



Further demonstration of running-based food avoidance learning in laboratory mice (*Mus musculus*)

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ABSTRACT

Voluntary wheel running has hedonically bivalent properties in laboratory rats and mice. While it works as a reward for instrumental performance such as bar pressing, it also functions as an aversive stimulus to establish Pavlovian conditioned avoidance of the paired stimulus. The present study focused on the latter case. Running in closed wheels hampered habituation of a reluctance to eat a target snack in rats (Experiment 1A) and mice (Experiment 1B) trained by pairing access to a target snack with confinement to a wheel attached to the cage. Experiment 2 successfully confirmed and extended this finding with mice running in both open and closed wheels. A differential conditioning procedure employed in Experiment 3 ensured that this phenomenon is specific to the snack paired with running, implying that it reflects Pavlovian conditioned flavor avoidance (CFA). Free exploration in cages without wheels, however, did not result in a CFA.

1. Introduction

Many species of rodents, including laboratory rats and mice, voluntarily run in activity wheels (Novak et al., 2012; Richter et al., 2014; Sherwin, 1998). Voluntary wheel running positively reinforces operant behavior, such as bar pressing, of rats (e.g., Belke, 1997; Collier and Hirsh, 1971; Iversen, 1993; Kagan and Berkun, 1954; Pierce et al., 2018) and mice (Belke and Garland, 2007). It also serves as a reward for maze performance (Livesey et al., 1972) and elicits "joyful" 50-kHz ultrasonic chirps (Heyse et al., 2015) at least in rats. These findings are consistent with the general notion that wheel running is "pleasurable" for rodents.

In the meantime, voluntary wheel running seems to induce an aversive inner state, because, in rats, it results in Pavlovian conditioned avoidance of the paired chamber (Masaki and Nakajima, 2008). In addition, a large number of studies have shown that voluntary wheel running works as an effective agent (i.e., an unconditioned stimulus) to generate Pavlovian conditioned avoidance of the paired flavor (e.g., Forristall et al., 2007; Lett and Grant, 1996; Hayashi et al., 2002; Heth et al., 2001; Nakajima, 2014; Nakajima et al., 2000; Salvy et al., 2004). Although the running-based conditioned flavor avoidance (CFA) have been mainly studied with rats (see Boakes and Nakajima, 2009, for a review), it was also reported to occur in mice (Nakajima and Oi, 2018) and golden hamsters (Masaki, 2009). Recently, a series of experiments have demonstrated running-based CFA in mice with snack food (cheese, chocolates, or marshmallows) as the target flavor cue (Nakajima,

2019b).

Because of the puzzling nature of the hedonically bivalent properties of voluntary wheel running, the present study attempts to replicate running-based CFA in mice. Mice are more suitable than rats in the availability of mutant or genetically engineered strains. Hence, collection of further information and quantification of running-based CFA in mice will pave a way for understanding of the mechanism of this phenomenon.

The present study consists of three experiments. The first experiment was designed to demonstrate running-based CFA in rats (Experiment 1A) and mice (Experiment 1B) with the identical procedure to determine the inter-species generality of this learning; the results were affirmative. Experiments 2 and 3 examined whether CFA is also generated by running in an open wheel. In a majority of research on running-based CFA in rats, animals are confined in the closed wheels. Some studies employed an open wheel with an adjacent cage to demonstrate running-based CFA in rats (e.g., Dobek et al., 2012; Salvy et al., 2004; Satvat and Eikelboom, 2006). Because the demonstration of CFA with an open wheel assures the voluntary nature of wheel running, an open wheel procedure was used in Experiments 2 and 3 with mice.

2. Experiments 1A and 1B: simple conditioning in non-deprived rats and mice

Experiments 1A and 1B were, respectively, executed with rats and

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mice. They not only shared an identical experimental design, but the specific parameters employed were also the same. A piece of processed cheese was chosen as a target snack, because a pilot study revealed that it is consumed to some extent even when rats and mice are not deprived of food. Each experiment compared animals given paired cheese–running trials with animals given access to cheese and unpaired running opportunities. If the former animals consume the cheese less than do the latter animals in later sessions of the conditioning phase, it can be claimed that running generates CFA in mice.

2.1. Method

2.1.1. Subjects and housing

The subjects were 16 male Slc:Wistar/ST rats and 16 male Slc:ICR mice purchased from a breeder (Japan SLC, Hamamatsu, Japan), when they were 8 weeks old. They were experimentally naïve and randomly assigned to one of two treatment groups of equal number (see Section 2.1.3 for the individual treatments) when they arrived at the vivarium, which was maintained on a 12:12 h light-dark cycle (lights on at 0900 h) with controlled temperature (23 °C) and humidity (55%). The animals were housed singly, because it was afraid that social interactions would affect CFA (e.g., Galef, 1989; Hishimura, 2015, 2018).

2.1.1.1. Experiment 1A (Rats). The rats were individually housed in suspended home cages (20 cm wide, 25 cm long, 18.7 cm high) set in two rows of the rack. The floor, ceiling, and front and back walls of each cage were made of wire mesh, and the side walls were made of metal plates. Tap water was freely available from a built-in metal nozzle protruding through a hole in the center of the back wall of each cage. The chow pellets (MF diet, Oriental Yeast, Tokyo, Japan) were freely available in a metal container hung from the cage ceiling with its end apertures 3.5 cm above the cage floor.

2.1.1.2. Experiment 1B (Mice). The mice were housed in four clear plastic home cages (KN-606, Natsume Seisakusho, Tokyo, Japan) with paper bedding, which was changed twice a week. Each cage was divided into four compartments (11 cm wide, 16 cm long, 13.5 cm high) by metal plates, and the mice were individually kept in the compartments. The chow pellets (MF diet, Oriental Yeast, Tokyo, Japan) were freely available from a metal container positioned at the cross section of the cage with its end aperture 1.5 cm above the bedding. Fresh tap water was freely available in each compartment from a plastic bottle fitted with a metal sipper tube positioned 6.5 cm above the bedding.

2.1.2. Apparatus

2.1.2.1. Experiment 1A (Rats). The rats were transferred from their home cages by a plastic case cart having individual compartments (10.5 cm wide, 13 cm long, 14.2 cm high) to a conventionally illuminated experimental room, which had 16 test chambers (copies of the home cages) on a table and 8 activity wheels on a wall. Each chamber had a metal cup (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. A full turn of each 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 compartments of the cart, the test chambers, and the wheels were cleaned with wet paper tissues after each session.

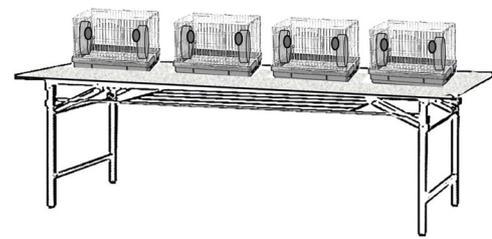


Fig. 1. The experimental setup for mouse running. The front wall of each cage has two wheel counters (small dark ovals). The right and left walls of each cage have wheels (one per wall). Mice were individually confined to the wheels by covering the wheel openings with an opaque plate (not shown for clarity).

2.1.2.2. Experiment 1B (Mice). The home cages of the mice were transferred on a metal trolley cart from the vivarium to another conventionally illuminated experimental room, which had 8 plastic test chambers with wire lids (9.5 cm wide, 14.3 cm long, 9.5 cm high) on a table. Each chamber had a plastic cup (2.8 cm in diameter, 1.5 cm deep) secured to the floor with velcro pad. Four commercial pet cages were located on another table (Fig. 1). Each cage had 2 opaque plastic activity wheels attached to its inside walls (one on the left and the other on the right), and thus there were 8 wheels in total. 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 also solid, while the other had three openings for animal entrance therein: these openings were covered with a metal/plastic plate for confinement, and thus the mice were not allowed to explore the pet cages. Furthermore, they had neither physical nor visual contact with each other. The wheels could be turned in both directions. A full turn of each wheel was counted automatically by a handcrafted 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 cage (Fig. 2). The test chambers and the wheels were cleaned with wet paper tissues after each session.

2.1.3. Procedure

2.1.3.1. Pretreatment for each experiment. A piece of processed cheese (\approx 5 g, QBB Candy Cheese, Rokko Butter, Kobe, Japan) was given to each animal in the home cage on the day of arrival (Day 0), and the leftover was collected on Day 1. Another piece of the same brand cheese

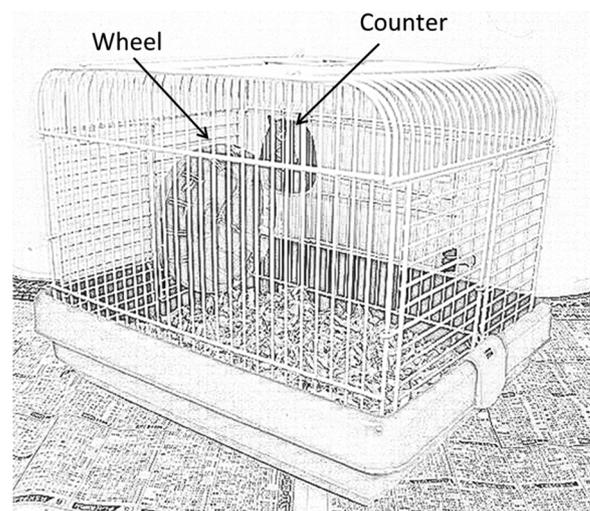


Fig. 2. A pet cage viewed from the rear. There is a wheel on a side wall and its counter on the front wall. This set up is for the mice trained to run in an open wheel (see Experiments 2 and 3). In Experiment 1B, the cage had an additional wheel on the opposite wall and its counter on the front wall. Not shown for clarity is an opaque plate covering the openings of the wheel.

Table 1
Treatments in the experimental room (Experiments 1A and 1B).

Group	Days 9, 11, 13, 15, and 18	Days 10, 12, 14, 16, and 17
Paired	cheese (15 min) → wheel (45 min)	no cheese (15 min) → cart (45 min)
Unpaired	cheese (15 min) → cart (45 min)	no cheese (15 min) → wheel (45 min)

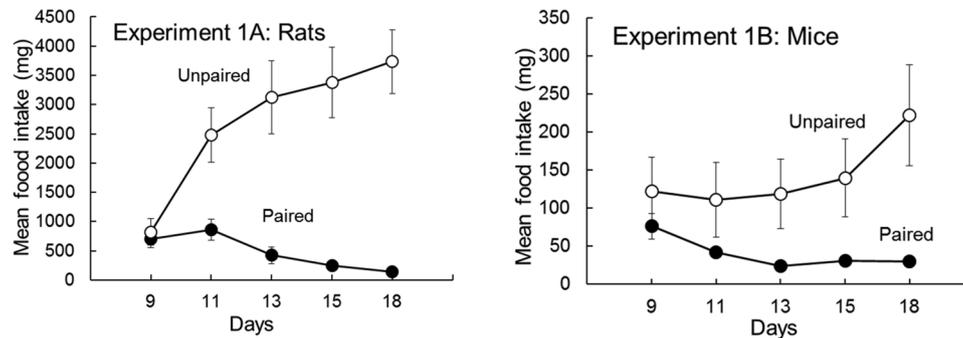


Fig. 3. Mean amount of cheese intake per 15 min period (when cheese was available) of Experiments 1A and 1B, separately shown for the animals who ran in wheels after consuming cheese (Paired) and those who ran in wheels on different day than when they consumed cheese (Unpaired). The bars indicate standard errors.

was given on Day 2, and the leftover was collected on Day 3. Thus, the animals had two opportunities of 24 h to become familiar with the cheese before the experiment proper began. These pretreatment was executed by the author, but the following experimental protocols were administered by laboratory assistants, who were blind to the aim of the experiments.

2.1.3.2. Experiment 1A (Rats). The experiment began on Day 8 and lasted until Day 18. On each day at 1015 h, all rats were moved together in the cart to the experimental room, where the rats were individually weighed by an electric balance (KS-251, Dretac, Koshigaya, Japan) to the nearest 1 g. Prior to the conditioning treatment, all rats were confined in the test chambers without any food for 15 min (Day 8).

The conditioning phase was consisted of 5 blocks of 2 days (Table 1). More specifically, each rat of Group Paired ($n = 8$) was given a 15-min access to a fresh piece of cheese in the cup of the test chamber, immediately followed by a 45-min confinement in the wheels on Days 9, 11, 13, 15, and 18; these rats spent 15 min with no cheese in the test chambers before directly returning to the cart and kept there for 45 min on Days 10, 12, 14, 16, and 17. For the remaining rats (Group Unpaired, $n = 8$), the cheese access and wheel confinement were administered on separate days: each rat was given a 15-min cheese access in the test chamber before directly returning to the cart and kept there for 45 min on Days 9, 11, 13, 15, and 18, and 15-min holding in the test chamber without cheese followed by a 45-min wheel confinement on Days 10, 12, 14, 16, and 17. Thus, the conditioning phase consisted of 5 blocks of 2 days with the sequence of CNCNCNCCNC (C = cheese day, N = no-cheese day). Notably, the order of cheese and no-cheese days was reversed for the final two days (i.e., NC instead of CN) to exclude the possibility that any group difference in cheese intake is ascribed to the difference in the treatments of the preceding day.

2.1.3.3. Experiment 1B (Mice). The experimental procedure for mice were the same as for rats: Groups Paired ($n = 8$) and Unpaired ($n = 8$) received 1-day adaptation to the test chamber and 10-day treatments according to the sequence noted above. An exception was that the mice experiment was conducted in two successive squads starting at 1305 and 1430 h, respectively, because the number of the test chambers was eight. The chambers were cleaned with wet paper tissues after each squad.

2.1.4. Measurement and statistical analysis

The amounts of cheese ingested (i.e., 15-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, Japan) to the nearest 0.01 g (i.e., 10 mg). The number of wheels turns were recorded by reading the digits shown in the display of each wheel counter. 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. For simplicity, the standardized effect size will be reported only when the main or interactive effect of the given ANOVA was significant ($P < 0.05$) or marginally significant ($0.05 \leq P < 0.10$). The measurement and statistical analysis of the following experiments were also carried out in the same manner.

2.1.5. Ethical considerations

All treatments of the experiments reported in this article 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. Experiment 1A (Rats)

The left panel of Fig. 3 shows that both groups of rats were at first somewhat reluctant to eat cheese, but the control rats (Group Unpaired) gradually increased the consumption over the experimental days. The decreasing cheese consumption in the experimental rats (Group Paired) presents intuitive evidence that wheel running yielded CFA, but a methodologically more sophisticated assessment is comparing the cheese consumptions of the paired and unpaired groups on later sessions of the training. A 2 (group) \times 5 (day) mixed-design ANOVA yielded significant main effects of group, $F(1, 14) = 22.51$, $P < 0.001$, $\eta_p^2 = 0.62$, and day, $F(4, 56) = 12.07$, $P < 0.001$, $\eta_p^2 = 0.46$, and most importantly their interaction, $F(4, 56) = 26.22$, $P < 0.001$, $\eta_p^2 = 0.65$. Post hoc simple main effect analyses of the interaction revealed that two groups significantly differed on Day 11 and onward, $F_s(1, 14) > 10.56$, $P_s < 0.006$. The simple day effect was highly significant for Group Unpaired, $F(4, 56) = 35.77$, $P < 0.001$, and marginally significant for Group Paired, $F(4, 56) = 2.52$, $P = 0.051$.

The number of wheel turns did not differ between the groups but

also across running days: a 2 (group) \times 5 (day) mixed-design ANOVA found no main or interactive effect, $F_s < 1$. The averages collapsed across the groups (\pm standard errors) were 189 ± 17 , 184 ± 18 , 178 ± 16 , 202 ± 17 , and 187 ± 13 turns per session, from the first to the fifth running days of the conditioning phase. In other words, the average rats ran 167–190 m per session in this experiment, because 100 turns of the rat wheel correspond to 94 m in running distance.

The average body weight gradually increased over the experimental days from 295.4 ± 1.9 g on Day 8 to 339.4 ± 3.1 g on Day 18. The two groups did not differ significantly. A 2 (group) \times 11 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(10, 140) = 259.18$, $P < 0.001$, $\eta_p^2 = 0.95$, but the main effect of group was far from significant, $F < 1$, and the interaction was only marginal, $F(10, 140) = 1.85$, $P = 0.057$, $\eta_p^2 = 0.12$, even with the large degrees of freedom for this computation.

2.2.2. Experiment 1B (Mice)

The mice were also initially reluctant to eat cheese as depicted in the right panel of Fig. 3. The control mice (Group Unpaired) gradually increased the consumption over the experimental days, while the experimental mice (Group Paired) showed a slight decrease. A 2 (group) \times 5 (day) mixed-design ANOVA yielded a marginally significant main effect of group, $F(1, 14) = 4.23$, $P = 0.059$, $\eta_p^2 = 0.23$. The main effect of day, $F(4, 56) = 4.83$, $P = 0.002$, $\eta_p^2 = 0.26$, and the group \times day interaction, $F(4, 56) = 7.82$, $P < 0.001$, $\eta_p^2 = 0.36$, were significant. Post hoc simple main effect analyses of the interaction revealed that the two groups marginally differed on the third, $F(1, 14) = 4.32$, $P = 0.057$, and fourth, $F(1, 14) = 4.38$, $P = 0.055$, cheese days. The group significantly differed on the fifth day, $F(1, 14) = 8.43$, $P = 0.012$. The simple day effect was highly significant for Group Unpaired, $F(4, 56) = 10.45$, $P < 0.001$, and marginally significant for Group Paired, $F(4, 56) = 2.20$, $P = 0.081$.

The number of wheel turns increased over the running days similarly for the experimental and control mice. A 2 (group) \times 5 (day) mixed-design ANOVA found a significant main effect of day, $F(4, 56) = 8.65$, $P < 0.001$, $\eta_p^2 = 0.38$. The main effect of group and the group \times day interaction were far from significant, $F_s < 1$. The averages collapsed across the groups (\pm standard errors) were 999 ± 99 , 1269 ± 118 , 1584 ± 135 , 1633 ± 181 , and 1681 ± 150 turns per session, from the first to the fifth running days of the conditioning phase. In other words, the average mice ran 410–689 m per session in this experiment, because 100 turns of the mouse wheel correspond to 41 m in running distance. Hence, the mice ran 2–4 times more than the rats.

The average body weight gradually increased over the experimental days from 38.7 ± 0.5 g on Day 8 to 41.1 ± 0.6 g on Day 18. The two groups did not differ significantly. A 2 (group) \times 11 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(10, 140) = 19.10$, $P < 0.001$, $\eta_p^2 = 0.58$, but the main effect of group, $F < 1$, and its interaction with the day, $F(10, 140) = 1.20$, $P = 0.299$, were nonsignificant.

3. Experiment 2

Experiment 1B successfully replicated the finding that wheel running yields avoidance of the paired snack in mice (Nakajima, 2019b). The novelty of Experiment 1B lies in the fact that the identical experimental procedure also yielded snack avoidance in rats (Experiment 1A), suggesting the same learning process is responsible for the behavior of these species. An intriguing difference between these species is that the mice ran 2–4 times more than rats. This aspect of mouse running wheel behavior was the stimulus for the next experiment.

Experiment 2 employed a simple conditioning design to examine whether snack avoidance is also generated by truly voluntary running in an activity wheel attached to the pet cage. The openings of the wheel

were uncovered to allow a mouse to freely enter, turn, and exit the wheel. In addition to the mice receiving this treatment (Group Open-Wheel), a conventional treatment (i.e., confinement in the closed wheel) was administered for a second group of mice (Group Closed-Wheel). The results of these two groups were contrasted with the performance of a third group of mice having no opportunity to run (Group No-Access).

The target snack in Experiment 2 was cheese as in Experiment 1B, but a different brand was employed for convenience and affordability. The specificity of snack avoidance was tested with other kinds of snacks (raisins and marshmallows).

3.1. Method

3.1.1. Subject

The subjects were 24 experimentally naïve male Slc:ICR mice purchased from the same breeder as those in Experiments 1A and 1B, when they were 8 weeks old. They were maintained as were those in Experiment 1B.

3.1.2. Apparatus

The test chambers were identical to those of Experiment 1B. The activity wheels were also identical to those of Experiment 1B, but their conditions differed as follows. On the adaptation days, 8 closed wheels were set in 4 pet cages on a table as in Experiment 1B. On the conditioning days, there were 6 pet cages on the table. Two of them had two closed wheels as before (4 wheels in total). Each of the remaining 4 cages had a single wheel with the uncovered openings, and thus the mice were allowed to freely explore the pet cages. The pet cages were made of wire grids coated with white vinyl resin and a green plastic tray (7.6 cm deep) with paper bedding of 2 cm. The inner dimension of each pet cage was 30 cm wide, 23 cm long, 26 cm high (see Fig. 2 to get an image of the cage inside), but the area for exploration was restricted by white plastic plates to 30 cm \times 15 cm \times 22 cm. The wheel was installed on one of the narrow walls of the pet cage with the lower end 1.5 cm above the bedding. Feces and urine in the wheels attached to the pet cages were wiped out after each session, but other parts of the pet cages were left untouched throughout the experiment.

3.1.3. Procedure

3.1.3.1. Pre-Treatment. A raisin (\approx 0.5 g, TON'S Brand California Raisins, Toyo Nuts, Kobe, Japan) was given to each mouse in the home cage on the day after arrival (Day 1), and the leftover was collected on Day 2. Another raisin was given on Day 6, and the leftover was collected on Day 7. A small marshmallow pellet (\approx 0.1 g, Mini Marshmallows Vanilla, Wisuc, Tokyo, Japan) was given to each mouse on Day 7, and the leftover was collected on Day 8. A shred of Gouda cheese (\approx 1 g, JCCU, Tokyo, Japan) was given to each mouse on Day 9, and the leftover was collected on Day 10. Thus, the animals had opportunities to become familiar with the raisin, marshmallow, and cheese before the experiment proper began. These snack familiarization pretreatments were executed by the author, but the following experimental protocols were administered by laboratory assistants, who were blind to the aim of the experiment.

3.1.3.2. Training. The experiment began on Day 10 and lasted until Day 25. The experimental protocols were conducted in two successive squads of 12 mice each, starting at 1340 and 1500 h, respectively. The test chambers and the compartments of the carrying cart were cleaned with wet paper tissues after each squad. All mice were moved together in the home cages on the cart to the experimental room, where they were individually weighed by an electric balance (KS-251, Dretex, Koshigaya, Japan) to the nearest 1 g.

Adaptation to the test chambers were administered for the first two days (Days 10 and 11): all mice were confined in the chambers without any food for 15 min. Immediately after the chamber confinement, mice

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

Group	Days 12–21, 23, and 24
Closed-Wheel	cheese (15 min) → wheel (45 min)
Open-Wheel	cheese (15 min) → pet cage w/ wheel (45 min)
No-Access	cheese (15 min) → home cages on cart (45 min)

of Groups Closed-Wheel and Open-Wheel were also adapted to the closed wheels for 45 min. During this running period, mice of Group No-Access were kept in the individual compartments of the home cages on the cart.

The conditioning phase started on Day 12 (Table 2). Each mouse was given a 15-min access to 1 or 2 fresh pieces of cheese (1–2 g) in the cup of each test chamber, immediately followed by a 45-min confinement in a closed wheel (Group Closed-Wheel), a 45-min confinement in the pet cages with an open wheel (Group Open-Wheel), or returning to the home cage on the cart for 45 min (Group No-Access).

As the mice were still reluctant to eat cheese after 5-day training, all food pellets in the containers of the home cages were removed when they were in the test chambers on the fifth training day (Day 16). The same training procedures were administered to the food-deprived mice for the next 5 days (Days 17–21). Supplemental feeding was given in the vivarium by putting a broken piece of the food pellet (≈ 2 g) in each compartment of the home cages immediately after each daily session.

3.1.3.3. Testing with raisins or marshmallows. A piece of cheese in the test chambers was replaced with 4 or 5 raisins (≈ 2 g) on Day 22. All mice were immediately returned to the vivarium without wheel running, and they were given supplemental feeding as before. After additional 2-day training with cheese (Days 23 and 24), a second test was administered with 10–15 marshmallows (≈ 1.2 g) per mouse on Day 25.

3.2. Results and discussion

3.2.1. Snack intake

The left section of Fig. 4 shows that all groups of mice consumed a small amount of cheese when they were not deprived of food (Days 12–16). A 3 (group) \times 5 (day) mixed-design ANOVA yielded a marginally significant main effect of day, $F(4, 84) = 7.31$, $P < 0.001$, $\eta_p^2 = 0.26$, reflecting a slight decrease in consumption. The main effect of group, $F(2, 21) = 1.27$, $P = 0.300$, and the interaction, $F < 1$, were not significant.

Food deprivation not only increased consumption of cheese but also

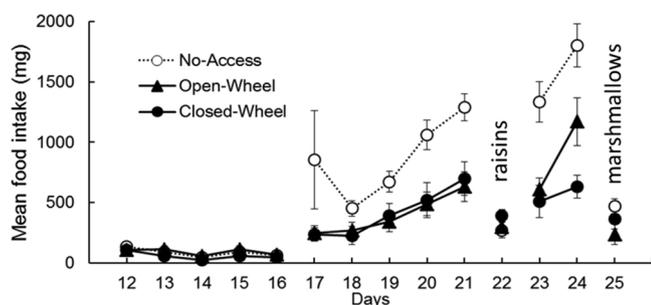


Fig. 4. Mean amount of snack intake on the conditioning and test days of Experiment 2. The snack was cheese on the conditioning days, while it was raisins or marshmallows on the test days (Days 22 and 25). The mice were food-deprived from Day 17 onward. After the cheese intake, a third of the mice were returned to the home cages on the cart without access to wheels. The remaining mice were allowed to run in open wheels or closed wheels. The bars indicate standard errors.

revealed otherwise latent running-based CFA as shown in the middle section of Fig. 4. An abrupt increase of cheese intake on Day 17 and following decrease on Day 18 reflect the mice's acute response to, and adaptation to, the food deprivation. A 3 (group) \times 5 (day) mixed-design ANOVA yielded significant main effects of group, $F(2, 21) = 8.02$, $P < 0.001$, $\eta_p^2 = 0.43$, and day, $F(4, 84) = 10.33$, $P < 0.001$, $\eta_p^2 = 0.32$, but the interaction was far from significant, $F < 1$. Holm-Sidak post hoc tests revealed that Group No-Access consumed significantly more than Groups Closed-Wheel and Open-Wheel, which did not differ from each other.

The three groups did not differ in the consumption of raisins, implying that the group difference in snack avoidance observed on Days 17–21 was specific to the cheese paired with running. A one-way ANOVA applied to the intake of raisins on Day 22 yielded no group difference, $F(2, 21) = 1.06$, $P = 0.364$.

Retraining with cheese on Days 23–24 manifested the difference between Groups Closed-Wheel and Open-Wheel, which had been equivalent on Days 17–21. A 3 (group) \times 2 (day) mixed-design ANOVA yielded significant main effects of group, $F(2, 21) = 14.42$, $P < 0.001$, $\eta_p^2 = 0.58$, and day, $F(1, 21) = 23.85$, $P < 0.001$, $\eta_p^2 = 0.53$. Holm-Sidak post hoc tests for the main effect of group revealed that Group No-Access consumed more than Group Open-Wheel, which in turn consumed less than Group Closed-Wheel. Although the group \times day interaction failed to reach significance, $F(2, 21) = 2.84$, $P = 0.081$, $\eta_p^2 = 0.21$, Fig. 4 clearly shows an interactive effect. Thus, additional analyses were executed for the group effect of each day. On day 23, Holm-Sidak post hoc tests revealed that Group No-Access consumed significantly more than Groups Closed-Wheel and Open-Wheel, which did not differ from each other. Similar tests for the data of on Day 24 revealed that Group No-Access consumed more than Group Open-Wheel, which in turn consumed more than Group Closed-Wheel.

The three groups did not differ in the consumption of marshmallows on Day 25: a one-way ANOVA yielded a nonsignificant group effect, $F(2, 21) = 2.32$, $P = 0.123$, replicating the specificity of snack avoidance based on wheel running.

3.2.2. Wheel turns

Group Closed-Wheel turned the activity wheels more than Group Open-Wheel, as expected and depicted in Fig. 5. A 2 (group) \times 5 (day) mixed-design ANOVA applied for the data of the food ad-lib. phase (Days 12–16) yielded significant main effects of group, $F(1, 14) = 15.48$, $P = 0.001$, $\eta_p^2 = 0.53$, and day, $F(4, 56) = 6.38$, $P < 0.001$, $\eta_p^2 = 0.31$. The group \times day interaction was also significant, $F(4, 56) = 3.69$, $P = 0.010$, $\eta_p^2 = 0.21$. Post hoc simple main effect analyses of the interaction revealed that two groups significantly differed on all days, $F_s(1, 14) > 5.24$, $P_s < 0.039$. The simple day effect was highly significant for Group Closed-Wheel, $F(4, 56) = 9.79$, $P < 0.001$, but it was not significant for Group Open-Wheel, $F < 1$.

The group difference was maintained in the food-deprived phase as well as the retraining phase. A 2 (group) \times 5 (day) mixed-design

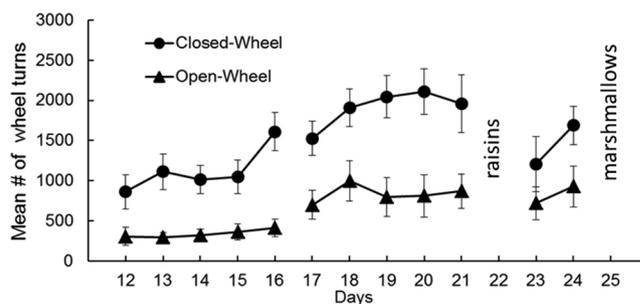


Fig. 5. Mean number of wheel turns on the conditioning days of Experiment 2. The mice were allowed to run in open wheels or closed wheels. The bars indicate standard errors.

ANOVA applied for the data of this phase (Days 17–21) yielded a significant main effect of group, $F(1, 14) = 13.55$, $P = 0.002$, $\eta_p^2 = 0.49$. The main effect of day, $F(4, 56) = 1.54$, $P = 0.203$, and their interaction, $F < 1$, were not significant. A 2 (group) \times 2 (day) mixed-design ANOVA applied for the data of the retraining phase (Days 23–24) yielded a marginally significant main effect of group, $F(1, 14) = 4.27$, $P = 0.058$, $\eta_p^2 = 0.23$. The main effect of day, $F(1, 14) = 2.45$, $P = 0.140$, and the group \times day interaction, $F < 1$, were not significant.

3.2.3. Body weight

The three groups did not differ significantly in body weight throughout Experiment 2. The average body weight gradually increased in the food ad-lib. phase from 40.4 ± 0.5 g on Day 12 to 41.6 ± 0.8 g on Day 16. A 3 (group) \times 5 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(4, 84) = 8.80$, $P < 0.001$, $\eta_p^2 = 0.30$, but the main effect of group, $F(2, 21) = 1.81$, $P = 0.188$, and its interaction with the day, $F < 1$, were nonsignificant.

The average body weight gradually decreased in the food-deprived phase from 35.6 ± 0.5 g on Day 17 to 32.8 ± 0.6 g on Day 21. A 3 (group) \times 5 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(4, 84) = 44.6$, $P < 0.001$, $\eta_p^2 = 0.68$, but the main effect of group, $F(2, 21) = 1.01$, $P = 0.380$, and its interaction with the day, $F < 1$, were nonsignificant.

The decreasing trend of body weight was maintained during the raisins test (33.4 ± 0.6 g on Day 22), the retraining phase (32.4 ± 0.6 g on Day 23 and 31.9 ± 0.6 g on Day 24), and the marshmallow test (31.9 ± 0.6 g on Day 25). A 3 (group) \times 4 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(3, 63) = 24.43$, $P < 0.001$, $\eta_p^2 = 0.54$, but the main effect of group failed to reach the significant level, $F(2, 21) = 2.76$, $P = 0.086$, $\eta_p^2 = 0.21$, and the group \times day interaction was nonsignificant, $F(6, 63) = 1.51$, $P = 0.189$.

4. Experiment 3

Experiment 2 employed a simple conditioning procedure to demonstrate CFA based on running in an open or closed wheel attached to the pet cage. Small cheese consumption of running mice, compared with no-running control mice, support the claim that wheel running generated CFA. The specificity of conditioning to the paired snack (cheese) was confirmed by showing that running and non-running mice consumed equivalent amount of other snacks (raisins and marshmallows), although this argument is not compelling for at least two reasons. First, the amounts of consumption were relatively low for these snacks, and thus the floor effect may have obscured any group differences. Second, the target snack was cheese and the test snack was raisins or marshmallows for all mice in Experiment 2: the physical identities of target and control snacks were not counterbalanced.

In Experiment 3, mice were trained in a differential conditioning paradigm with two kinds of snacks (cheese and raisin) as flavor cues: one of them was paired with an opportunity to run in an open wheel, while the other was not. Thus, assessment of conditioning was between these counterbalanced snacks (i.e., a within-subject design). The second aim of Experiment 3 was to examine whether it is possible to establish CFA by pet cage confinement without any opportunity to run in a wheel. For this purpose, a second set of mice were trained with the pet cages having no wheels.

4.1. Method

4.1.1. Subjects and apparatus

The subjects were 16 experimentally naïve male Slc:ICR mice purchased from the same breeder as those in the preceding experiments, when they were 7 weeks old. They were maintained as were those in

the preceding experiments.

The test chambers were 16 translucent plastic storage containers (7.8 cm wide, 11.5 cm long, 10 cm high) located in racks on a table. The left side of each chamber was painted white in such a way to prevent visual contact with conspecifics. The chambers had no food cups. The activity wheels were identical to those of the preceding experiments. On the adaptation days, a closed wheel was set in each of 8 pet cages on a table. In the conditioning phase, the mice were allowed to freely explore the entire area of the pet cages (30 cm wide, 23 cm long, 26 cm high: the partition plates employed in Experiment 2 were not used here). Four of the 8 pet cages had no wheels. Each of the remaining 4 pet cages had a wheel on a side wall with the lower end of the wheel 1.5 cm above the paper bedding of the pet cage. The test chambers were cleaned with wet paper tissues after each session. Feces and urine in the wheels attached to the pet cages were also wiped out after each session, but other parts of the pet cages were left untouched throughout the experiment.

4.1.2. Procedure

4.1.2.1. Pre-treatment. A piece of processed cheese for pet dogs (≈ 1 g, hello! DiaCut Cheese, Doggy Man H.A., Osaka, Japan) was given to each mouse in the home cage on 6 days after arrival (Day 6), and the leftover was collected on Day 8. A raisin (≈ 0.5 g, TON'S Brand California Raisins, Toyo Nuts, Kobe, Japan) was given to each mouse on Day 8, and the leftover was collected on Day 10. Thus, the animals had an opportunity of 48 h to become familiar with these snacks before the experiment proper began. These snack familiarization pretreatments were executed by the author, but the following experimental protocols were administered by laboratory assistants, who were blind to the aim of the experiment.

4.1.2.2. Training. All food pellets in the containers of the home cages were removed on Day 12. The experiment began on Day 13 and lasted until Day 26 under mild food deprivation. Supplemental feeding was given in the vivarium by putting a broken piece of the food pellet (≈ 2 g) in each compartment of the home cages immediately after each daily session. The experimental protocols were conducted in a single squad starting at 1340 h. All mice were moved together in the home cages on the cart to the experimental room, where they were individually weighed by an electric balance (KS-251, Dretex, Koshigaya, Japan) to the nearest 1 g.

Adaptation to the test chambers were administered for two days (Days 13 and 14): all mice were confined in the chambers without any food for 15 min. Immediately after the chamber confinement, a group of 8 mice were also adapted to the closed wheels for 60 min. During this running period, the other group of 8 mice were kept in the individual compartments of the home cages on the cart.

On the next 12 days (differential conditioning phase: Days 15–26), each mouse was given access to either 2 pieces of cheese or 4–5 raisins placed directly on the floor of the test chamber. The access to one of the two kinds of snacks was always followed by 60-min pet cage confinement. It is notable that the groups of mice which had been adapted to the closed wheels were now in the pet cages with wheels, while the other group of mice were in the pet cages without wheels. Access to the other kind of snack was followed by returning to the home cages on the cart. The specific snack that preceded the pet cage confinement was counterbalanced across mice, and the conditioning consisted of 6 blocks of 2 days with the snack sequence of CRCRCRCRRRC (C = cheese, R = raisins) as shown in Table 3. Hence, on the first conditioning day, half of the mice (#1–4) of each group received access to cheese followed by pet cage confinement, while the others (#5–8) were given access to cheese without pet cage confinement. On the second conditioning day, the former mice received access to raisins without pet cage confinement, while the latter mice were confined to the pet cages after access to raisins. The same procedures were performed for the remaining days. Notably, the order of cheese and raisins was reversed

Table 3
Treatments in the experimental room (Experiment 3).

Mice	Days 15, 17, 19, 21, 23, and 26	Days 16, 18, 20, 22, 24, and 25
#1–4	cheese (15 min) → pet cage (60 min)	raisin (15 min) → home cage on cart (60 min)
#5–8	cheese (15 min) → home cage on cart (60 min)	raisin (15 min) → pet cage (60 min)

for the final two days (i.e., RC instead of CR) to ensure that performance of the mice truly reflected the snack identities.

4.2. Results and discussion

4.2.1. Mice in the pet cages with wheels

The left panel of Fig. 6 shows that the snack, which was unpaired with pet cage confinement, was gradually consumed over days, suggesting habituation of snack neophobia. The consumption of the snack paired with pet cage confinement was small and constant across days, implying that the habituation of neophobia was counteracted by acquisition of CFA. A 2 (snack) × 6 (day) repeated measures ANOVA yielded a significant main effect of day, $F(5, 35) = 10.88$, $P < 0.001$, $\eta_p^2 = 0.61$, and its interaction with snack, $F(5, 35) = 3.73$, $P = 0.008$, $\eta_p^2 = 0.35$. The main effect of snack was not significant, $F(1, 7) = 3.02$, $P = 0.126$. Post hoc simple main effect analyses of the interaction revealed that the paired and unpaired snacks significantly differed on Days 23–24, $F(1, 7) = 6.24$, $P = 0.041$. The difference on Days 25–26 was statistically marginal, $F(1, 7) = 5.45$, $P = 0.052$. The simple day effect was highly significant for the unpaired snack, $F(5, 35) = 9.73$, $P < 0.001$, but it was nonsignificant for the paired snack, $F(5, 35) = 1.34$, $P = 0.271$.

The mean numbers of wheel turns per session (\pm standard errors) were 1572 \pm 433, 1883 \pm 396, 2711 \pm 475, 2784 \pm 452, 2496 \pm 546, and 2644 \pm 629, from the first running day (Day 15 or 16) to the sixth running day (Day 25 or 26) of the differential conditioning phase. A one-way ANOVA yielded a significant day effect, $F(5, 35) = 4.65$, $P = 0.002$, $\eta_p^2 = 0.40$.

4.2.2. Mice in the pet cages without wheels

Confinement in the pet cages without wheels yielded no snack avoidance, as shown in the right panel of Fig. 6. Habituation of neophobia was equivalent for the unpaired and paired snacks. A 2 (snack) × 6 (day) repeated measures ANOVA yielded a significant main effect of day, $F(5, 35) = 17.82$, $P < 0.001$, $\eta_p^2 = 0.72$, but the main effect of snack, $F < 1$, and the interaction, $F(5, 35) = 1.10$, $P = 0.380$, were far from significant.

4.2.3. Body weight

The first session of wheel adaptation was conducted on Day 13 with

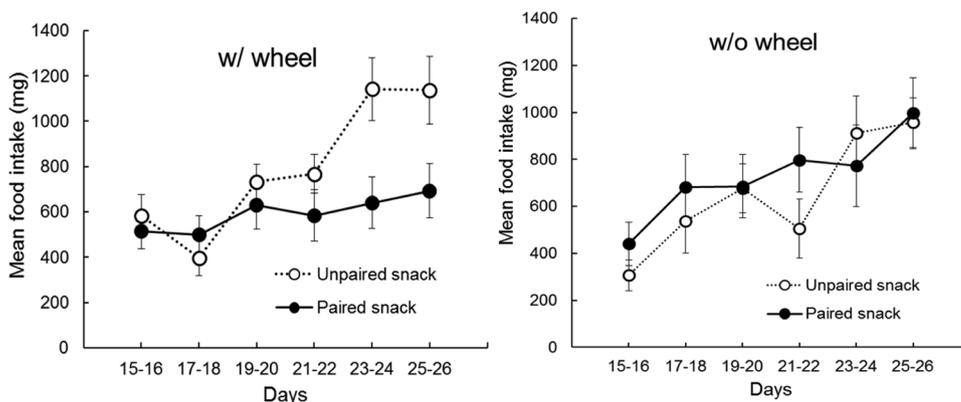


Fig. 6. Mean amount of snack intake on the conditioning days of Experiment 3, separately shown for the snack paired with pet cage confinement and the snack unpaired with pet cage confinement. Each block consists of a day for the paired snack and a day for the unpaired snack. The differential conditioning was successful in the mice trained in the pet cages with open wheels (left panel) but not in those trained in the pet cages without wheels (right panel). The bars indicate standard errors.

non-deprived mice of 39.3 ± 0.6 g. The food pellets in the home cages were removed when they were in the test chambers, and thus the average decreased to 34.4 ± 0.6 g on the next day (Day 14). The two groups (the mice to be confined in the pet cages with wheels and those in the pet cages without wheels) did not differ in body weight on these days. A 2 (group) × 2 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(1, 14) = 526.35$, $P < 0.001$, $\eta_p^2 = 0.97$, but the main effect of group and the interaction were far from significant, $F_s < 1$.

The body weight gradually decreased over the differential conditioning days from 33.3 ± 0.6 g on Day 15 to 30.6 ± 0.7 g on Day 26. A 2 (group) × 12 (day) mixed-design ANOVA yielded a highly significant main effect of day, $F(11, 154) = 15.70$, $P < 0.001$, $\eta_p^2 = 0.53$, but the main effect of group, $F < 1$, and its interaction with the day, $F(11, 154) = 1.02$, $P = 0.431$, were nonsignificant.

5. General discussion

Wheel running hampers habituation of neophobic reaction in mice to the snack consumed before the running (Nakajima, 2019b). A plausible interpretation of this phenomenon is that the mice learn avoidance of that snack by Pavlovian conditioning with wheel running as an unconditioned stimulus inducing an aversive inner state. This claim is supported by the fact that a similar procedure generates the same phenomenon in rats (Nakajima, 2019a), which are standard animals for demonstrating running-based CFA (Boakes and Nakajima, 2009). Experiments 1A and 1B of the present research give further credence to such a contention, because the same phenomenon was observed in rats and mice with an identical procedure. Another piece of evidence for running-based CFA in mice is intuitively more appealing: there are modestly decreasing curves of snack consumptions over training days in these experiments as in Nakajima (2019a,b).

It is noteworthy that non-associative (i.e., snack-nonspecific) factors of 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; Lamanna et al., 2019; Pierce, 2001, for reviews) have been ruled out in the previous studies by the use of an unpaired control group or differential conditioning procedures (Nakajima, 2019a, b). This is also the case for Experiment 3 of the present research, which employed a differential conditioning procedure. Furthermore, comparable body weights between the running and non-running animals in Experiments 1A, 1B, 2, and 3 buttress the claim that the intake suppression is specific to the snack paired with wheel running.

Another contribution of the present research is the finding that mice develop CFA by running in open activity wheels, although the amount of effect was smaller than the one generated with a closed wheel in the retraining phase of Experiment 2. Notably, successful demonstration of running-based CFA with open wheels has been reported with rats (e.g., Dobek et al., 2012; Salvy et al., 2004; Satvat and Eikelboom, 2006).

Although such an "optional" running procedure was not effective to establish CFA in my laboratory (Masaki and Nakajima, 2006), only a single conditioning trial was employed in that research.

Running-based CFA with open wheels was replicated in Experiment 3. A notable feature of the apparatus employed in Experiments 2 and 3 is that the area for exploration was large, compared with the side cage adjacent to the open wheel employed in the aforementioned rat studies. Hence, one may argue that exploring the large space would be also effective to generate CFA in mice. This was not the case in Experiment 3, where exploring the pet cage without wheels had no effect on the snack consumption. This possibility, however, should be further investigated, because there is a report that activity in a flat circular alley works as a weak agent to generate CFA in rats (Lett et al., 1999).

As in the previous research on running-based CFA in mice (Nakajima, 2019a), the number of wheel turns increased over the running days in Experiments 1B, 2, and 3 of the present study. Furthermore, wheel running positively reinforces instrumental responses in mice (Belke and Garland, 2007). Accordingly, it is reasonable to infer that wheel running is pleasurable for mice, and thus, we conclude that wheel running has hedonically bivalent properties in mice as well as in rats. The remaining enigma is why wheel running generates CFA even though it is pleasurable. Because drugs of abuse also positively reinforce operant behavior and generate CFA in rats (see Hunt and Amit, 1987; Verendeev and Riley, 2012, for reviews), Lett and Grant (1996) have claimed that wheel running activates mesolimbic rewarding system and the altered inner state of the rats' body functions as an aversive unconditioned stimulus to generate CFA.

Wheel running is pleasurable for rodents. However, it is a physical stressor, because it activates both the sympathetic nervous system, resulting in epinephrine production, and the hypothalamic-pituitary-adrenal (HPA) axis, resulting in glucocorticoid production and HPA axis feedback (Mul, 2018). Furthermore, as pointed out by Hundt and Premack (1963), all self-initiated behaviors including wheel activity are self-terminated, and the "on-off systems" of these behaviors are capable of generating both positive and negative reinforcement. In other words, running has a delightful start with a distressful ending. Thus, the hedonically bivalent nature of wheel running might be related to the biphasic nature of running: the onset of running is pleasurable, but aversive property of running gradually overcomes the pleasant nature of running.

What kind of aversive property of running is responsible for running-based CFA? Wheel running causes kaolin clay ingestion in rats (Nakajima, 2016, 2018, ; Nakajima and Katayama, 2014), which is a marker of nausea in rats (Andrews and Horn, 2006). Taken together with other indirect pieces of evidence (Dwyer et al., 2008; Eccles et al., 2005; Nakajima et al., 2006), it is plausible that the internal state induced by running is nausea (or visceral discomfort similar to nausea) in rats. Because the kaolin test is also applicable to mice as a measure of nausea induced by strong treatments such as a cisplatin injection, irradiation, and hypergravity (Santucci et al., 2002; Yamamoto et al., 2002, 2005; Yamamoto and Yamatodani, 2018), future studies should be developed to determine the exact kaolin consumption induced by wheel running despite the difficulties due to its relative weakness in causing CFA.

So far, we have considered the obtained results as flavor avoidance based on the aversive property of running. Another interpretation of the results, however, is anticipatory contrast: a reduction of responses towards a substance when it is successively followed by a more valued substance (Flaherty, 1982, 1996). For example, in an experiment with rats, the intake of a 0.15% saccharin solution remained suppressed for 7 daily sessions when it was followed by strongly preferred a 32% sucrose solution, but the saccharin intake gradually increased over the days when it was followed by nothing (Flaherty and Checke, 1982). This is a kind of Pavlovian conditioning between the saccharin CS and the sucrose US: the saccharin CS was avoided in anticipation of the imminent availability of the attractive sucrose US (Flaherty and Grigson, 1988).

Anticipatory contrast is not specific to consummatory behavior. Grigson (1997, 2008) has interpreted CFA based on drug of abuse as a case of anticipatory contrast: anticipation of the availability of the highly preferred drug lowers the hedonic valence of the flavor CS. An experimental support comes from a report that a saccharin-morphine pairing led to a reduction in rats' saccharin intake, which was accompanied by a conditioned blunting of the accumbens dopamine response to the saccharin cue (Grigson and Hajnal, 2007). We can apply the same explanation to the effect reported in the present study: mice were reluctant to consume a target snack because they favorably anticipated wheel running. This possibility is open to evaluation.

Leaving aside consideration of the specific mechanism of the obtained results, there are at least two limitations in this study that could be addressed in future research. First, replication of the present findings in the dark period is warranted, because mice are nocturnal animals: they eat and run mainly in the dark period of the day (e.g., Fuss et al., 2010; Harri et al., 1999; Possidente and Birnbaum, 1979; Ticher and Ashkenazi, 1995). Second, demonstration of running-based CFA in other strains of mice is necessary to broaden the generality of this learning, since there are strain differences in wheel-running behavior in mice (e.g., Ebihara and Tsuji, 1976; Festing, 1977). These and other inquiries are currently under investigation.

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