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FULL PAPER

Physiology

Dietary L-serine modifies free amino acid composition of maternal milk and lowers the body weight of the offspring in mice

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ABSTRACT. The growth of offspring is affected not only by the protein in maternal milk but also by the free amino acids (FAAs) contained in it. L-Serine (L-Ser) is known as an important FAA for the development of the central nervous system and behavioral activity. However, it is not clear whether L-Ser is transported into the pool of FAAs contained in milk and thereby affects the growth of offspring. Using mice, the current study investigated the effects of dietary L-Ser during pregnancy and lactation on milk and plasma FAA composition, as well as on growth, behavior, and plasma FAAs of offspring. Dietary L-Ser did not significantly affect the maternal, anxiety-like, or cognitive behaviors of either the dam or the offspring. The FAA composition notably differed between plasma and milk in dams. In milk, dietary L-Ser increased free L-Ser levels, while glutamic acid, L-alanine, D-alanine and taurine levels were decreased. The body weight of the offspring was lowered by dietary L-Ser. The concentrations of plasma FAAs in 13-day-old offspring (fed only milk) were not altered, but 20-day-old offspring (fed both milk and parental diet) showed higher plasma L-Ser and D-Ser concentrations as a result of the dietary L-Ser treatment. In conclusion, the present study found that dietary L-Ser transported easily from maternal plasma to milk and that dietary L-Ser treatment could change the FAA composition of milk, but that an enhanced level of L-Ser in milk did not enhance the plasma L-Ser level in the offspring.

KEY WORDS: free amino acid, growth, L-serine, maternal milk, mouse

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Milk is derived from the mother's blood constituents. Maternal milk is the only source of nutrition for mammalian species during the suckling period. In terms of the nitrogenous compounds in milk, both protein and free amino acids (FAAs) are important nutrients. Recent reports have indicated that nutrition in early infancy may affect a child's later health [14, 32]. Thus, some changes in the contents of a mother's milk may influence the health and growth of her offspring.

L-Serine (L-Ser) is one of the important components of maternal milk, either functioning as a free form or being incorporated to form protein. L-Ser is classified as a non-essential amino acid for animals and is biosynthesized from a glycolytic intermediate by phosphoglycerate dehydrogenase (PHGDH). It is clear from the evidence of patients with PHGDH deficiency who suffer from severe neurological symptoms that L-Ser is an important nutrient for brain function and development [5, 10]. Additionally, results from PHGDH null knockout mice showed that the PHGDH-dependent pathway of *de novo* synthesis was the principal source of L-Ser in developing embryos. This pathway is essential for normal embryonic development, especially for brain morphogenesis [33]. L-Ser also affects the neuronal system. In cultured rat hippocampal neurons it has been found to increase the viability of dendrites and promote their growth [15], and it has also been found to serve as a major astroglia-derived trophic factor for cerebellar Purkinje neurons [8]. L-Ser also exerts a neuroprotective effect on the ischemic-reperfused brain [31].

L-Ser and its related compounds also influence locomotor activity and stressful behavior in animals. Intracerebroventricular (i.c.v.) injection of L-Ser induced sedative and hypnotic effects [2, 3, 25] through acting on γ-amino butyric acid $(GABA)_A$ receptors [25] under an acute stressful condition in neonatal chicks, and it reduced the locomotor activity of socially isolated rats [26]. Moreover, central administration of phosphatidylserine (1,2-diacyl-sn-glycerol-(3)-L-phosphoserine), a phospholipid which is comprised of L-Ser and a lipid molecule, attenuates stress-induced isolation behavior in chicks [13].

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However, it is not clear whether dietary L-Ser can be efficiently transferred into maternal milk to influence the growth and behavior of offspring. Thus, using mice, the present study investigated the effects of chronic intake of L-Ser from the pregnancy period to weaning on the contents of FAAs in maternal milk and in plasma in dams and offspring. The body weight and behavior of dams and offspring were also investigated.

MATERIALS AND METHODS

Animals

Male and female ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Their offspring, raised until they were 13 weeks old, were used in the experiment. Offspring were reared in a group. Water and standard diet for laboratory rodents were given *ad libitum* (MF, Oriental Yeast, Tokyo, Japan). The mice were maintained on a 12 hr light/dark cycle (lights on at 8:00, lights off at 20:00) at a room temperature of 23 ± 1°C. All animal experiments reported here were conducted in accordance with the Guidelines for Animal Experiments in the Faculty of Agriculture at Kyushu University, as well as with Law no. 105 and Notification no. 6 of the Japanese government.

Experimental procedure

Fifteen female mice and 15 male mice (all of them 13 weeks old) were mated at random (i.e. there were 15 mating groups). The day of mating was considered "day zero of pregnancy" (G0), and this assumption was corroborated by observing the length of gestation. All mice used in the experiment were pregnant at G0. At day G10, the pregnant mice were separated into two groups (n=7–8). One group was fed MF as a control diet (Con group) and another group was fed MF with 2% L-Ser (Ser group). The dietary treatment was continued until the mice were euthanized. The pregnant mice had free access to food and water and their food consumption was estimated by weighing the remaining food every 3 days. The body weight of the mother was measured every 3 days from day 10 of gestation.

The behavioral tests were conducted during the light periods and were recorded by a video capture system. On postnatal day 1 (P1), the maternal behavior test was performed. The open field test (OFT) was performed on maternal mice on P12, when milking was also performed. Litter sizes during suckling were culled to 7–8 pups, and for all experiments, only male offspring from each litter were tested (n=8 /group). The body weights of dams and offspring were measured every 5 days after birth of the offspring. All offspring in both treatments were weaned at 20 days of age. After weaning, the offspring had free access to the same food as the mother, and to water. The OFT was performed on the offspring at P17 and the object recognition test (ORT) was performed on them at P19. The mothers (at P20) and eight male offspring from each group (at P13 or P20) were anesthetized with isoflurane (Escain®, Mylan, Osaka, Japan) and trunk blood samples were obtained by decapitation. The blood samples obtained were centrifuged at 3,000 × *g* for 15 min at 4°C (KUBOTA 3740), and stored at −80°C until analysis took place. Milk samples were stored at −80°C until analysis took place.

Milking

Before milking, the maternal mice were isolated from their offspring for more than 5 hr in order to obtain a sufficient amount of milk for analysis. Then the mice were injected with 0.1 m*l* (1 oxytocin unit) oxytocin (ZENOAQ, Fukushima, Japan) subcutaneously to promote the milk release. Twenty min after the injection, the mice were anesthetized using isoflurane and milked using KN-591 milking equipment for mice and rats (Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 10 min. Milk (1.0–1.3 m*l*) was obtained from each mouse. After milking, the mice were put into their home cages.

Behavioral tests

The maternal behavioral test was performed according to a previously described method [18] to evaluate motivation to rear offspring. At the beginning of the test, each mother mouse was moved outside of the home cage and her 3 offspring were taken out from their nest, located in the home cage, and placed singly at each of three corners of the cage. Next, each mother was returned to her nest and her maternal behaviors observed by video camera recording. The maternal behaviors, such as sniffing, licking, crouching, nursing, retrieving, and nest-building, were recorded every 15 sec for 10 min.

The OFT was performed to evaluate locomotor activity and anxiety-like behavior in a novel environment, using a circular arena (diameter 60 cm, height 35 cm) made of black-colored wood. The test was performed under light conditions of 100 lux. At the beginning of the test, each mouse was transferred from its home cage to the center of the open field area. After each trial, the arena was cleaned with 10% ethanol solution to unify the conditions of all of the tests. Open field behavior was analyzed with ANY-maze software (Stoelting Co., Wood Dale, IL, U.S.A.) by dividing the field into 3 areas (with diameters of 20, 40 and 60 cm). The total distance moved, the distance moved in the central area (diameter 40 cm) and the number of entries into the central area were measured.

The ORT was performed as reported previously [29]. On the first day, animals were acclimated in the apparatus for 10 min within an open field, which was the same circular arena as used in the OFT (diameter 60 cm, height 35 cm), under light conditions of 100 lux. Twenty-four hr after the acclimation trial, training was conducted for 5 min by placing mice individually into the field, in which two identical objects (buckets, 9.5 cm in diameter and 9 cm in height) were positioned in two adjacent corners, 15 cm from the walls. Twenty-four hr after the training, mice explored two dissimilar objects, a familiar one (the bucket) and a novel one (a watering can, 7.5 cm in diameter and 7 cm in height), for 3 min as a test trial. All objects were similar in texture, color and size

but were different in shape. To avoid preference for the novel object, the novel object (the watering can) was switched with the familiar object (the bucket) for half of the mice (i.e. four mice from each group); this procedure was repeated with the remaining mice, except that the watering can was used as the familiar object and the bucket as the novel object. A recognition index calculated for each mouse was expressed by the ratio $T_N/(T_F+T_N)$. T_F means time spent exploring the familiar object, and T_N means time spent exploring the novel object. The objects and arena were cleaned with 10% ethanol solution between each trial. Exploration was defined as sniffing or touching the object with the nose and/or forepaws, as recorded by a video capture system. Sitting on the object was not considered exploration behavior.

Amino acid analysis

To investigate the effects of chronic L-Ser supplementation on amino acid metabolism, FAA concentrations in the maternal milk and plasma as well as in the offspring plasma were analyzed. The samples of milk and plasma were prepared by centrifuging at 14,000 × g for 20 min at 4°C, and then they were deproteinized through an ultrafiltration tube (Millipore, Bedford, MA, U.S.A.). Both the L- and the D-amino acid contents were measured by Ultra Performance Liquid Chromatography (UPLC) (the Acquity™ UPLC system, comprised of Waters Binary Solvent Manager, Water Sample Manager and Waters FLR Detector) with an ACCQTAG™ ULTRA C18, 1.7 μm, 2.1 × 100 mm column (Waters Corporation, Milford, MA, U.S.A.). The excitation and emission wavelengths for fluorescent detection of amino acids were 350 nm and 450 nm, respectively. The system was operated with a flow rate of 0.25 m*l*/min. The UPLC gradient system (A=50 mM sodium acetate (pH 5.9), B=methanol) was 10–20% B over 3.2 min, 20% B for 1 min, 20–40% B over 3.6 min, 40% B over 1.2 min, 40–60% B over 3.8 min, 60% B for 1 min, and 60–10% B for 0.01 min. Just before the UPLC analysis was carried out, each sample (10 μl) was transferred to a UPLC tube, and NAC/OPA (20 μl) and a borate buffer (70 μl) were added; then it was left for 2 min in a dark room. The same method was used for the standard solutions containing 16 L-amino acids, 16 D-amino acids, taurine (Tau) and so on. The milk and plasma FAA concentrations were expressed as pmol/μl. Glutamic acid and asparagine could not be distinguished as L- and D-types.

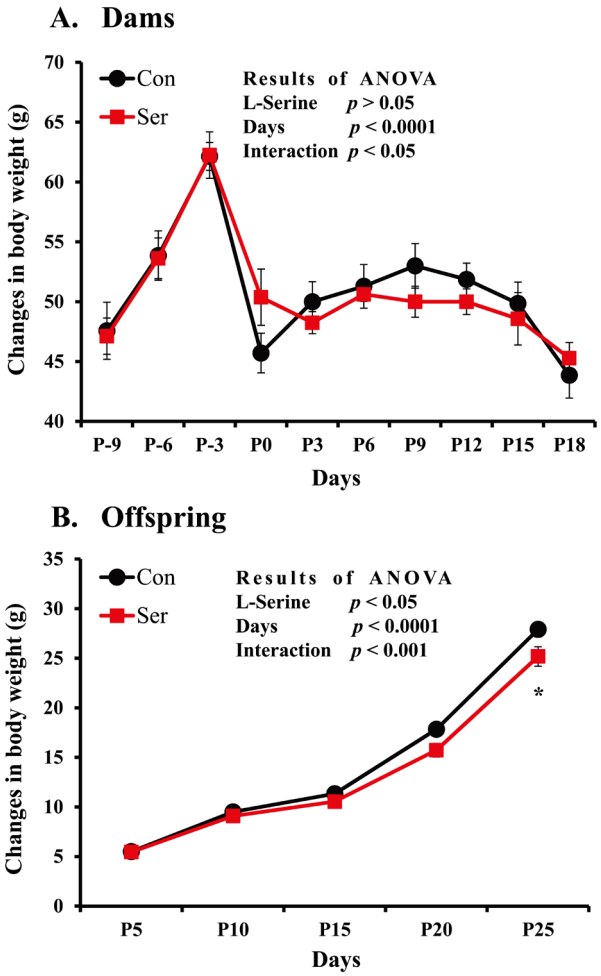

Fig. 1. Effect of dietary L-serine on body weight. Body weight of mothers (A) and offspring (B) was measured every 3 days. Means ± SEM, n=7–8 for mothers and n=8 for offspring. P, postnatal day after delivery or birth.

Statistical analysis

All data in each group were first subjected to a Thompson's rejection test to eliminate outliers (P<0.01), and the remaining data were used for the analysis among groups. All data were expressed as means ± SEM; FAA levels in the maternal plasma and milk, and the results of the behavioral test were analyzed by t test. Body weight and food intake were analyzed by a two-way repeated-measures ANOVA, and the results of the OFT were analyzed by a one-way ANOVA. The Tukey-Kramer test was applied as a *post hoc* analysis when a significant interaction between days and L-serine treatment was detected. Differences were considered significant at P<0.05. All analyses were performed with Stat View (version 5, SAS Institute, Cary, NC, U.S.A., 1998).

RESULTS

Effects of dietary L-Ser on body weight and food intake

Changes in the body weight of the dams and offspring are shown in Fig. 1A and 1B, respectively. There was no significant difference in the body weight of dams by treatment. A significant (P<0.05) interaction between treatment and days was detected for dams. The body weight of offspring was significantly (P<0.05) lower in the Ser group than in the Con group. A significant (P<0.001) interaction between treatment and days was detected for offspring, suggesting that the difference between the two groups widened with the progress of time. Post hoc analysis clarified that the body weight of offspring in the Ser group was significantly lower than that in the Con group at P25 (P<0.05).

Changes in the mothers' food intake are shown in Fig. 2. A significant (P<0.001) interaction between treatment and days was

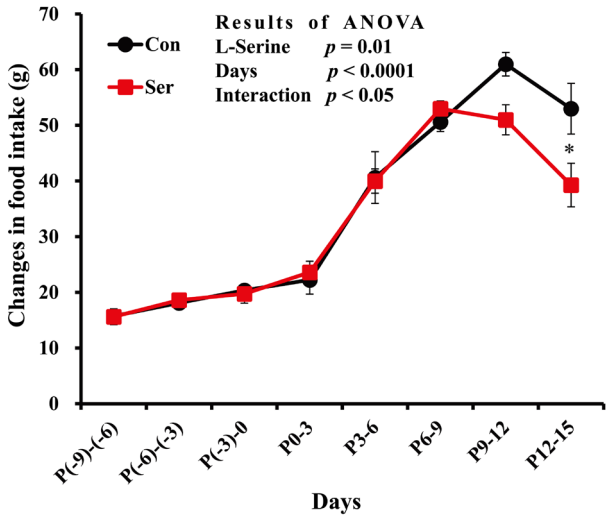

Fig. 2. Effect of dietary L-serine on food intake of mother. Food intake was examined every 3 days. Means ± SEM, n=7–8. P, postnatal day after delivery or birth.

observed, implying that food intake was comparable between the two groups without any reduction in the Ser group except for P12–15. Post hoc analysis clarified that food intake in the Ser group was significantly lower than that in the Con group at P12–15 ($P<0.05$).

Effects of dietary L-Ser on the behavioral parameters

There was no significant difference between the two groups in the maternal behavioral test (data not shown). In the dams at P12, total distance, number of entries into the central area, and ratio of inner distance/total distance were not significantly different between the two treatments (data not shown). In the offspring, total distance for the Ser group was significantly ($P<0.05$) higher than that for the Con group (Fig. 3A). However, no significant differences were found in the number of entries into the central area or in the ratio of inner distance/total distance (Fig. 3B and 3C). No significant difference was found between the two groups in the ORT (data not shown).

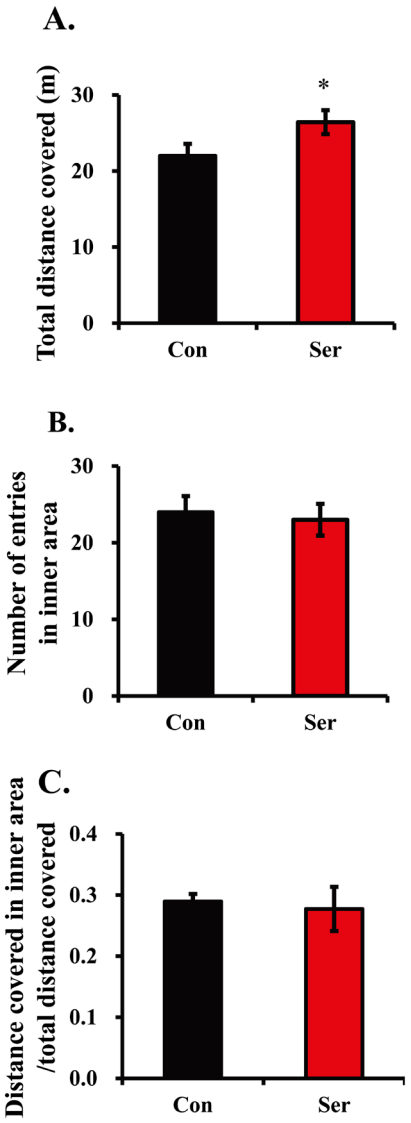

Fig. 3. Effect of dietary L-serine on open field test in terms of total distance (A), number of entries into central area (B), and ratio of distance in inner area/total distance (C) for 17-day-old offspring. Means ± SEM, n=8 in offspring.

Effects of dietary L-Ser on FAA composition of maternal milk and plasma

The concentrations of FAAs in the maternal plasma are shown in Fig. 4A, and in the maternal milk in Fig. 4B. In the maternal plasma, a significant effect was detected only in D-Ser levels, although the levels were relatively low compared with other FAAs. D-Ser increased slightly ($P<0.05$) in the Ser group compared with the Con group. In maternal milk, L-Ser levels increased significantly ($P<0.001$) in the Ser group, but levels of glutamic acid (Glu), Tau, L-alanine (L-Ala), and D-Ala significantly ($P<0.05$) decreased. Plasma FAAs of offspring at P13 and P20 are shown in Fig. 5A and 5B, respectively. No significant differences were observed in any FAAs between the groups at P13, while both L-Ser and D-Ser were found to have increased significantly ($P<0.05$ and $P<0.01$, respectively) at P20.

DISCUSSION

Food intake of the dams significantly decreased in the Ser group mainly due to the reduction that occurred during P12–15. The reduction might have been caused by changes in the brain levels of D-Ser, a metabolite of L-Ser, which suppresses intake of high-preference food [23]. In rats chronically supplemented with L-Ser, D-Ser levels in the brain increased, while L-Ser in the brain, as well as L-Ser and D-Ser in the plasma, did not increase [17]. These data are comparable with our data showing the concentration of L-Ser in plasma not to have changed. This may also be the case for offspring, since the body weight gain tended to decrease when offspring took both maternal milk and the maternal diet.

The results of the OFT in the dams indicated that chronic supplementation of L-Ser did not affect total distance traveled, number of entries into the inner area or ratio of distance in inner area/total distance. Total distance was used as an index of locomotor

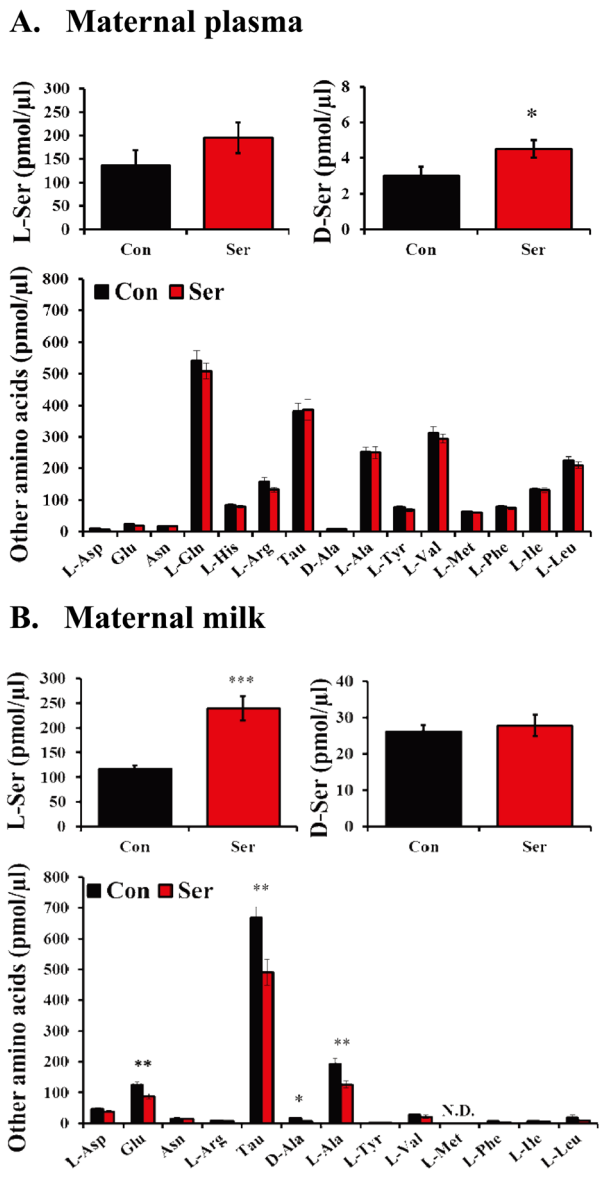

Fig. 4. Effect of dietary L-serine on concentration of free amino acids in maternal plasma (A) and maternal milk (B). Means ± SEM, n=7–8 in mother. **P*<0.05; ***P*<0.01; ****P*<0.001. N.D.: L-Met was under the detection limit.

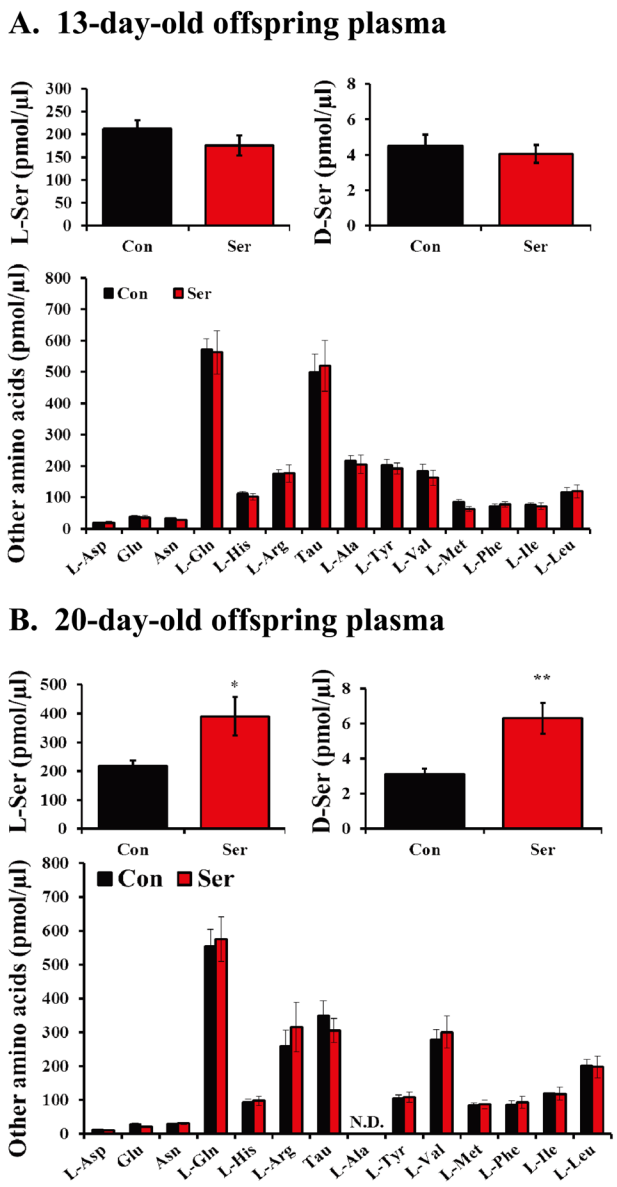

Fig. 5. Effect of dietary L-serine on concentration of free amino acids in plasma of 13-day-old offspring (A) and 20-day-old offspring (B). Means ± SEM, n=8 in both offspring groups. **P*<0.05; ***P*<0.01. For plasma of 20-day-old offspring, there is one missing value for L-Leu in the Ser group, and there are two missing values for L-Phe, in the Ser group and the Con group, as they were under the detection limit. N.D.: L-Ala was judged as not detectable, since the peak was overlapped to another peak.

activity, and number of entries into the inner area and ratio of distance in inner area/total distance were used as indexes of anxiety-like behavior. These results were contrary to those of a previous study [26], which reported that chronic administration of 2% L-Ser in distilled water decreased locomotor activity of isolated male rats in their home cages during the dark period. I.c.v. injection of L-Ser also induces sedative and hypnotic effects in neonatal chicks under stressful conditions [25]. However, the present study examined locomotor activity using the OFT during the suckling period in female mice, which were fed L-Ser through the diet, rather than through the drinking water. In addition, the test was performed during the light period under the novel environment of the OFT. These differences in experimental conditions may explain the different results. In addition, in the case of acute treatment, the concentration of L-Ser in plasma was found to have increased eightfold 30 min after the oral administration, and that in the hippocampus and the cortex also increased [25]. On the other hand, as has been reported in previous research using rats [17], chronic treatments do not affect L-Ser levels in the plasma and brain. Thus, there is a broad difference in metabolism and behaviors

between single and chronic L-Ser treatments.

The results of the OFT performed on the offspring indicated that the administration of L-Ser increased locomotor activity in the offspring, which was contrary to the findings of a previous study [26]. This increase may have been due to the L-Ser-induced activation of pyruvate kinase M2 (PKM2), an isoenzyme of the glycolytic enzyme [4], in the Ser group which positively influenced the energy metabolism to increase locomotor activity. However, the relationship between energy metabolism and locomotor activity has not yet been studied.

The composition of FAAs in the mothers differed notably between the plasma and the milk. In maternal plasma, most amino acids were detected, but significant differences between the Con and the Ser group were only barely detected. On the other hand, Tau, L-Ala, L-Ser, Glu, and L-aspartic acid (L-Asp) were mainly detected in maternal milk. These amino acids have also been detected in the milk of other species, such as dairy cattle, humans and rats [12, 21]. This result showed that FAAs in milk are selected through mammary epithelial cells or as the residue of milk protein synthesis. The number of amino acids that are abundant in plasma but undetectable in milk may have been decreased by the L-amino-acid oxidase expressed in the mammary glands [7, 16, 27].

Although L-Ser levels did not change significantly in the plasma of the Ser group, their levels in maternal milk were high. These data suggested that plasma L-Ser transferred efficiently to milk. The increased levels of L-Ser, along with the decreased levels of other amino acids (Glu and D/L-Ala), in the milk of the Ser group may be related to the functions of amino acid transporters in the mammary gland [1]. Amino acid transporters are important for carrying each FAA from plasma to the milk, and thus it is possible that more L-Ser went through the transporters, with the effect that several other amino acids (but not L-Ser) decreased in the Ser group. The ASC system is a major component of the Na^+-dependent transportation of amino acids, which prefers linear dipolar amino acids, such as L-Ser, L-Ala, L-Gln, L-cysteine (L-Cys) and so on. ASCT2 was found in the rat mammary gland [1]. Cys is important for synthesis of Tau, and L-Gln is translated into L-Glu by glutamate synthase. Thus, it is possible that L-Ser competes against L-Ala, L-Cys and L-Gln, resulting in the decreased levels of Ala, Tau and Glu in the milk. Moreover, Glu is one of the main FAAs in maternal milk and Ala can be transported easily through the mammary gland [30]. Therefore, the presence of these amino acids in milk may be largely affected by L-Ser treatment.

Body weight of the offspring was significantly lower in the Ser group. However, the parental behavioral test showed that there was no significant difference in motivation to rear between the groups. Thus, there are three possible reasons that might explain the lower body weight in the Ser group. First, the amount of maternal milk and its intake by offspring may have decreased as a result of the reduced food intake by the dams in the Ser group. Second, the reduction of Tau in the milk may have caused the decline in offspring growth, as Tau is a key factor in offspring growth through its multiple functions related to digestion, absorption, and hormone regulation [9]. In agreement with our data, L-Ser has been found to reduce the concentration of Tau in the extracellular fluid of chick brain slices [24]. Although Tau levels in the plasma of offspring were not altered in the Ser group, this may have been the result of the reduced transportation of Tau into tissue, including the liver, spleen and kidneys [19]. Third, food preference was changed by increasing the levels of D-Ser [23], which was also done with the mothers, although the increment was rather small. Further research is necessary to clarify the relationship between chronic L-Ser administration and frequency of feeding.

The differences between P13 and P20 in terms of concentration of FAAs in the offspring may have been derived from the fact that the P13 offspring were fed only milk, while the P20 offspring were fed both milk and the same diet as the mother. This explanation is supported by the data showing that, compared with the other FAAs, the overall concentration of Tau in the plasma of P13 offspring was particularly affected by the high content of Tau in the maternal milk, because it was almost five times higher at P13 than it was at P20 in offspring plasma. These data confirm that Tau is essential for growth during the early stages of life [9]. Additionally, the composition of FAAs in offspring plasma at P20 may have been caused by ingestion of the maternal diet, given that plasma L-Ser and D-Ser levels increased in the Ser group at P20, but not at P13. Thus, it is considered that the enhanced level of L-Ser in the milk at P13 was not sufficient to increase plasma L-Ser in the offspring. For comparison, P20 offspring were fed approximately 3g of a 2% L-Ser diet, which included 570 nmol of L-Ser; on the other hand, the concentration of L-Ser in milk in the Ser group was approximately 250 pmol/μl, and the offspring took approximately 250 μl milk/day (milk production is 2 g/day in dams, divided by 8 offspring) [28], which includes 62.5 nmol of L-Ser. Thus, the L-Ser intake in offspring at P13 was far less than that in offspring at P20. However, this comparison has some limitations: first, the concentrations of plasma and milk FAAs also changed according to the point in the lactation period during which feeding occurred [6, 11, 20, 22]. Second, because the point at which the offspring started to eat the maternal diet during the suckling period was not clear, the effect of the maternal diet on offspring plasma could not be analyzed precisely. Thus, in future studies, milking and sampling time should be carefully controlled. In conclusion, the present study found that L-Ser was efficiently transported from maternal plasma to milk and that dietary L-Ser can change the FAA composition of milk. However, an increased L-Ser level in milk did not affect plasma L-Ser in suckling offspring.

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