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Intraperitoneal injection of D-serine inhibits high-fat diet intake and preference in male mice

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ABSTRACT

D-serine is a co-agonist of the N-methyl D-aspartate (NMDA) receptor, an important modulator of glutamatergic excitatory synaptic transmission. We previously reported that oral D-serine ingestion inhibited the intake of highly preferred food and promoted the intake of less preferred food in mice. Here, we analyzed the effects of intraperitoneal (IP) D-serine injections on feeding behavior in mice. We assessed the effects of D-serine during both the acquisition and maintenance of a preference for high-fat diets (HFDs). Aversiveness of IP D-serine was analyzed in the conditioned taste aversion paradigm. The effects on food intake were assessed by providing liquid meals with different fat contents. Finally, we measured brain D-serine and L-serine levels after D-serine administration. We found that IP-injected D-serine effectively inhibited the acquisition of a HFD preference, but failed to prevent expression of a previously learned HFD preference. IP-injected D-serine was not sufficient to condition taste aversion. The effect on HFD preference acquisition was associated with increases in D-serine levels in the cerebral cortex, hypothalamus, and cerebellum. IP-injected D-serine most effectively inhibited the intake of liquid meals with high fat content. This effect was dose-dependent, but the responses varied significantly among male C57BL/6J mice. The differential responses to D-serine were consistent among multiple trials in each mouse. In summary, IP-injected D-serine inhibited HFD intake and the acquisition of an HFD preference. Individual mice with the same genetic background showed different sensitivities to D-serine; thus, D-serine sensitivity may be associated with unidentified traits.

1. Introduction

Organisms are made of what they ingest. Therefore, it would make sense from homeostatic point of view for organisms to preferentially ingest nutritionally well-balanced diet. However, in reality, both humans and experimental animals prefer diet rich in fat over diet with proper macronutrient balance. Diet rich in fat promotes weight gain by increasing caloric intake (Thaler et al., 2012) and altering feeding patterns (Kohsaka et al., 2007), and excessive consumption of high-fat diet is detrimental to health. To overcome the issue, better understandings on the molecular and neural mechanisms that controls diet selection are necessary.

D-serine is a co-agonist for the N-methyl-D-aspartate (NMDA) receptor; thus, it facilitates excitatory glutamatergic neurotransmission at synapses (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Papouin et al., 2012). Co-agonism of NMDA receptors is the only known *in vivo* function of D-serine (Hashimoto & Oka, 1997), and its activity is necessary for the NMDA receptor to function as an ion channel (Cheriyan, Mezes, Zhou, Balsara, & Castellino, 2015). D-serine production and degradation are regulated *in vivo* by serine racemase (SR) (Kartvelishvily, Shleper, Balan, Dumin, & Wolosker, 2006; Wolosker et al., 1999; Yoshikawa et al., 2007) and D-amino acid oxidase (Weimer & Neims, 1977), respectively. D-serine is also supplied from dietary sources, such as fermented foods, microorganisms, plants, and marine invertebrates (Friedman, 1999). The cerebral cortex of SR-knockout mice had only 10% of the D-serine levels present in wild-type mice (Basu et al., 2009; Horio et al., 2011). This finding indicated that 90% of brain

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D-serine was maintained by endogenous production and 10% was supplied from the gastrointestinal tract.

We previously reported that, in mice, oral ingestion of D-serine inhibited the intake of highly preferred food (Sasaki et al., 2015). That effect depended on co-agonism of the NMDA receptor, but it was independent of any particular macronutrient, sensory input, or intact leptin receptor signaling. Oral D-serine ingestion resulted in a reverse preference (chow preferred over high-fat food), when given at the time that mice acquired a food preference, and it prevented expression of a previously learned food preference, when given at later times. In the absence of food choices (access provided to only one food type), oral D-serine ingestion inhibited food intake. However, it took approximately 2 days for oral D-serine ingestion to affect feeding behaviors; thus, we could not rule out any potential effects mediated by incretions and gut microbiota. In that study, the central nervous system was the implied main target of orally administered D-serine, because D-serine maintained effectiveness in mice treated with capsaicin to remove sensory afferent transmission. However, we lacked evidence to show that D-serine levels changed within the central nervous system with D-serine administration.

Here, we aimed to resolve these unaddressed issues by analyzing the effects of intraperitoneal injections of D-serine on feeding behavior in mice. In addition, we measured D-serine and L-serine concentrations in the brain. We analyzed the effects of D-serine on a high-fat diet (HFD) preference, during both the acquisition and the expressed phases. Effects on food intake were assessed by providing liquid meals with different fat contents. We also assessed the aversiveness of IP D-serine to condition taste aversion. Better time resolution of IP D-serine results over PO D-serine results unexpectedly revealed variability in responses even in age-matched isogenic B6 male mice. Therefore, we also assessed the consistency of the differential responses over multiple trials.

2. Materials and methods

2.1. Animals

All mice (male C57BL/6J) used in experiments were purchased from CLEA Japan (Tokyo, Japan). All experiments, except for the conditioned taste aversion (CTA) experiment, were performed with mice between eight to ten weeks of age and different cohorts of mice were used in each experiment. To reduce number of animals, male C57/BL6J mice between twelve to fourteen-weeks of age, which had been mice used in other studies (open-field and sucrose chow intake tests), were subjected to CTA experiment with multiple conditioning paradigm. For the duration of this study, all mice were housed in individual cages in a temperature-controlled facility on a 12-h light/dark cycle (6:00 a.m. - 6:00 p.m.) and had *ad libitum* access to food and water. Body weight (BW) was measured daily. Cages and water bottles were changed on a weekly basis. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committees at Gunma University and Osaka University.

2.2. Food

Normal chow (CE-2, here referred to as NC) and high-fat diet chow (HFD32, here referred to as HFD) were purchased from CLEA Japan. We decided to use these food because it was used in our previous study assessing the effect of oral D-serine ingestion (Sasaki et al., 2015), and the use of these food allowed us to compare the results between oral versus intraperitoneal administration of D-serine. We also used the following liquid meal to manipulate the fat content of the diet. SanET2.0 liquid meal was purchased from Sanwa Kagaku Kenkyusho (Nagoya, Japan). IntraLipos lipid emulsion was purchased from Otsuka Pharmaceutical Factory (Naruto, Japan). Based on the preliminary experiments testing various mixture ratio of SI liquid meals (data not shown), we decided to perform the experiments with the following liquid meals: one was prepared by mixing SanET2.0 and distilled water at a 1:1 ratio (SW), the other liquid meal was prepared by mixing SanET2.0 and IntraLipos at a 1:1 ratio (SI). Liquid meal intakes were measured with drink bottles (SN-950 H) purchased from Shinano (Tokyo, Japan). A separate water bottle was provided in each cage during the liquid meal studies. The calorie-based macronutrient composition of the food used in this study is shown in Table 1.

2.3. D-serine effect on the acquisition phase of HFD preference

To test the dose dependence of intraperitoneal D-serine injection on feeding behavior, we chose to test it on the acquisition phase of a HFD preference, during which orally ingested D-serine showed the strongest effect in our previous study. We used the Feeding, Drinking, and Activity Monitoring System for mice (FDAMS, Shinfactory, Fukuoka, Japan) to monitor food intake, food access, water intake, and locomotor activity every minute (Sasaki et al., 2015), and these data were analyzed in 1-h bins. C57Bl/6J male mice were acclimated to FDAMS, and received two food trays that both contained NC (NC vs. NC). After three-day acclimation, on the day of the experiment, mice were given an intraperitoneal injection of vehicle (water) or D-serine (1, 2, or 4 g/kg BW) at 5:45 p.m., just before the start of the dark cycle at 6:00 p.m., and subsequently, mice were given two foods to choose from: NC and HFD (two-food choice). Observation was continued for the next 24 h. We analyzed 6, 7, 8, and 7 mice for 0, 1, 2, and 4 g/kg BW D-serine doses, respectively.

2.4. Conditioned taste aversion experiment

To assess if D-serine injection conditioned avoidance of novel food (HFD), we performed the conditioned taste aversion experiment using saccharine solution as conditioned stimulus (CS) and lithium chloride injection as unconditioned stimulus (US). Male C57/BL6J (twelve to fourteen-week old at beginning of experiment) mice were placed on a 20-h water deprivation schedule (from 17:00 to 13:00 the next day) in which they received one-bottle access to distilled water for 10 min starting at 13:00 each day and additional 1-h water access from 16:00 to 17:00 to avoid dehydration throughout behavioral procedure. The animals received the one-bottle training for 5 days to stabilize the 10-min water intake. The subjects were then divided into two groups to match water intake. For conditioned aversion to saccharin, all mice were given 10-min access to 5 mM sodium saccharin (Sigma, UK) as a conditioned stimulus (CS) instead of distilled water for 10 min followed by an intraperitoneal (i.p.) injection of either 0.15 M lithium chloride (LiCl, 2% of BW, $n = 8$) or D-serine (2 g/kg BW, 0.1 ml/10 g BW, $n = 9$) for conditioning on day 1 (C1). Each group received the same conditioning procedure on additional two days (days 2 and 3) (C2 and C3, respectively). On day 4 (retention test 1, T1), all mice received access to the same saccharin solution to test conditioned aversion to the CS. Both groups received the same CTA test (T2) on

Table 1
Calorie-based macronutrient composition of the food used in this study.

	Protein	Fat	Carbohydrate	Calories
Normal chow (CE2)	28.9%	12.0%	59.1%	3.50 (kcal/g)
High-fat diet (HFD32)	20.1%	56.7%	23.2%	5.08 (kcal/g)
SW (SanET2.0/water)	16.0%	34.0%	50.0%	1.00 (kcal/ml)
SI (SanET2.0/IntraLipos)	8.0%	67.0%	25.0%	2.00 (kcal/ml)

the next day without any injection. Intake of the saccharin CS was measured over the conditioning and testing days. LiCl injection was used as an unconditioned stimulus (US), because it is known to cause CTA (Sasaki et al., 2015). We analyzed 9 and 8 mice for D2 group and LiCl group, respectively.

2.5. Measurements of D-serine and L-serine in brain samples

To test if the altered feeding behavior caused by D-serine administration was associated with increases in brain D-serine levels, we measured the levels of D-serine and L-serine in the cerebral cortex, hypothalamus, and cerebellum, before and after D-serine administration. Based on the FDAMS data, we chose a 2 g/kg dose (D2) for the intraperitoneal injection (Fig. 1). The samples were collected at 2 h and 24 h after the injection. For oral administration, the samples were collected after 48 h of drinking 1.5% D-serine *ad libitum*, the condition in which mice previously showed an altered HFD preference (Sasaki et al., 2015). Nine week old male C57Bl6/J mice fed NC *ad libitum* (N = 3 per group) were used for tissue harvesting. The mice were sacrificed between 8:00 a.m. and 10:30 a.m. with pentobarbital anesthesia. The tissues (cerebral cortex, hypothalamus, and cerebellum) were rapidly excised, weighed, and stored at −80 °C. These samples were sent to Shiseido (Tokyo, Japan), where D-serine and L-serine were measured with enantioselective two-dimensional high-performance liquid chromatography (Miyoshi et al., 2011).

2.6. D-serine effect on the expression of a previously learned HFD preference

Six mice per group were acclimated to the FDAMS for 2 days with NC only; then, they were given a two-food choice between NC and HFD for 5 days to acquire HFD preference. HFD was changed daily. Food intake, food access, water intake, and locomotor activity were recorded every minute and analyzed in 1-h bins. On the day of the experiment, at 5:45 p.m., mice were given an intraperitoneal injection of vehicle (water) or D-serine (2 g/kg BW). Observation was continued for the next 24 h, and results were compared over 24 h before and after the injection. We used a single intraperitoneal injection of D-serine (2 g/kg BW, D2), because this had been sufficient to inhibit the acquisition of the HFD preference, without affecting locomotor activity or water intake (Fig. 1).

2.7. Experiments with one-food access

To test if intraperitoneally-injected D-serine inhibit food intake under the single-food access paradigm, and if the effect is dependent on the protein: fat: carbohydrate (PFC) balance of the food, we prepared liquid meals, of which PFC balance can be easily manipulated. We diluted the SanET2.0 (2 kcal/ml) liquid meal 2-fold, either with water (SW meal) or with a 2 kcal/ml lipid emulsion (IntraLipos; SI meal). A liquid meal (either SW or SI) was given in a drinking bottle, and intake was measured daily at 8:00 a.m. The drinking bottles that contained the liquid meal were changed every day to provide fresh liquid meals daily. Six mice per group received an intraperitoneal injection of vehicle (water) or D-serine (1, 2, or 4 g/kg BW) daily at 8:00 a.m. Because the degree of SI intake inhibition varied quite remarkably among the mice tested (Fig. 5), we conducted a sub-analysis of the D-serine-injected groups by defining “responders” as mice that showed less than 40% caloric intake compared to the pre-injection day in any given day during the 3-day D-serine intraperitoneal-injection period.

2.8. Experiments testing the consistency of the responses to D-serine

The sub-analyses of the result in Fig. 5 implied that the response threshold toward D-serine may vary among individual mice even within the age-matched isogenic male mice. To determine whether the sensitivity to D-serine was consistent across days in individual mice, we studied another cohort of age-matched isogenic C57BL6/J male mice. In this cohort, we conducted D-serine intraperitoneal injections/SI presentation experiment three times with 1-week intervals, and asked if the degree of responses are consistent within each individuals over multiple trials. Mice underwent the following 1-week experimental paradigm for a total of three times. For the first 3 days, mice were given the SW liquid meal. On the morning of the fourth day, mice received an intraperitoneal injection of vehicle (water) or D-serine (2 g/kg BW) at 8:00 a.m. After the injection, the liquid meal was changed from SW to SI, and mice received liquid SI meals for one day only. On the fifth day, liquid meal intake was measured, the liquid meal was removed, and the diet was changed to NC for next three days. The same experiments were repeated two more times. The SI intake was compared to the average SW intake of the three previous days of the same week. The same mice received either vehicle (n = 8) or D-serine (n = 16) for all three trials.

2.9. Body weight measurement

BWs were measured daily at 5:30 p.m. for the two-food choice studies and at 8:00 a.m. for the one-food access studies.

2.10. Data analysis

Data are expressed as the mean ± SEM. A p-value <0.05 was considered significant. For multiple group comparisons, data were analyzed with a one-way analysis of variance (ANOVA) and post-hoc Bonferroni correction. For the CTA experiment data in Fig. 2, data were analyzed by a two-way ANOVA with repeated measures with between-subjects factor of Group (D-serine vs. LiCl) and within-subjects Day (C1-C3, T1-2). For multiple comparison, Spjotvoll-Stoline (Tukey's test for unequal sample sizes) post hoc tests were performed when appropriate. Paired t-test was used to compare responses before and after IP injections at each corresponding time of the day in Fig. 4. For sub-analyses of SI-fed mice that received a D-serine IP injection in Fig. 5, data were compared to day 0 and analyzed with the paired t-test.

3. Results

3.1. D-serine intraperitoneal injection inhibited acquisition of a high-fat diet preference

The dose dependence of injected D-serine effects on the acquisition phase of a HFD preference was tested by comparing the patterns of NC ingestion and HFD ingestion. Vehicle-injected mice acquired a preference for HFD within the first 2–3 h after the choices were presented (Fig. 1*A*), while intraperitoneal injections of D-serine inhibited the acquisition of a HFD preference (Fig. 1, *B-D*). At the 2 g/kg dose (D2), D-serine effectively reversed the preference, where mice preferred NC over HFD, without significantly affecting locomotor activity or water intake (Fig. 1, *E* and *F*, and Table 2). At the 4 g/kg dose (D4), D-serine suppressed intake of both foods, locomotor activity, and water intake over the first 6 h (Fig. 1, *D-F*). Although 24-h cumulative intake of NC was not significantly different among four groups ($F(3,24) = 2.773$, $p = 0.063$), 24-h HFD intake was significantly suppressed by the 2 and 4 g/kg doses of D-serine ($F(3,24) = 5.039$, $p = 0.008$) (Fig. 1*G*). The decrease in HFD

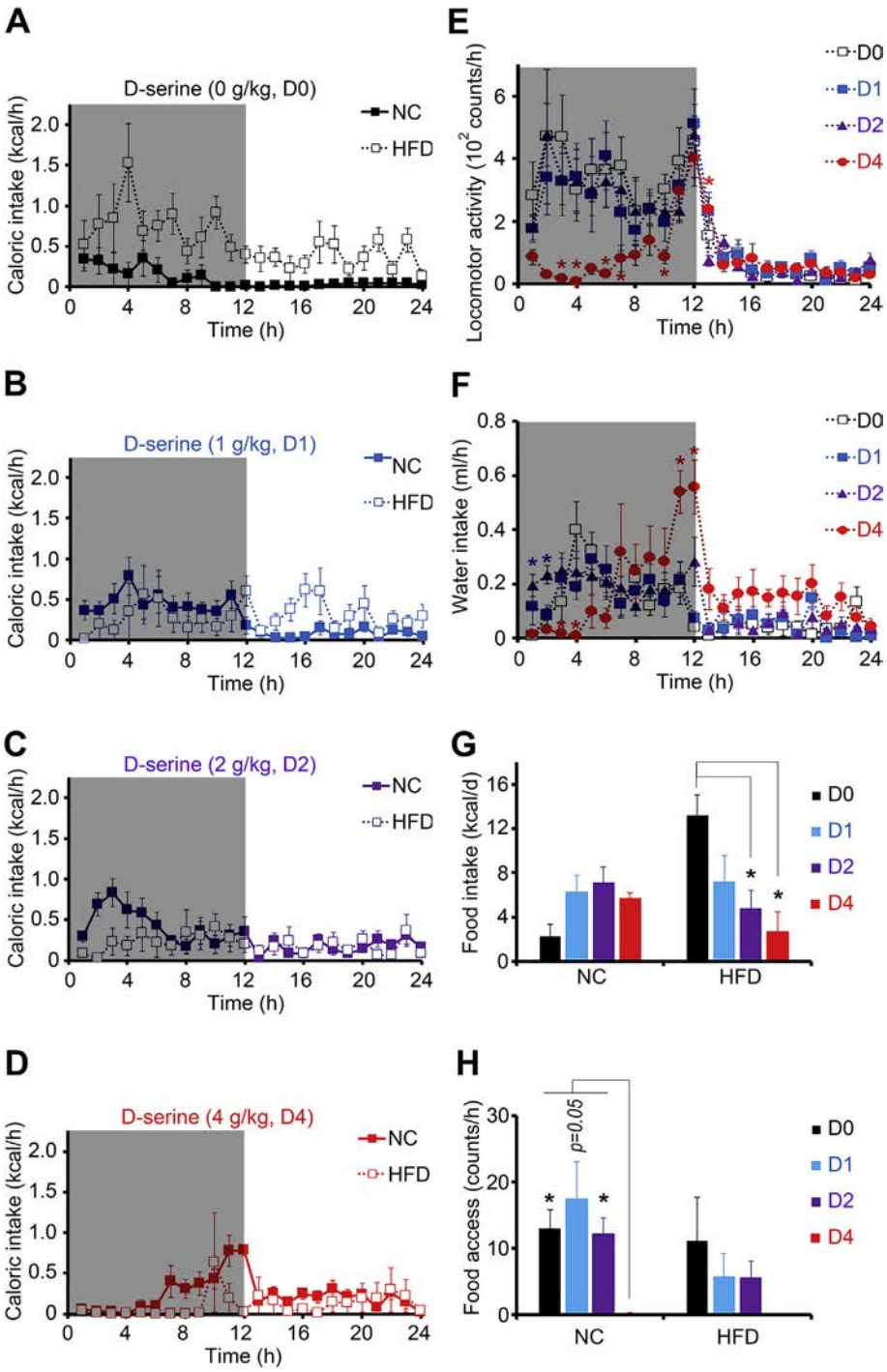

Fig. 1. Intraperitoneal (IP) injection of D-serine suppressed acquisition of a high-fat diet (HFD) preference. *A-D*: The effects of D-serine IP injections on NC intake and HFD intake during the acquisition phase of a HFD preference over the first 24 h after two food choices were presented. The D-serine doses increased as indicated from 0 to 1, 2, and 4 (g/kg BW). Solid squares with solid line: NC intake; open squares with dotted lines: HFD intake. *E–G*: Effect of D-serine IP injections on locomotor activity (*E*), water intake (*F*), and 24-h food intake (*G*). Black open squares: D-serine (0 g/kg BW, D0); blue solid squares: D-serine (1 g/kg BW, D1); purple solid triangles: D-serine (2 g/kg BW, D2); red solid circles: D-serine (4 g/kg BW, D4). Dark shades indicate the dark cycle. *H*: Access counts to NC and HFD during the first hour after two food choices were presented. Data represent the mean ± SEM. We analyzed 6, 7, 8, and 7 mice for 0, 1, 2, and 4 g/kg BW D-serine doses, respectively. In *E-G*, data were analyzed with one-way ANOVA and a post-hoc Bonferroni correction. $^{*}p < 0.05$ compared to the 0% D-serine group. HFD, high-fat diet; NC, normal chow.

intake was unlikely to be due to food neophobia, because the access to HFD did not significantly differ among four groups during the first hour of the dark cycle ($F(3,24) = 1.551$, $p = 0.227$) (Fig. 1*H*), although the highest dose suppressed the access to any food and locomotor activity (Fig. 1, *E* and *H*). Therefore, intraperitoneally-injected D-serine at the 2 g/kg dose (D2) suppressed the acquisition of a HFD preference without affecting locomotor activity or water intake.

Table 2
F values for locomotor activity and water intake at every hour in Fig. 1E–F.

Time	Locomotor	Water intake
1	$F(3,24) = 1.940$, $p = 0.150$	$F(3,24) = 4.723$, $p = 0.010$
2	$F(3,24) = 2.935$, $p = 0.054$	$F(3,24) = 10.75$, $p = 0.0001$
3	$F(3,24) = 4.226$, $p = 0.016$	$F(3,24) = 3.080$, $p = 0.047$
4	$F(3,24) = 3.121$, $p = 0.045$	$F(3,24) = 5.549$, $p = 0.005$
5	$F(3,24) = 2.386$, $p = 0.094$	$F(3,24) = 2.088$, $p = 0.128$
6	$F(3,24) = 3.665$, $p = 0.026$	$F(3,24) = 1.593$, $p = 0.217$
7	$F(3,24) = 2.362$, $p = 0.097$	$F(3,24) = 0.882$, $p = 0.455$
8	$F(3,24) = 0.754$, $p = 0.531$	$F(3,24) = 0.852$, $p = 0.479$
9	$F(3,24) = 1.094$, $p = 0.371$	$F(3,24) = 0.975$, $p = 0.421$
10	$F(3,24) = 3.865$, $p = 0.022$	$F(3,24) = 0.584$, $p = 0.570$
11	$F(3,24) = 0.782$, $p = 0.515$	$F(3,24) = 5.041$, $p = 0.003$
12	$F(3,24) = 0.240$, $p = 0.868$	$F(3,24) = 10.01$, $p = 0.0002$
13	$F(3,24) = 3.663$, $p = 0.026$	$F(3,24) = 3.408$, $p = 0.034$
14	$F(3,24) = 2.674$, $p = 0.070$	$F(3,24) = 1.560$, $p = 0.202$
15	$F(3,24) = 0.580$, $p = 0.634$	$F(3,24) = 1.312$, $p = 0.294$
16	$F(3,24) = 1.038$, $p = 0.394$	$F(3,24) = 1.858$, $p = 0.164$
17	$F(3,24) = 0.798$, $p = 0.507$	$F(3,24) = 2.459$, $p = 0.087$
18	$F(3,24) = 0.178$, $p = 0.910$	$F(3,24) = 1.124$, $p = 0.359$
19	$F(3,24) = 1.098$, $p = 0.369$	$F(3,24) = 3.441$, $p = 0.033$
20	$F(3,24) = 0.900$, $p = 0.456$	$F(3,24) = 2.734$, $p = 0.066$
21	$F(3,24) = 0.468$, $p = 0.707$	$F(3,24) = 1.954$, $p = 0.148$
22	$F(3,24) = 1.204$, $p = 0.330$	$F(3,24) = 3.338$, $p = 0.036$
23	$F(3,24) = 1.307$, $p = 0.295$	$F(3,24) = 2.609$, $p = 0.075$
24	$F(3,24) = 1.963$, $p = 0.147$	$F(3,24) = 0.702$, $p = 0.560$

3.2. D-serine intraperitoneal injection was insufficient to condition taste aversion in mice

We performed the conditioned taste aversion experiment using saccharine solution as conditioned stimulus (CS) and lithium chloride injection as unconditioned stimulus (US), and compared the potency of intraperitoneal injection of D-serine (2 g/kg dose, D2) to condition taste aversion. D2 was insufficient to condition taste aversion in mice (Group (US), $F(1,15) = 10.66$, $p = 0.005$; Day, $F(4,60) = 7.98$, $p = 0.00003$; US x Day, $F(4,60) = 2.05$, $p = 0.099$) (Fig. 2). No difference was found in the CS intake between C1 and C3 or T1 days within D-serine group (C3, $p = 0.821$; T1, $p = 0.997$; Spjotvoll-Stoline test), while the CS intake on C3 and T1 in the LiCl-injected group was significantly reduced in comparison to that on C1 day (C3, $p = 0.016$; T1, $p = 0.0095$; Spjotvoll-Stoline test).

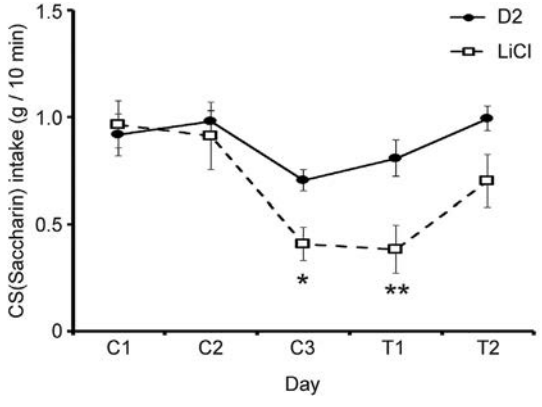

Fig. 2. D-serine does not cause conditioned taste aversion in male mice. 5 mM saccharin solution was used as a conditioned stimulus (CS), and the intraperitoneal injection of lithium chloride (LiCl) was used as an unconditioned stimulus (US). Intraperitoneal injection of D-serine (2 g/kg BW, D2) was assessed for its ability to condition taste aversion. C1-C3, conditioning days. T1-T2, test days. Results of the conditioned taste aversion experiment indicated by the CS intake over the 10-min presentation period. We analyzed 9 and 8 mice for D2 group and LiCl group, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the CS intake of C1 in the corresponding group by Spjotvoll-Stoline test.

3.3. Brain D-serine levels were increased by oral and intraperitoneal D-serine administration

We measured the levels of D-serine and L-serine in the cerebral cortex, hypothalamus, and cerebellum, before and after D-serine administration. Both intraperitoneal and oral administrations of D-serine significantly raised the brain D-serine levels in the cerebral cortex ($F(3,8) = 36.3$, $p = 5.2 \times 10^{-5}$), hypothalamus ($F(3,8) = 121.7$, $p = 5.2 \times 10^{-7}$), and cerebellum ($F(3,8) = 26.3$, $p = 1.7 \times 10^{-4}$). Injected mice exhibited D-serine levels several fold higher than the endogenous D-serine levels observed in pre-injected mice, in the parts of the brain measured (Fig. 3*A*). In the cerebral cortex and the hypothalamus, at 24 h after the intraperitoneal injection, D-serine levels remained significantly higher than the endogenous levels in pre-injected brains. In the cerebellum, endogenous D-serine levels were low and exogenous D-serine was rapidly cleared. These findings may have been due to the high expression levels of D-amino acid oxidase, which degrades D-serine (Horio et al., 2011). With oral administration, after 48 h, brain D-serine was raised to levels comparable to those observed at 2 h after the intraperitoneal injection. In all conditions tested, administrations of D-serine did not affect the levels of L-serine in the cerebral cortex ($F(3,8) = 3.094$, $p = 0.090$), hypothalamus ($F(3,8) = 4.031$, $p = 0.051$), and cerebellum ($F(3,8) = 2.189$, $p = 0.167$) (Fig. 3*B*).

3.4. A single intraperitoneal D-serine injection was insufficient to prevent expression of a previously learned HFD preference

The effect of D-serine on the expression of a previously learned

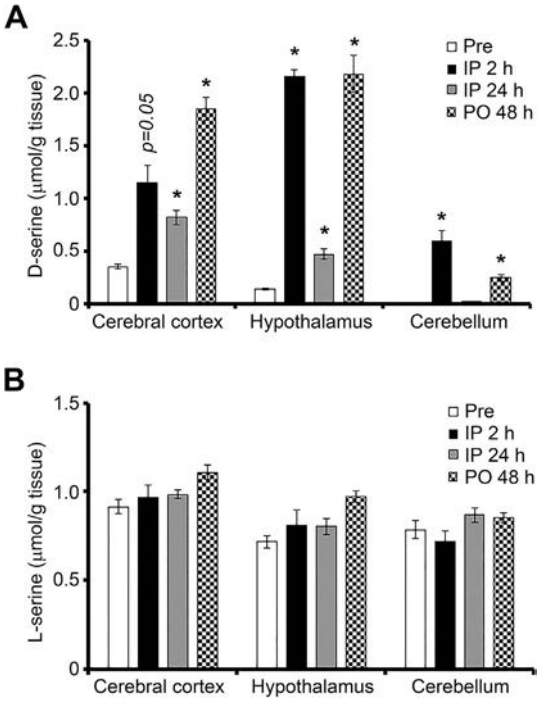

Fig. 3. Brain D-serine and L-serine levels after D-serine administration. *A-B*: Levels of D-serine (*A*) and L-serine (*B*) were measured in the cerebral cortex, hypothalamus, and cerebellum before and after D-serine administration by intraperitoneal injection (2 g/kg BW) or oral administration in 1.5% (w/v) D-serine water, taken *ad libitum*. Open bars (Pre), before D-serine administration; black bars (IP 2h), 2 h after D-serine IP injection; gray bars (IP 24h), 24 h after D-serine IP injection; checkered bars (PO 48h), 48 h after *ad libitum* intake of D-serine water. Data represent the mean ± SEM (n = 3 per group). Data were analyzed with one-way ANOVA and post-hoc Bonferroni correction. $^{*}p < 0.05$, compared to the corresponding tissue from the pre-injection group. BW, body weight; IP, intraperitoneal; PO, per os.

HFD preference was tested. We found that 24-h intake of NC and HFD was not significantly different between before and after the single intraperitoneal injection of vehicle or D-serine (A, t = 1.259, df = 5, $p = 0.264$; B, t = −0.453, df = 5, $p = 0.670$; C, t = 1.867, df = 5, $p = 0.121$; D, t = 2.304, df = 5, $p = 0.069$) (Fig. 4, *A-D*). Locomotor activities were not affected by the injection of vehicle (t = −0.512, df = 5, $p = 0.630$) or D-serine (t = 1.844, df = 5, $p = 0.124$), but water intake was unexpectedly by the injection of vehicle (t = 2.723, df = 5, $p = 0.042$) but not D-serine (t = 0.344, df = 5, $p = 0.745$) (Fig. 4, *E-H*).

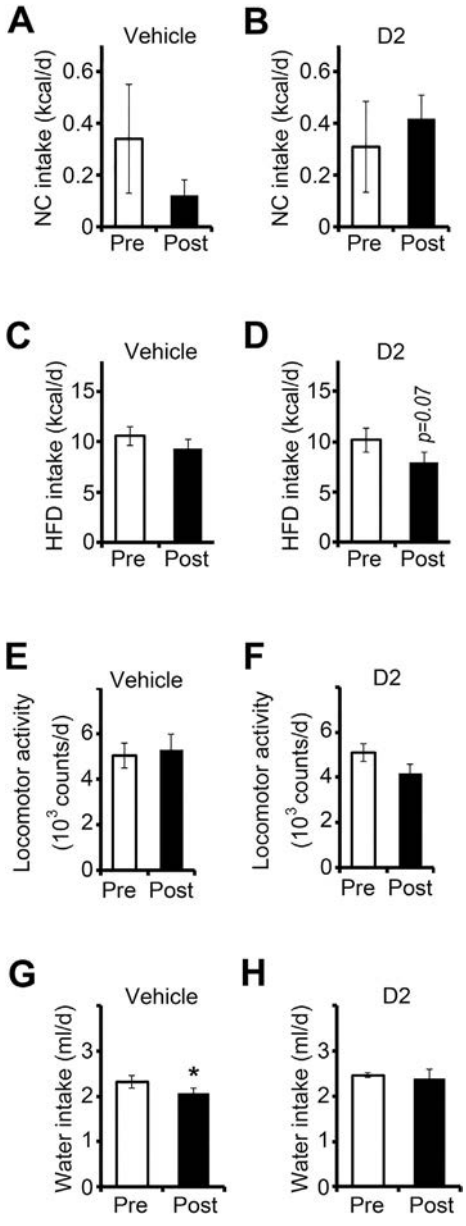

Fig. 4. A single intraperitoneal (IP) injection of D-serine was not sufficient to cancel an existing high-fat diet (HFD) preference. *A-D*: Effect of an IP injection of vehicle or D-serine (2 g/kg BW, D2) on NC intake (*A* and *B*) or HFD intake (*C* and *D*) in mice that had expressed a preference for HFD. *E–H*: Effect of an IP injection of vehicle or D-serine (2 g/kg BW, D2) on locomotor activity (*E* and *F*) and water intake (*G* and *H*) in mice that had expressed a preference for HFD. Open bars: pre-injection data; black bars, post-injection data. Data represent the mean ± SEM (n = 6 per group). $^*p < 0.05$ between two groups based on paired *t*-test. HFD, high-fat diet; NC, normal chow.

3.5. Daily intraperitoneal D-serine injections effectively inhibited high-fat liquid meal intake

We tested the effect of intraperitoneally-injected D-serine under the single-food access paradigm using liquid meals with different protein: fat: carbohydrate balance. We diluted the SanET2.0 (2 kcal/ml) liquid meal 2-fold, either with water (SW meal) or with a 2 kcal/ml lipid emulsion (IntraLipos; SI meal). We found that daily intraperitoneal injections of D-serine dose-dependently inhibited the intake of both SW and SI, with a stronger effect on the higher fat-containing (67%) SI (Fig. 5*B*: 1st day, $F(3,20) = 5.708$, $p = 0.0054$; 2nd day, $F(3,20) = 7.460$, $p = 0.0015$; 3rd day, $F(3,20) = 3.552$, $p = 0.033$) than the lower fat-containing (34%) SW (Fig. 5*A*: 1st day, $F(3,20) = 4.795$, $p = 0.011$; 2nd day, $F(3,20) = 9.708$, $p = 3.7 \times 10^{-4}$; 3rd day, $F(3,20) = 10.88$, $p = 1.9 \times 10^{-4}$). The decrease in liquid meal intake was associated with body weight loss, showing more pronounced weight loss in the SI group (Fig. 5*G*: 1st day, $F(3,20) = 8.544$, $p = 7.5 \times 10^{-4}$; 2nd day, $F(3,20) = 9.690$, $p = 3.7 \times 10^{-4}$; 3rd day, $F(3,20) = 8.082$, $p = 0.010$) than the SW group (Fig. 5*F*: 1st day, $F(3,20) = 7.075$, $p = 0.0020$; 2nd day, $F(3,20) = 13.70$, $p = 4.4 \times 10^{-5}$; 3rd day, $F(3,20) = 17.16$, $p = 9.4 \times 10^{-6}$). However, the degree of SI intake suppression varied quite remarkably among the mice tested.

3.6. The effect of D-serine injection on suppressing high-fat liquid meal intake varied among mice

The sub-analysis of the D-serine-injected groups revealed that the responders in each dosing group showed similar degrees of intake suppression on all three days, but the number of responders increased with increasing doses of D-serine (Fig. 5, *C-E*, Table 3). The high D-serine dose also marginally suppressed SI intake on the first day in "non-responder" mice, but the effects became weak by the 3rd day. The degree of BW loss was associated with SI intake (Fig. 5, *H-J*, Table 3).

3.7. The differential responses to D-serine were consistent among multiple trials

We conducted D-serine intraperitoneal injections/SI presentation experiment three times with 1-week intervals, and asked if the degree of responses are consistent within each individuals over multiple trials. We identified six non-responders and ten responders among the 16 mice tested with D-serine. Overall, D-serine became more effective over the three trials, both in non-responders and responders in inhibiting caloric intake (1st injection, $F(2,21) = 13.389$, $p = 1.8 \times 10^{-4}$; 2nd trial, $F(2,21) = 120.9$, $p = 3.0 \times 10^{-12}$; 3rd injection, $F(2,21) = 47.91$, $p = 1.5 \times 10^{-8}$) (Fig. 6, *A-D*), and causing weight loss 1st injection, $F(2,21) = 8.431$, $p = 0.021$; 2nd trial, $F(2,21) = 80.80$, $p = 1.8 \times 10^{-9}$; 3rd injection, $F(2,21) = 44.15$, $p = 3.0 \times 10^{-8}$) (Fig. 6, *E-H*). However, the non-responders were less responsive than the responders in all three trials; moreover, the responders remained responders in all three trials, indicating that each mice might have had a unique and consistent sensitivity threshold to D-serine.

4. Discussion

In the present study, we found that intraperitoneal-injected D-serine effectively suppressed the acquisition of a HFD preference. The decrease in HFD intake was unlikely to be due to food neophobia, because the access to HFD did not significantly differ among four groups during the first hour of the dark cycle (Fig. 1*H*). Meanwhile, IP D-serine (2 g/kg BW dose) did not produce aversive visceral discomfort to induce conditioned taste aversion to saccharin CS in

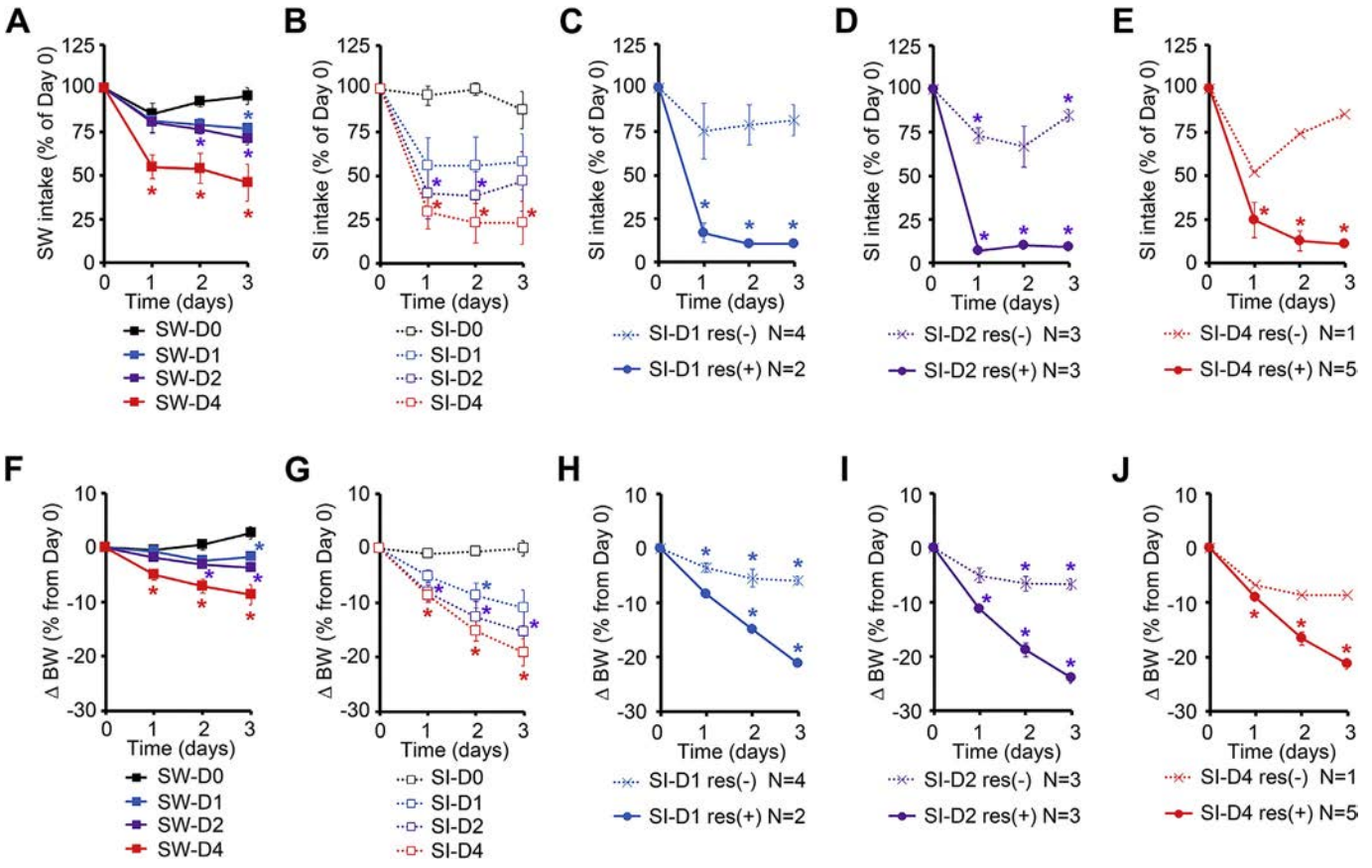

Fig. 5. Daily intraperitoneal (IP) injections of D-serine suppressed intake of high-fat liquid meals more effectively than standard liquid meals. *A-B*: Effects of daily D-serine injections on intakes of SW (*A*) and SI (*B*). Black squares (D0): D-serine 0 g/kg BW; blue squares (D1): D-serine 1 g/kg BW; purple squares (D2): D-serine 2 g/kg BW; red squares (D4): D-serine 4 g/kg. Data represent the mean ± SEM (n = 6 per group). Data were analyzed with one-way ANOVA and post-hoc Bonferroni correction. $^*p < 0.05$ compared to the D0 group at each time point. *C-E*: Sub-analyses of SI-fed mice that received a D-serine IP injection of 1 g/kg BW (*C*), 2 g/kg BW (*D*), or 4 g/kg (*E*). Circles with solid line (res+): responders; crosses with dotted lines (res-): non-responders. Data represent the mean ± SEM (n is indicated in each figure). Data were compared to day 0 (after 1 wk of SW, before injection), and analyzed with the paired *t*-test. $^*p < 0.05$ compared to day 0. *F-G*: Effect of daily D-serine injections on BW changes in mice fed SW (*F*) and or SI (*G*). Color coding is the same as that shown in (*A*). Data represent the mean ± SEM (n = 6 per group). Data were analyzed with one-way ANOVA and post-hoc Bonferroni correction. $^*p < 0.05$ compared to the D0 group at each time point. *H-J*: Sub-analyses of SI-fed mice that received a D-serine IP injection of 1 g/kg BW (*H*), 2 g/kg BW (*I*), and 4 g/kg (*J*). Color and symbol coding are the same as those shown in (*C*). Data represent the mean ± SEM (n is indicated in each figure). Data were compared to day 0 and analyzed with the paired *t*-test. $^*p < 0.05$ compared to day 0. ΔBW: changes in body weight; res: response; SI: SanET2.0/IntraLipos liquid meal; SW: SanET2.0/water liquid meal.

Table 3
Summary of statistics on sub-group analyses in Fig. 5C–E and H-J.

Percent caloric intake of each day compared to day 0			
	Day 1	Day 2	Day 3
D1 res (−)	t = 1.557, df = 3, $p = 0.217$	t = 1.836, df = 3, $p = 0.164$	t = 2.068, df = 3, $p = 0.130$
D1 res (+)	t = 14.72, df = 1, $p = 0.043$	t = 192.0, df = 1, $p = 0.0032$	t = 307.7, df = 1, $p = 0.0021$
D2 res (−)	t = 6.122, df = 2, $p = 0.026$	t = 2.788, df = 2, $p = 0.108$	t = 4.420, df = 2, $p = 0.048$
D2 res (+)	t = 187.3, df = 2, $p = 2.9 \times 10^{-5}$	t = 109.4, df = 2, $p = 8.3 \times 10^{-5}$	t = 159.0, df = 2, $p = 4.0 \times 10^{-5}$
D4 res (−)	N.D.[1]	N.D.[1]	N.D.[1]
D4 res (+)	t = 7.335, df = 4, $p = 0.0018$	t = 15.34, df = 4, $p = 0.00011$	t = 95.21, df = 4, $p = 7.3 \times 10^{-8}$
Percent change in body weight of each day compared to day 0			
	Day 1	Day 2	Day 3
D1 res (−)	t = 4.251, df = 3, $p = 0.024$	t = 3.354, df = 3, $p = 0.044$	t = 7.592, df = 3, $p = 0.0047$
D1 res (+)	t = 12.01, df = 1, $p = 0.053$	t = 39.38, df = 1, $p = 0.016$	t = 320.2, df = 1, $p = 0.0020$
D2 res (−)	t = 3.733, df = 2, $p = 0.065$	t = 4.780, df = 2, $p = 0.041$	t = 6.732, df = 2, $p = 0.021$
D2 res (+)	t = 24.76, df = 2, $p = 0.0016$	t = 15.14, df = 2, $p = 0.0043$	t = 25.20, df = 2, $p = 0.0016$
D4 res (−)	N.D.[1]	N.D.[1]	N.D.[1]
D4 res (+)	t = 6.195, df = 4, $p = 0.0035$	t = 10.70, df = 4, $p = 0.00043$	t = 13.10, df = 4, $p = 0.00020$

[1]N.D., not done.
There was only 1 mouse in the D4 res (−) group, so there is no statistical data for this group.

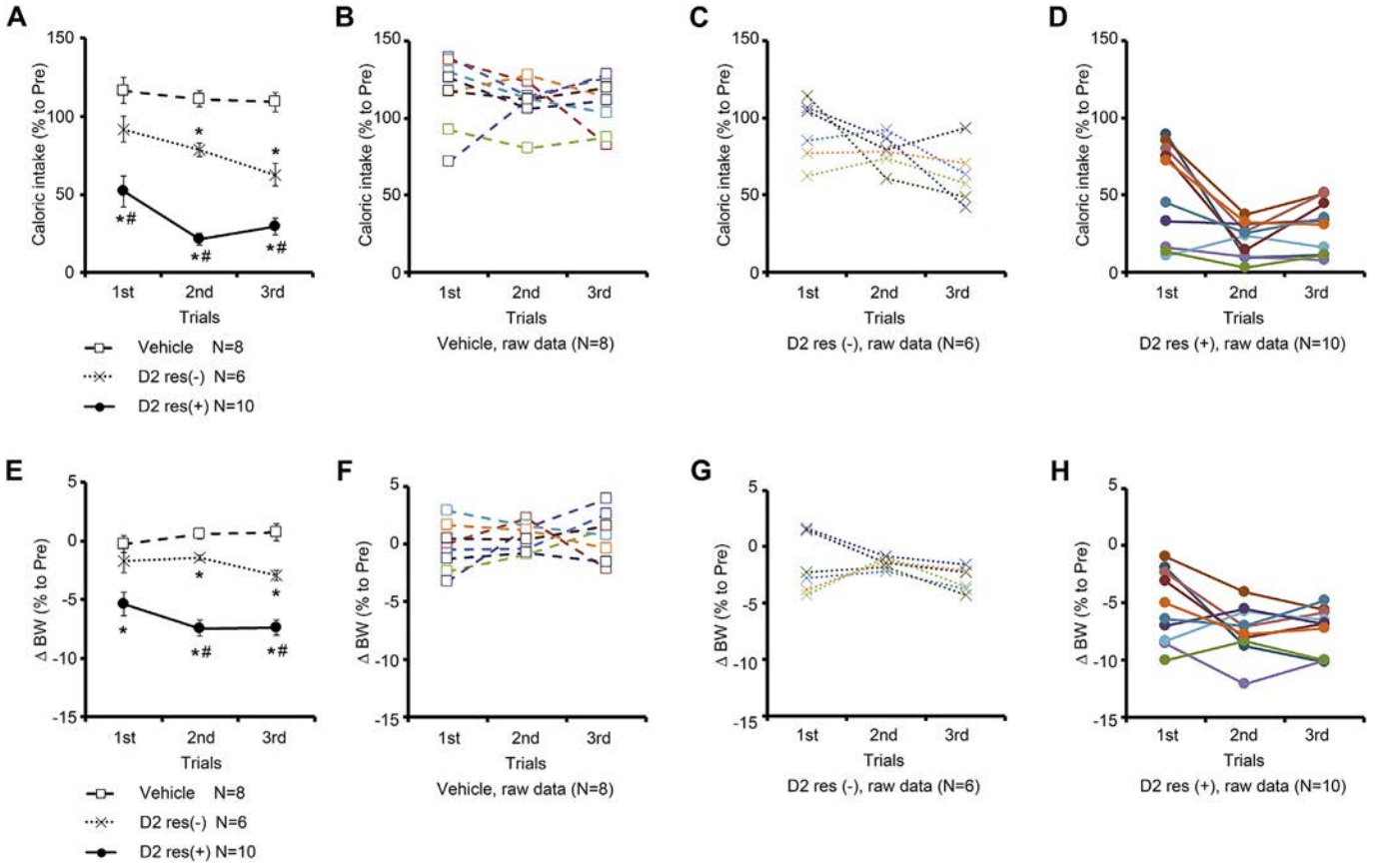

Fig. 6. Varied response to D-serine is reproducible among multiple trials. *A*: Mean 24-h caloric intakes after intraperitoneal injection of vehicle or D-serine (2 g/kg BW, D2). Mice were presented with SI liquid meals, and the trials were repeated three separate times. Data were normalized to the average SW intake over the three days prior to the injection for each trial. Open squares with dashed lines: vehicle; crosses with dotted lines: D2 mice with low responses (non-responders; res-); black circles with solid lines: D2 mice with high responses (responders, res+). Data represent the mean ± SEM (n = 6–10 per group). Data were analyzed with one-way ANOVA and post-hoc Bonferroni correction. $^{*}p < 0.05$ compared to the vehicle group for each trial. $\#p < 0.05$ compared between the D2 responders and non-responders for each trial. *B-D*: Plots of raw data from the experiment shown in (*A*). Vehicle group (*B*, n = 8), D2 non-responders (*C*, n = 6), and D2 responders (*D*, n = 10). Each paired line and symbol represents a single mouse. *E*: 24-h BW changes after intraperitoneal injections of vehicle or D-serine in the experiments described above. Data were normalized to the BW just before the injection for each trial. Color coding is the same as that shown in (*A*). Data represent the mean ± SEM (n = 6–10 per group). Data were analyzed with one-way ANOVA and post-hoc Bonferroni correction. $^{*}p < 0.05$ compared to the vehicle group for each trial. $\#p < 0.05$ compared between D-serine responders and non-responders for each trial. *F–H*: Plots of raw data from the experiment described in (*E*). Vehicle group (*F*, n = 8), D-serine injected non-responders (*G*, n = 6), and D-serine injected responders (*H*, n = 10). Each paired line and symbol represents a single mouse. ΔBW, changes in body weight; res, response.

one-bottle test, suggesting that D-serine does not have aversive property (Fig. 2). Nevertheless, the inhibited acquisition of HFD preference in the D2 group cannot be explained simply by conditioned taste aversion or food neophobia, and we can hypothesize that HFD intake in mice with D-serine is reduced by drug-induced reduction of preference for HFD. The reversal of preference (NC preferred over HFD) during the acquisition phase was associated with an increase in D-serine levels in the cerebral cortex, hypothalamus, and cerebellum (Fig. 3). However, a single intraperitoneal injection of 2 g/kg D-serine, which was sufficient to suppress the acquisition of a HFD preference, was insufficient to prevent expression of a previously learned HFD preference (Fig. 4). We expected a cancellation, and not a reversal, of a previously learned HFD preference, because orally administered D-serine prevented expression of a previously learned HFD preference (but it did not reverse the preference) in our previous study (Sasaki et al., 2015).

Intraperitoneally-injected D-serine most effectively suppressed the high-fat liquid meal intake; and this effect was dose-dependent, but the responses varied significantly among male C57BL/6J mice of the same age (Fig. 5). Over multiple trials, individuals consistently showed consistent degree of responses, indicating that each mice might have a unique sensitivity threshold to D-serine (Fig. 6). In summary, intraperitoneally-injected D-serine inhibited HFD intake and preference acquisition. The sensitivity to D-serine varied among mice with the same genetic background; thus, sensitivity may be associated with unidentified traits.

The results of the current study (intraperitoneal administration) and the previous study (oral administration) are summarized in Table 4. Under the one-food access paradigm, oral D-serine did not affect NC intake, but intraperitoneally-injected D-serine reduced SW intake (Fig. 5). In the latter case, mice had been consuming the SW liquid meal prior to the D-serine injection; thus, the result could

Table 4
Summary of the feeding behavior phenotypes observed after D-serine administration through oral (PO) and intraperitoneal (IP) routes.

	PO	IP
One-food access	NC: →	SW: ↓
	HFD: ↓↓	SI: ↓↓
HFD preference	Acquisition: suppressed	Acquisition: suppressed
	Maintenance: cancelled	Maintenance: no effect

PO data are from our previous study (Sasaki et al., 2015).
HFD, high-fat diet; IP, intraperitoneal; NC, normal chow; PO, per os; SI, SanET2.0/IntraLipos liquid meal; SW, SanET2.0/water liquid meal.

not be explained by an effect on the acquisition phase of food preference. However, it might have been due to the difference in the PFC balance between NC and the SW liquid meal (Table 1). The SW liquid meal contained more fat (34%) than NC (12%) and, considering that the effect of D-serine was more pronounced on SI intake (67% of calories were fat), we can speculate that D-serine affected the SW liquid meal intake, because it contains more fat and thus was preferred more than NC.

In the previous study, exogenous D-serine was administered by delivering D-serine in water and allowing mice to drink *ad libitum*. In that study, we observed effects on both the acquisition of a HFD preference and the maintenance of a previously learned HFD preference (Table 4). Conversely, in the present study, an intraperitoneal-injection of D-serine affected only the acquisition phase, not the maintenance phase of a HFD preference. This difference suggested that prolonged D-serine action may be required to override a previously learned HFD preference. Alternatively, the sensitivity threshold may be different for each phase of the HFD preference, because the phases may involve different neural circuits; indeed, the occupancy of co-agonist binding sites on NMDA receptors varies among synapses (Mothet et al., 2000).

In the previous study, we noticed that oral D-serine had different degrees of potency in its effects on the acquisition of a HFD preference among different strains of mice; the strength of the effects declined in the following order: C57BL6/J, ICR, and BKS (Sasaki et al., 2015). The effectiveness in those experiments was inversely correlated with the variability in the data; the highest variability was observed in BKS mice and the lowest was observed in C57BL6/J. However, in the present study, we also observed variable sensitivity to injected D-serine in a cohort of isogenic C57BL6/J mice. Therefore, both inter-strain differences and intra-strain differences may affect the sensitivity to D-serine's effects on feeding behavior. Substantial intra-strain variability was previously shown to exist in various inbred mouse strains. For instance, sociability and corpus callosum development were shown to be highly variable in BALB/c mice (Fairless et al., 2008). Large individual differences in the extent of food restriction-evoked hyperactivity were also reported within single litters of C57BL6 mice, in a study that generated activity-based anorexia models (Aoki, Chowdhury, Wable, & Chen, 2017). Nevertheless, genetic factors can influence food preferences (Pirastu et al., 2012). These factors accounted for approximately 20% of variation in carbohydrate and fat preferences in humans (Faith, Rha, Neale, & Allison, 1999). Genetic polymorphisms at *TAS1R3* (a component of the sweet receptor) and *GNAT3* (the taste-specific G alpha protein subunit gustducin) were associated with variations in sucrose sensitivity in humans (Fushan, Simons, Slack, & Drayna, 2010; Fushan, Simons, Slack, Manichaikul, & Drayna, 2009). Therefore, unidentified traits and/or genetic polymorphisms in C57BL6/J mice could be a cause for the variability we observed in D-serine responses.

In conclusion, both oral and IP administered D-serine influenced feeding behavior. We observed different thresholds for the effects of D-serine on the acquisition and maintenance phases of food preference. Individual variability among phenotypes might be associated with unidentified traits. Although the model used in this study requires pharmacological dose of D-serine and therefore artificial in its nature, it nevertheless provides a new tool for dissecting the neural circuits responsible for differential responses to food cues. It may provide clues to understanding why some people overeat while others may become anorectic even under the similar circumstance.

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