Research report

Monosodium glutamate ingestion during the development period reduces aggression mediated by the vagus nerve in a rat model of attention deficit-hyperactivity disorder

Ruriko Nishigaki a,1, Yoshihiro Yokoyama a,b,1, Yuko Shimizu a, Ryosuke Marumoto a, Sachiyo Misumi a, Yoshitomo Ueda a, Akimasa Ishida a, Yasuyuki Shibuya b, Hideki Hida a,⇑

a Department of Neurophysiology & Brain Science, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan
b Department of Neurophysiology & Oral Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

1 These authors contributed equally to this paper.

E-mail addresses: nishiga2@med.nagoya-cu.ac.jp (R. Nishigaki), yoshihironico@yahoo.co.jp (Y. Yokoyama), tsakura@med.nagoya-cu.ac.jp (Y. Shimizu), smisumi38@yahoo.co.jp (S. Misumi), yueda@med.nagoya-cu.ac.jp (Y. Ueda), a-ishida@med.nagoya-cu.ac.jp (A. Ishida), shibuya@med.nagoya-cu.ac.jp (Y. Shibuya), hhida@med.nagoya-cu.ac.jp (H. Hida).

Abstract

We used an umami substance, monosodium glutamate (MSG), as a simple stimulant to clarify the mechanism of the formation of emotional behavior. A 60 mM MSG solution was fed to spontaneously hypertensive rats (SHR), used as a model of attention-deficit hyperactivity disorder, from postnatal day 25 for 5 weeks kept in isolation. Emotional behaviors (anxiety and aggression) were then assessed by the open-field test, cylinder test and social interaction test. MSG ingestion during the developmental period resulted in a significant reduction in aggressive behavior but had few effects on anxiety-like behavior. Several experiments were performed to identify the reason for the reduced aggression with MSG intake. Blood pressure in the MSG-treated SHR was comparable to that of the controls during development. Argyrophil III staining to detect the very early phase of neuronal damage revealed no evidence of injury by MSG in aggression-related brain areas. Assessment of plasma amino acids revealed that glutamate levels remained constant (~80 μM) with MSG ingestion, except for a transient increase after fasting (~700 μM). However, lactate dehydrogenase assay in an in vitro blood-brain barrier model showed that cell toxicity was not induced by indirect MSG application even at 700 μM, confirming that MSG ingestion caused minimal neuronal damage. Finally, vagotomy at the sub-diaphragmatic level before MSG ingestion blocked its effect on aggressive behavior in the isolated SHR. The data suggest that MSG ingestion during the developmental period can reduce aggressive behavior in an attention deficit-hyperactivity disorder model rat, mediated by gut-brain interaction.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Early life experiences have long-term consequences for emotional control, contributing to the development of psychopathologies in later life (Baldini et al., 2013; Botanas et al., 2016; de Carvalho et al., 2010; Ishikawa et al., 2015; Pamplona et al., 2009; Urakawa et al., 2013). Aggression is an important indicator of multiple mental health and psychosocial problems (Nguyen et al., 2016). Early life experiences can influence aggression in humans and other species (Meyer et al., 2016). In animals, aggressiveness was shown to increase in adult animals that were submitted to post-weaning social isolation (Toth et al., 2012). The neural background of normal (rivalry) aggression has been investigated using c-Fos immunochemistry (Haller et al., 2006): positive cells were shown in the medial amygdala, the bed nucleus of the stria terminalis (BNST), the hypothalamic attack area including the ventromedial hypothalamus, the hypothalamic paraventricular nucleus, and the periaqueductal gray.

External environmental stimuli during development can affect the formation of emotions such as anxiety, social behavior, and aggression (Kloke et al., 2013; Saylor and Amann, 2016). Numerous...
studies have shown that environmental enrichment influences emotional formation and stress reactivity (Chapillon et al., 2002; de Carvalho et al., 2010; Pamplona et al., 2009). Recently, we reported that environmental enrichment from postnatal day 25 (P25) to P60 reduced anxiety and aggression in the inbred spontaneously hypertensive rat (SHR), which exhibits more anxious characteristics than the control strain. However, the mechanisms underlying the formation of emotions remain unclear.

Juvenile SHR that show hyperactivity, inattention, and impulsivity are commonly used as a behavioral model of attention-deficit hyperactivity disorder (ADHD) (Hopkins et al., 2009; Ohno et al., 2012; Sagvolden, 2000), a neurodevelopmental disorder (Wilens et al., 2002). Impulsive aggression is a clinically distinct and common behavior in ADHD and autism, with 54% of ADHD patients showing clinical aggression (Saylor and Amann, 2016). Although ADHD is known to have a strong genetic component (Faraone et al., 2005), several biological and environmental factors are proposed as risk factors for ADHD, including food additives and diet, lead contamination, maternal smoking during pregnancy, and low birth weight (Banerjee et al., 2007).

As environmental enrichment contains many factors that increase motor and sensory stimuli as well as exploration, it is rather difficult to identify the mechanism underlying the formation of emotions. Therefore, we have used one of the five basic tastes, savory umami (Iwatsuki et al., 2012; Uematsu et al., 2010), as a simple stimulant to examine how emotional behaviors such as anxiety and aggression are induced by external sensory stimulation during rat development.

In the present study, we used an umami substance, monosodium L-glutamate (MSG), which is used to improve the sensory quality of many foods. SHR were fed an MSG solution for 5 weeks from P25 to P60, followed by assessments of emotional behaviors such as anxiety, exploration, and aggression. To further investigate how MSG changes emotional behavior, we measured blood pressure and plasma amino acids, conducted in vitro and in vivo cell toxicity assays, and performed vagotomy to analyze the gut-brain interaction. The results clearly revealed that MSG ingestion during the developmental period reduced aggressive behavior in the ADHD model rat, mediated by gut-brain interaction.

2. Results

2.1. Physiological results in the H2O-treated control group and 60 mM MSG-treated group

In the H2O-treated control group, the body weight at postnatal day (P) 60 was 243.1 ± 3.0 g (n = 11), the total food intake over 5 weeks was 568.1 ± 8.1 g (n = 13), and the total drinking volume over 5 weeks was 855.3 ± 39.3 g (n = 13). Mean blood pressure (MBP) was 104.8 ± 2.9 mmHg at P40 (n = 6) and 139.7 ± 2.2 mmHg at P64 (n = 5) in the H2O-treated control group (Fig. 3). Plasma free glutamate (Glu) levels were 78.1 ± 8.9 μM (n = 3, Fig. 4A).

In the MSG-treated group, the body weight at P60 was 241.1 ± 3.5 g (n = 13), and the total food intake over 5 weeks was 554.7 ± 15.6 g (n = 7). The total drinking volume over 5 weeks was 1331.75 ± 58.9 g (n = 13) which was significantly higher compared with

---

Fig. 1. The effect of MSG ingestion on anxious behavior. After 5 weeks of MSG treatment (from P25 to P60), the OFT (A) and CYT (B) were performed to assess anxious behavior. (A) In the OFT, there were no differences in the total distance travelled in the arena (upper graph) and the number of entries into the center area (lower graph) between the H2O-treated (*n = 15) and MSG-treated rats (n = 15). (B) For the CYT, the total inactivity time was not significantly different between the control group (n =12) and MSG-treated group (n = 12) (upper graph). However, the MSG-treated group spent a significantly longer time inactive in the second time of the test (lower graph). *P < 0.05 for Student’s t-test comparison between control and MSG groups.
controls \((P < 0.001)\). MBP in the MSG-treated group was \(111.8 \pm 2.7\) mmHg at P40 \((n = 6)\) and \(142.7 \pm 3.6\) mmHg at P64 \((n = 6)\) (Fig. 3). Glu levels were \(73.7 \pm 5.3\) \(\mu M\) \((n = 3)\) in the 60 mM MSG-intake group (Fig. 4A).

2.2. Effect of MSG on emotional behavior

We assessed the effects of 5 weeks of MSG treatment (from P25 to P60) on emotional behavior using the open-field test (OFT) (which assesses anxious behavior induced by novel circumstances) and the cylinder test (CYT) (another test for anxious behavior) (Fig. 1).

On the OFT, there were no differences in the total distance covered in the arena or the number of entries into the center area between the control group \((n = 15)\) and the MSG-treated group \((n = 15)\) (Fig. 1A). For the CYT, there were no significant differences in the total inactivity time among the groups, although an increasing tendency was shown in the MSG group (Fig. 1B, upper graph). The inactivity time in the second CYT was significantly longer in the MSG-treated group \((n = 12)\) than the control group \((n = 12)\) (Fig. 1B).

We also assessed the effect of MSG ingestion on the social interaction test (SIT) (which assesses active interaction with an unfamiliar animal) performed following habituation in a test box for 3 successive days (Fig. 2). Total sniffing time in 5 min, a measure of exploratory behavior, was significantly lower in the MSG group \((n = 12)\) than the control group \((n = 12)\) (Fig. 2A). Each sniffing period in every minute quickly decreased in the MSG group, and by the second minute the time was significantly shorter \((P < 0.001; P < 0.0001\) for Student’s t-test comparison between control and MSG groups.

![Fig. 2.](image)

**Fig. 2.** The effect of MSG ingestion on social interaction. The SIT was performed to assess social behavior toward an unfamiliar animal. (A) MSG ingestion for 5 weeks after weaning resulted in significantly reduced sniffing time (upper graph) in the MSG group \((n = 12)\) compared with the control group \((n = 12)\). Although the sniffing time was similar among groups during the first minute, it significantly decreased in the second minute in the MSG group. (B) Aggressive behaviors such as riding, scratching, biting, and kicking were also assessed. The MSG group showed a significant decrease in the total number of riding behaviors, and the number in each minute was lower from the beginning of the test (upper graph). The MSG group also displayed significantly fewer strongly aggressive behaviors (scratching, biting, kicking) (lower graph). \(P < 0.05\), \(P < 0.01\) and \(P < 0.0001\) for Student’s t-test comparison between control and MSG groups.

![Fig. 3.](image)

**Fig. 3.** Effect of MSG ingestion on blood pressure in SHR. The possibility that MSG ingestion increases blood pressure was investigated at P40 and P64. Mean blood pressure (MBP), a parameter of blood pressure, developmentally increased in all three groups: H2O-treated \((n = 6)\), 60 mM MSG-treated \((n = 6)\), 60 mM NaCl-treated \((n = 6)\) groups. There were no significant between-group differences in MBP at P40 and P64.
Aggressive behaviors such as riding, scratching, biting, and kicking were also assessed in the SIT (Fig. 2B). The total number of riding instances was significantly decreased in the MSG group (7.1 ± 1.5, n = 12) compared with the control group (18.2 ± 3.3, n = 12), and the number of instances was lower from the beginning of the test in the MSG group (Fig. 2B upper graph). Strongly aggressive behaviors such as scratching, biting, and kicking were also significantly decreased in the MSG-treated group (Fig. 2B lower graph).

2.3. Effect of MSG on blood pressure during the developmental period

To investigate the possibility that MSG ingestion induced an increase in blood pressure that culminated in the change in emotional behavior, we measured MBP at P40 and P64 in the 60 mM MSG-treated group and 60 mM NaCl-treated groups (Fig. 3). Similar to controls, developmental increases in MBP were detected in the MSG group and NaCl group (P40: 103.2 ± 4.1 mm Hg, n = 6; P64: 138.5 ± 2.8 mmHg, n = 6). There were no significant differences in MBP among the three groups at either P40 or P64.

2.4. Effect of MSG on plasma amino acids

To investigate the possibility that MSG ingestion increases plasma free Glu, and the concomitant neuronal damage results in the change in emotional behavior, we first measured plasma Glu levels in MSG-treated rats (Fig. 4). Various doses of MSG solution (0, 60, 180 mM) were ingested with standard chow, followed by plasma free amino acid (FAA) assessment (Fig. 4A). No differences were found in Glu levels between the groups at 0 mM MSG, 60 mM MSG, or 180 mM MSG (74.0 ± 7.2 µM, n = 4). Similarly, no significant differences among three groups were shown in any other free amino acids (Fig. 4A).

To investigate whether there was a transient increase in plasma free Glu after fasting, the rats were fasted for 16 h followed by 30 min of self-intake of 180 mM MSG, and then plasma FAA concentrations were measured (Fig. 4B). The volume drunk in 30 min was 7.84 ± 0.56 ml (n = 7), which was rather high compared with that of 60 mM MSG (6.23 ± 0.36 ml, n = 4; p = 0.057, t-test). The plasma Glu level was significantly elevated after MSG intake (686.9 ± 55.9 µM, n = 4) compared with the basal level (74.0 ± 7.2 µM, n = 3) (Fig. 4B). After MSG intake following fasting, concentrations of glycine, aspartate, and isoleucine were significantly higher and that of proline was significantly lower compared with basal levels (Fig. 4B).

2.5. Effect of MSG on cell damage in the in vitro blood-brain barrier (BBB) model

A BBB model culture system was used to investigate whether a rapid increase in Glu can induce cell toxicity in neurons (Fig. 5). Direct administration of MSG (100 µM), a dose comparable to plasma Glu concentrations, to cultured neurons without a BBB induced significant cell damage (Fig. 5B, upper panel). In contrast, there was no neuronal damage when MSG was administered to the BBB cultures (Fig. 5B, lower panel). Cell toxicity measured by the lactate dehydrogenase (LDH) assay was significantly increased after 100 µM of MSG was added to neurons without a BBB.
Fig. 5. Neuronal cell death reduced by indirect MSG application via blood brain barrier (BBB) in vitro. To investigate the possibility that a rapid Glu increase induces cell death in cultured neurons, an in vitro BBB model system was used. (A) The BBB was composed of blood vessel endothelial cells on a polyester membrane-filter transwell, with astroglial cells under the membrane. Neurons from the embryonic (E17) rat brain were cultured in a 24-well dish containing the BBB transwell. (B) Direct application of MSG (100 μM) to cultured neurons without a BBB caused significant neuronal damage (upper right panel). However, no neuronal damage was observed following MSG treatment via the BBB (lower right panel). (C) The lactate dehydrogenase (LDH) assay showed a significant increase in neuronal toxicity with the addition of 100 mM MSG in cultures without a BBB (left graph). However, cell toxicity was completely blocked by the presence of a BBB, even with the application of 700 μM MSG, comparable to the Glu levels observed after fasting (right graph).

2.6. Effect of MSG on neuronal cell death

To rule out the possibility that the rapid increase in Glu after fasting affects neurons in the brain, we used argyrophil III staining to detect the very early phase of neuronal damage in vivo (Fig. 6). Although typical argyrophil III-positive cells showed high binding to silver in the cytosol of excitotoxic-positive controls, there were no obvious argyrophil III-positive cells at 6 h after the Glu increase in the medial prefrontal cortex, central nucleus of the amygdala, ventromedial hypothalamus, paraventricular nucleus, lateral septum, nucleus solitarius of the tractus (NTS), hippocampus, periaqueductal gray or medial amygdala (Fig. 6). No argyrophilic-damaged neurons were detected at 3 h or 12 h after rapid Glu increase (data not shown).

2.7. MSG effect is blocked by sub-diaphragmatic vagotomy

Vagotomy of the dorsal and ventral trunks of the vagus nerve on the lower esophagus at the sub-diaphragmatic level was carried out to investigate the effect of MSG on the gut-brain nervous system interaction (Fig. 7A). Vagotomy caused a significant decrease in body weight at P60 (Fig. 7B) in the H2O-treated group (sham: 241.7 ± 7.5 g, n = 5; vago: 183.9 ± 7.9 g, n = 6) as well as the MSG-treated group (sham: 240.0 ± 3.0 g, n = 9; vago: 184.7 ± 5.4 g, n = 10) (Fig. 7B). Total food intake over 5 weeks was decreased by vagotomy in the H2O-treated group (sham: 575.8 ± 16.7 g, n = 5; vago: 401.6 ± 19.5 g, n = 6) as well as the MSG-treated group (sham: 584.4 ± 20.1 g, n = 9; vago: 439.2 ± 15.1 g, n = 10) (Fig. 7C). Total drinking volume was decreased by vagotomy in the H2O-treated group (sham: 845.1 ± 34.2 ml, n = 5; vago: 511.1 ± 36.3 ml, n = 6) and the MSG-treated group (sham: 1499.0 ± 36.7 ml, n = 9; vago: 938.3 ± 24.5 ml, n = 10) (Fig. 7D).

Following vagotomy at the sub-diaphragmatic level to block the input of the vagus nerve to the NTS (an afferent of gut-brain interaction), the MSG-treated SHR showed emotional sniffing behavior (Fig. 8A) and aggressive behavior, such as riding (Fig. 8B), which was comparable to H2O-treated controls.

3. Discussion

In the present study, we used an umami substance, MSG, as a simple external stimulant that can induce emotions such as anxi-
ety, social behavior, and aggression. MSG was ingested by socially isolated, post-weaning SHR (an ADHD model animal) for 5 weeks from P25, and the mechanism of MSG action in the change in emotional behavior was investigated in several experiments: blood pressure measurement, plasma amino acids assessment, in vitro and in vivo cell toxicity assay, and gut-brain interaction analysis by vagotomy. Our main findings were that MSG during development reduced emotional behavior, especially aggression, in adulthood, whereas vagotomy at the sub-diaphragmatic level clearly blocked the effect of MSG on emotional behavior, suggesting that the action is mediated at least in part by gut-brain interaction. In addition, we revealed that the effects of MSG on aggression in SHR during development were not due to neuronal damage in brain areas with a known role in aggression.

3.1. Effect of MSG on emotional behavior

Although there was no significant difference in the OFT, there was a faint but significant difference in the CYT, another test for anxiety, suggesting that MSG has a minor effect on anxiety. However, exploratory behavior (sniffing) and aggressive behavior (riding, strong aggression) were significantly decreased in MSG-treated animals. It should be note that sniffing time was significantly reduced after the second minute. The fact that inactivity time was longer and sniffing time was shorter at the second time of the test implies enhanced adaptation to the new situation or tolerance toward unfamiliar animals, rather than less anxiety.

The finding that anxiety-like behavior in the OFT was unchanged by MSG ingestion while environmental enrichment reduced anxiety-like behavior (Baldini et al., 2013; Benaroya-Milshtein et al., 2004) also indicates that the effect of MSG seems to be very weak. This seems to be supported by our preliminary data showing that MSG’s action on aggression is masked by group housing (two animals per cage). Thus, it is very likely that the effect of MSG (a simple stimulus) on emotional behavior was weaker than that of group housing or environmental enrichment, which contains more complex sensory stimuli.

The SHR seemed to be more anxious in a novel environment than the control WKY rats that are known to be vulnerable to anxiety (McAuley et al., 2009). Therefore, we cannot rule out the possibility that the effect of MSG is shown only in SHR with aggressive characteristics (McFie et al., 2012; Potegal and Myers, 1989). We failed to detect similar effects of MSG (less aggression) in isolated Wistar rats in our preliminary data, because WKY did not exhibit aggression in the SIT. To address this possibility, the experimental conditions of the SIT should be changed in future (Toth et al., 2012): aggression should be investigated in the dark (animal-active) phase instead of the later light phase (between 5 p.m. and 8 p.m.). The investigation of emotional behavior in the animal-active phase will enhance aggression levels in both Wistar and SHR.

3.2. Effect of MSG ingestion on the brain

We investigated three possible mechanisms by which administration of a simple stimulus such as MSG during development might affect emotional behavior in rats: blood pressure, brain damage caused by plasma glutamate, and gut-brain interaction.

We first checked blood pressure in the MSG-treated SHR, because aggressive individuals are much more likely to have hypertension. Although MSG ingestion would cause an increase in blood pressure in adulthood (Kondoh and Torii, 2008; Sontag

Fig. 6. Brain regions related to aggression show no obvious neuronal damage after MSG ingestion. To rule out the possibility that a transient Glu increase after fasting causes neuronal damage in vivo, argyrophil III staining was performed to detect the very early phase of neuronal damage. No evidence of neuronal damage (argyrophil III staining-positive cells) was observed in the medial prefrontal cortex, central nucleus of the amygdala, or the ventromedial nucleus of the hypothalamus at 6 h after fasting, although a yellowish-brownish background was broadly observed. No neuron damage was shown in the paraventricular nucleus, lateral septum, hippocampus, periaqueductal gray, nucleus of solitary tract, or medial amygdala. Note that damaged neurons with argyrophilic neurites (dark neurons) were evident in the hippocampus that received excitotoxic ibotenic acid (positive control).
et al., 2010), no significant increase was shown in the young MSG-treated SHR in our experiment. Thus, the possibility that predisposition to hypertension in SHR may contribute to the altered aggression following MSG treatment is almost ruled out by the blood pressure assessment.

Many studies have reported only small changes in plasma Glu levels following administration of large quantities of MSG (Hawkins, 2009). To investigate whether oral MSG intake increases plasma Glu to a level that could induce neuronal damage in the brain, plasma FAA were measured and neuronal damage was investigated in in vitro and in vivo experiments. The level of plasma Glu in animals receiving 60 mM MSG for 5 weeks was similar to previously reported levels (Kondoh and Torii, 2008; Uneyama et al., 2006), although a rapid ~10-fold increase in plasma Glu (~700 μM) was shown in SHR after receiving 180 mM MSG after fasting. An experiment with an in vitro BBB model clearly revealed that the in vivo plasma Glu level (700 μM) was insufficient to induce neuronal toxicity in our in vitro BBB system. In preliminary studies, we also found that MSG had no effects on astrocyte or oligodendrocyte survival in cultures with and without a BBB. These in vitro data are consistent with our findings of no apparent neuronal damage in the brain after exposure to oral MSG, which suggests that neuronal damage does not explain the reduced aggression. Our in vivo studies strongly suggest that oral intake of MSG was not toxic to neurons in the brain. Thus, the most likely reason is that the BBB prevents Glu permeability, thus inhibiting the direct neurotoxic effect of MSG (Goldsmith, 2000; Hawkins, 2009; Walker and Lupien, 2000). However, it is notable that extremely high levels of MSG following subcutaneous or intravenous administration can cause excitatory brain injury (Foran et al., 2017a,b; O’Brien and Cairns, 2016; Walker and Lupien, 2000). In our preliminary data, the Glu level was 7.73 ± 0.53 mM (n = 3) at 30 min after subcutaneous injection of 10% MSG, although it was 126.6 ± 41.0 μM (n = 3) after oral intake of 60 mM MSG at P25.

Recent studies suggest that gut-brain communication is involved in affection, motivation, and higher cognitive functions linked to emotion (Cryan and Dinan, 2012; Klarer et al., 2014; Mayer, 2011). There are numerous taste receptors in enteroeendocrine cells and in the taste buds, including metabotropic glutamates type 1 (mGluR1) and mGluR4 and taste receptors 1 (T1R1) and T1R3 (Daly et al., 2013; Kurihara, 2015; San Gabriel et al., 2007; Yasumatsu et al., 2015), which signal to the brain via the vagus nerve. The presence of umami receptors on enteroendocrine cells is supported by the fact that MSG ingestion induced amygdala activation, which was then abolished after sub-diaphragmatic total vagotomy (Kitamura et al., 2011; Tsurugizawa et al., 2009, 2011; Uematsu et al., 2010). To determine the role of the gut-brain axis in the reduced aggression, sub-diaphragmatic total vagotomy before MSG administration was performed. The results clearly revealed the involvement of the gut vagus nerve in the reduction. However, we cannot completely rule out the possibility that hormonal factors such as ghrelin, gastrin, insulin, testosterone, and oxytocin are involved in the MSG effect.

3.3. Possible mechanism of reduced aggression by gut-brain interaction

How is MSG in the gut linked to reduced aggression? MSG binds to umami receptors on the enteroeendocrine cells such as mGluR1, mGluR4, and T1R1/T1R3, probably releasing the neurotransmitter 5-HT (Iwatsuki et al., 2012; Uneyama et al., 2006). The neural signal is transmitted to the NTS via the vagus nerve, which is con-
nected to the amygdala and prefrontal cortex, both of which are strongly involved in aggression. The medial amygdala, bed nucleus of the striatal terminalis, ventromedial hypothalamus, paraventricular nucleus, periaqueductal gray, and central nucleus of the amygdala are also involved in the mechanisms of aggression (Haller et al., 2006; Hong et al., 2014; Lin et al., 2011; Nelson and Trainor, 2007). It is reasonable to conclude that cell damage in the aggression-related brain areas is not involved in the altered aggression, as no evidence of argyrophil III-positive neurons in these brain areas was shown even after self-intake of MSG after fasting (resulting in a transient increase in plasma Glu up to 700 μM).

In our preliminary data, direct MSG administration into the stomach by probe after fasting resulted in c-Fos expression in the NTS and amygdala as previously reported (Otsubo et al., 2011). Interestingly, expression was not detected with other amino acids such as lysine, arginine and aspartic acid, indicating that the vagus nerve activation is specific to MSG. Thus, specific Glu binding to umami receptors on enteroendocrine cells increased vagal activity related to c-Fos expression in the NTS and amygdala.

We cannot rule out the possibility of the signal from taste receptors on the taste buds of the tongue, although the MSG effect was almost completely blocked by the vagotomy. As the microbiota in the gut is strongly affected by food (Bravo et al., 2011; Cryan and Dinan, 2012; Hsiao et al., 2013; Mayer, 2011; Yano et al., 2015), it is very likely that MSG ingestion changed the pattern of microbiota, which influenced the activity of the vagus nerve.

3.4. Limitations and future experimental issues

Although we have shown an effect of MSG on aggression during a developmental period, it is not known if a similar effect of MSG on reduced aggression is found in humans. As free Glu is rich in human breast milk (Zhang et al., 2013), dietary Glu is an important substance for normal development. Therefore, it is likely that an appropriate amount of Glu ingestion during development has some effect on normal development in young children.

To clarify the detailed mechanism of the gut-brain interaction following MSG ingestion, several experiments should be performed. As reported by other groups showing enhancement of vagal activity by MSG (Kitamura et al., 2011; Uneyama et al., 2006), we will examine the relationship between Glu binding to taste receptors in the enteroendocrine cells and vagus nerve activity at the sub-diaphragmatic level in the future. In addition, the detailed neural circuits of the brain involved in the reduced aggression by MSG should also be clarified in the future: the NTS-prefrontal cortex pathway, the NTS-amygdala pathway, and the prefrontal cortex-amygdala pathway may be more activated by MSG ingestion. Loss of function by selective blocking of the pathways using double-virus transfection (Ishida et al., 2016) may help to elucidate the mechanism of reduced aggressive behavior.

3.5. Conclusions

We used an umami substance, MSG, as a simple stimulant to determine how emotional behavior is formed during the develop-
mental period in SHR. MSG ingestion during development resulted in significant reduction of aggressive behavior, probably mediated by the vagus nerve (gut-brain interaction), but had little effect on anxiety-like behavior. Our data sheds light on the importance of appropriate simple stimulant on the formation of emotional behavior such as aggression during development.

4. Experimental procedure

4.1. Animals

Male SHR (Hoshino Laboratory Animals Inc., Ibaragi, Japan) and Wistar rats (Japan SLC Inc., Shizuoka, Japan) were used in these experiments. Animals were housed under temperature-controlled conditions (23–25 °C) with a 12-h light/dark cycle (light on at 8:00 a.m.). Animal care and handling were performed according to the guidelines of the Institute for Experimental Animal Sciences, Nagoya City University Medical School. All experimental procedures were approved by the animal experimentation committee of Nagoya City University Medical School, and all efforts were made to minimize the number of animals used and their suffering.

Male SHR was housed individually in a standard cage (40 × 23 × 18 cm) from P25 to P60, with free access to standard rodent diet that contains Glu of 3.75 mg/100 g diet (MFG; Oriental Yeast Co., Ltd.) and a drinking bottle containing a 60 mM MSG solution (gift from Ajinomoto Co., Tokyo Japan; MSG group: n = 13) or water (control group, n = 12). The drinking bottle was changed twice a week. Body weight, food intake, and drinking volume were measured three times a week.

4.2. Behavioral tests

The OFT was performed at P60 and the CYT at P61 to assess anxiety-like behavior after exposure to a novel situation in a wide arena or narrow space. The SIT was performed at P65 following a 3-day habituation (P62–64) in a test box, and involved interaction with an unfamiliar Wistar rat. The rats weighed 245 ± 5 g on average at the beginning of the tests. Behavioral tests were conducted between 5:00 p.m. and 8:00 p.m.

4.2.1. OFT

Each animal was placed in the center of the black circular arena (60-cm diameter × 50-cm height) under normal light conditions (350 lux), and 10 min of free movement was recorded on a video camera attached directly above the field. After each test, the floor of the field was cleaned with water to remove odors. A video camera was mounted directly above the open arena to record behavior for later scoring. The distance traveled, the velocity of locomotion, and the number of entries into the center area (30-cm diameter) of the arena were measured using Smart software (Bio Research Center Inc., Aichi, Japan) (Otero et al., 2010).

4.2.2. CYT

The rats were placed in a transparent cylinder (20-cm diameter × 30-cm height) under normal light conditions (350 lux), and 5 min of free movement was recorded on a video camera beside the field. After each test, the floor of the field was cleaned with water to remove odors. Rearing behavior, standing vertically inclined with the forelimb raised, was assessed. To assess anxious behavior induced by the unfamiliar narrow space, the grooming time (licking and mouthing of fur with occasional use of forepaws), location, and inactivity (amount of time without any voluntary movement) were assessed. The total activity time and time per minute spent on each activity, including rearing, grooming, walking, and other movements, were measured by an observer who was blind to the experimental conditions.

4.2.3. SIT

Before the SIT test, each rat was habituated to the test box (20-cm wide × 60-cm long × 50-cm high) for 5 min over 3 consecutive days. On the test day, the experimental rat and an unfamiliar Wistar rat (220–230 g) were placed at either end of the test box. The behavior of the experimental rat was recorded for 5 min on a video camera attached directly above the box. The number of episodes of sniffing (the experimental target rat sniffed any part of the body, including the anogenital area, of the unfamiliar Wistar rat) and aggression (the experimental rat rode on, scratched, bit, or kicked the body of the unfamiliar Wistar rat) were counted. In this SIT, the Wistar rats treated with H2O (n = 6) or MSG (n = 7) were gentle and showed no aggression.

4.3. Measurement of blood pressure

At P40 and P64, MBP was measured in conscious rats by the noninvasive tail-cuff method (BP-98A-L, Softron Tokyo, Japan). Briefly, the animals were pre-warmed in a special cylindrical net covered with a special fabric pocket at 37 °C for more than 5 min until stable. Systolic blood pressure, diastolic blood pressure, MBP, and heart rate were automatically calculated from the tail-cuff probe. Data were obtained from the average of three consecutive measurements of MBP and only considered valid when each MBP did not differ by more than 10 mmHg. The experiments were performed between 3:00 pm and 8:00 p.m.

4.4. Measurement of plasma free glutamate

To measure FAA, a blood sample (~5 ml) was collected transectionally under deep pentobarbital-mediated anesthesia and transferred into a plastic tube containing ethylenediaminetraacetic acid disodium salt (1–1.5 mg/ml) on ice. The samples were then centrifuged at 3000 rpm at 4 °C for 15 min to separate the plasma from the blood cells. The supernatants (plasma fractions) were carefully collected and kept at −80 °C until analysis (Nakamura et al., 2014). The concentrations of 20 basic FAA in the plasma were measured by high-performance liquid chromatography (NDTS Inc., Hokkaido, Japan) following the derivatization procedure using the EZ-Fast amino acid analysis kit (Phenomenex®, Shimadzu GLC Inc., Tokyo, Japan) (Fonteh et al., 2007).

To measure FAA after fasting, SHR were acclimated to 180 mM of MSG solution for at least 7 days. Before blood sampling, no food or water was given for 16 h (~5 p.m.–9 a.m.). During the fast, rats were placed in individual cages with a wire net to prevent them eating protein-rich feces. At the end of the fast, the animals were allowed to drink 180 mM of MSG solution for 30 min.

The levels of free Glu before the fast (n = 4) and for 30 min after MSG intake (n = 4) were measured.

4.5. Argyrophil-III silver staining

Argyrophil III silver staining was performed as described previously (Ishida et al., 2011; Misumi et al., 2016). In brief, under deep pentobarbital-mediated anesthesia, animals were transcardially perfused with 0.1% (weight per volume [w/v]) cacodylate buffer solution containing 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde. The brains were removed at 24 h after perfusion and immersed in the same fixative for at least 1 week at room temperature. Serial 50-μm thick coronal sections were cut on a cooled microtome. The sections were esterified in 1-propanol containing 1.2% sulfuric acid and 2% distilled water for 16 h, and then pro-
cessed in a silicotsungstate physical developer. As a positive control, ibotenic acid (25 mM; Sigma Inc., Kanagawa, Japan) dissolved in phosphate buffer saline was injected unilaterally into the hippocampal cornus ammonis 1 region (3.0 mm caudal to the bregma; 2.0 mm left from the mid-line; 3.5 mm below the skull surface) at a rate of 0.2 µl/min for 5 min.

4.7. Cell culture

4.7.1. Neuronal culture

Rat cortical neurons were cultured as previously described (Hida et al., 2003). In brief, fetal brains at embryonic day 17 were rapidly removed and placed in ice-cold saline. The cerebral cortices were dissected and digested with 0.25% trypsin-ethylenediaminetetra cetic acid for 15 min at 37 °C, followed by trituration with micro-pipettes. Cells were plated on poly-i-lysine-coated 24-well plates at a density of 2.0 × 10^5/cm², and then cultured with Neurobasal A medium (Thermo Fisher Scientific K. K., Kanagawa, Japan) supplemented with B27 (Thermo Fisher Scientific K. K., Kanagawa, Japan) (Neurobasal B27) at 37 °C in a humidified 5% CO₂ atmosphere incubator. The medium was changed every other day starting from in vitro day 1, and was used for studies at in vitro day 7.

4.7.2. Blood-brain barrier (BBB) in vitro model

We used the BBB kit (RBE-12 kit; Farmaco-cell Co. Ltd., Nagasaki, Japan) composed of blood vessel endothelial cells plated on a polyester membrane filter (0.4 µm), with astroglial cells under the membrane (Nakagawa et al., 2009). The polyester membrane filters were cultured with medium for 4 days to obtain a BBB function with transendothelial electrical resistance of greater than 150 Ωcm². The insert was then transferred to a dish of cultured neurons, producing a BBB in vitro model comprising blood vessel endothelial cells, astroglial cells, and neurons. Various doses of MSG were then added into the insert well, and neuronal death was assessed using the LDH assay.

4.7.3. Measurement of Glu in the BBB in vitro model

To assess the concentrations of FAA in the BBB in vitro model, the medium inside and outside the insert well was collected after 12 h of co-culture, followed by centrifuging at 3000 rpm at 4 °C for 5 min. The concentration of FAA in the supernatant were measured in the same way as the plasma assessment. Results are expressed as mean ± SEM.

4.8. LDH measurement of neuronal cell death

LDH was measured using the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 100 µl of culture medium was collected in a 96-well plate, mixed with 100 µl of reaction solution containing non-colored WST-8, and incubated for 30 min. LDH was measured by its reduction activity from WST-8 to colored form, and then measured using the Neurobasal B27 medium. Data are presented as the percentage of cell toxicity using the following equation: cell toxicity (%) = (test sample − medium)/(high control − medium).

4.9. Vagotony

Vagotomy was carried out at the sub-diaphragmatic level using SHR at P25, according to our previous report with some modifications (Uematsu et al., 2010). Briefly, after overnight food restrictions, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and laid on a thermo-pad to maintain a rectal temper-
