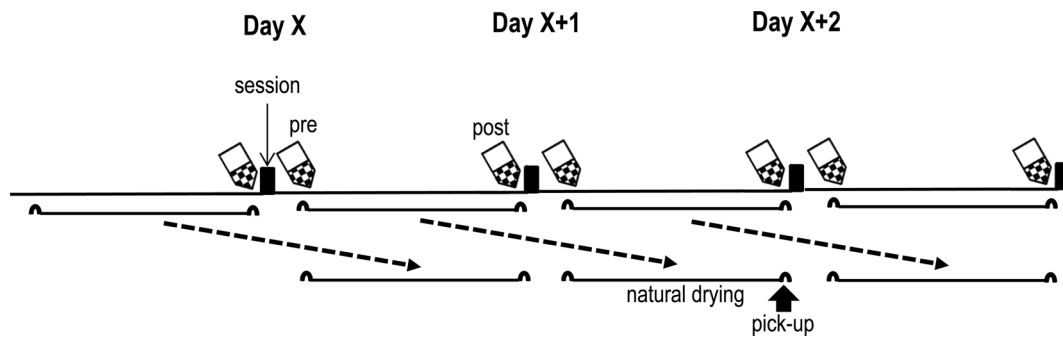
Fig. 1. Measurement of consumption of kaolin and chow pellets. The treatment session was conducted in the experimental room, while chow and kaolin pellets were available in the vivarium. The house-shaped pentagons designate the pellet containers attached to the home cages. The tray under each cage was replaced with a new one on the next day, and splinters in the original tray were dried for a day before weighing in order to obtain the correct amount of consumption in the following form: consumption = pre − (post + pick-up).

Procedure: On each day, all rats were transferred by a cart from their home cages to the experimental room for a treatment session starting at 10:15 h. On the first day, the rats were adapted to the test cages without any food in the bowls for 15 min. On the next 10 days (conditioning phase), each rat was given access to either 3 raisins (≈1.5 g) of the same brand as those in Experiment 1 or a piece of processed cheese (≈5 g, QBB Candy Cheese, Rokko Butter Co., Ltd., Kobe, Japan) in the bowl for 15 min. Access to one of these two kinds of snacks was always followed by 45-min wheel running, while the rats were kept in the compartments of the cart for the same period after access to the other kinds of snacks. The specific snack that preceded wheel running was counterbalanced across rats, and the conditioning consisted of 5 blocks of 2 days with the snack sequence of RCRCRCRCCR (R=raisins, C=cheese). Hence, on the first conditioning day, half of the rats (Group A) received access to raisins followed by running, while other half of the rats (Group B) were given access to raisins without running. On the second conditioning day, Group A received access to cheese without running, while Group B ran in the wheels after access to cheese. The same procedures were performed for the remaining days. Notably, the order of raisins and cheese was reversed for the final two days (i.e., CR instead of RC) to ensure that performance of the rats truly reflected the snack identities.

Measurement and analysis: The amounts of raisins and cheese ingested in the test cages (i.e., 15-min intakes) were measured by weighing them before and after the eating period as in Experiment 1. Also measured were the number of wheel turns in the 45-min period and the body weights of the rats. An ANOVA with within-subject factors of snack and day was applied to the conditioning data. The running data and body weights were assessed by one-way repeated measures ANOVAs. The alpha level was identical to that of Experiment 1, $P<0.05$.

Results

Experiment 1

Raisin intake in the test cage: Figure 2 illustrates that the non-running rats gradually increased consumption of raisins, implying habituation of neophobic reactions to unfamiliar food. The consistently low consumption of raisins in the running rats thus suggests that the increasing effect was counteracted by food aversion learning based on wheel running. A 2 (group) × 6 (day) ANOVA yielded significant main effects of group ($F(1, 14)=18.66$, $P<0.01$) and day ($F(5, 70)=4.80$, $P<0.01$), and most importantly their interaction ($F(5, 70)=6.08$, $P<0.01$). Subsequent simple main effect analyses of the interaction with separate error terms revealed significant group differences on the third conditioning day and onward (Fs $(1, 14)>6.41$, Ps<0.03). The simple day effect was significant for the non-running rats ($F(5, 70)=10.53$, $P<0.01$) but not for the running rats ($F<1$).

Kaolin intake in the home cage: As in previous studies conducted in my laboratory [52–55] and elsewhere [65], the rats consumed some kaolin when they first encountered it, reflecting exploratory sampling of novel objects [5]. However, consumption gradually decreased to a near-zero level, as shown in the left section in Fig. 3: a
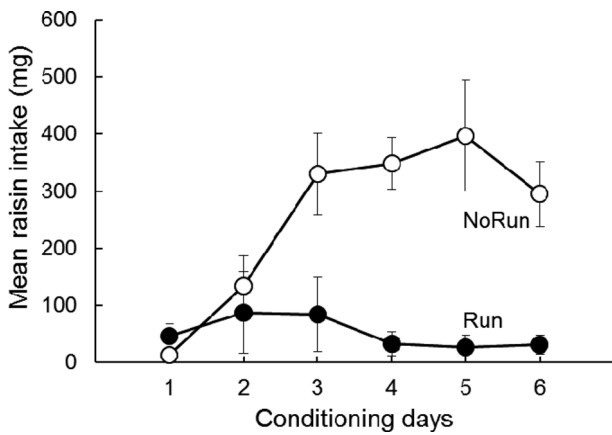
Fig. 2. Mean amounts of raisin intake in the conditioning phase for the rats having a 45-min running opportunity after the raisin intake and the rats with no running opportunity. The bars indicate SEs.
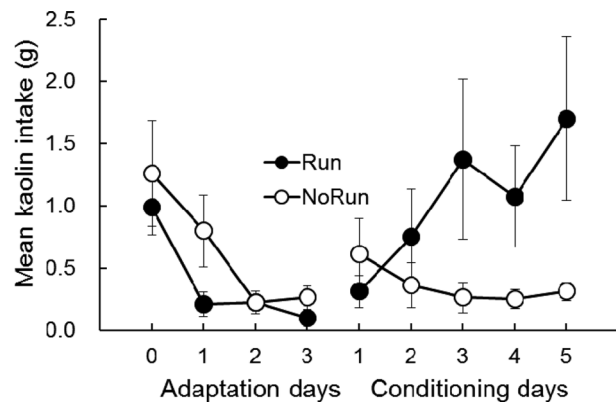

Fig. 3. Mean amounts of kaolin intake across the successive days. The first data plot is on Day 0, because the kaolin pellets were available from the day before the beginning of the adaptation treatment. The two groups received different treatments in the conditioning phase (see the caption of Fig. 2). The kaolin pellets were available for 23 h in the home cages. The bars indicate SEs.

2 (group) × 4 (day) ANOVA applied to the adaptation phase data yielded a significant main effect of day ($F(3, 42)=13.75$, $P<0.01$). The main effect of group ($F(1, 14)=1.45$, $P=0.25$) and the interaction ($F(3, 42)=1.12$, $P=0.35$) were nonsignificant. Wheel running gradually generated pica behavior as depicted in the right section of Fig. 3: a 2 (group) × 5 (day) ANOVA yielded a significant interaction of group × day ($F(4, 56)=4.73$, $P<0.01$). The main effects of group ($F(1, 14)=2.46$, $P=0.14$) and day ($F(4, 56)=2.01$, $P=0.11$) were nonsignificant. Subsequent simple main effect analyses of the interaction with separate error terms revealed marginally significant group differences on the fourth ($F(1, 14)=3.99$, $P=0.07$) and fifth ($F(1, 14)=4.41$, $P=0.05$) running days. The simple day effect was significant for the running rats ($F(4, 56)=6.27$, $P<0.01$) but not for the non-running rats ($F<1$). These results imply that wheel running induced mild nausea in the rats as demonstrated in previous reports [52, 54, 55].

Chow intake in the home cage: The two groups did not differ in the amount of chow intake throughout the experiment. A 2 (group) × 4 (day) ANOVA applied to the adaptation phase data yielded no significant main or interactive effects: group, $F<1$; day, $F(3, 42)=1.58$, $P=0.21$; group × day, $F(3, 42)=1.21$, $P=0.32$. The averages (and SEs) collapsed across the groups were 22.4 ± 0.4, 21.5 ± 0.4, 22.7 ± 0.4, and 21.7 ± 0.8 g, for the day before the experiment, the first, second, and third days of the adaptation phase. The consumptions of chow pellets dropped slightly on the fourth running day for un-

known reason. A 2 (group) × 5 (day) ANOVA applied to the running phase data yielded no significant main effect of group or its interaction with day ($Fs<1$), but the main effect of day was significant ($F(4, 56)=4.44$, $P<0.01$). The averages collapsed across the groups were 21.6 ± 0.5, 21.7 ± 0.4, 21.6 ± 0.5, 20.6 ± 0.4, and 21.7 ± 0.4 g, respectively, from the first to fifth running days. However, the most important point of the chow intake data is that the running rats consumed the chow pellets as readily as the non-running control rats, suggesting that effect of wheel running was not of the kind attributable to activity anorexia [12, 25].

Wheel turns: A one-way repeated measures ANOVA applied for the data of the running rats showed that the number of wheel turns was statistically unchanged throughout the conditioning phase ($F<1$). The averages were 130 ± 17, 143 ± 16, 127 ± 12, 133 ± 16, 142 ± 20, and 132 ± 14 turns per session from the first to the sixth conditioning days. The maintained amount of running across days suggests an intrinsic reward value of this behavior. Thus, taken together with the raisin and kaolin data, one may conclude that wheel running have bivalent effects in that they can act as both hedonic and aversive stimuli.

Body weights: The two groups showed gradual and equivalent increases in their weights throughout the experiment. A 2 (group) × 5 (day) ANOVA applied to the adaptation phase data yielded no significant main effect of group or its interaction with day ($Fs<1$), but the main
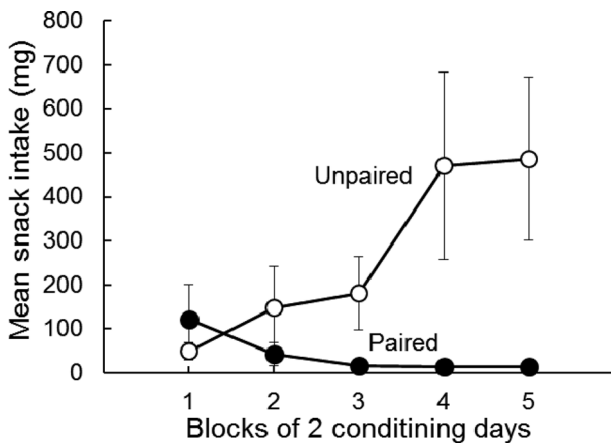
Fig. 4. Mean amounts of snack intake in the conditioning phase for the snack followed by a 45-min running opportunity and the snack unpaired with the running opportunity. The bars indicate SEs.

effect of day was significant ($F(3, 42)=85.96$, $P<0.01$). The averages collapsed across the groups were 316 ± 2, 323 ± 2, 327 ± 2, and 330 ± 2 g, respectively, for the day before the experiment and the first, second, and third days of the adaptation phase. A 2 (group) × 6 (day) ANOVA applied to the running phase data also yielded no significant main effect of group or its interaction with day ($Fs<1$), but the main effect of day was significant ($F(5, 70)=121.17$, $P<0.01$). The averages collapsed across the groups were 335 ± 2, 338 ± 2, 342 ± 3, 347 ± 3, 350 ± 3, and 354 ± 3 g, respectively, from the first to sixth running days.

Experiment 2

Snack intake in the test cage: Figure 4 illustrates that wheel running suppressed consumption of the paired snack (raisins or cheese, counterbalanced across rats) throughout the experiment. The unpaired snack, however, was gradually consumed by the rats over the treatment days. A 2 (snack: paired vs. unpaired) × 5 (2-day block) ANOVA yielded a marginally significant main effect of snack ($F(1, 7)=5.29$, $P=0.05$) and a reliably significant main effect of block ($F(4, 28)=3.34$, $P=0.02$). Most importantly, the snack × block interaction was quite significant ($F(4, 28)=4.70$, $P<0.01$). Subsequent simple main effect analyses of the interaction with separate error terms revealed a significant snack difference in the fifth block of days ($F(1, 7)=6.44$, $P=0.04$). Notably, the snack differences in the third ($F(1, 7)=4.05$, $P=0.08$) and fourth ($F(1, 7)=4.66$, $P=0.07$) blocks were statistically

marginal. The simple day effect was significant for the unpaired snack ($F(4, 28)=4.38$, $P<0.01$) but not for the paired snack ($F(4, 28)=1.96$, $P<0.13$).

Wheel turns: The averages numbers of wheel turns per session were 221 ± 9, 207 ± 15, 172 ± 10, 166 ± 16, and 195 ± 16 from the first to the fifth running days. A one-way repeated measures ANOVA applied for these data yielded a significant effect of day ($F(4, 28)=6.17$, $P<0.01$). The reasons for these unsystematic oscillations are unknown.

Body weights: The rats gradually gained weight throughout the experiment. A one-way repeated measures ANOVA yielded a significant effect of day ($F(10, 70)=241.10$, $P<0.01$). Body weight increased from 294 ± 3 g on the day before the experiment to 350 ± 3 g on the last day of the experiment.

Discussion

In the present study, unfamiliar food presented in the experimental room was avoided by the rats maintained with chow and water available *ad libitum* in their home cages. The rats then gradually increased consumption of the unfamiliar food over the course of the daily sessions. This consumption increase was, however, suppressed if voluntary running followed the access to that food. These results were demonstrated in both a between-groups design (Experiment 1) and a within-subject design (Experiment 2), and taken together, they imply that habituation of neophobic reactions to the unfamiliar food was counteracted by running-based food aversion learning. Aversive conditioning based on voluntary running of the non-deprived rats has already been reported by Lett *et al.* [41], but they used a water-deprivation procedure in the pretraining phase. The rats in the present study, on the other hand, were completely non-deprived.

According to Dawkins [21], excluding disease or injury, most animal suffering can be classified into either presence of aversive stimuli or deprivation of suitable stimuli. The conditioning procedure documented in this article (i.e., CFA based on voluntary running in non-deprived rats) was free from the aversive stimulation and deprivation, and thus, it is a more humane method than the conventional preparation, in which water-deprived animals are made sick by irradiation or emetic drugs [27]. Notably, according to the framework of the Scientists Center for Animal Welfare [67], one may classify the experimental procedures employed by the present

study into Category B (i.e., experiments on vertebrate animal species that are expected to produce little or no discomfort), while the conventional technique would be classified into Category D (i.e., experiments that involve significant but unavoidable stress or pain to vertebrate animal species).

Voluntary wheel running is seemingly pleasant, because it derives from the animals' volition by definition [28], evokes the voice of pleasure [34], and works as a positive reinforcer [8, 19, 37, 38]. It is nonetheless operationally aversive in the context of taste learning, as reported here and elsewhere (see Boakes and Nakajima [13] for a review). Because wheel running generates kaolin clay ingestion as demonstrated in Experiment 1 of the present study as well as in the previous reports [52, 54, 55], it should be concluded that the running induces a mild gastrointestinal discomfort such as nausea. Accordingly, the conditioning procedure documented in this article is not completely devoid of aversive stimulation. However, the discomfort experienced by rats is definitely less for running than for the conventional conditioning agents such as irradiation and emetic drugs.

As noted in the introduction of this article, the purpose of the present research was to provide a convenient tool for studying aversive conditioning with minimized discomfort in laboratory rats. The physiological process of this aversive learning remains to be explored. Several hypotheses have been proposed for the physiological cause of running-based CTA, which include activation of the mesolimbic dopamine system [40], motion sickness by rocking movement of wheels [30], energy expenditure by physical exercise [57], and general stress [58]. Unfortunately, all of these accounts have their own inconvenient truths [43, 50, 52, 56].

Despite the fact that a large number of studies have been conducted regarding wheel running by rodents including rats (see Novak, Burghardt, and Levine [59] for a review), its biological significance has not yet been fully clarified. In fact, it shares many features with pathological behavior such as stereotypy and addiction [62, 68]. However, we must remember that the period of running was short in the present research: 5 or 6 days of 45-min running. Accordingly, any health problems which arise on wheel running would be minimal, at least regarding the technique proposed here.

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