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Maternal Stress During the Lactation Period Rather than the Gestation Period Strongly Influences the Amino Acid Composition in Milk and Affects Growth and Behaviour in Offspring

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It is well known that maternal stress during the gestation and lactation periods induces abnormal behavior in the offspring and causes a lowering of the offspring's body weight. However, the negative effects of maternal stress on the mother's milk, which is one of the most important connecting factors between mothers and their offspring, are not yet fully understood. The present study aimed to investigate how free amino acids in milk, as well as the growth and behavior of offspring, are changed by maternal stress during the gestation and lactation periods. Although maternal stress during the gestation period did not affect the growth of offspring or the free amino acid composition in the milk, the offspring in the stressed group showed lower locomotor activity. However, maternal stress during the lactation period caused a reduction in body weight in the offspring following the stress load. It increased the concentration in the milk of several amino acids, such as taurine, aspartic acid and β–alanine, while it decreased the concentration of most of the free amino acids in the maternal plasma. Notably, the body weight of offspring at postnatal age 11 days was negatively correlated with taurine concentration in the milk. These results show that maternal stress during the lactation period may have a great impact on the free amino acid composition of the milk, which may partly, but not entirely, be the cause of lower body weight in the offspring.

Key words: Free amino acids, Gestation period, Lactation period, Maternal stress, Milk

INTRODUCTION

It is well known that pregnant women are vulnerable to stress during the gestation period and that various mental diseases can develop as a result of excessive stress (Steiner, 1979). For instance, it has been recognized that excessive stress can increase the risk of depression after delivery—termed "postpartum depression" (Brummelte and Galea, 2010; Becker *et al*., 2016). Maternal stress during the gestation period not only influences the affective state of the mother, it also affects the psychiatric and mental health of her children. Recent studies have shown that maternal stress during the gestation period induces various changes in the behavior of animal offspring, leading to, for example, depression–like behavior (Zheng *et al*., 2016), anxiety–like behavior (Zheng *et al*., 2016), behaviors related to schizophrenia (Koenig *et al*., 2002) and learning disorders (Ishiwata *et al*., 2005), and deficits in memory recognition (Wilson and Terry, 2013). Similar observations are reported in human children, as maternal stress during the gestation period has been shown to elevate the risk of attention deficit hyperactivity disorder (Ronald *et al*., 2011) and autism (Gillott and Standen, 2007). In addition, Baker *et al*. (2008) observed that the body–weight gain in rat offspring after birth, especially after weaning, was decreased by maternal restraint stress during the gestation period.

Maternal stress during the lactation period also affects the growth and behavior of both the mother and her children. Gao *et al*. (2011) showed that in mice, maternal stress during the lactation period caused a decrease in the body–weight gain of the offspring, and its effect continued for 15 weeks after their birth. Stephen *et al*. (2006) showed that acute maternal stress during the lactation period caused an increase in aggressive behavior of maternal mice. Various stressors, including psychological stress, nursing stress, and nutritional status during the lactation period, decreased the amount of immune substances such as secretory immunoglobulin A (sIgA) in human milk (Miranda *et al*., 1983; Lovelady *et al*., 2003; Fetherston *et al*., 2006), which seems to cause immune deficiency in human children.

Although maternal stress during the gestation and lactation periods is a serious social issue, little is known about the mechanisms by which maternal stress is transmitted by a mother to her children. Champagne and Meaney (2006) reported that maternal stress altered the parental behavior of maternal mice, including licking and grooming, and increased anxiety–like behavior in their offspring, but very few papers have shown the effects of maternal stress on the composition of milk, which is the most important nutrition for offspring. Therefore, the current study examined how maternal stress during the gestation and lactation periods affects the composition of milk, with particular reference to free amino acids. We

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focused on free amino acids because milk free amino acids rather than milk protein are rapidly available for offspring. In order to determine the effects of maternal stress during the gestation and lactation periods on the growth and behavior of offspring in mice, we measured the body weight of mice offspring after birth and performed the open field test (OFT) on offspring. Therefore, two experiments were carried out: on maternal stress during the gestation period (Experiment 1); and on maternal stress during the lactation period (Experiment 2).

MATERIALS AND METHODS

Animals

In Experiment 1, ICR mice (males: 7 weeks old; females: 6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The female mice were mated in a group and subsequently delivered their offspring, which were raised until they were 7 weeks of age. The mice were housed in a group of 6 females with 3–4 males, and were mated in a large cage (25 cm × 40 cm × 20 cm) placed in a room at a temperature of 23 ± 1°C and with humidity at 60%. They were maintained on a 12–h light/dark cycle (lights on at 08:00, lights off at 20:00). The day of mating was considered "day zero of pregnancy" (G0), and this assumption was corroborated by observing the length of gestation. Water and a standard diet for laboratory rodents (MF, Oriental Yeast, Tokyo, Japan) were available *ad libitum*. At day G10, the pregnant dams were separated into two groups: 1) control group (n = 8); and 2) stress group (n = 8), with individual housing in a cage (12 cm × 30 cm × 14 cm) suitable for breeding. Pregnant dams in the stress treatment group were subjected to restraint stress as described below. When their offspring were delivered, the sexes of the offspring were checked, and the number of offspring was standardized to 8 in order to make the nutritional condition the same among the dams. The male–to–female ratio varied among the litters. The delivery day was regarded as "P0". All the offspring were housed with their dams, where drinking water and a standard diet for laboratory rodents were available *ad libitum*. The OFT was performed on the offspring at P21, P28 or P35. Blood samples in dams were collected at P20 and centrifuged at 3,000 × g for 15 min at 4°C (KUBOTA 3740), and stored at –80°C until analysis. After weaning, the body weights of the offspring were measured every 5 days from day 5 after birth (P5) to P30. Only male offspring were used in all analyses. Milk samples in dams were collected at P10 and stored at –80°C until analysis took place.

In Experiment 2, ICR mice (8 weeks old for both males and females) were purchased from Japan SLC (Hamamatsu, Japan). They were housed in separate groups of either males or females for acclimation over the course of a week. Then they were housed in a group of 5–6 females with 2–3 males and allowed to mate in a large cage (25 cm × 40 cm × 20 cm) placed in a room at a temperature of 23 ± 1°C and with humidity at 60%. They were maintained on a 12–h light/dark cycle (lights on at 08:00, lights off at 20:00). When the dams were confirmed to be pregnant, they were then housed individually in a cage suitable for breeding, as described in Experiment 1. After 19–26 days from the start of mating, all the female mice delivered between 9 and 18 offspring. At day P0, the sexes of the offspring were checked, and the number of offspring was standardized to 7 in order to make the nutritional conditions the same among the dams. The male–to–female ratio varied among the litters. The dams were separated into two groups: 1) control group (n = 7); and 2) stress treatment group (n = 7). Those in the stress treatment group were subjected to restraint stress as described below. Water and a standard diet for laboratory rodents (MF, Oriental Yeast, Tokyo, Japan) were available *ad libitum* for dams and offspring throughout this experiment. The body weights of offspring were measured every 3 days from P5 to P35. Male and female offspring were used in the analysis between P5 and P20 (i.e. before weaning), but only male offspring were used in the analysis after weaning. We collected milk samples from dams at P12, and maternal blood samples at P20. Milk samples were collected after 60 min of stress treatment and maternal blood samples were collected after 5 min of stress treatment. Blood samples were centrifuged at 3,000 × g for 15 min at 4°C (KUBOTA 3740), and stored at –80°C until analysis. Milk samples were stored at –80°C until analysis took place. We also conducted the OFT on offspring at P35.

The present study was performed according to the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and conformed to Law No. 105 and Notification No. 6 of the Japanese government.

Maternal stress procedure

In Experiment 1, the pregnant dams in the stress treatment group were subjected to a daily stress session starting at 9.30 a.m. and lasting for 30 min, between G10 and G18. They were kept on a plastic board with a bandage wrapped around their body. The control pregnant dams were left undisturbed in their own cages and were gently handled only when the cages were cleaned once a week.

In Experiment 2, the dams in the stress treatment group were subjected to a daily stress session starting at 9.30 a.m. and lasting for 10 days in total, between P2 and P20. They were wrapped in wire mesh and fixed for 30 min so that they were not able to move freely in their own cages with their offspring. The control pregnant dams were left undisturbed in their own cages with their offspring and were gently handled only when the cages were cleaned once a week.

OFT

In Experiment 1, the motor activity and anxiety–like behavior of male offspring in each group (control: n = 8; stress: n = 8) were evaluated using the OFT on P21, P28 and P35. This test was performed using apparatus consisting of a black circular base (diameter 60 cm) with walls that were 35 cm high. Offspring were tested during the light period and were kept in a closed room at a constant temperature (23 ± 1°C). Each test was recorded on a video recording system for analysis. The test was performed under light conditions of 100 lx. At the beginning of the test, a mouse was placed in the center of the apparatus and then allowed to move freely for 5 min. After each trial, the arena was cleaned with 10% ethanol solution to standardize the conditions of all the tests. Open field behavior was analyzed with ANY–maze software (Stoelting Co, Wood Dale, IL) by dividing the field into two zones: an inner zone and an outer zone. The total distance traveled, the number of inner entries and the amount of time spent in the inner zone were measured. The distance traveled was considered the parameter for motor activity, and the amount of travel occurring in the inner zone as a proportion of the total distance traveled (i.e. distance traveled in inner zone/total distance traveled) was considered the parameter for anxiety–like behavior.

In Experiment 2, the motor activity and anxiety–like behavior of male offspring in each group (control: n = 12; stress: n = 12) were evaluated on P35 using the OFT as described in Experiment 1.

Milking

In Experiment 1, milk samples were collected on P10 from the maternal mice in each group (control: n = 8; stress: n = 5), and were used for the analysis of the free amino acid level. We started the stress treatment with 8 animals, but 3 dams in the stress treatment group died during the stress treatment. Maternal mice and their offspring were separated 6 h before milking in order to collect enough milk. After 6 h, maternal mice were anesthetized with isoflurane and were injected subcutaneously with 0.1 ml of oxytocin to promote the secretion of milk. 20 min after the injection, they were anesthetized with isoflurane again and milked for 10–15 min using a KN–591 milking machine for mice and rats (Natsume Seisakusho Co. Ltd, Tokyo, Japan).

In Experiment 2, milk samples were collected on P12 from the maternal mice from each group (control: n = 6; stress: n = 6), and were used in the analysis of the free amino acid level as described above. We started the experiment with 7 animals per group, but one dam in the control group and one dam in the stress treatment group died during milking.

Analysis of free amino acids

In Experiment 1, ultra–performance liquid chromatography (UPLC) (using the AcquityTM UPLC system, comprised of Waters Binary Solvent Manager, Waters Sample Manager and Waters FLR Detector) with an ACCQTAGTM ULTRA C18 1.7 μm 2.1 × 100 mm column (Waters Corporation, USA) was used to quantify the concentration of each amino acid (both the L– and the D–type) in the plasma and maternal milk samples. These samples were deproteinized through an ultrafiltration tube (Millipore, Bedford, USA). The excitation and emission wavelengths for the fluorescent detection of amino acids were 350 nm and 450 nm, respectively. The system was operated with a flow rate of 0.25 ml/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 for 1.2 min, 40–60% B over 3.8 min, 60% B for 1 min, and 60–10%B over 0.01 min. Just before the UPLC analysis took place, each sample (10 μl) was transferred to a UPLC tube, and then NAC/OPA (20 μl) and a borate buffer (70 μl) were added to the tube. After being left for 2 min in a dark room, it was then transferred to the UPLC machine. Standard solutions containing 16 L–amino acids, 16 D–amino acids, taurine and so on were analyzed in the same way as the samples. L– and D–type Glutamate and Asparagine could not be distinguished from each other, and both amino acids were therefore given without prefixes.

In Experiment 2, free amino acid levels in the maternal plasma and milk were analyzed by HPLC. In Experiment 1, D–type amino acids were not detected. A different type of HPLC was therefore applied in Experiment 2 and abbreviations of the amino acid names were given without the L– and D– prefixes. Plasma samples were filtrated through an ultrafiltration tube (Millipore, Bedford, USA). Milk was also filtrated through an ultrafiltration tube. Each 10 μl sample of plasma and milk was completely dried under reduced pressure. The dried residues were dissolved in 10 μl of 1 M sodium acetate–methanol–triethylamine (2:2:1) and re–dried under reduced pressure, then dissolved in 20 μl of methanol–distilled water–triethylamine–phenylisothiocyanate (7:1:1:1) (derivatization solution). 20 min after the phenylisothiocyanate had finished reacting with the amino groups at room temperature, the samples were dried again under reduced pressure and then dissolved in 100 μl of buffer A [70 mM sodium acetate (pH 6.45 with 10% acetic acid)–acetonitrile (975:25)]. The same methods were used on standard solutions that were prepared by diluting a commercially available L–amino acid solution (type ANII, type B, L–asparagine, L–glutamine and L–tryptophan; Wako, Osaka, Japan) with HCl solution. A Waters HPLC system (Pico–tag free amino acid analysis column (3.9 mm × 300 mm), Alliance 2690 separation module, 2487 dual–wavelength UV detector, and Empower 2 chromatography manager; Waters, Milford, USA) was applied to the samples. They were then equilibrated with buffer A and eluted with a linear gradient of buffer B [water–acetonitrile–methanol (40:45:15)] (0, 3, 6, 9, 40 and 100%) at a flow rate of 1ml/min at 46ºC. The absorbance at 254nm was applied to determine the concentrations of the free amino acids. Triethylamine and sodium acetate trihydrate were purchased from Wako (Osaka, Japan), while the other drugs, for which no manufacturer is noted, were purchased from Sigma (St Louis, USA). The concentration of free amino acids in the plasma and maternal milk samples was expressed as pmol/μl.

Analysis of corticosterone

Maternal plasma samples collected at P20 were analyzed for total corticosterone concentrations, using a corticosterone enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol, except for the use of Steroid Displacement Reagent (2.5%, Enzo Life Science, Farmingdale, NY) in the plasma dilution step.

Statistical analysis

All data were expressed as means ± SEM; free amino acid levels in the maternal plasma and milk, the results of the OFT in Experiment 2, and the results of the corticosterone measurement were analyzed by t test. Body weight of offspring was analyzed by two–way repeated measures ANOVA, and the results of the OFT in Experiment 1 were analyzed by two–way ANOVA. Differences were considered significant at $P<0.05$. All analyses were performed with Stat View (version 5, SAS Institute Cary, United States, SAS 1998). Outlying data were eliminated by Thompson's test criterion for outlying observations ($P<0.01$).

RESULTS

Experiment 1

Body weight of offspring

The effects of maternal stress during the gestation period on the growth of offspring after birth are shown in Fig. 1. No significant effect of maternal stress was detected on the body weight of offspring. The body weight gradually increased after birth in offspring reared only with maternal milk; however, it sharply increased after P15 in offspring reared with both mother's milk and standard diet.

OFT

The effects on offspring of maternal stress during the gestation period in terms of the parameters in the OFT are shown in Fig. 2. Maternal stress significantly ($F(1,41) = 7.424$, $P<0.01$) decreased the distance traveled, but no significant effects of days and no interactions between stress and days were detected. On the other hand, distance traveled in the inner zone/total distance traveled significantly ($F(2,41) = 8.157$, $P<0.001$) increased with the progress of days, but no significant effects of stress and no interactions between stress and days were observed.

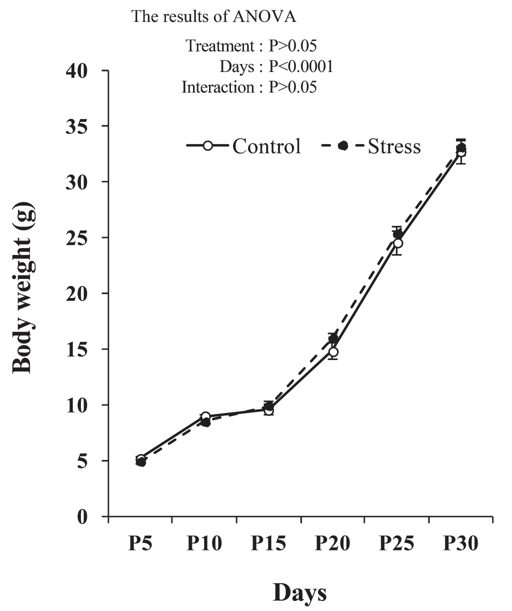

Fig. 1. The effects of maternal stress during the gestation period on growth of offspring. Body weight of male offspring was examined. Values are expressed as means ± SEM; n = 13–18 in the control group; n = 8–10 in the stress group. P: postnatal age (day).

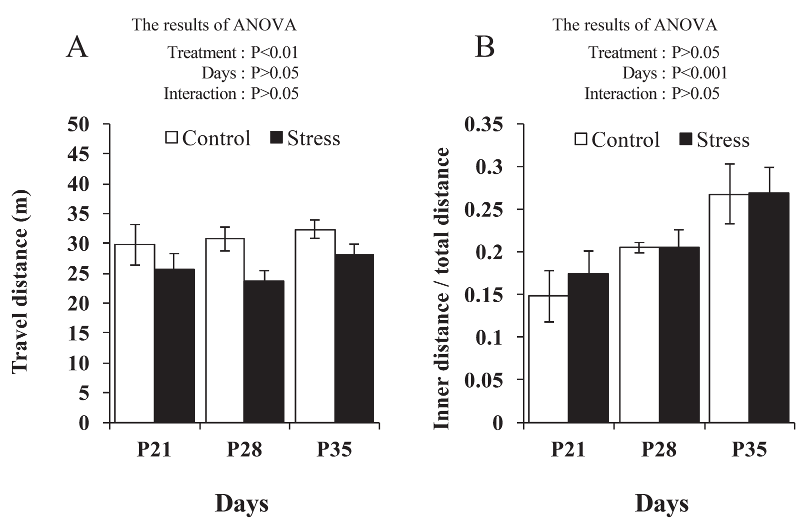

Fig. 2. The effects of maternal stress during the gestation period on behavior of offspring. Distance traveled (A) and distance traveled in inner zone/total distance traveled (B) were analyzed. Values are expressed as means ± SEM; n = 8 in the control group; n = 8 in the stress group. P: postnatal age (day).

Table 1. The effects of maternal stress during the gestation period on free amino acid concentrations in maternal plasma and milk

Amino acids	Maternal plasma			Milk		
	Control	Stress	P value	Control	Stress	P value
L–Aspartate	12.0±1.0	9.3±0.6	NS	43.0±1.9	46.8±4.9	NS
Glutamate	27.0±2.5	18.7±1.5	<0.05	126±11	148±36	NS
Asparagine	27.6±1.9	17.8±1.8	<0.01	21.1±2.4	19.8±3.3	NS
D–Serine	ND	ND		32.5±2.2	34.9±6.5	NS
L–Serine	172±7	115±10	<0.001	121±12	121±16	NS
L–Glutamine	713±40	463±24	<0.001	15.2±2.7	16.1±2.6	NS
L–Histidine	100±5	76±1	<0.05	ND	ND	
L–Arginine	247±19	185±21	NS	14.7±1.4	14.5±1.9	NS
Taurine	382±20	333±82	NS	987±69	982±57	NS
D–Alanine	ND	ND		16.7±1.6	17.0±2.5	NS
L–Alanine	ND	ND		230±29	235±2	NS
L–Tyrosine	91.5±10	78.2±11	NS	ND	ND	
L–Valine	467±44	363±97	NS	60.7±9.7	54.9±9.2	NS
L–Methionine	88.0±5.5	70.0±6.3	NS	25.3±2.8	22.8±2.5	NS
L–Phenylalanine	98.3±8.5	84.8±7.4	NS	ND	ND	
L–Isoleucine	188±19	180±22	NS	15.5±2.3	13.7±2.3	NS
L–Leucine	324±35	273±35	NS	33.5±4.4	33.9±7.7	NS

The values for the concentrations for each amino acid are expressed as means ± SEM in pmol/μl sample volume; n = 8 in control group; n = 5 in stress treatment group. NS, not significant; ND, not detectable.

Free amino acid concentration in maternal plasma and milk

The effects of maternal stress during the gestation period on the free amino acid composition in maternal plasma and milk are shown in Table 1. Plasma glutamate (P<0.05), asparagine (P<0.01), L–serine (P<0.001), L–glutamine (P<0.001), and L–histidine (P<0.05) significantly decreased with stress treatment. However, no significant effects of maternal stress during the gestation period were observed on any of the amino acids occurring in the analysed milk. Some amino acids were detected only in the maternal plasma and some were detected only in the milk samples. Almost all of the detected amino acids showed quite different concentrations between the maternal plasma and the milk; for example: taurine (in maternal plasma, control = 382 ± 20, stress = 333 ± 82; in milk, control = 987 ± 69, stress = 982 ± 57).

Experiment 2

Body weight of offspring

The effects of maternal stress the during lactation period on the growth of offspring after birth are shown in Fig. 3. Fig. 3A shows the changes in body weight during P5 to P20 in both male and female offspring, and the right panel (Fig. 3B) only shows the changes in male offspring. A significant (F (1,5) = 2.333, P<0.05) interaction between stress treatment and days after birth was observed in the body weight of offspring from P5 to P20, implying that growth was retarded due to the maternal stress. Although the stress group showed a trend of low body weight gain (P = 0.054) for the male offspring after weaning, no significant main effects of stress treatment were found.

OFT

The effects of maternal stress during the lactation period on the parameters in the OFT are shown in Fig. 4. Maternal stress significantly (P<0.05) increased the distance travelled (Fig. 4A). However, no significant effects of maternal stress on distance traveled in the inner zone/total distance traveled were observed (Fig. 4B).

Free amino acid concentration in plasma and milk

The effects of maternal stress during the lactation period on the free amino acid composition of the maternal plasma and milk are shown in Table 2. Plasma aspartate (P<0.01), serine (P<0.001), asparagine (P<0.001), glycine (P<0.001), gltamine (P<0.001), histidine (P<0.05), threonine (P<0.001), alanine (P<0.001), arginine (P<0.01), proline (P<0.01), tyrosine (P<0.01), valine (P<0.001), methionine (P<0.001), isoleucine (P<0.001), leucine (P<0.001), phenylalanine (P<0.01), ornithine (P<0.001), and lysine (P<0.001) were found to have significantly decreased, but gamma–aminobutyric acid (GABA) (P<0.01) was found to have significantly increased as a result of the stress treatment. Irrespective of great decreases in plasma free amino acid concentrations, only three amino acids (asparagine (P<0.001), β–alanine (P<0.001), and taurine (P<0.05)) were, conversely, found to have increased in milk. Some amino acids were detected only in the maternal plasma

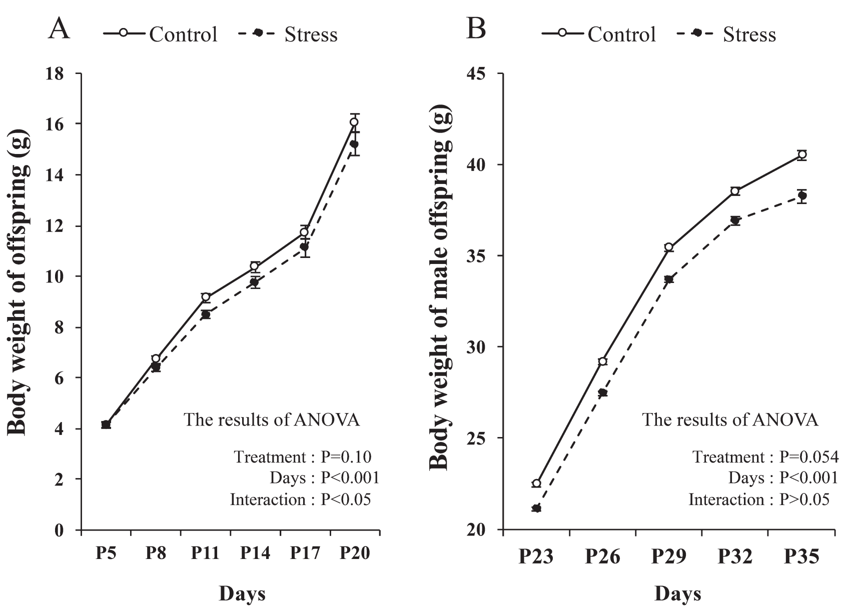

Fig. 3. The effects of maternal stress during the lactation period on growth of offspring. Body weight of male and female offspring was examined before weaning (P5–P20); n = 42 in the control group; n = 42 in the stress group, and body weight of male offspring only was examined after weaning (P23–P35); n = 26 in the control group; n = 24 in the stress group. Values are expressed as means ± SEM.; n = 13–18 in the control group; n = 8–10 in the stress group. P: postnatal age (day).

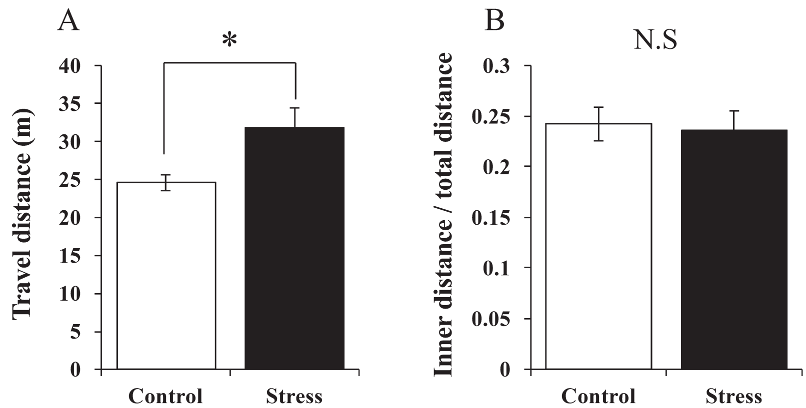

Fig. 4. The effects of maternal stress during the lactation period on behavior of offspring. Total distance traveled (A) and distance traveled in inner zone/total distance traveled (B) were analyzed. Values are expressed as means ± SEM; n = 12 in the control group; n = 12 in the stress group. *Significantly different at P<0.05.

and some amino acids were detected only in the milk samples. Almost all the amino acids that were detected showed quite different concentrations between the maternal plasma and the milk (e.g. taurine (in maternal plasma, control = 220 ± 12, stress = 225 ± 27; in milk, control = 425 ± 13, stress = 686 ± 82).

Corticosterone concentration in maternal plasma

Corticosterone levels in the maternal plasma tended to increase (control = 9.6 ± 1.6, stress = 21.0 ± 7.9 ng ml) as a result of restraint stress during the lactation period, although the increase was not significant (P = 0.07).

DISCUSSION

Few studies have demonstrated the effects of maternal stress during the gestation and lactation periods on the free amino acid composition of milk. The present study examined the effects of maternal stress during the gestation and lactation periods on the growth and behavior of offspring, as well as on the amino acid composition of maternal plasma and milk. Moreover, we compared the effects of maternal stress during the gestation period with the effects of maternal stress during the lactation period.

Table 2. The effects of maternal stress during the lactation period on free amino acid concentrations in maternal plasma and milk

Amino acids	Maternal plasma			Milk		
	Control	Stress	P value	Control	Stress	P value
Aspartate	17.8±1.0	12.6±1.1	<0.01	38.5±3.4	42.2±6.2	NS
Glutamate	42.9±0.9	43.8±3.7	NS	134±9	181±26	NS
Serine	122±8	61.9±4	<0.01	33.9±2.4	37.0±4.0	NS
Asparagine	56.9±5.7	24.9±1.8	<0.001	14.3±0.5	23.6±0.9	<0.001
Glycine	155±2	77±6	<0.001	53.1±3.7	62.0±3.3	NS
Glutamine	656±24	425±14	<0.01	10.1±1.1	11.0±1.2	NS
β–Alanine	ND	ND		26.7±0.4	41.4±2.6	<0.001
Taurine	220±12	225±27	NS	425±13	686±82	<0.05
Histidine	70.5±0.9	54.6±5.3	<0.05	ND	ND	
GABA	89±5	115±5	<0.01	ND	ND	
Threonine	261±6	104±4	<0.001	11.5±0.9	10.8±1.9	NS
Alanine	320±6	184±12	<0.001	98±9	106±8	NS
Arginine	272±8	199±13	<0.01	ND	ND	
Proline	149±16	68±6	<0.01	87.0±9.6	110±11	NS
Tyrosine	86.7±5.2	60.5±5.9	<0.01	6.09±0.43	8.92±1.08	NS
Valine	379±21	209±3	<0.001	21.7±2.5	32.0±4.4	NS
Methionine	77.8±4.7	40.8±2.0	<0.001	2.57±0.12	2.78±0.09	NS
Cystathionine	230±12	244±12	NS	189±11	161±23	NS
Cysteine+Cystine	15.7±1.0	12.8±1.1	NS	6.99±0.84	8.79±0.94	NS
Isoleucine	137±10	74±4	<0.001	6.63±0.67	8.20±1.01	NS
Leucine	248±11	127±11	<0.001	2.95±0.44	3.37±0.87	NS
Phenylalanine	102±6	70±7	<0.01	ND	ND	
Tryptophan	71.4±3.4	69.5±6.5	NS	5.18±1.28	7.11±1.88	NS
Ornithine	98.1±7.3	37.9±3.7	<0.001	4.58±0.55	6.30±1.29	NS
Lysine	308±20	142±9	<0.001	11.4±0.8	12.1±0.4	NS

The values for the concentrations for each amino acid are expressed as mean ± SEM in pmol/μl sample volume; n=6 in control group; n=6 in stress treatment group. NS not significant; ND not detectable.

Corticosterone concentrations were only measured in Experiment 2 due to an assumption in Experiment 1 that corticosterone in maternal plasma cannot serve as a biomarker of stress. It has been reported that the concentration of plasma corticosterone was much higher after 15 min of stress treatment and that it returned to the basal concentration after 60 min of stress treatment (Muir and Pfister, 1987). In Experiment 1, maternal dams were stressed during the gestation period but the blood samples were collected at P20. In Experiment 2, however, corticosterone levels in the maternal plasma tended to be increased by stress treatment during the lactation period, although the increase was not significant (P = 0.07). Thus, the dams seemed to be in a state of mild stress.

Maternal stress during the gestation period did not affect the early development of offspring. This finding was in accordance with a previous study (Amugongo and Hlusko, 2014). However, body–weight gain of offspring after weaning tended to be lowered by maternal stress during the lactation period. Gao *et al*. (2011) showed that immobilization stress during the lactation period decreased the body weight of offspring from weaning to 15 weeks of age, and this result was in accordance with the present results. The decreased body weight in offspring might be explained as follows. A first possibility would be that the alteration in the composition of milk amino acid caused the decrease in the body weight of offspring. In fact, a significant increase in some free amino acids in the milk was observed following maternal stress in Experiment 2. For instance, the relationship between the concentration of taurine in milk at P12 and the body weight of offspring at P11 was negatively correlated (P<0.05) (Fig. 5). One previous study has reported that the deficiency of taurine may disturb offspring growth (Hu *et al*., 2000). The present study suggest that an excessive amount of taurine might also disturb offspring growth, and thus an appropriate level of taurine is necessary for normal growth. Second, it could be assumed that the amount of insulin–like growth factor (IGF)–1 present in the milk (Savino *et al*., 2009) could have decreased due to the maternal malnutrition caused

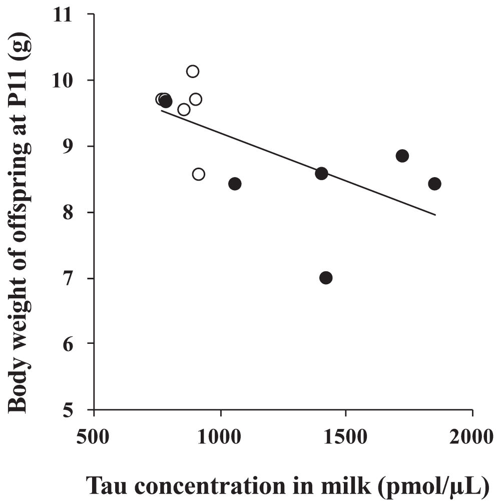

Fig. 5. Correlation between taurine concentration in milk (pmol/μl) and body weight of offspring at postnatal age 11. r = 0.64 (P<0.05).

by the stress–induced decrease in food intake (Jeong *et al.*, 2013). Indeed, stress–induced malnutrition caused by restraint has been found to decrease the amount of IGF–1 in the serum (Gao *et al.*, 2011).

Maternal stress during the gestation period resulted in lower motor activity of offspring in the OFT (i.e. a reduced emotional response in a novel environment). Previous studies have reported similar effects of maternal stress on the motor activity of offspring in pigs (Emack *et al.*, 2008), rats (Fujioka *et al.*, 2001), and mice (Shiota and Kayamura, 1989); the mechanism appears to involve Fos expression in the amygdala (Fujioka *et al.*, 2001). The values for distance traveled in the inner zone/total distance traveled, which is the parameter for anxiety–like behavior, increased gradually with the progression of days. Because P21 (3 weeks of age) was the time of weaning, at this point offspring felt maternal separation stress strongly. It has been reported that maternal separation stress increased anxiety–like behavior in offspring (Sánchez *et al.*, 2001; Daniels *et al.*, 2004). However, maternal stress during the lactation period induced an increase in motor activity in offspring. This might be due to an increased level of taurine in the milk as a result of maternal stress treatment, as observed in our study. In agreement with this hypothesis, previous studies have shown that taurine supplementation induced an increase in the total distance traveled in mice (Idrissi *et al.*, 2009), and neonatal taurine supplementation led to an increase in total distance traveled in rats (Francisco *et al.*, 2015).

The concentration of some free amino acids in the maternal plasma decreased as a result of stress during the gestation period. Similarly, previous studies using male rats exposed to acute restraint stress reported that some of the free amino acids in the plasma decreased as a result of stress treatment (Kennett *et al.*, 1986). The reduction might have occurred through catabolization of the amino acids by plasma catecholamines, which are enhanced by stress (Taylor *et al.*, 1989; De Boer *et al.*, 1990), because Shamoon *et al.* (1980) showed that adrenaline decreased levels of most amino acids in human plasma. However, despite what was reported previously (Kennett *et al.*, 1986; Nagasawa *et al.*, 2012), the reduction in free amino acids was not extreme. This seems to be due to a time lag between the final stress treatment at gestational age 18 days and blood sampling at postnatal age 20 days. We confirmed the important fact that the levels of most of the free amino acids in the milk were very low compared with those in the maternal plasma in both Experiments 1 and 2 (Tables 1 and 2). Nagaoka *et al.* (2009) reported that the free amino acids in mouse milk were converted into keto acids, ammonia, and hydrogen peroxide by high activity of L–amino acid oxidase in the mammary glands, with the result that the free amino acids in milk were then decreased.

Maternal stress during the gestation period did not affect any of the free amino acids in the milk. We do not know the reason for this result because milk is synthesized on the basis of blood components in the mammary glands. In the case of brain amino acid concentrations, a similar tendency was found, whereby amino acid concentrations either remained unchanged or increased under stressful conditions (Kennett *et al.*, 1986). It has been found that the presence of amino acid transporters such as SNAT–2 seems to maintain homeostasis of free amino acids in milk (Velázquez–Villegas *et al.*, 2015). Thus, amino acid transporters may play some role in the unchanged free amino acid concentrations in the milk.

On the other hand, almost all the amino acids in the maternal plasma decreased significantly as a result of maternal stress during the lactation period. This result might be due to the influence of catecholamines, as discussed above (Shamoon *et al.*, 1980; Taylor *et al.*, 1989; De Boer *et al.*, 1990). However, only GABA significantly

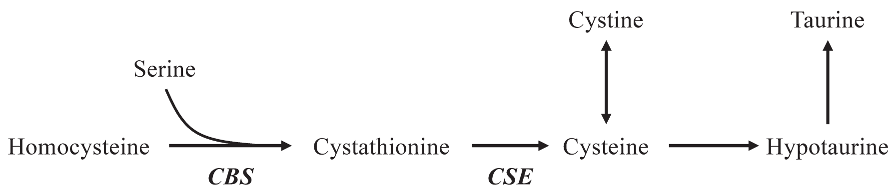

Fig. 6. Metabolic pathway of sulfur amino acids. Cystathionine is synthesized from serine and homocysteine by cystathionine β–synthase (CBS) and metabolized to cysteine by cystathionine γ–lyase (CSE). Taurine is synthesized from cysteine through hypotaurine.

increased as a result of stress treatment. That increase might have been due to increased GABA synthesis with stress. Tsukidate *et al*. (2012) demonstrated that stress treatment increased the synthetic amount of GABA in the liver through the increased expression of glutamic acid decarboxylase 65 (GAD65), which codes the key enzyme synthesizing GABA from glutamate, allowing GABA to then be transferred into the blood.

The metabolic pathway of sulfur amino acids is shown in Fig. 6. Cystathionine is metabolized from serine and homocysteine by the catabolic action of cystathionine β–synthase (CBS), and metabolized to cysteine by cystathionine γ–lyase (CSE) (Aitken *et al*., 2011; Yamada *et al*., 2012). In Experiment 2, taurine levels in milk were significantly higher in the stress treatment group compared with the control group ($P<0.05$). It is assumed that the majority of taurine *in vivo* is synthesized in the liver, given that the enzymatic activity of CBS is highest in the liver (Zhao *et al*., 2013). However, the expression of a taurine transporter, rB16a, in the mammary glands was lower during the lactation period than during the gestation period (Alemán *et al*., 2009). Therefore, the amount of taurine transported from plasma to milk may be low and it may also be synthesized in the mammary glands. Previous studies have shown that stress treatment decreased the transcription of CBS (Zhao *et al*., 2013), and increased the transcription of CSE, in the liver (Dickhout *et al*., 2012). These results suggest that stress treatment can alter the gene expression of CBS and CSE in the mammary glands. Consequently, the metabolism of sulfur amino acids in the mammary glands might be altered, and these changes might cause the increased taurine concentration in the milk.

Although the amino acid composition of milk was not changed by maternal stress during the gestation period, stress during the lactation period significantly increased some amino acid levels and caused a change in milk amino acid composition. In conclusion, maternal stress during the lactation period has a greater impact on the amino acid composition of milk than stress during the gestation period. These changes might result in lower body weight of offspring in the stress treatment group. Further studies are needed to explain the growth suppression in offspring and its relationship to the amino acid composition of milk. The present study may contribute to elucidating the effects of maternal stress during the gestation and lactation periods on the free amino acid compositions of milk and on the growth of offspring, and provides an insight into the cause of these effects.

AUTHOR CONTRIBUTIONS

T. Nishigawa designed the study, performed the research and data analysis, and wrote the manuscript. S. Nagamachi, M Takakura, H Ikeda, M Kodaira, and T Yamaguchi performed the research and data analysis. V.S. Chowdhury and S. Yasuo wrote the manuscript. M. Furuse designed the study and wrote the manuscript.

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