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Food avoidance learning based on voluntary wheel running in laboratory mice *(Mus musculus)*

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ABSTRACT

Mice show a reluctance to eat unfamiliar food, when they first encounter it. This neophobic reaction is conventionally habituated by repeated trials: the mice gradually increase their consumption of the novel food. The new finding reported here is that the consumption remains low in mice that voluntarily run in activity wheels after the novel food access. This effect implies that running yields Pavlovian conditioned flavor aversion, which suppresses, otherwise increasing, consumption of the novel food. In the present research, the effect was demonstrated with a between-group design by pitting experimental mice receiving cheese-running paired treatment against cheese/running unpaired control mice (Experiment 1). The running-based food avoidance in mice was also shown in a differential conditioning paradigm, where one of two novel snacks (chocolate and marshmallow) was paired with running while the other was not, in non-deprived animals (Experiment 2 A) and food-deprived animals (Experiment 2B). These results concord with those previously reported in rats, indicating the generality of the phenomenon.

1. Introduction

Voluntary wheel running has hedonically bivalent properties for laboratory rats: it elicits appetitive 50-kHz ultrasonic calls (Heyse et al., 2015) and positively reinforces instrumental responses, such as lever pressing (e.g., Belke, 1997; Collier and Hirsh, 1971; Iversen, 1993; Kagan and Berkun, 1954) and maze performance (Livesey et al., 1972), while it also works as an aversive agent to establish Pavlovian conditioned avoidance[1] of the preceding flavor (e.g., Lett and Grant, 1996; Hayashi et al., 2002; Heth et al., 2001; Nakajima et al., 2000).

Although conditioned flavor avoidance based on running is well established in rats (see Boakes and Nakajima, 2009, for a review), this learning phenomenon has not been well studied in other species. A few exceptions include a brief report with human participants (Havermans et al., 2009) and a conference article reporting a weak effect in golden hamsters (Masaki, 2009). Although we have recently reported an experiment suggesting running-based flavor avoidance in mice (Nakajima and Oi, 2018), its experimental design allows an alternative interpretation due to nonassociative factors. In order to exclude such nonassociative factors, flavor/running uncorrelated control is necessary. The present series of experiments aims to demonstrate running-based conditioned flavor avoidance in mice to broaden the generality of this learning. Mice were chosen as subjects in the present research, not only because they are the most commonly used vertebrate species in laboratory studies, but they are good runners (Novak et al., 2012; Richter et al., 2014; Sherwin, 1998). Furthermore, they press a lever for an opportunity to run in wheels (Belk and Garland, 2007), suggesting wheel running is pleasurable for them as well as for rats.

Running-based taste avoidance in rats has been conventionally conducted with food or water deprived animals to facilitate consuming flavored food or water, which is a to-be conditioned cue. However, I have recently reported a technique to establish running-based flavor avoidance in non-deprived rats, where retardation of habituation of neophobic reaction to novel food is indexed as acquisition of Pavlovian conditioned flavor aversion (Nakajima, 2019). Because of fast metabolic and water turnover in mice comparted with rats (Rowland, 2007), this new technique would be employed in the present investigation (except for Experiment 2B) for considering animal welfare.

2. Experiment 1: simple conditioning

Based upon a pilot study, a piece of processed cheese was chosen as a target food in Experiment 1, because it is consumed to some extent even when non-deprived mice first encounter it. Experiment 1 compares mice given paired cheese–running trials with mice given unpaired presentations of the cheese and running. Running-based food avoidance

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[1] In this article, I use "avoidance" to describe a reluctance to consume a target flavor (or food) and "aversion" to refer to the animals' unpleasant inner state evoked by the target.

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learning is demonstrated, if the former mice consume the cheese less than do the latter mice in later sessions.

2.1. Method

2.1.1. Subjects and apparatus

The animals were 16 experimentally naïve male Slc:ICR mice purchased from a breeder (Japan SLC, Hamamatsu, Japan), when they were 8 weeks old. The vivarium was maintained on a light–dark cycle of 12–12 h (lights on at 0900 h) with controlled temperature (23 ∘C) and humidity (55%). The mice were housed singly, because it was afraid that interactions with conspecific animals in the home cages would attenuate food aversions (cf. Hishimura, 2015). Specifically, each of two clear plastic home cages (KN-606, Natsume Seisakusho, Tokyo) was separated crosswise into four compartments by metal steel plates, and the mice were individually housed in the compartments (11 cm wide, 16 cm long, 13.5 cm high) with wood chip bedding of 2 cm. The bedding was changed twice a week. The chow pellets (MF diet; Oriental Yeast, Tokyo) were placed in a metal container positioned at the cross section of the cage with its apertures 1.5–6.5 cm above the bedding. Fresh tap water was available in each compartment from a bottle fitted with a metal sipper tube positioned 6.5 cm above the bedding. The animals were adapted to this housing condition for a week, before the experimental procedure began.

A conventionally illuminated experimental room had 8 plastic test cages (9.5 cm wide, 14.3 cm long, 9.5 cm high) with wire lids and 8 activity wheels on the table. The travelling time from the vivarium to the experimental room was around 2 min. The activity wheels were made of plastic and set in pet den cages (two wheels per den cage). Each wheel had an internal width of 4.5 cm and a diameter of 13 cm. The running surface (i.e., the wheel floor) was solid. One side of the wheel was solid, while the other had three openings for animal entrance therein: these openings were covered with sheet metal or plastic for confinement. The wheels could be turned in both directions. A full turn of each wheel was counted automatically by a system consisting of a small magnet on the outer rim of the wheel and a commercial wheel counter (HM-101, Ai Electronic Industry, Ohtawara, Japan) on the wire bars of the den.

2.1.2. Procedure

Laboratory assistants, who were blind to the hypothesis of the study, administered the experimental protocols. On each day at 1030 h, all home cages were moved together to the experimental room, where the mice were individually weighed by an electric balance (KS-251, Dretec, Koshigaya, Japan) to the nearest 1 g.

Prior to the conditioning treatment, all mice were confined in the test cages without any food for 20 min (Day 0). Because the number of the test cages was eight, this adaptation training was conducted in two successive squads. After training of the second squad, all mice were returned to the vivarium.

The conditioning phase was constituted by five cycles of two-day treatments (Table 1). All mice were tested once a day in a single squad during this phase. On a day of each cycle, mice of Group Paired (n = 8) were given a 20-min access to a cubic piece of processed cheese (Belcube, Bel Japon, Tokyo) of 5.2 g on the floor of each test cage, immediately followed by 60-min confinement in the wheels; on the other day, these mice spent 80 min with no event in the home cages on the table in the experimental room. For the remaining mice (Group Unpaired, n = 8), cheese access and wheel confinement were administered on separate days: these mice were given a 20-min cheese access in the test cages followed by 60-min holding in the home cages on a day, and 20-min holding in the home cages followed by 60-min wheel confinement on the other day. The daily order of these two-day treatments was counterbalanced within each group. Namely, the running day was the first of the 2 consecutive days for half of each group of mice (#1–4), while it was the second day for the other mice (#5–8).

2.1.3. Measurement and statistical analysis

The amounts of cheese ingested (i.e., 20-min intakes) were measured by weighing the cheese before and after the eating period using tweezers and an electric balance (HT-120, A & D Company, Tokyo) to the nearest 0.01 g (i.e., 10 mg). In this study, all statistical decisions concerning analyses of variance (ANOVAs) were based on an alpha level of 0.05.Post hoc simple main effect analyses of significant interactions were executed with separate error terms and the uncorrected alpha level.

2.1.4. Ethical considerations

All treatments of this and following experiments were approved by the Animal Care and Use Committee of Kwansei Gakuin University, based on a Japanese law and the guideline published by the Science Council of Japan in 2006.

2.2. Results and discussion

2.2.1. Cheese intake

Fig. 1 shows that both groups of mice were at first somewhat reluctant to eat cheese, but the control mice (Group Unpaired) gradually increased the consumption over days after an inexplicable drop on the second day. The slightly decreasing cheese consumption in the experimental mice (Group Paired) suggests that taste aversion was established by wheel running. A 2 (group) × 5 (day) mixed-design ANOVA yielded significant main effects of group, F (1, 14) = 26.75, $P < 0.001$, $\eta_p^2 = 0.66$, and day, F (4, 56) = 3.45, $P = 0.014$, $\eta_p^2 = 0.20$, and most importantly their interaction, F (4, 56) = 4.76, $P = 0.002$, $\eta_p^2 = 0.25$. Post hoc simple main effect analyses of the interaction revealed a marginal group difference on the second cheese day, F (1, 14) = 3.80, $P = 0.072$, $\eta_p^2 = 0.21$. The group significantly differed on the third day and onward, Fs (1, 14) > 12.25, Ps < 0.004, η_p^2s > 0.46. The simple day effect was significant not only for Group Unpaired, F (4, 56) = 5.20, $P = 0.001$, $\eta_p^2 = 0.27$, but also for Group Paired, F (4, 56) = 3.01, $P = 0.026$, $\eta_p^2 = 0.18$.

2.2.2. Wheel turns

The number of wheel turns gradually increased over the conditioning phase: a 2 (group) × 5 (day) mixed-design ANOVA yielded a significant main effect of day, F (4, 56) = 6.38, $P < 0.001$, $\eta_p^2 = 0.31$. The main effect of group, F (1, 14) = 2.91, $P = 0.110$, and the interaction, $F < 1$, were nonsignificant. The averages collapsed across the groups (± standard errors) were 1581 ± 140, 1777 ± 142,

Table 1
Treatments in the experimental room (Experiment 1).

Mice	Days 1, 3, 5, 7, and 9	Days 2, 4, 6, 8, and 10
Paired #1-4	cheese (20 min) → running (60 min)	HC stay (80 min)
Paired #5-8	HC stay (80 min)	cheese (20 min) → running (60 min)
Unpaired #1-4	cheese (20 min) → HC stay (60 min)	HC stay (20 min) → running (60 min)
Unpaired #5-8	HC stay (20 min) → running (60 min)	cheese (20 min) → HC stay (60 min)

Note. HC = home cage.

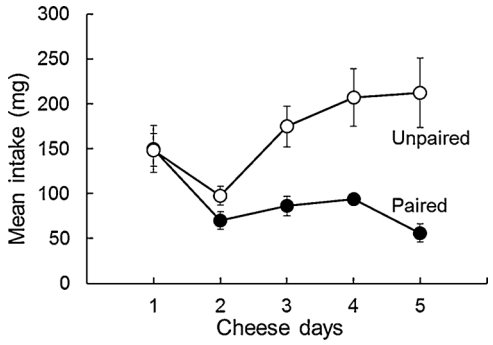

Fig. 1. Mean amount of cheese intake on the cheese days of Experiment 1, separately shown for the mice having a cheese-running paired treatment and the mice receiving a cheese/running unpaired treatment. The bars indicate standard errors.

1791 ± 151, 2167 ± 179, and 2044 ± 216 turns per session, from the first to the fifth running days.

3. Experiments 2A and 2B: differential conditioning

Experiment 1 employed a simple conditioning procedure for food avoidance learning. In a new set of two experiments, we trained mice in a differential conditioning paradigm with two novel snacks (chocolate and marshmallow) as flavor cues: one of them was paired with running, while the other was not. Thus, assessment of conditioning was between these flavors (i.e., a within-subject design). The first of the two experiments (Experiment 2 A) was conducted with non-deprived mice as was Experiment 1, but the second (Experiment 2B) was administered with food-deprived mice in order to show that the effect is not specific to non-deprived animals.

3.1. Method

3.1.1. Subjects and apparatus

The current sets of experiments used experimentally naïve male Slc:ICR mice purchased from the same breeder as in Experiment 1, when they were 8 weeks old. The numbers of subjects were 8 and 16, respectively, for Experiments 2 A and 2B. They were maintained as in Experiment 1 for two weeks (Experiment 2 A) or one week (Experiment 2B) before the onset of the experimental treatment: the between-experiment difference in the maintaining period was simply due to a personnel matter of hired experimenters, who were unaware of the research aim as in Experiment 1. All subjects of Experiment 2 A and a half of the subjects of Experiment 2B were individually housed in small compartments of the home cages as in Experiment 1. The remaining animals of Experiment 2B were housed singly in clear plastic home cages (KN-600, Natsume Seisakusho, Tokyo) measuring 22 cm wide, 32 cm long, 13.5 cm high with wood chip bedding of 2 cm and the wire ceiling having chow pellets. Because the housing condition had no significant effects on the data of our interest, this factor was collapsed for the analyses reported below. Although the chow pellets were freely available in the vivarium for the subjects of Experiment 2 A like in Experiment 1, animals of Experiment 2B were given a post-session feeding with chow pellets in the home cages to maintain at 85% of their body weights throughout the experimental treatment noted below.

The test cages and the wheels in the experimental room were identical to those of Experiment 1, except that each test cage has a white plastic food cup (2.8 cm in diameter) attached to the cage floor with Velcro pads.

3.1.2. Procedure

3.1.2.1. Experiment 2A. On each day, all home cages were transferred from the vivarium to the experimental room for a treatment session starting at 1030 h. Prior to the conditioning treatment, mice were adapted to the test cages without any food in the food cups for 20 min (Day 0). On the next 10 days (conditioning phase), each mice was given access to either chocolate pellets (Choco Baby Jumbo, Meiji, Tokyo) or marshmallow pellets (Mini Mini Marshmallows Vanilla, Wisuc, Tokyo) in the food cup of the test cage. These two pellets were the same size (0.5 cm diameter × 0.7 cm long), but they differed in weight per pellet (≈300 and ≈100 mg, respectively, for the chocolate and marshmallow pellets). The access to one of these two kinds of snacks was always followed by 60-min wheel confinement, while the mice were kept in the home cages for the same period after access to the other kind of snacks. The specific snack that preceded wheel running was counterbalanced across mice, and the conditioning consisted of 5 blocks of 2 days with the snack sequence of CMCMCMCMMC (C = chocolate, M = marshmallow). Hence, on the first conditioning day, half of the mice (#1–4) received access to chocolate followed by running, while the others (#5–8) were given access to chocolate without running. On the second conditioning day, the former mice received access to marshmallow without running, while the latter mice ran in the wheels after access to marshmallow. The same procedures were performed for the remaining days (Table 2). Notably, the order of chocolate and marshmallow was reversed for the final two days (i.e., MC instead of CM) to ensure that performance of the mice truly reflected the snack identities.

3.1.2.2. Experiment 2B. Because of the large number of mice employed in Experiment 2B, they were tested in two squads starting at 1250 h for the first squad and 1415 h for the second squad. The treatment procedures were identical to those of Experiment 2 A except for two details. Firstly, the snack access period was now 15 min, and secondly, the wheel confinement was now 45 min. These modifications were performed for convenience to administer two squads of mice within the limited working hours of the experimenters. One of the mice was excluded from the experiment because of ill health, leaving 15 animals for data analysis.

3.2. Results and discussion

3.2.1. Experiment 2A

3.2.1.1. Snack intake. Fig. 2 depicts the consumptions of novel food by all mice over 5 opportunities to eat each snack, separately shown for its functional role (i.e., paired or unpaired) regardless of its physical identity (chocolate or marshmallow): the between-group factor of physical identity was not included in this figure and the following analyses, because this factor had no significant main or interactive effect on the results in a preliminary 2 (physical identity) × 2 (snack: CS + vs. CS-) × 5 (block) mixed-design ANOVA. Although the mice were initially reluctant to consume the snacks employed in this experiment, the consumption of the unpaired snack increased on the

Table 2
Treatments in the experimental room (Experiment 2 A).

Mice	Days 1, 3, 5, 7, and 10	Days 2, 4, 6, 8, and 9
#1-4	chocolate (20 min) → running (60 min)	marshmallow (20 min) → HC stay (60 min)
#5-8	chocolate (20 min) → HC stay (60 min)	marshmallow (20 min) → running (60 min)

Note. HC = home cage.

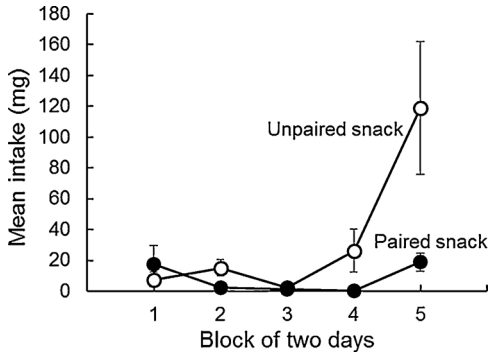

Fig. 2. Mean amount of snack intake on the conditioning days of Experiment 2 A with non-deprived mice, separately shown for the snack paired with running in a wheel and the snack unpaired with the running. Each block consists of a day for the paired snack and a day for the unpaired snack. The bars indicate standard errors.

last two blocks. A 2 (snack) × 5 (block) repeated ANOVA yielded significant main effects of snack, $F(1, 7) = 7.60$, $P = 0.028$, $\eta_p^2 = 0.52$, and block, $F(4, 28) = 6.67$, $P < 0.001$, $\eta_p^2 = 0.49$, and most importantly their interaction, $F(4, 28) = 4.73$, $P = 0.005$, $\eta_p^2 = 0.40$. Post hoc simple main effect analyses of the interaction revealed a marginal snack difference on the second block, $F(1, 7) = 4.07$, $P = 0.083$, $\eta_p^2 = 0.37$. The simple main effect of snack was also marginal on the fourth block, $F(1, 7) = 3.58$, $P = 0.100$, $\eta_p^2 = .34$, and it was reliably significant on the fifth block, $F(1, 7) = 5.73$, $P = 0.048$, $\eta_p^2 = 0.45$. The simple block effect was significant for the unpaired snack, $F(4, 28) = 6.07$, $P = 0.001$, $\eta_p^2 = 0.46$, suggesting habituation of neophobic reaction to the novel food. A weak increasing trend for the paired snack failed to reach the significance, $F(4, 28) = 2.36$, $P = 0.078$, $\eta_p^2 = 0.25$. The aforementioned small intake of the paired snack, compared with the unpaired snack, on the fifth block implies that running-based flavor aversion prevented the habituation of food neophobia.

3.2.1.2. Wheel turns. The number of wheel turns was gradually increased over the conditioning phase: a one-way repeated ANOVA yielded a significant main effect of running day, $F(4, 28) = 12.72$, $P < 0.001$, $\eta_p^2 = 0.65$. The averages were 1029 ± 284, 1450 ± 339, 2074 ± 370, 2254 ± 328, and 1869 ± 473 turns per session, from the first to the fifth running days.

3.2.2. Experiment 2B

3.2.2.1. Snack intake. As we expected, snack intake was considerable for food-deprived mice. Fig. 3 shows an increasing trend for the unpaired snack and constant low consumption of the paired snack, supporting the claim that running-based food aversion interfered habituation of neophobic reaction to the novel food. Notably, the between-group factor of physical identity of snack was not included in this figure and the following analyses, because this factor had no significant main or interactive effect in a preliminary 2 (physical identity) ×2 (housing condition, see Section 3.1.1) × 2 (snack: CS + vs. CS-) × 5 (block) mixed-design ANOVA.

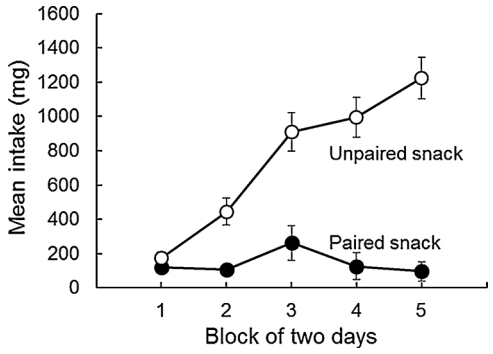

Fig. 3. Mean amount of snack intake on the conditioning days of Experiment 2B with food-deprived mice, separately shown for the snack paired with running in a wheel and the snack unpaired with the running. Each block consists of a day for the paired snack and a day for the unpaired snack. The bars indicate standard errors.

A 2 (snack) × 5 (block) repeated ANOVA applied to the data summarized in Fig. 3 yielded significant main effects of snack, $F(1, 14) = 56.93$, $P < 0.001$, $\eta_p^2 = 0.80$, and block, $F(4, 56) = 27.65$, $P < 0.001$, $\eta_p^2 = 0.66$, and most crucially their interaction, $F(4, 56) = 22.74$, $P < 0.001$, $\eta_p^2 = 0.62$. Post hoc simple main effect analyses of the interaction revealed that the unpaired snack was greater in consumption than the paired snack on the second block and onward, Fs $(1, 14) > 22.54$, Ps < 0.001, η_p^2s $> .61$. Habituation of neophobic reaction to the unpaired snack was statistically supported by the simple block effect, $F(4, 56) = 37.48$, $P < 0.001$, $\eta_p^2 = 0.73$. The intake of paired snack was unchanged across blocks, $F(4, 56) = 1.77$, $P = 0.148$.

3.2.2.2. Wheel turn. The number of wheel turns was gradually increased over the conditioning phase: a one-way repeated ANOVA yielded a significant main effect of running day, $F(4, 56) = 11.14$, $P < 0.001$, $\eta_p^2 = 0.44$. The averages were 929 ± 115, 1479 ± 169, 1429 ± 102, 1561 ± 111, and 2011 ± 113 turns per session, from the first to the fifth running days.

4. General discussion

Animals are reluctant to eat an unfamiliar edible (target food) when they are not very hungry, but this food neophobia is conventionally habituated by repeated trials (Schachtman et al., 2018)[2]. The habituation, however, is hampered if the access to the target food is followed by an opportunity to run in activity wheel. This maintained suppression of target food intake has been reported by Nakajima (2019) with laboratory rats as subjects, and the present series of experiment successfully replicated the phenomenon with laboratory mice. In addition to the maintained suppression of target food intake, a more direct piece of evidence for running-based flavor aversion comes from that the novel food consumptions decreased modestly, but statistically reliably, over training trials in the paired mice of Experiment 1.

Presumably, the underlying mechanism of these results is Pavlovian conditioned flavor aversion based on the association between the flavor of the target food and physiological effect of wheel running. Nonassociative factors, such as exercise-induced suppression of eating (e.g., Katch et al., 1979; Mayer et al., 1954; Nance et al., 1977) and activity-based anorexia (e.g., Routtenberg and Kuznesof, 1967; see Boakes, 2007; Pierce, 2001, for reviews), were ruled out by the use of an unpaired control group (Experiment 1) or a differential conditioning procedures (Experiments 2 A and 2B). In other words, the intake suppression was specific to the food paired with wheel running.

In the present research, wheel running was self-reinforcing because the number of wheel turns increased over the running days. In addition, as noted in the introduction (Section 1), wheel running positively reinforces instrumental responses in mice (Belk and Garland, 2007). These properties of wheel running imply that it is pleasurable for mice as well as for rats. Taken together with the aforementioned running-based flavor aversion, we conclude that wheel running has hedonically bivalent properties in mice as well as in rats.

Why does wheel running cause flavor aversion even though it is

[2] One may regard the increase of food consumption observed here as flavor preference learning based on caloric outcome (Fedorchak, 1997) rather than habituation of neophobia. The latter position is taken in this article, because it is a simpler account of behavior.

pleasurable? Several accounts have been proposed for running-based flavor aversion learning in rats. According to Lett and Grant (1996), activation of mesolimbic rewarding system plays a major role in this learning. A support of this hypothesis comes from a subsequent report that wheel running simultaneously establishes taste aversion and place preference in rats (Lett et al., 2001), suggesting that running activates the rewarding system when it is acting as aversive agent for flavor conditioning. However, the temporal sequence of their conditioning procedure was flavor → running → place. In other words, Pavlovian forward flavor conditioning and backward place conditioning were executed in their study. Because a forward sequence of place → running causes place aversion in rats (Masaki and Nakajima, 2008), the mesolimbic system hypothesis for running-based flavor aversion should be reexamined.

Other hypotheses on running-based flavor aversion include energy expenditure by physical exercise (Nakajima and Masaki, 2004), motion sickness by rocking movement of wheels (Grant et al., 2012), and general physiological stress (Nakajima et al., 2006). These hypotheses have also been questioned, because energy supply does not alleviate running-based flavor aversion (Nakajima, 2011), running in wheels without rocking movement establish flavor aversion (Nakajima, 2016), and stressful conspecific fighting yields no flavor aversion (Nakajima et al., 2012).

John Garcia proposed in a personal communication to Lett et al. (1999) that gastrointestinal discomfort (i.e., nausea) induced by running activity is a physiological cause for running-based flavor aversion in rats. This hypothesis has been buttressed by several lines of research (Dwyer et al., 2008; Eccles et al., 2005; Nakajima et al., 2006). This hypothesis is also supported by a new finding that kaolin clay intake, a marker of nausea in rats (Andrews and Horn, 2006), is generated by wheel running (Nakajima, 2016, 2018; Nakajima and Katayama, 2014). Since the kaolin clay ingestion is

also applicable as a measure of nausea in mice (Yamamoto et al., 2002), future study may use this measure for running activity of mice, although a highly special technique is necessary to determine the exact kaolin consumption for this species due to the minuscule amount of consumption.

It is nevertheless notable that mice are superior to rats in terms of cost, space to cage, and the availability of mutant or genetically engineered strains. Hence, the demonstration of running-based food avoidance learning in mice will be conductive to further research into the nature and mechanism of this learning. In the present research, ICR mice were chosen as subjects because of convenience: they are gentle, have good appetite, and inexpensive. Future research should replicate the finding with other mouse strains to confirm its generality. It is notable that rats' running-based flavor aversion has been demonstrated in many strains (Nakajima, 2014).

In closing, it should be mentioned that the conditioning technique I presented here is based on voluntary wheel running in non-deprived animals, and thus seemingly more humane than conventional flavor aversion established by emetic drugs (Nakajima, 2019). Hence, from an animal welfare point of view, this technique is recommended for future use as a substitute for the conventional emetic-based flavor aversion procedure in experiments with rats and mice.

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