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N-methyl-D-aspartate receptor coagonist D-serine suppresses intake of high-preference food

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Sasaki T, Kinoshita Y, Matsui S, Kakuta S, Yokota-Hashimoto H, Kinoshita K, Iwasaki Y, Kinoshita T, Yada T, Amano N, Kitamura T. *N*-methyl-D-aspartate receptor coagonist D-serine suppresses intake of high-preference food. *Am J Physiol Regul Integr Comp Physiol* 309: R561–R575, 2015. First published July 8, 2015; doi:10.1152/ajpregu.00083.2015.—D-Serine is abundant in the forebrain and physiologically important for modulating excitatory glutamatergic neurotransmission as a coagonist of synaptic *N*-methyl-D-aspartate (NMDA) receptor. NMDA signaling has been implicated in the control of food intake. However, the role of D-serine on appetite regulation is unknown. To clarify the effects of D-serine on appetite, we investigated the effect of oral D-serine ingestion on food intake in three different feeding paradigms (one-food access, two-food choice, and refeeding after 24-h fasting) using three different strains of male mice (C57Bl/6J, BKS, and ICR). The effect of D-serine was also tested in leptin signaling-deficient *db*/*db* mice and sensory-deafferented (capsaicin-treated) mice. The expression of orexigenic neuropeptides [neuropeptide Y (*Npy*) and agouti-related protein (*Agrp*)] in the hypothalamus was compared in fast/refed experiments. Conditioned taste aversion for high-fat diet (HFD) was tested in the D-serine-treated mice. Under the one-food-access paradigm, some of the D-serine-treated mice showed starvation, but not when fed normal chow. HFD feeding with D-serine ingestion did not cause aversion. Under the two-food-choice paradigm, D-serine suppressed the intake of high-preference food but not normal chow. D-Serine also effectively suppressed HFD intake but not normal chow in *db*/*db* mice and sensory-deafferented mice. In addition, D-serine suppressed normal chow intake after 24-h fasting despite higher orexigenic gene expression in the hypothalamus. D-Serine failed to suppress HFD intake in the presence of L-701,324, the selective and full antagonist at the glycine-binding site of the NMDA receptor. Therefore, D-serine suppresses the intake of high-preference food through coagonism toward NMDA receptors.

anorexia; food preference

D-SERINE IS AN *N*-methyl-D-aspartate (NMDA) receptor coagonist and facilitates excitatory glutamatergic neurotransmission at synapses within the nervous system (29, 35, 52). D-Serine is abundant in the forebrain, and the concentration of D-serine within the brain correlates with the density of NMDA receptor (24, 25, 48, 62). NMDA receptor is a heteromeric cation channel made of two GluN1 subunits and two GluN2 subunits (51). L-Glutamate binds to GluN2 subunits, whereas coagonists D-serine and glycine bind to GluN1 subunits (29, 35, 46). Coagonism is necessary for the heteromeric pairing of ligand-binding domains of NMDA receptor, which is an essential step for forming functional ion channels (12).

Several reports indicate that NMDA signaling regulates food intake. Studies have suggested that NMDA agonist suppresses food intake, and NMDA inhibitors promote food intake (21, 55, 64, 66, 68). Interestingly, another NMDA receptor coagonist, glycine, is the only amino acid found at low levels in the plasma of obese subjects (16). Conversely, anorexia nervosa patients have been reported to have high plasma glycine levels (47, 50).

Glycine has its own cognate glycine receptors that mediate inhibitory neurotransmission (43) and has multiple modes of action outside the nervous system. For example, it is a major source/component of collagen, glutathione, and bile acid conjugation, and it is also used for biosynthesis of heme, creatine, nucleic acids, and uric acid (73). On the other hand, D-serine is known to function only as an NMDA receptor coagonist in vivo (26). Furthermore, D-serine has a stronger coagonist effect than glycine both in vitro and in vivo (4, 44). D-Serine fully occupies the glycine sites of NMDA receptors at some synapses while not saturating at other sites (46). D-Serine is found in fermented foods, microorganisms, plants, and marine invertebrates (17). Although most of us eat these foods without any apparent consequence on appetite, oral ingestion of food rich in D-serine may modulate D-serine concentration in vivo (especially where the endogenous D-serine level is not high enough to saturate the glycine sites of NMDA receptors) and affect appetite.

In this study, we orally administered D-serine (or L-serine, which lacks the coagonism toward NMDA receptors) to mice to test the effect on appetite. We investigated the effect of oral D-serine ingestion on food intake in three different feeding paradigms (one-food access, two-food choice, and refeeding after 24-h fasting) using three different strains of male mice (C57Bl/6J, BKS, and ICR). The effect of D-serine was also tested in leptin signaling-deficient *db*/*db* mice and sensory-deafferented (capsaicin-treated) mice. The expressions of orexigenic neuropeptides [neuropeptide Y (*Npy*) and agouti-related protein (*Agrp*)] in the hypothalamus were compared in fast/refed experiments to address the effect of D-serine on the relationship between food intake and metabolic needs for

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energy of mice. Conditioned taste aversion for high-fat diet (HFD) was tested in the D-serine-treated mice. Finally, we tested if coagonism toward NMDA receptors is required for the effect of D-serine on appetite using L-701,324, the selective and full antagonist at the glycine-binding site of the NMDA receptor.

MATERIALS AND METHODS

Animals. Male ICR mice and C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). For the *db*/*db* study, *db*/+ mice purchased from CLEA Japan were bred in-house to obtain male *db*/*db* mice and control littermates. For the duration of the studies, the mice were housed in individual cages in a temperature-controlled facility on a 12:12-h light-dark cycle (6:00 A.M.–6:00 P.M.) and had ad libitum access to food and water. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committees at Gunma University and Shinshu University.

Body weight and food intake measurements. Body weight and food intake were measured daily between 8:00 and 9:00 A.M. unless otherwise indicated. Cages and water bottles were changed on a weekly basis. Mice were acclimated to feeding from multifeeders (Shinfactory, Fukuoka, Japan). For the experiments using the one-food-access paradigm, one multifeeder was placed in a cage. For the experiments using the two-food-choice paradigm, two multifeeders were placed in a cage, and the positions of the multifeeders were changed every day. Normal chow (NC, CE-2) and HFD (HFD32) were purchased from CLEA Japan. High-sucrose diet (HSD, F2HScD), which consists of 50% sucrose and 50% standard diet (MF), was purchased from Oriental Yeast (Tokyo, Japan). High-protein diet (HPD) was custom-made by Oriental Yeast based on the components of HPD used by another group (56). The calorie-based macronutrient composition of the food used in this study is provided in Table 1. For one-food-access experiments, either NC, HFD, HSD, or HPD was given. For two-food-choice experiments, NC and either HFD, HSD, or HPD were given. D-Serine and L-serine (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in water at the concentration of 0.5, 1.0, or 1.5% (wt/vol) and given to mice in the form of drinking water.

Sensory deafferentation by capsaicin. Sensory deafferentation by capsaicin was performed as previously described (27, 28). Capsaicin (Wako Pure Chemical Industries) was solubilized in vehicle (10% ethanol-10% Tween 80-80% saline). The capsaicin solution was subcutaneously injected in the backs of 6-wk-old male ICR mice on *day 1* (40 mg/kg) and *day 3* (50 mg/kg) under anesthesia with tribromethanol (Sigma Aldrich, Tokyo, Japan). On *day 5*, 5 mg/kg capsaicin were injected intraperitoneally without anesthesia. Mice were deafferented by *day 5* and did not show any sign of distress upon intraperitoneal injection of capsaicin. To confirm the effect of capsaicin, mice underwent an eye-wipe test on *day 7* and cholecystokinin-8 (CCK8) test on *day 8*. For the eye-wipe test, 5 μl of 0.5 mM capsaicin solution (in 5% DMSO-10% Tween 80–85% saline) were applied to one eye, and the number of wiping movements was counted for a minute; mice with <10 wipes were used. For the preprocedure CCK8 test, mice fasted for 8 h were intraperitoneally injected with 4 ng/g CCK8 (Peptide Institute, Osaka, Japan) just before the onset of the dark cycle, and their 1-h NC intake was measured to check for a loss of feeding reduction by CCK8. We used <20% reduction in feeding by CCK as a cut-off for the loss of CCK8 effect (27). Mice underwent a postprocedure CCK8 test for which a 24-h fasting period was used. Data from mice without food intake suppression by CCK8 were analyzed. Among 22 capsaicin-treated mice, 1 mouse was excluded from the study.

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

	Protein, %	Fat, %	Carbohydrate, %	Calorie, kcal/g
Normal chow (CE2)	28.9	12.0	59.1	3.449
High-fat diet (HFD32)	20.1	56.7	23.2	5.076
High-sucrose diet	13.4	6.4	80.3	3.670
High-protein diet	40.9	17.4	41.6	3.700

Tissue sampling. After mice were killed with pentobarbital sodium overdose, brain samples were quickly removed, and hypothalamic samples were harvested. Bilateral epididymal white adipose tissues (eWAT) were harvested, and their weights were measured.

Real-time polymerase chain reaction. RNA extraction, cDNA synthesis, and real-time PCR were performed using standard protocols (60). *Npy* and *Agrp* expression levels were normalized to β-actin (*Actb*). The primers used in the study were as follows: *Actb*, AGCCTTCCTTCTTGGGTA (forward), GAGCAATGATCTTGATCTTC (reverse); *Agrp*, GAGTTCCCAGGTCTAAGTCTGAATG (forward), ATCTAGCACCTCCGCCAAAG (reverse); and *Npy*, TACTCCGCTCTGCGACACTA (forward), TCTTCAAGCCTTGTTCTGGG (reverse).

Conditioned taste aversion test. To test if mice find HFD aversive when ingesting D-serine, we paired saccharine [conditioned stimulus (CS)] with either NC or HFD (test stimulus) to mice fed NC so that these mice were exposed to HFD with CS for the first time. To provide a positive control for nausea, we added the intraperitoneal injection of lithium chloride (sickness-inducing substance) as unconditioned stimulus (US). To control for the intraperitoneal injection, both NC and HFD groups received saline instead of lithium chloride injection. First, to accustom mice to a water-deprivation schedule, 8-wk-old male C57BL/6J mice were allowed access to two 15-ml water bottles (purchased from SHINANO, Tokyo, Japan) containing 1.5% D-serine for the first 2 h of the dark cycle (6:00 P.M.–8:00 P.M.) for 5 days with NC feeding. On the 6th day (conditioning day), mice were given two bottles of 1.5% D-serine containing 0.15% saccharine for 30 min with either NC or HFD, and then received intraperitoneal injection of lithium chloride (0.15 M, 20 ml/kg, US) or saline (10 ml/kg, as a control injection). As a result, we generated the following three experimental groups: intraperitoneal saline with NC (negative control), intraperitoneal lithium chloride with NC (positive control), and intraperitoneal saline with HFD (test group). Next, they were allowed to access 1.5% D-serine water again for 90 more minutes with NC. The 7th day was the rest day when mice were given 2 h of access to two bottles of 1.5% D-serine with NC. The 8th-10th days were test days when two-bottle preference tests (1.5% D-serine with or without 0.15% saccharine) were performed for 30 min with NC to avoid the effect of HFD itself on the test days. Conditioned taste aversion was determined as the saccharine preference ratio, saccharine intake/total intake.

L-701,324 test. To test the effect of blocking the glycine-binding site of NMDA receptors, mice were first acclimated to the Feeding, Drinking, and Activity Monitor System for Mouse (Shinfactory) for 3 days with NC only. Next, mice were assigned to either the water drinking group or the 1.5% D-serine drinking group and given two-food choices between NC and HFD, while receiving intraperitoneal injection of L-701,324 (Sigma Aldrich) at 2.5 mg/kg concentration or vehicle (1% DMSO in PBS) two times a day (at 5:30 P.M. and 8:00 A.M.). The dose of the selective and full antagonist L-701.324 at the glycine-binding site of the NMDA receptor was decided based on a previous publication by others (83). Food intake, drinking, locomotor activity, and access to food were measured every minute and analyzed per hour or per period. The position of the food was switch on the 2nd day of the test just before the onset of the dark cycle (at 1800) so that the data would represent the preference for food and not the preference for the side of the cage.

Data analysis. Data are expressed as mean values ± SE. Significance was assessed using Student's *t*-test. A P value <0.05 was

considered significant. For multiple-group comparison, data were analyzed by one-way ANOVA with post hoc Bonferroni correction.

RESULTS

D-Serine, but not L-serine, suppresses HFD intake without aversion. First, to test the dose response of D-serine on NC intake and HFD intake, we monitored the food intake of C57Bl/6J male mice for 2 wk with free access to water containing 0, 0.5, 1.0, or 1.5% (wt/vol) D-serine after 1-wk acclimation to feeding from the multifeeder (Fig. 1*A*). D-Serine minimally affected food intake, body weight, and eWAT weight when mice were fed NC (Fig. 1, *B–D*). However, 1.0 and 1.5% D-serine significantly suppressed food intake when mice were fed a HFD, leading to significant weight loss and almost total loss of eWAT (Fig. 1, *E–G*). There was no additional decline in body weight between *day 6* and *day 10*, but the difference in body weight was maintained most likely due to the difference in the caloric intake from *days 0* and *5*. The suppression of food intake was strong enough to cause starvation in one, two, and two out of six mice receiving 0.5, 1, and 1.5% D-serine, respectively. There was no difference in water intake (~10 ml/day, including the drip-off into the cage) over the 2-wk study period among the groups. To exclude the possibility that mice find HFD aversive when ingesting D-serine, we performed a conditioned taste aversion test with mice drinking 1.5% D-serine. There was no difference in saccharine preference between mice that were given intraperitoneal saline injection with NC feeding and HFD feeding, whereas mice that were given intraperitoneal lithium chloride injection with NC feeding showed aversion from saccharine-containing D-serine water (Fig. 1, *H–I*). Therefore, mice do not find HFD aversive when ingesting D-serine.

Coagonism toward NMDA receptor is specific to the D form of serine (78). To test if NMDA receptor coagonism is required for the observed effect on food intake, 1.5% (wt/vol) L-serine was given to mice fed NC or HFD. L-Serine did not affect food intake, body weight, or eWAT weight (Fig. 2, *A-G*). Therefore, D-serine suppresses HFD intake and L-serine does not, indicating that the coagonism toward NMDA receptor is required for the effect.

D-Serine suppresses HFD, HSD, and HPD intake. To investigate if the suppression of food intake by D-serine is specific to fat, we tested the effect of 1.5% D-serine on HSD and HPD intake (Fig. 3*A*). The intake of HSD and HPD was suppressed by 1.5% D-serine, which was accompanied by decreased body weight and eWAT weight (Fig. 3, *B–F*). D-Serine caused starvation in three out of six HSD-fed mice and two out of six HPD-fed mice. Mice that showed starvation were not lethargic and moved freely within the cage until they were found dead. There was no apparent sign of distress or other signs of gastrointestinal maladies, such as diarrhea. Thus, D-serine suppressed the intake of HFD, HSD, and HPD, causing starvation in some cases, without any effect on NC intake. Therefore, the D-serine effect is not specific to a particular macronutrient.

D-Serine inhibits the intake of preferred foods in the two-food-choice paradigm. We hypothesized that two-food choices (NC vs. HFD, HSD, or HPD) may rescue mice from starvation by allowing mice to eat NC while ingesting D-serine. Mice were acclimated with two multifeeders, both containing NC. After 1 wk, we switched one of the multifeeders for a multifeeder containing HFD, HSD, or HPD and started giving 1.5% D-serine (Fig. 4*A*). We found that mice preferred to eat HFD, HPD, and HSD (in this order) over NC and that the ingestion of D-serine reversed the food preference (Fig. 4, *B–D*). The reversal effect on food preference was stronger for food that was more preferred in the absence of D-serine. Because D-serine-ingesting mice ate NC instead of high-preference food, they did not suffer from starvation and death (Fig. 4, *E–G*). Suppression of HFD intake was enough to significantly reduce total caloric intake and prevent the weight gain caused by an HFD (Fig. 4, *E–J*). Therefore, D-serine inhibited the intake of preferred foods in the two-food-choice paradigm when the choices were presented simultaneously with D-serine ingestion. This finding indicates that D-serine can prevent mice from acquiring a food preference.

Next, we tested whether D-serine can reverse the expression of food preference, with two-food choices for 5 days before the ingestion of D-serine. The mice were given ad libitum access to D-serine in drinking water for 5 days and observed for five more days after discontinuation of D-serine to determine if the effect persists (Fig. 5*A*). D-Serine suppressed the intake of HFD, HSD, or HPD but promoted NC intake (Fig. 5, *B–D*). The effect was observed from the 2nd day after beginning D-serine treatment. The degree of suppression of high-preference food intake was not as strong as when D-serine was started simultaneously with two-food choices. However, total caloric intake and body weight decreased during D-serine treatment because the compensatory increase in NC was also not as strong (Fig. 5, *E–J*). The preference was reversed to that of *day 5* (i.e., before D-serine treatment) within 5 days of discontinuing D-serine (Fig. 5, *B–D*). Taken together, the results show that D-serine can suppress both the acquisition of food preference and the expression of food preference. The effect is stronger for food that has higher preference, and the effect is lost when D-serine ingestion is terminated, indicating no memory effect. Furthermore, these data exclude the food neophobia as the mechanism for suppressing the intake of preferred foods.

Sensory inputs and intact leptin receptor signaling are not required for D-serine suppression of HFD intake. We investigated if D-serine can inhibit obesity caused by deficient leptin receptor signaling. Leptin is an adipokine and suppresses food intake (22). The *db* mutation in the leptin receptor gene causes abnormal splicing, leading to receptor dysfunction, hyperphagia, and obesity in *db*/*db* mice (11, 40). We presented two-food choice (NC vs. HFD) to either male *db*/*db* mice or their male littermate control mice with or without 1.5% D-serine (Fig. 6*A*). These mice had a BKS background (as opposed to C57Bl/6J in the previous studies). D-Serine suppressed the preference for and intake of HFD, decreased total caloric intake, and prevented weight gain in both control littermates and *db*/*db* mice (Fig. 6, *B–G*). Although control littermates ingesting D-serine showed slight increase in total caloric intake on *day 7*, they gained less weight because the HFD intake was decreased. Therefore, the effect of D-serine is independent of leptin receptor signaling.

Food preference can be affected by various sensory inputs, such as smell and taste. Sensation of nutrients is also conveyed by the vagus nerve innervating the digestive tract to the solitary tract nucleus (NTS) in the brain stem via the NMDA receptor (7, 8, 19, 55). Therefore, to test if D-serine requires various

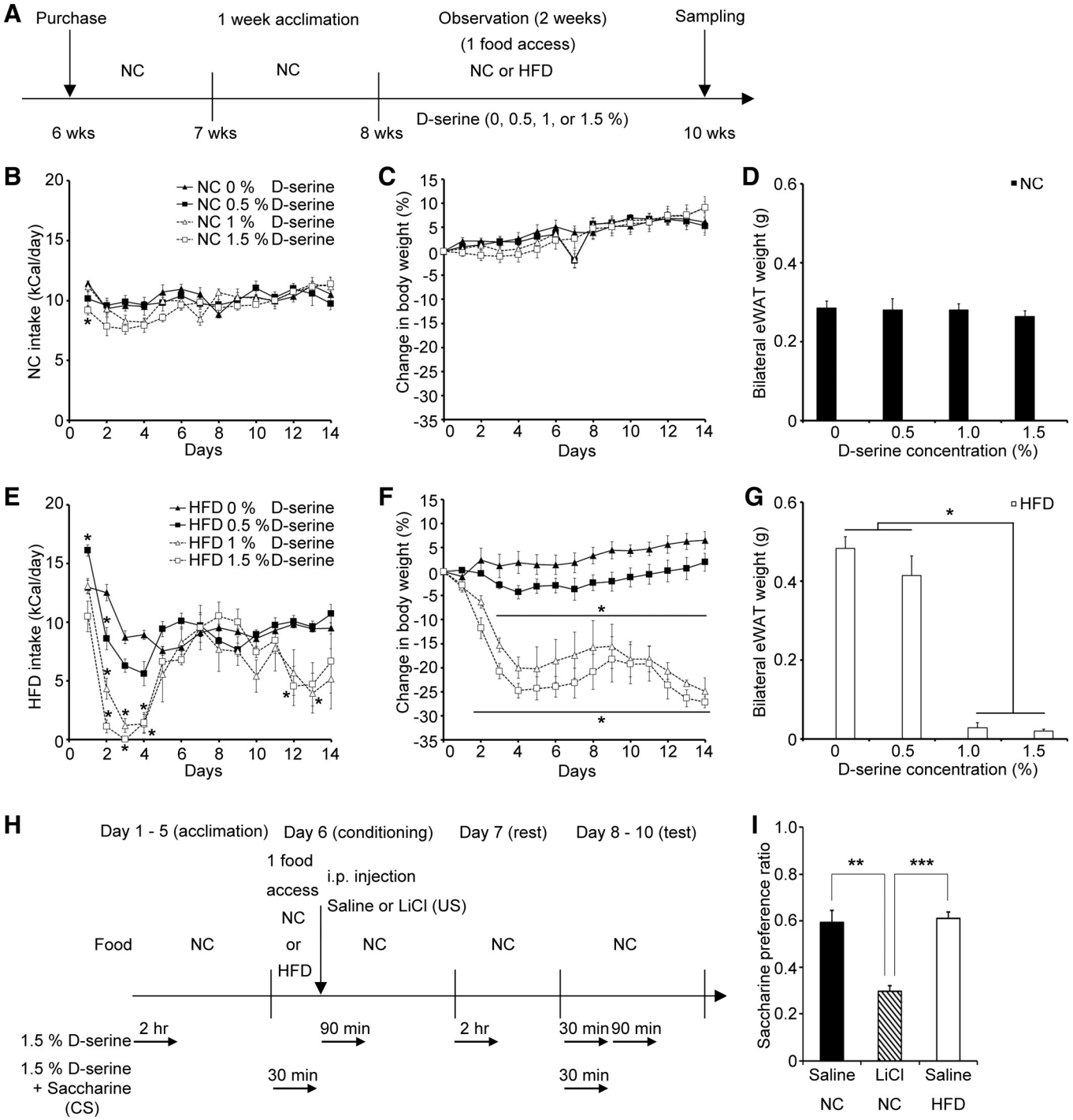


Fig. 1. D-Serine suppresses high-fat diet (HFD) intake without aversion. *A*: experimental design. *B* and *C*: effect of D-serine (0–1.5%) on intake of normal chow (NC) (*B*) and body weight (*C*). ▲, 0% D-serine; ■, 0.5% D-serine; △, 1.0% D-serine; □, 1.5% D-serine. *D*: effect of D-serine on epididymal white adipose tissue (eWAT) weight during NC feeding. *E* and *F*: effect of D-serine (0–1.5%) on intake of HFD (*E*) and body weight (*F*). *G*: effect of D-serine on eWAT weight during HFD feeding. Data are means ± SE (n = 4–6 mice/group). Data only from survived mice were analyzed and shown. Data were analyzed by 1-way ANOVA with post hoc Bonferroni correction. *$P < 0.05$ compared with the 0% D-serine group. *H* and *I*: conditioned taste aversion test. The experimental design (H) and average saccharine preference measured on the 9th and 10th day (*I*) (3 and 4 days after ip injection of saline or lithium chloride). Data are means ± SE (n = 4/group). Data were analyzed by 1-way ANOVA with post hoc Bonferroni correction. **$P < 0.01$ and ***$P < 0.001$. CS, conditioned stimulus; LiCl, lithium chloride; US, unconditioned stimulus.

sensory inputs to the central nervous system to suppress the intake of high-preference food, male ICR mice were treated with capsaicin to cause sensory deafferentation (5). We presented two-food choice (NC vs. HFD) to sensory-deafferented ICR mice and control ICR mice with or without 1.5% D-serine (Fig. 7*A*). Although sensory-deafferented mice were slower to acquire HFD preference compared with control ICR mice, HFD intake was suppressed by D-serine as the preference for

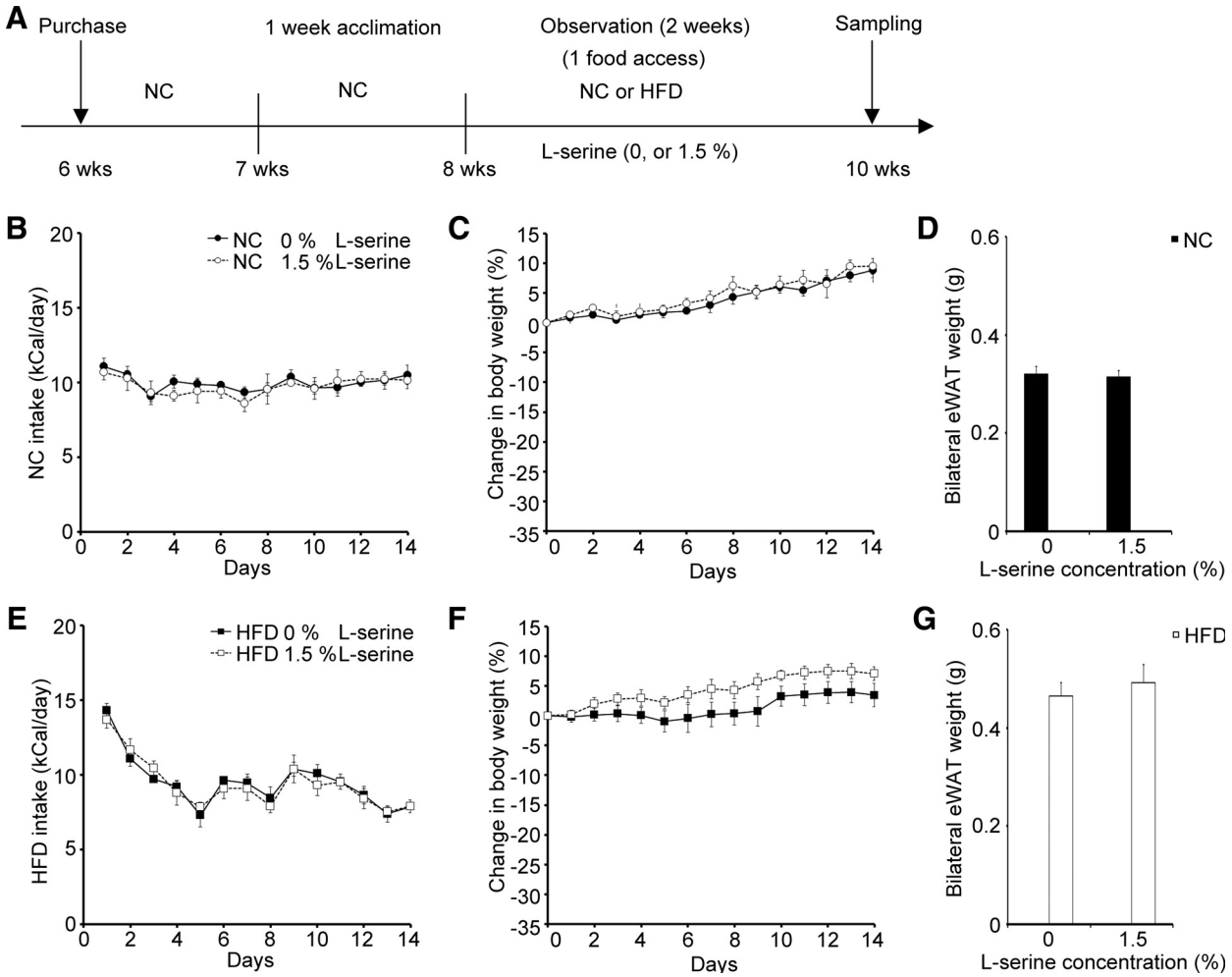


Fig. 2. L-Serine does not suppress HFD intake. *A*: experimental design. *B–D*: effect of L-serine on NC intake (*B*), body weight (*C*), and eWAT weight (*D*) during NC feeding. ●, 0% L-serine; ○, 1.5% L-serine. *E–G*: effect of L-serine on HFD intake (*E*), body weight (*F*), and eWAT weight (*G*) during HFD. ■, 0% L-serine; □, 1.5% L-serine. Data are means ± SE (n = 5–6/group). Data were analyzed by Student's t-test.

HFD was acquired, leading to reduced total caloric intake (Fig. 7, *B–E*). The effectiveness of sensory deafferentation was confirmed by the CCK8 test (Fig. 7*F*). Therefore, D-serine was effective in suppressing HFD preference under the two-food-choice paradigm in three different strains of mice (C57Bl/6J, BKS, and ICR) (Figs. 4*B*, 6*B*, and 7*B*). Sensory inputs are not required for the effect of D-serine, implying that at least one target responsible for the effect is located within the central nervous system.

D-Serine suppresses the intake of palatable food despite metabolic needs for food being unmet. What mice find palatable depends on their feeding status. Mice usually prefer HFD over a high-carbohydrate diet when fed ad libitum, but they prefer a high-carbohydrate diet over HFD after 24-h fasting, presumably because carbohydrates can be used as an instant energy source (65). NC is a high-carbohydrate diet compared with HFD (Table 1); therefore, we tested if pretreatment with D-serine suppresses NC intake after 24-h fasting. Because D-serine starts to take effect on the 2nd day after beginning ingestion, mice were given access to 1.5% D-serine for 2 days and refed with either NC or HFD after 24-h fasting (Fig. 8*A*). D-Serine suppressed the intake of NC and HFD for the 1st h of refeeding, but the suppression was greater for NC (Fig. 8*B*). By the 3rd h of refeeding, D-serine no longer affected NC intake and suppressed only HFD intake. These data indicate that D-serine suppresses the intake of what mice find palatable at a given time.

We also measured the hypothalamic expression of orexigenic neuropeptides (*Npy* and *Agrp*), indicators for metabolic needs for food, after 24-h fasting and 1-h NC refeeding with or without D-serine treatment. *Npy* expression significantly declined after 1 h of refeeding in the D-serine(−) group, but no decline was observed in the D-serine(+) group (Fig. 8*C*). No difference was found in *Npy* expression between the D-serine(−) and D-serine(+) group after 24-h fasting. Although there were no significant differences in *Agrp* expression among four groups (P = 0.087, 1-way ANOVA), the pattern was similar to that of *Npy* (Fig. 8*D*). Therefore, mice in the D-serine(+) group ate less NC within the 1st hour of refeeding after 24-h fasting even though their metabolic needs for food were unmet.

Coagonism toward NMDA receptor is required for D-serine to suppress HFD preference. To assess if D-serine alters HFD preference through coagonism toward NMDA receptor, we analyzed the effect of D-serine on acquiring HFD preference in the presence of L-701,324, the selective and full antagonist at

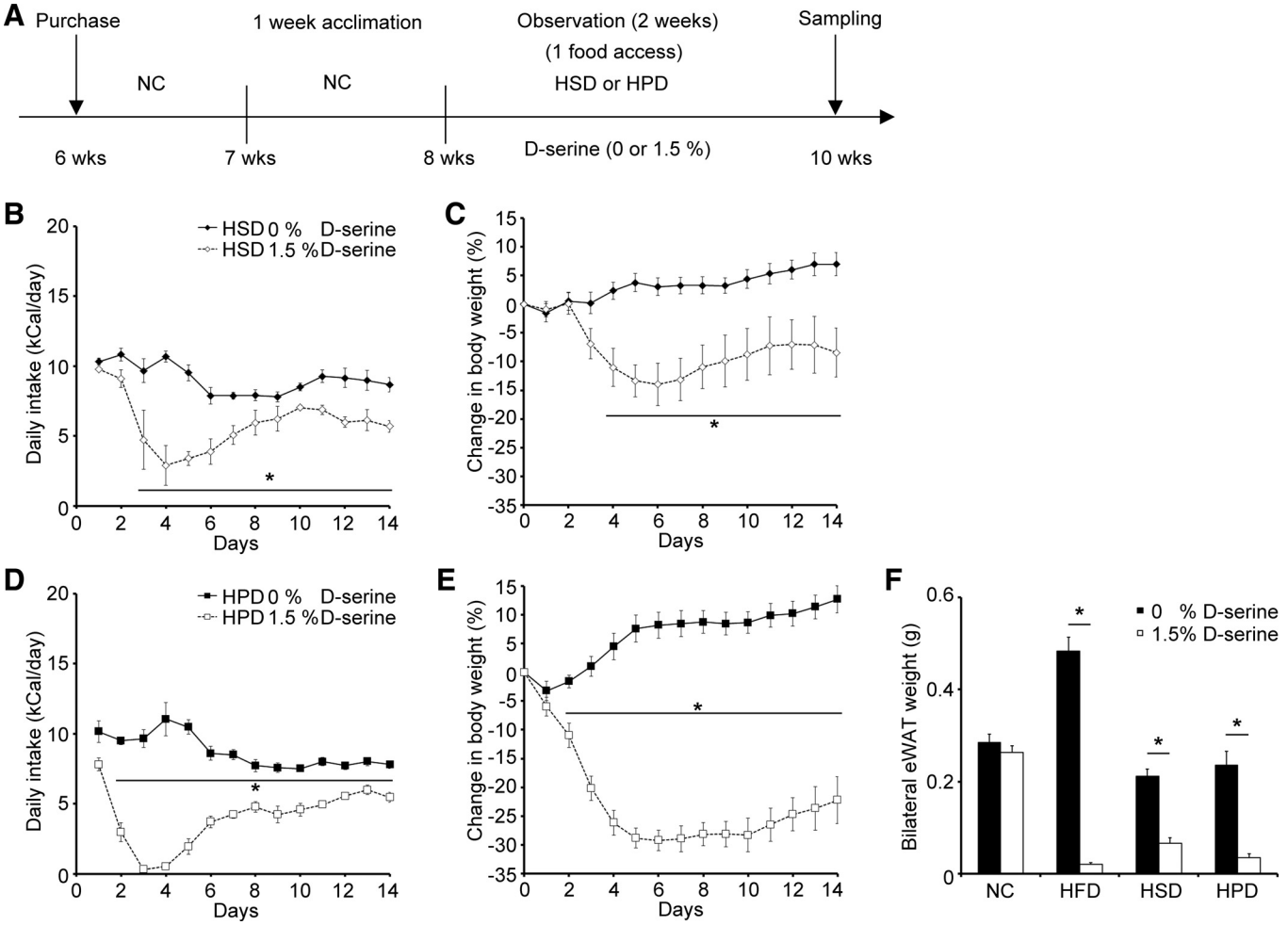


Fig. 3. D-Serine suppresses the intake of HFD, high-sucrose diet (HSD), and high-protein diet (HPD), but not NC intake. *A*: experimental design. *B* and *C*: effect of D-serine on food intake (*B*) and body weight (*C*) during HSD feeding. ◆, 0% D-serine; ◇, 1.5% D-serine. *D* and *E*: effect of D-serine on food intake (*D*) and body weight (*E*) during HPD feeding. ■, 0% D-serine; □, 1.5% D-serine. *F*: effect of D-serine on eWAT weight under each feeding condition. Data for NC and HFD are from Fig. 1, *D* and *G*. Black bar, 0% D-serine; white bar, 1.5% D-serine. Data only from survived mice were analyzed and shown. Data are means ± SE (n = 3–6/group). *$P < 0.05$ at each time point between 2 groups by Student's t-test.

the glycine-binding site of the NMDA receptor (Fig. 9*A*). We also analyzed the time course of acquiring HFD preference more in detail while simultaneously analyzing water intake, food access patterns, and locomotor activity. Mice drank 2–3 ml/day of water, and the drinking patterns were not affected by D-serine and L-701,324 (Fig. 9*B*). Although D-serine did not affect HFD intake and HFD preference at the first 6 h, mice gradually reduced HFD intake and HFD preference over the course, and they were significantly reduced by the 2nd day compared with the remaining groups (Fig. 9, *C* and *G*). The reduced HFD intake and HFD preference coincided with the significant increase in NC intake (Fig. 9*E*). The intraperitoneal injection of L-701,324 blocked these effects caused by D-serine, whereas L-701,324 alone did not affect any of the parameters measured (Fig. 9, *B–H*). Therefore, coagonism toward NMDA receptor is required for D-serine to suppress HFD preference.

Throughout the observed period, the patterns of accessing NC and HFD were not different among the groups (Fig. 9, *D* and *F*). These data argue against the idea that mice develop phobia toward HFD when drinking D-serine. During the 6th to 12th h of the observation, HFD intake was significantly reduced without apparent decrease in NC intake (Fig. 9, *C* and *E*). No alteration in locomotor activities was observed (Fig. 9*H*). These data do not support the idea that mice avoid HFD because they are nauseated. Overall, data argue against the idea that D-serine makes mice avoid high-preference diet because mice find the diet aversive.

DISCUSSION

The present study supports the idea that oral D-serine administration modulates food intake in a way that it suppresses the intake of what mice find palatable at the time. D-Serine suppressed the intake of high-preference food under the two-food-choice paradigm. D-Serine did not affect NC intake with one-food access when fed ad libitum but suppressed it when mice fasted for 24 h. Fasting for 24 h increases the desire for food, especially high-carbohydrate diet, such as NC. The results indicate that the effect is not dependent on a particular macronutrient in the food. Furthermore, hypothalamic gene expression indicated that D-serine-treated mice had similar

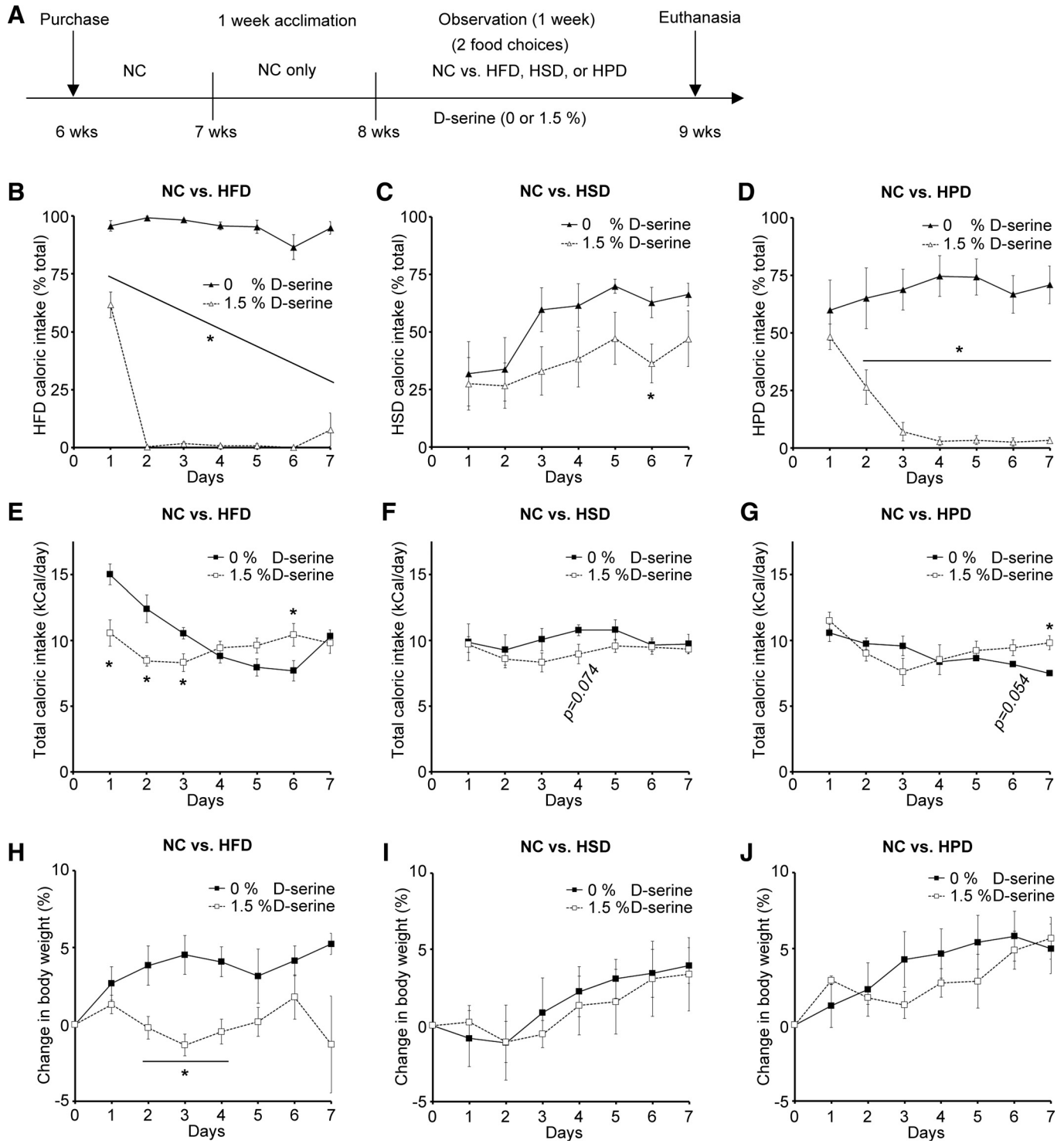


Fig. 4. D-Serine inhibits the intake of preferred foods in the 2-food choice paradigm. *A*: experimental design. *B–D*: percentage of calories consumed with the HFD (*B*), HSD (*C*), and HPD (*D*) under the 2-food choice paradigm. ▲, 0% D-serine; △ 1.5% D-serine. *E–G*: total caloric intake during the 2-food choice paradigm when given choices between NC and HFD (*E*), HSD (*F*), or HPD (*G*). *H–J*: percent changes in body weight during the two-food-choice paradigm when given choices between NC and HFD (*H*), HSD (*I*), or HPD (*J*). ■, 0% D-serine; □, 1.5% D-serine. Data are means ± SE (n = 5–6/group). *$P < 0.05$ between 2 groups by Student's t-test.

levels of metabolic needs for food after fasting, but they ate less when they were refed after fasting despite their metabolic needs being unmet. Finally, we show that coagonism toward NMDA receptor is required for D-serine to suppress HFD preference by using L-701,324, the selective and full antagonist at the glycine-binding site of the NMDA receptor, and L-serine, which lacks coagonism toward NMDA receptor.

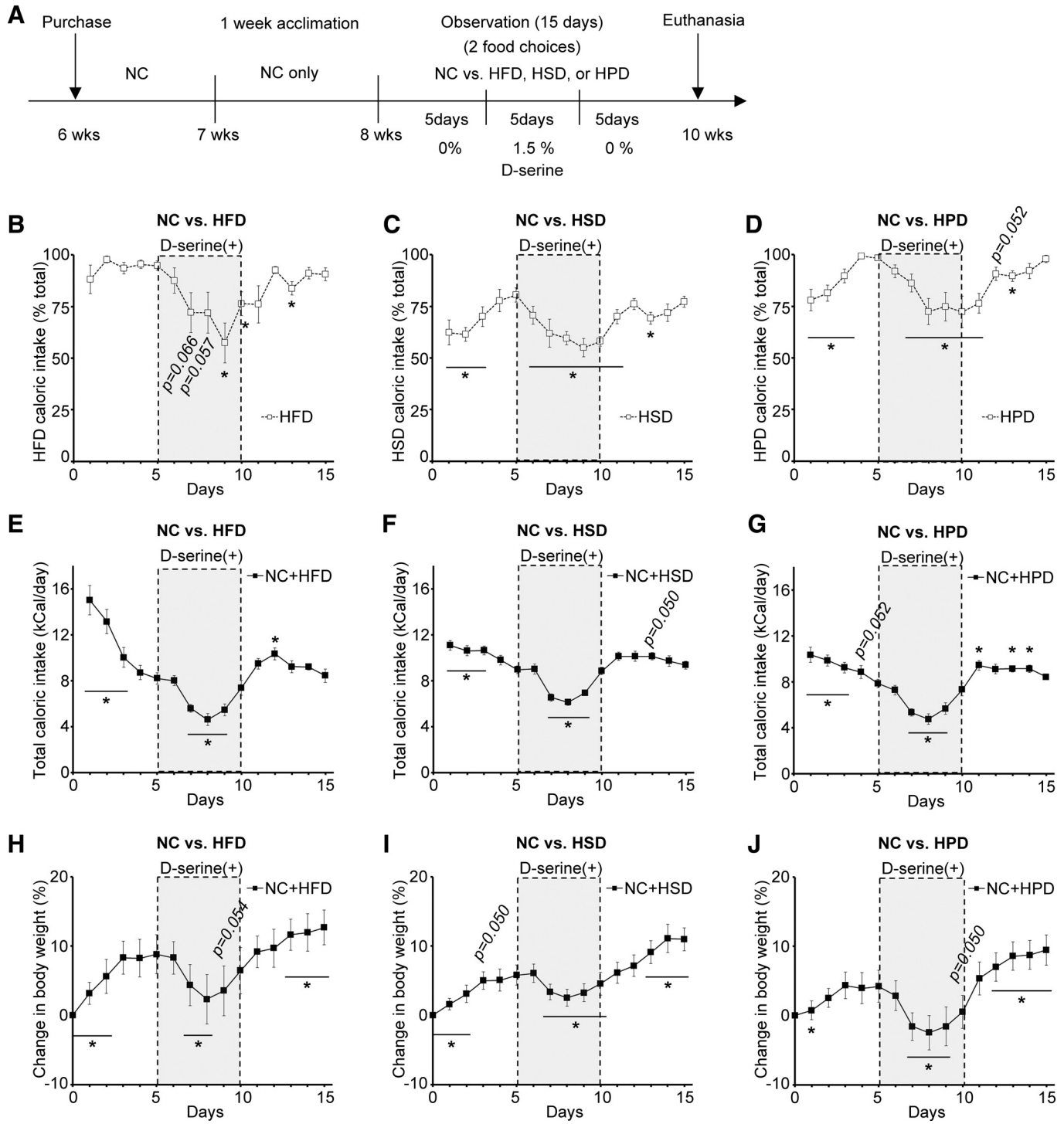


Fig. 5. D-Serine suppresses the established preference for palatable food in the 2-food choice paradigm. *A*: experimental design. *B–D*: percentage of calories consumed with the HFD (*B*), HSD (*C*), and HPD (*D*) under the 2-food choice paradigm. *E–G*: total caloric intake during the experiment, NC vs. HFD (*E*), HSD (*F*), or HPD (*G*). *H–J*: percent changes in body weight during the experiment, NC vs. HFD (*H*), HSD (*I*), or HPD (*J*). Data are means ± SE ($n = 6–8$/group). *$P < 0.05$ between data from *day 5* and each time point by Student's paired *t*-test. The gray hashed area indicates days (*days 5-10*) when D–serine was given to mice.

Vagal afferents have been shown to be necessary for fat preference (14, 49), conditioned flavor preference (70, 81), and taste aversion (82). Although capsaicin treatment as a model for vagal sensory deafferentation may have limits, including the possibility that a substantial proportion of vagal and spinal afferents may not be destroyed by capsaicin, we found that D-serine was equally effective in suppressing the intake of HFD in sensory-deafferented mice by capsaicin. Interestingly, sensory-deafferented mice took more time (3 days) to develop the preference for HFD compared with the other mice, which

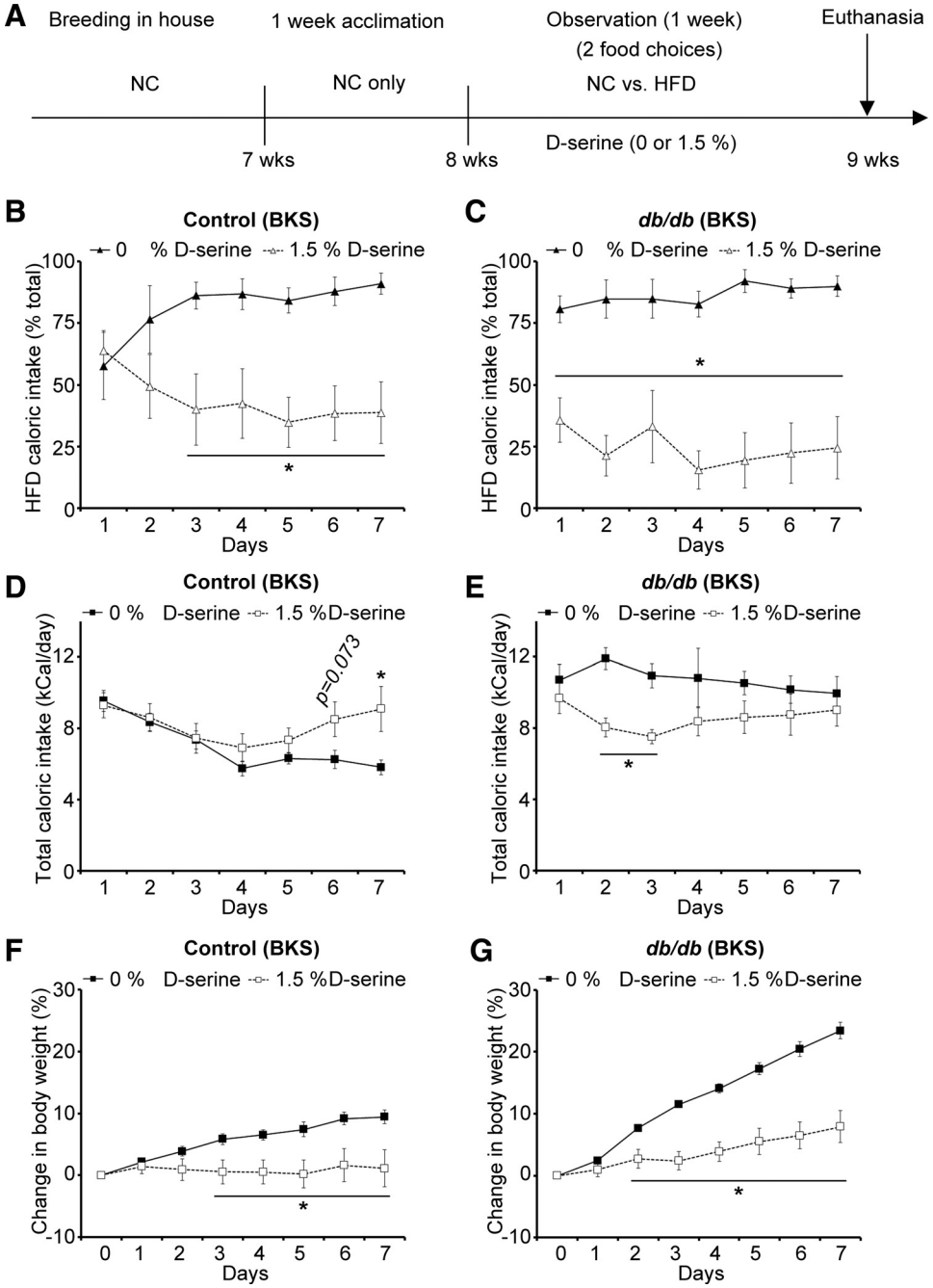


Fig. 6. Intact leptin receptor signaling is not required for the suppression of HFD intake by D-serine. *A*: experimental design. *B and C*: percentage of calories consumed with the HFD in control mice (*B*) and *db*/*db* mice (*C*). ▲, 0% D-serine; △, 1.5% D-serine. *D* and *E*: total caloric intake of control mice (*D*) and *db*/*db* mice (*E*). ■, 0% D-serine; □, 1.5% D-serine. *F* and *G*: body weight change for control mice (*F*) and *db*/*db* mice (*G*) during the study. Data are means ± SE ($n = 6–8$/group). *$P < 0.05$ between 2 groups by Student's *t*-test.

generally preferred HFD from *day 1*. The development of preference may require both sensory input and the metabolic effects of food; the former may be crucial for the instant development of a preference, whereas the latter may be sufficient for later preference development. D-Serine could suppress the intake of high-preference food in the absence of sensory input that is essential for the development of conditioned taste aversion, indicating that aversion is not essential for the D-serine effect.

The effect of D-serine on food intake was observed from the second day after ingestion, not immediately after, implying that the effect is unlikely to be mediated by the instant modulation of sensory input mediated by excitatory glutamatergic neurotransmission. Nevertheless, we cannot completely rule out the possibility that D-serine also modulates sensory input and suppresses the intake of high-preference food. Sensory inputs from vagal afferents and excitatory glutamatergic neurotransmission do not impinge on the same neural pathway (5). Although mice developed aversion to saccharine-containing D-serine water in the lithium chloride-injected group, saccharine preference in the saline-injected groups was somewhat milder than expected. Therefore, we cannot totally rule out that D-serine might affect taste sensation and make palatable food less appealing.

D-Serine has not been reported to affect food preference in previous studies in which either the NMDA receptor or D-ser-

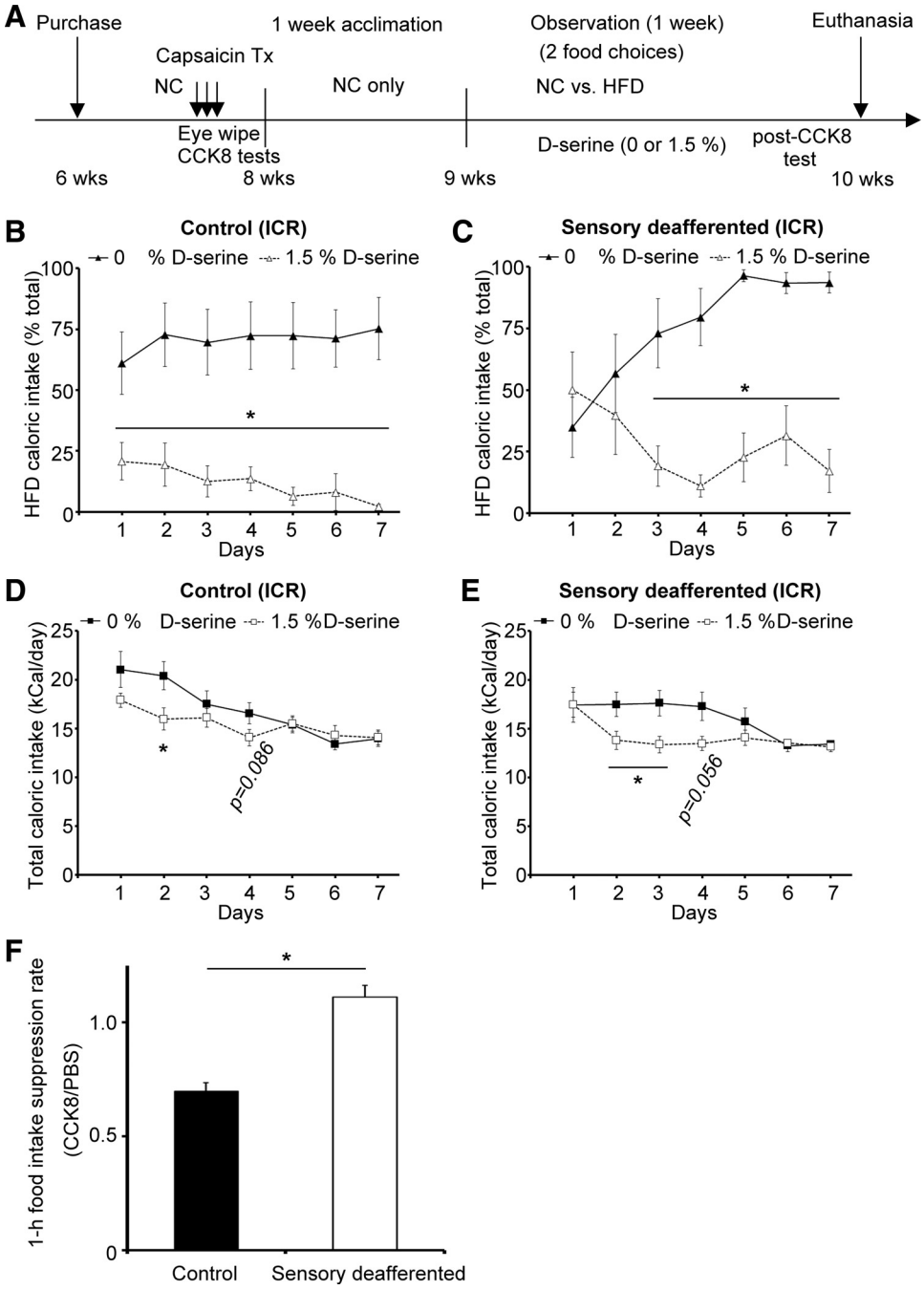
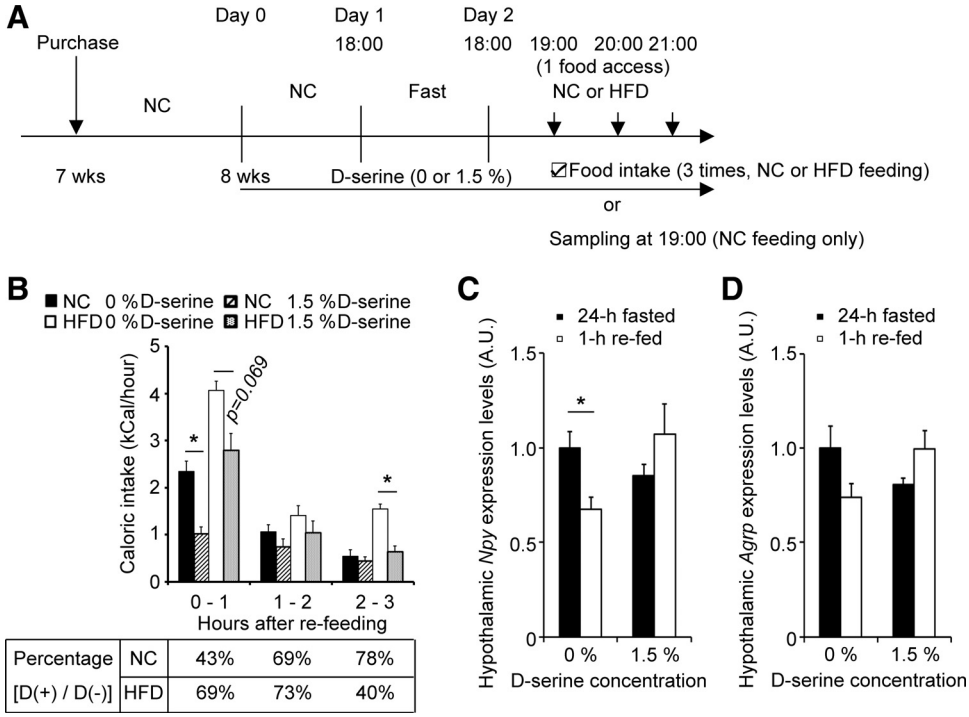


Fig. 7. Sensory inputs are not required for the suppression of HFD intake by D-serine. *A*: experimental design. *B* and *C*: percentage of calories consumed with the HFD in control ICR mice (*B*) and sensory-deafferented mice (*C*). ▲, 0% D-serine; △, 1.5% D-serine. *D* and *E*: total caloric intake of control ICR mice (*D*) and sensory-deafferented mice (*E*). ■, 0% D-serine; □, 1.5% D-serine. *F*: effect of cholecystokinin-8 (CCK8) on 1-h food intake after 24-h fasting in control mice (black bar) and sensory-deafferented mice (white bar). Data are means ± SE (n = 6–11/group). *$P < 0.05$ between 2 groups by Student's t-test.

ine system was genetically modified. For instance, mice harboring hypoactivating mutations in the NMDA receptor (D481N, K483Q) at the glycine-biding site (72) present with abnormal long-term potentiation, hyperactivation of the dopaminergic and serotonergic system, drug-resistant nonhabituating hyperactivity, and altered anxiety-like behaviors (1, 33, 38). D-Serine production and degradation in vivo are regulated by serine racemase (SR) (32, 76, 80) and D-amino acid oxidase (DAAO) (74), respectively. SR knockout mice have been reported to have defects in long-term potentiation, anxiety-like behavior, and increased locomotor activity (3, 13). On the other hand, neither DAAO mutant mice nor rats have been reported to exhibit gross abnormalities (36, 59). Considering that D-serine regulates cerebellar long-term depression and motor coordination through the δ_2-glutamate receptor during development in the cerebellum (30), analyzing the effect of genetic manipulation of the D-serine system on food preference would be difficult.

NMDA signaling has been shown to regulate food intake (21, 55, 64, 66, 68). NMDA receptors in the hindbrain suppress food intake (7, 20, 77). Furthermore, glutamatergic projection from NTS to the parabrachial nucleus contributes to the suppression of food intake (9, 79). NMDA receptors in ventromedial nucleus and paraventricular nucleus of the hypothalamus have also been reported to contribute to the suppression of food intake (54, 68). On the other hand, NMDA receptor signaling



Percentage [D(+) / D(-)]				
	NC	43%	69%	78%
	HFD	69%	73%	40%

Fig. 8. D-Serine suppresses the intake of palatable food despite metabolic needs for food being unmet. *A*: experimental design. *B*: hourly intake of NC and HFD during the 1st 3 h of refeeding after 24-h fasting. Black bar, NC 0% D-serine; hatched bar, NC 1.5% D-serine; white bar, HFD 0% D-serine; gray bar, HFD 1.5% D-serine. D(−) and D(+) indicate 0 and 1.5% D-serine, respectively. Data are means ± SE (n = 6/group). Data were analyzed by 1-way ANOVA with post hoc Bonferroni correction. *$P < 0.05$. *C* and *D*: hypothalamic expression of neuropeptide Y (*Npy*, *C*) and agouti-related protein (*Agrp*, *D*) after 24-h fasting or 1-h refeeding of NC after 24-h fasting. Black bar, 24-h fasted; white bar, 1-h refed with NC after 24-h fasting. Data are means ± SE (n = 9/group). Data were analyzed by 1-way ANOVA with post hoc Bonferroni correction. *$P < 0.05$.

has also been reported to increase food intake in the lateral hypothalamus (15, 34, 67). NMDA receptor is required for the activation of orexigenic AgRP neuron to promote food intake (42). Glutamatergic projection from the lateral hypothalamus to ventral tegmental area, via NMDA receptor, promotes eating-induced dopamine release and reward-based feeding (63, 75). Because the occupancy of glycine-binding sites of NMDA receptors varies among synapses (46), exogenous D-serine may facilitate excitatory glutamatergic neurotransmission only at NMDA receptors when and where the occupancy of the glycine-binding sites by endogenous coagonists is low. Therefore, detailed dissection of neural circuits affected by oral D-serine ingestion is crucial to understand the mechanisms responsible for the suppression of high-preference food intake.

Results of the present study imply that D-serine could be used for appetite control in the context of obesity. Peripheral administration (oral, ip, or sc) of D-serine has been tested in the context of schizophrenia, in which reduced D-serine level has been implicated, with improvements in some of the symptoms (2, 23, 39, 69). Acute administration of 30 mg/kg D-serine significantly increased serum D-serine level without any significant side effect, and D-serine given orally with a 30- to 120-$mg \cdot kg^{-1} \cdot day^{-1}$ dose regimen improved schizophrenia symptoms in humans (31, 41). One intraperitoneal injection of 10 mmol/kg (1,052 mg/kg) D-serine in rats showed significantly increased D-serine level throughout the central nervous system for 24 h, whereas D-serine in the periphery was rapidly cleared, indicating that exogenously administered D-serine somehow tends to remain within the central nervous system (23). Based on the daily consumption (2–10 ml) of 1.5% D-serine by mice in the current study, mice received ~1.5–7 g/kg of D-serine orally per day, which is higher than the above studies. Although we did not measure the D-serine concentration in the bloodstream, it is plausible that orally ingested D-serine may have increased D-serine concentration within the central nervous system of the mice, enough to provide D-serine where the endogenous D-serine level is not high enough to saturate the glycine sites of NMDA receptors. However, using D-serine for treating obesity requires caution because increased D-serine level has been implicated as a potential cause for amyotrophic lateral sclerosis (45, 53, 57, 58).

Alternatively, the D-serine-administered mice could serve as a model for anorexia nervosa. Voluntary reduction in food intake despite unmet metabolic needs is one of the early symptoms observed in patients with anorexia nervosa (18) and was also exhibited by D-serine-ingesting mice. In addition, some anorexia patients starve themselves to death, which was also observed in some of the D-serine-treated mice under the one-food-access paradigm. Fat avoidance in anorexia nervosa is considered to be based primarily on cognitive factors because these patients do not have a markedly greater ability to taste fat (61). The lack of a requirement of sensory input for the suppression of high-preference food intake by D-serine is consistent with this finding in anorexia nervosa patients.

Genetic studies of anorexia nervosa have been hampered by the heterogeneity of the patient population. Although a correlation between the GRIN2B (NR2B) single-nucleotide polymorphism and anorexia nervosa has been reported (37), more recent genomewide association studies of anorexia nervosa patients have failed to identify any loci with genomewide significance in this disorder (6). Therefore, a genetic approach to elucidate the pathogenesis of anorexia nervosa has not yet been successful. At least four rodent models of anorexia nervosa are available: dietary restriction model, stress-induced appetite loss, stress-induced hyperactivity, and activity-based anorexia model (71). In all of these current models, successful establishment of the model is defined by decreased body weight. However, one of the culprits that makes anorexia nervosa research complicated is that it is difficult to decipher if the observed symptoms and phenotypes are the cause of

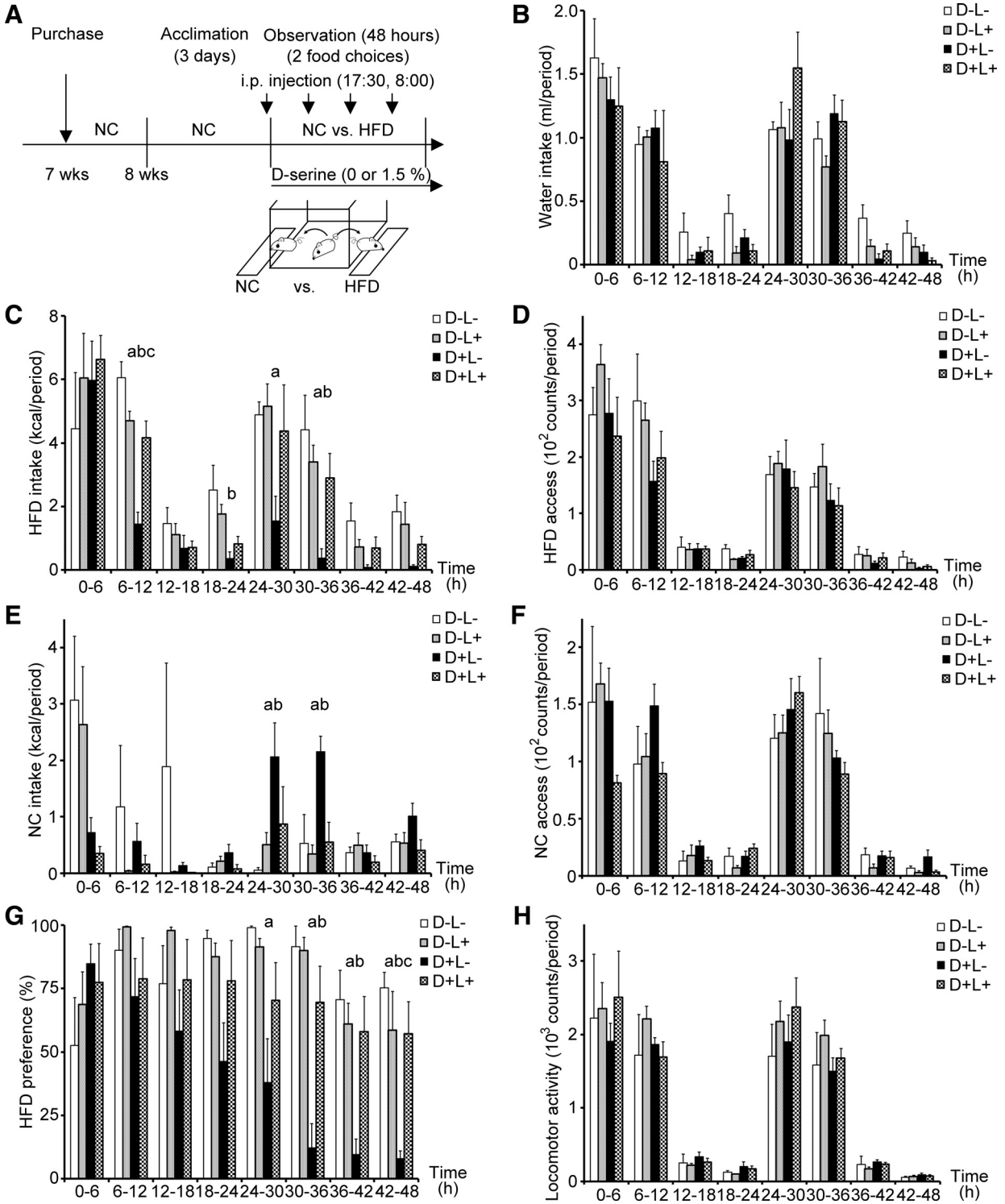


Fig. 9. Coagonism toward *N*-methyl-D-aspartate (NMDA) receptor is required for D-serine to suppress HFD preference. *A*: experimental design. *B*: water intake over the observation period. *C–G*: feeding patterns during the 48-h observation period. HFD intake (*C*), HFD access (*D*), NC intake (*E*), NC access (*F*), and HFD preference (*G*) were summarized for every 6-h period. *H*: locomotor activity during the 48-h observation period. White bar, 0% D-serine with vehicle injection (D−L−); gray bar, 0% D-serine with L-701,324 injection (D−L+); black bar, 1.5% D-serine with vehicle injection (D+L−); checker bar, 1.5% D-serine with L-701,324 injection (D+L+). Data are means ± SE (n = 5/group). Data were analyzed by 1-way ANOVA with post hoc Bonferroni correction. When statistically significant difference ($P < 0.05$) was observed between the D+L− group and either the D−L− group, the D−L+ group, and/or the D+L+ group, they are indicated as "a," "b," or "c," respectively.

anorexia or the consequences of weight loss. Numerous endocrine changes that affect virtually every regulatory system are observed in anorexia patients, reflecting the body's adjustment to prolonged undernutrition and malnutrition (10). The D-serine ingestion model could be a way to overcome this issue because the model is not defined by weight loss, and weight loss could be rescued by forced caloric intake via oral gavage.

Perspectives and Significance

D-Serine may suppress the desire for food, whether it is induced by energetic need or by a "hedonic" mechanism. It could be by facilitating nutrient sensing and making mice hypersensitive to nutrient cues. That can be a way to trick mice to feel that their demands were met without actually fulfilling the needs. The current study also indicates the possibility that modulation of excitatory glutamatergic neurotransmission through synaptic NMDA receptor coagonists may be a contributing factor in the pathophysiology of anorexia nervosa. The sensitivity to D-serine, D-serine metabolism, the sensitivity to glutamatergic neurotransmission, or the overall tone of the neural circuit responsible for the D-serine effect may be altered in anorexia nervosa patients. The D-serine administration model may prove to be a useful tool to tease out the causes of anorexia and the consequence of weight loss accompanying anorexia. Further investigation into the mechanism by which D-serine suppresses palatable food intake is warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.S., Y.K., and T. Kinoshita conception and design of research; T.S., Y.K., S.M., H.Y.-H., and K.K. performed experiments; T.S., Y.K., and S.M. analyzed data; T.S., Y.K., S.K., Y.I., T.Y., N.A., and T. Kitamura interpreted results of experiments; T.S. and Y.K. prepared figures; T.S. and Y.K. drafted manuscript; T.S. and Y.K. edited and revised manuscript; T.S., Y.K., and S.M. approved final version of manuscript.

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