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Taste Preference and Nerve Response to 5′-Inosine Monophosphate Are Enhanced by Glutathione in Mice

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Abstract

Previous human sensory evaluation studies have shown that glutathione (GSH) enhances deliciousness, accompanied by thickness, mouthfulness, and continuity feeling, which is known as "kokumi" in Japanese, in an umami solution containing monosodium glutamate and 5′-inosine monophosphate (IMP). We conducted behavioral and electrophysiological experiments to explore possible interactions of taste effectiveness between GSH and umami substances in mice. The 2-bottle preference test revealed that the mice preferred GSH at concentrations ranging from 1 to 10 mM. When GSH was added to IMP or a mixture of IMP and monopotassium glutamate (MPG), the mice showed increased preference for these solutions over the individual IMP or the binary mixture of IMP and MPG in both short-term and long-term tests. The addition of GSH to MPG, however, did not increase preference. Neural responses of the chorda tympani and glossopharyngeal nerves to the mixture of IMP and GSH showed synergism, whereas synergism was not observed in the mixture of MPG and GSH in either taste nerve. Another behavioral study with the use of the conditioned taste aversion paradigm showed that aversions to MPG generalized moderately to GSH, but aversions to GSH did not generalize to MPG. The present study suggests that GSH enhances preference for umami solutions containing 5′-ribonucleotide rather than glutamate. On the basis of these results, we discuss possible receptors involved for the action of GSH.

Key words: electrophysiology, food additives, kokumi, preference, synergism, taste

Introduction

Deliciousness has an important role to play in enhancing consumption of food. Japanese people use the word "koku" on a daily basis when they evaluate the deliciousness of food. They often use this word in phrases such as, "this food is very delicious because of the koku in it." Koku is a conceptual word used for edibles implying strong deliciousness accompanying "thickness," "continuity," and "mouthfulness" in the flavors and textures (Ueda et al. 1990). Thickness refers to rich complexity, continuity refers to long-lasting sensory effects or an increase of aftertaste, and mouthfulness refers to sensory reinforcement or the increment of sensation throughout the whole mouth. Koku can be induced by rich chemical compositions contained in foodstuffs. It is said, for example, that cheddar cheese aged 9 months has more koku than that aged 2 months because the former has rich chemical reactions or decomposition products. Likewise, vintage wines have more koku than young wines such as Beaujolais Nouveau because the former have rich compositions, and soup cooked 6 h has more koku than soup cooked 1 h, for the same reason. Thus, koku is the most appropriately used when we enjoy the odor, texture, and color as well as the taste of food containing complex compounds after maturation.

The term "kokumi" ("mi" refers to taste in Japanese) has been suggested by previous scientists (Ueda et al. 1990, 1994, 1997; Fuke and Konosu 1991) when they refer to the concept

of koku in terms of taste component only rather than more complex components mentioned above, and this is the term we use in the present study. Although the definition of kokumi has not yet been accepted scientifically, it is noted here that kokumi does not refer to an independent taste quality like umami but instead refers to taste reinforcement accompanied again by thickness, continuity, and mouthfulness. In this case, however, these terms may be expressed in more specific ways. One way to describe thickness, continuity, and mouthfulness for human taste evaluation is as follows: 1) thickness refers to increased taste intensity evaluated 5 s after tasting, 2) continuity or long-lasting taste refers to persistent taste intensity measured 20 s after tasting, and 3) mouthfulness refers to the increment of taste sensation throughout the whole mouth (N. Miyamura, personal communication). Kokumi is used not only by Japanese but also, recently, has begun to be used by some European researchers (Dunkel et al. 2007; Toelstede et al. 2009) and by well-known American chefs (Kasabian D and Kasabian A 2005).

If you add a specific key substance instead of complex compounds to food as a seasoning and obtain a similar taste reinforcement effect, the substance can be called a kokumi-inducing substance. Among possible kokumi-inducing substances, such as glycogen, fat, oil, alliin, glutathione (GSH), sulfur-containing compounds, some specific peptides, amino acids, heated products of gelatin, and tropomyosin (Maga 1983; Ueda et al. 1990, 1994, 1997; Fuke and Konosu 1991; Kuroda and Harada 2004; Dunkel et al. 2007; Toelstede et al. 2009), GSH is a food candidate originally investigated by Ueda et al. (1997). GSH (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide with glutamic acid, cysteine, and glycine that is widely included in foodstuffs such as meat, seafood, and wine. In a human sensory test, Ueda et al. (1997) reported through their simplified experimental paradigm that this peptide increased flavor characteristics of an umami solution containing 0.05% (about 1 mM) each of monosodium glutamate (MSG) and 5′-inosine monophosphate (IMP), but it did not affect the intensity of basic tastes, such as sweetness, saltiness, sourness, and umami. They reported that the increased flavor (or enhanced deliciousness) of the umami solution could be expressed by such terms as continuity, thickness, and mouthfulness, which are collectively called kokumi (or ''kokumi flavor'' or ''kokumi taste,'' depending on the researchers), as described above.

The physiological mechanisms of kokumi are still a matter of speculation, for example, there are no answers to the question of whether kokumi is elicited among the chemical ingredients of food by a similar synergistic effect as that occurs in mixtures of umami substances (Yamaguchi 1967; Rifkin and Bartoshuk 1980; Kawamura and Kare 1987; Li et al. 2002). Yamaguchi (1987, 1998) also showed that umami was very important in increasing the deliciousness of food. To our knowledge, there is no report about the taste characteristics of GSH studied by the use of electrophysiological and behavioral techniques in animals. In the present study, therefore, we designed behavioral and electrophysiological experiments to examine possible interactions of taste effectiveness between GSH and umami substances in C57BL/6 mice. Part of the present study has been reported in abstract form (Watanabe and Yamamoto 2004).

Materials and methods

General procedure

Animals

Adult male C57BL/6-CrSLC mice, 8 weeks old at the beginning of the experiment, were used. They were housed in individual home cages in a temperature- (25 °C) and humidity (60%)-controlled room on a 12:12 h light/dark cycle. Animals had free access to food (dry pellets, MF) and tap water, except when deprived for training and testing as described below. All the experiments were carried out following the Guidelines for Ethical Treatment of Laboratory Animals in Osaka University and Asahi University.

Behavioral experiment

On the first day, the mice were put on a schedule of water deprivation for 8 h/day. The training period was from the second to the sixth days. In this period, each animal was trained to drink distilled water (dw) from 2 bottles. After the training period, the 2-bottle preference test was carried out. Each of the 2 bottles filled with a different taste stimulus (or dw) was presented simultaneously to each mouse on each test day. The order of presentation of test stimuli was randomized. The positions of the 2 bottles were switched every 24 h of the 48-h test session to avoid positional preference in the long-term test, and the positions of the bottles were switched every 1 min of the 10-min presentation period in the short-term test. The volume of intake for each solution in each bottle was measured.

Electrophysiological experiment

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight), and supplementary injections were given as needed to maintain a surgical level of anesthesia. A tracheal cannula was implanted, and the animal was secured by a head holder. The chorda tympani (CT) nerve was cut near its entrance into the tympanic bulla and dissected free from the underlying tissues. The glossopharyngeal (GL) nerve was also dissected free and cut near its entry to the posterior lacerated foramen. An indifferent electrode was positioned nearby in the wound. The whole-nerve activities were amplified, displayed on an oscilloscope, and monitored with an audio amplifier. The amplified signal was passed through an integrator with a time constant of 0.3 s and displayed on a slip chart recorder.

Taste nerve responses to test stimuli were recorded. Each stimulus solution and rinsing water flowed for 15 s at a constant flow rate (0.5 ml/s) controlled by a syringe pump at room temperature (25 ± 2 °C). The magnitude of the whole-nerve response was measured as the height of the integrated response from the baseline at 10 s after the onset of stimulation to avoid the tactile effects. Responses to taste stimuli were expressed as relative magnitudes of responses, when the magnitude of response to 0.1 M NH_4Cl was taken as the standard.

Experiment 1: preference test between GSH and dw

A total of 14 mice were used. Mice were subjected to the 2-bottle preference test between GSH and dw. Concentrations of GSH were 0.1, 0.3, 1, 3, 10, and 30 mM. We compared the total volume of intake per 30 g body weight for 48 h. The degree of preference was expressed as a preference score (=intake of GSH/sum of intake of GSH and dw).

Experiment 2: long-term preference for umami solutions with and without GSH

A total of 20 mice were used. They were divided into 2 groups and were subjected to a long-term 2-bottle preference test between dw and several taste solutions, including GSH, umami substances, and their mixtures. As taste stimuli, 1 mM GSH, 0.1 M monopotassium glutamate (MPG), 1 mM IMP, and their mixtures were used for one group and 1 mM GSH, 0.01 M MPG, 0.01 M IMP, and their mixtures were used for another group. It is noted here that the mixture contains the same concentrations of the individual components. We compared the total volume of intake per 30 g body weight for 48 h.

Experiment 3: short-term preference for umami solutions with and without GSH

A total of 10 mice were used. They were put on a schedule of water deprivation of 20 h/day. Each animal was placed in a test box and given free access to dw from 2 drinking bottles with stainless steel spouts for 10 min. Each spout contained a ball at the tip for the purpose of preventing spillage. We switched the bottles manually at the alarm sound of a timer set every 1 min. Supplemental water was available for 3 h in the home cage. After this training for a week, animals were subjected to a short-term 2-bottle preference test between dw and several taste solutions, including GSH, umami substances, and their mixtures. As taste stimuli, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures were used. We compared the volume of intake per 30 g body weight for 10 min.

Experiment 4: long-term preference test after denervation

A total of 24 mice were used. They were randomly divided into 4 groups ($n = 6$, each): naive control mice and mice with transection of either the bilateral chorda tympani nerve (CTx) or bilateral glossopharyngeal nerve (GLx) and mice with transection of both nerves (CTx + GLx). Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg). The ear ossicles through which the CT is running were crushed. The GL under the hypoglossal nerve was excised by tweezers. After the suture ligature, the mice were injected with penicillin G sodium (100 mg/kg) to avoid infection. All mice were allowed 6 days of postoperative recovery prior to any experimental manipulation. Each group was subjected to the long-term 2-bottle preference test between one of the taste solutions and dw. As taste stimuli, 0.1 M MPG with or without 1 mM GSH and 1 mM IMP with or without 1 mM GSH were used. The solutions were presented randomly to each mouse. The preference score was calculated for each taste solution in each animal, and the mean of the group was compared with each other. After the experiment, histological sections of the tongue were examined microscopically.

Experiment 5: conditioned taste aversion test

A total of 24 mice were used. They were randomly divided into 4 groups ($n = 6$, each) consisting of 2 experimental groups, GSH–LiCl and MPG–LiCl, and 2 control groups, GSH–NaCl and MPG–NaCl.

The mice were put on a schedule of water deprivation of 20 h/day. On the first training day, each animal was placed in a test box and given free access to dw for 1 h from a single drinking tube via a circular window. Supplemental water was available for 3 h in the home cage. The spout of polyethylene tubing (4 mm inner diameter) was located 2 mm outside the window. This arrangement prevented the spout from coming into contact with the animals' lips. Licks were detected by a lickometer equipped with a photo sensor. From the second to the fifth days, the training time was reduced from 1 h to 30 min. During this period, the animal was trained to drink dw on an interval schedule, consisting of 20-s presentations of dw with 30-s intertrial intervals, resulting in 30–50 trials during each 30-min session. On the sixth day, each animal was given access to either 0.1 M MPG for MPG–LiCl group or 0.01 M GSH for GSH–LiCl group as the conditioned stimulus and then given an intraperitoneal injection of 0.15 M LiCl (2% of the body weight) as an unconditioned stimulus that induces malaise with gastrointestinal distress. Control mice in MPG–NaCl group and GSH–NaCl group were injected with physiological saline instead of LiCl after ingestion of the MPG and GSH solutions, respectively. The seventh day was a recovery day. On the eighth day, the number of licks of each of the test stimuli was counted for 10 s after the first lick of each stimulus. Each test solution was presented randomly. The interval between each test solution was 30 s. The mean number of licks was obtained for each of the test stimuli in each mouse. Test stimuli were dw, 0.1 M MPG, 0.01 M GSH, 0.01 M IMP, 0.5 M sucrose(S), 0.1 M NaCl (N), 0.01 M HCl (H), and 0.0001 M quinine hydrochloride (Q).

Experiment 6: recording of CT and GL responses to taste stimuli

A total of 12 mice were used for recording of CT (6 mice) and GL (6 mice) responses to test stimuli. As test stimuli, 0.01 M GSH, 0.1 M MPG, 0.01 M IMP, 3 kinds of binary mixtures, such as 0.1 M MPG and 0.01 M IMP (MPG + IMP), 0.1 M MPG and 0.01 M GSH (MPG + GSH), 0.01 M IMP and 0.01 M GSH (IMP + GSH), and a trinary mixture of 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH (MPG + IMP + GSH) were used. Note that the concentrations of GSH and IMP were 10 times higher than those used for the behavioral experiments because we wanted enough responses to these stimuli for quantitative analyses.

The synergistic effects were shown as the potentiation ratio (response to a mixture solution/arithmetic sum of responses to the individual stimuli in the mixture). A ratio exceeding 1.0 suggests the occurrence of synergism.

Statistical analyses

All data were analyzed using STATISTICA (Ver 5.5) software, and a result was considered significant if $P < 0.05$.

Results

Preference for GSH (Experiment 1)

Mean amounts of intake ± standard error (SE) (milliliter for 48 h per 30 g body weight) for 0.1, 0.3, 1, 3, 10, and 30 mM GSH versus dw were 7.8 ± 1.5 and 6.4 ± 0.7, 6.6 ± 0.5 and 5.4 ± 0.4, 10.1 ± 0.9 and 4.1 ± 0.5, 8.8 ± 0.9 and 3.6 ± 0.3, 10.0 ± 0.9 and 3.7 ± 0.4, and 6.8 ± 0.8 and 4.7 ± 0.5, respectively. The corresponding mean reference scores ± SE are shown in Figure 1. The preference scores at concentrations ranging from 1 to 10 mM were about 0.7, and these values were statistically significantly ($P < 0.001$, *t*-test) higher than the score 0.5 level. Preference scores for 0.1, 0.3, and 30 mM GSH stayed near the level of 0.5 ($P > 0.05$).

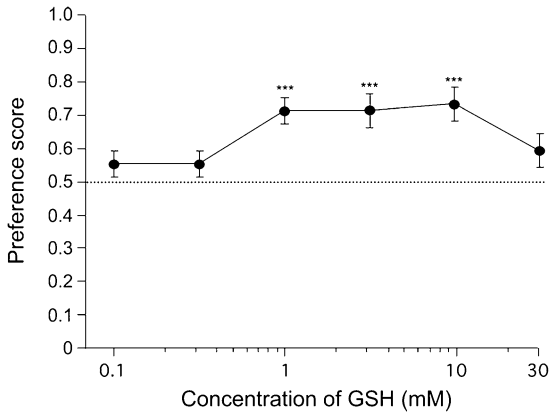

Figure 1 Mean preference scores ± SE for 6 concentrations of GSH. Asterisks indicate a significant difference; ***$P < 0.001$, *t*-test.

Long-term (48 h) preference test (Experiment 2)

Figure 2 shows the volume of intake (per 30 g body weight) for dw and 2 types of umami substances and their mixtures in the long-term 2-bottle preference test. When the volume of intake for 0.1 M MPG was compared with that of dw, MPG was preferred over dw significantly ($P < 0.05$, *t*-test) (Figure 2A). The binary mixture containing 0.1 M MPG and 1 mM GSH (MPG + GSH) was also preferred over dw ($P < 0.001$) (Figure 2B). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$) (Figure 2C).

IMP solution (1 mM) was preferred over dw ($P < 0.05$) (Figure 2D). The binary mixture of 1 mM IMP and 1 mM GSH (IMP + GSH) was strongly preferred ($P < 0.001$) (Figure 2E). When the volume of intake for IMP and that for IMP + GSH were compared, the mixture was preferred over IMP ($P < 0.001$) (Figure 2F).

The binary mixture of 0.1 M MPG and 1 mM IMP (MPG + IMP) was preferred over dw ($P < 0.001$) (Figure 2G). When 1 mM GSH was added to this mixture, this new mixture (MPG + IMP + GSH) was greatly preferred over dw ($P < 0.001$) (Figure 2H). When the volume of intake

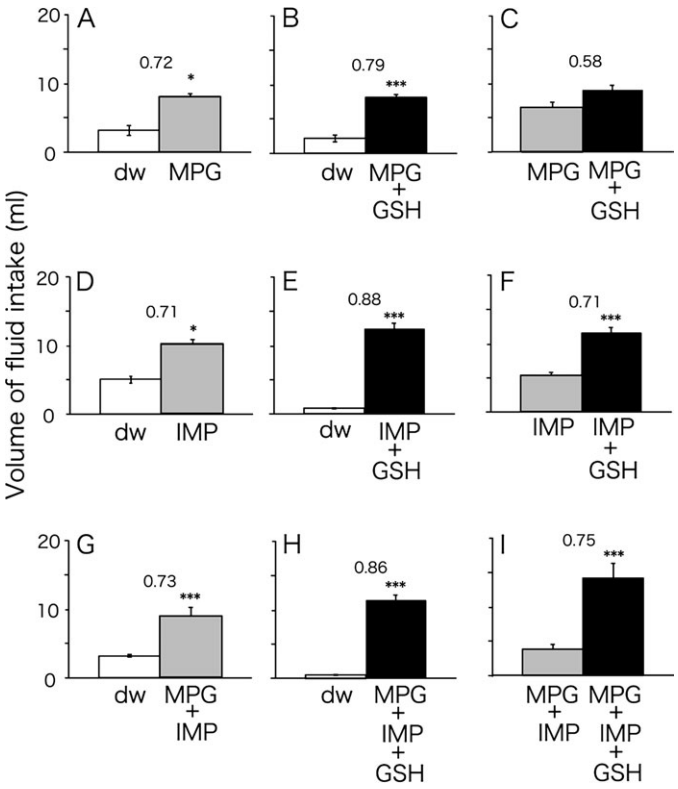

Figure 2 Mean volume of intake ± SE per 30 g body weight per 48 h for dw, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures in the long-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 48 h. Preference scores are shown in each graph. Asterisks indicate a significant difference; *$P < 0.05$, ***$P < 0.001$, *t*-test.

for MPG + IMP and that for MPG + IMP + GSH were compared, the latter was more preferred than the former ($P < 0.001$) (Figure 2I).

To examine the above finding that the addition of 1 mM GSH to 1 mM IMP, but not to 0.1 M MPG, increased preference, we did the same preference test with 1 mM GSH, 0.01 M IMP, and 0.01 M MPG. We obtained essentially the same result. As shown in Figure 3, when GSH was added to IMP, the mice showed increased preference to this mixture over IMP alone ($P < 0.01$, t-test). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$).

Short-term (10 min) preference test (Experiment 3)

Figure 4 shows the volume of intake (per 30 g body weight for 10 min) for dw and 2 types of umami substances and their mixtures in the short-term 2-bottle preference test. When the intake of 0.1 M MPG was compared with that of dw, MPG was preferred over dw significantly ($P < 0.01$, t-test) (Figure 4A). However, when the volume of intake for MPG and that for MPG + GSH were compared, there was no significant difference between these 2 solutions ($P > 0.05$) (Figure 4B), which is consistent with the result obtained in the long-term test. IMP solution (1 mM) was preferred over dw ($P < 0.05$) (Figure 4C). When the volume of intake for IMP and that for IMP + GSH were compared, the mixture was preferred over IMP alone ($P < 0.001$) (Figure 4D), indicating similar results to those of the long-term test.

Long-term preference test after denervation (Experiment 4)

We examined the effect of the bilateral transection of either one or both of the CT and GL on the long-term preference. Because the CT and GL innervate taste buds on the anterior and posterior tongue, respectively, the transection of these nerves would reduce a substantial portion of gustatory information to the brain, although taste buds on the nasoincisal, palatal, and pharyngeal regions are spared. The transection was confirmed by verifying microscopically the loss of taste buds on the tongue.

Figure 5 shows mean preference scores for MPG with and without GSH (Figure 5A) and those for IMP with and without GSH (Figure 5B) in naive control mice and mice with transection of the CT (CTx), GL (GLx), and both CT and GL (CTx + GLx). Two-way (Nerve × GSH) analysis of variance (ANOVA) for MPG revealed significant main effect of Nerve, $F(3, 38) = 0.006$, $P < 0.01$, but no significant main effect of GSH and a Nerve × GSH interaction. On the other hand, the ANOVA for IMP revealed significant main effects of Nerve, $F(3, 38) = 7.63$, $P < 0.001$, and GSH, $F(1, 38) = 61.14$, $P < 0.001$, but no Nerve × GSH interaction. Further analysis of the data using Tukey's honestly significant difference (HSD) test showed that the preference scores for IMP with GSH were statistically significantly larger than those for IMP without GSH in control, CTx, and GLx mice, but there was no significant difference in CTx + GLx mice (Figure 5B).

Conditioned taste aversion test (Experiment 5)

Figure 6 shows generalization of aversion across 8 test stimuli including dw after establishment of aversions to either 0.1

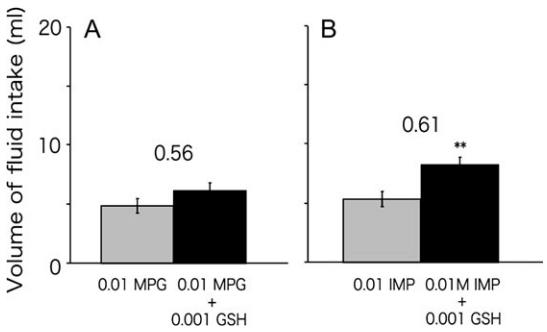

Figure 3 Mean volume of intake ± SE per 30 g body weight per 48 h for 0.01 M MPG and 0.01 M IMP with or without 1 mM GSH in the long-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 48 h. Preference scores are shown in each graph. Asterisk indicates a significant difference; $^{**}P < 0.01$, t-test.

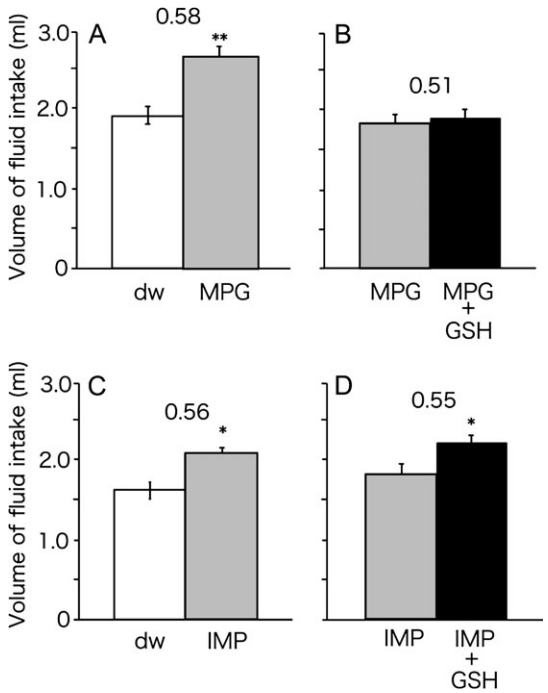

Figure 4 Mean volume of intake ± SE per 30 g body weight per 10 min for dw, 1 mM GSH, 0.1 M MPG, 1 mM IMP, and their mixtures in short-term 2-bottle preference test. A pair of liquids in each graph was presented simultaneously for 10 min with their positions changed every 1 min. Preference scores are shown in each graph. Asterisks indicate a significant difference; $^{*}P < 0.05$, $^{**}P < 0.01$, t-test.

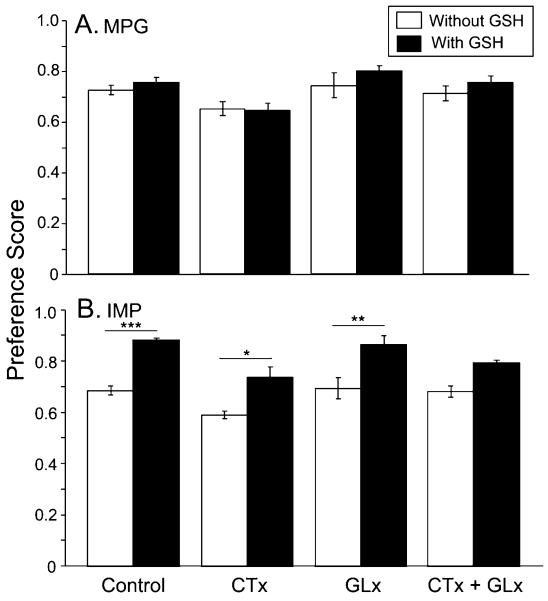

Figure 5 Mean preference scores ± SE for 0.1 M MPG with or without 1 mM GSH (A) and for 1 mM IMP with or without 1 mM GSH (B) in control and denervated mice. CTx, GLx, and CTx + GLx denote that only the CT, only the GL, and both nerves were transected, respectively, before the long-term preference test. Asterisks indicate a significant difference; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Tukey's HSD test.

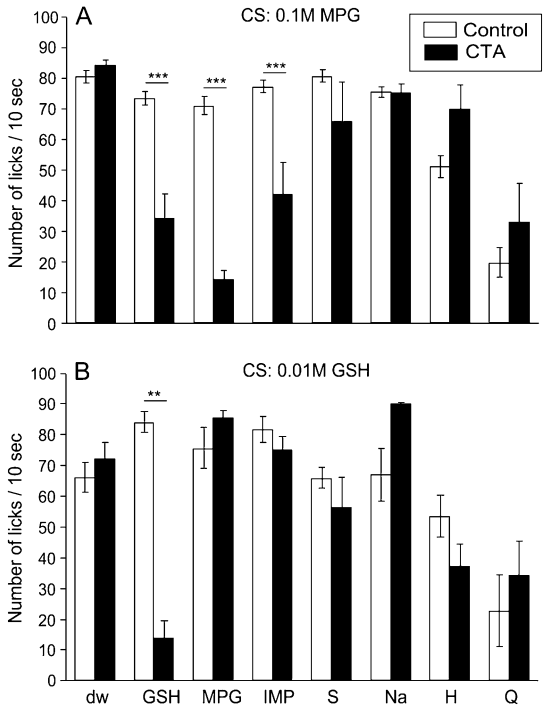

Figure 6 Mean numbers of licks ± SE for 8 test stimuli after conditioned taste aversions to 0.1 M MPG or 0.01 M GSH used as the conditioned stimulus (CS) in experimental group (CTA) and saline-injected control group (control). Suppression of licking was shown to MPG, GSH, and IMP after aversive conditioning to MPG, whereas suppression was shown only to GSH after aversive conditioning to GSH. $**P < 0.01$, $***P < 0.001$, Tukey's HSD test.

M MPG or 0.01 M GSH as expressed by mean numbers of licks during the first 10 s after the first lick. The mice conditioned to avoid MPG showed decreased licks to GSH and IMP as well as MPG. On the other hand, the animals conditioned to GSH showed decreased licks only to GSH, that is, no significant difference was observed to other taste stimuli and water between control and conditioned taste aversion (CTA) mice.

A 2-way (Group × Solution) ANOVA for conditioning to MPG revealed significant main effects of Group, $F(1, 112) = 28.07$, $P < 0.001$, and Solution, $F(7, 112) = 25.03$, $P < 0.001$, and a Group × Solution interaction, $F(7, 112) = 13.83$, $P < 0.001$. Post hoc analysis of the data using Tukey's HSD test showed that the numbers of licks to GSH, MPG, and IMP were significantly ($P < 0.001$) smaller than those in control group. A 2-way (Group × Solution) ANOVA for conditioning to GSH revealed significant main effects of Group, $F(1, 92) = 3.96$, $P < 0.05$, and Solution, $F(7, 92) = 15.13$, $P < 0.001$, and a Group × Solution interaction, $F(7, 92) = 13.03$, $P < 0.001$. Post hoc analysis of the data using Tukey's HSD test showed that the number of licks to GSH was significantly ($P < 0.01$) smaller than that in control group.

Taste nerve responses (Experiment 6)

Figure 7 shows sample records for umami, GSH, and their mixtures. Enhanced responses to the mixtures of GSH and umami substances as well as the enhanced response to the mixture of MPG and IMP were noted in both CT and GL. Quantitative analyses for these mixture effects are shown in Figure 8.

Figure 8A shows the relative responses to GSH, umami substances, and their mixtures in the CT. The mean magnitudes of responses (±SE, $n = 6$) to 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH were 0.42 ± 0.11, 0.14 ± 0.01, and 0.36 ± 0.04, respectively. In the mixture solutions, the magnitudes of responses to MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 0.98 ± 0.12, 0.60 ± 0.04, 1.10 ± 0.12, and 1.01 ± 0.15, respectively. Potentiation ratios for MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 1.94 ± 0.26, 0.87 ± 0.16, 2.26 ± 0.13, and 1.19 ± 0.26, respectively. Responses to both MPG + IMP and IMP + GMP were significantly higher than those to the arithmetic sum of the individual components ($P < 0.05$ and $P < 0.01$, respectively, t-test), indicating the existence of synergism. There was no significant difference between the response to MPG + GSH and the arithmetic sum of the MPG and GSH responses ($P > 0.05$).

Figure 8B shows the relative responses to GSH, umami substances, and their mixtures in the GL. The mean magnitudes of responses (±SE, $n = 6$) to 0.1 M MPG, 0.01 M IMP, and 0.01 M GSH were 0.56 ± 0.05, 0.23 ± 0.02, and 0.47 ± 0.13, respectively. In the mixture solutions, the magnitudes of responses to MPG + IMP, MPG + GSH, IMP + GSH, and

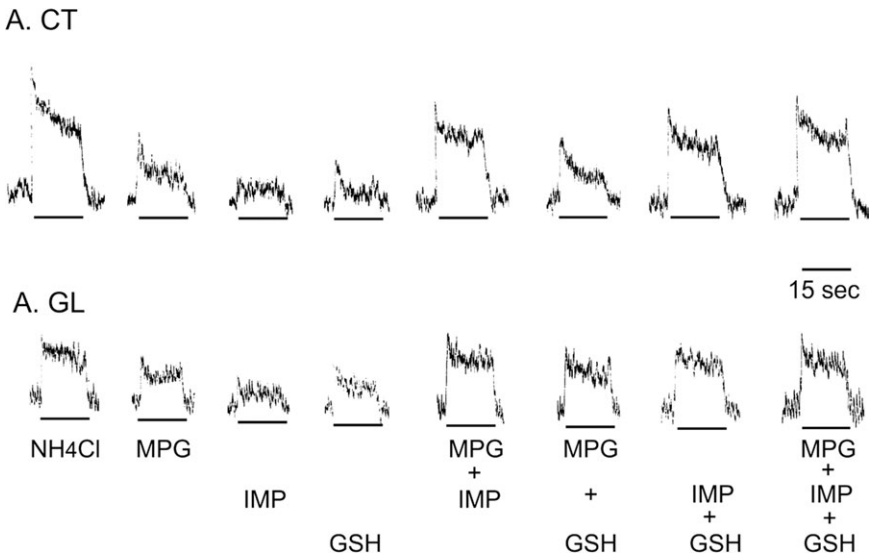

Figure 7 Representative integrated responses of the CT and the GL nerves to 0.1 M NH_4Cl, 0.1 M MPG, 0.01 M IMP, 0.01 M GSH, and 4 kinds of mixtures.

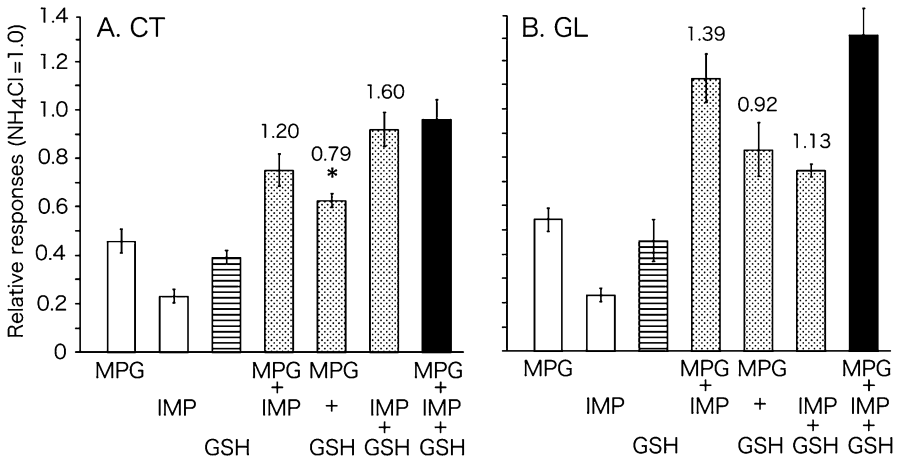

Figure 8 Mean relative responses ± SE to GSH, umami substances, and their mixtures in the CT and GL nerves. Values above each bar show potentiation ratios. The mixtures, MPG + IMP and IMP + GSH, showed large potentiation ratios, that is, the mixture responses were significantly larger than the arithmetic sum of the responses to the individual components. $*P < 0.05$, $**P < 0.01$, *t*-test.

MPG + IMP + GSH were 1.37 ± 0.17, 0.91 ± 0.15, 1.13 ± 0.13, and 1.30 ± 0.16, respectively. Potentiation ratios for MPG + IMP, MPG + GSH, IMP + GSH, and MPG + IMP + GSH were 1.71 ± 0.20, 1.02 ± 0.27, 1.66 ± 0.10, and 1.12 ± 0.21, respectively. Responses to both MPG + IMP and IMP + GMP were significantly higher than those to the arithmetic sum of the individual components ($P < 0.05$, *t*-test), indicating the occurrence of synergism. There was no significant difference between the response to MPG + GSH and the arithmetic sum of the MPG and GSH responses ($P > 0.05$).

In both nerves, the potentiation ratios for the trinary mixture exceeded 1.0, indicating a tendency of synergism. However, the values were not statistically significant in comparison to the arithmetic sum of the 3 component responses, possibly because of the ceiling effect.

Discussion

Ueda et al. (1997) found in a human sensory test that GSH induced a characteristic taste reinforcement in terms of thickness, continuity, and mouthfulness when it was added to umami solutions or a model beef extract. In other words, under the action of GSH, hedonically positive aspect of umami taste is enhanced, continues, and spreads within the whole mouth. Such characteristics are collectively called kokumi in Japanese. Kokumi may be a result of processing of afferent information within the higher center of the gustatory system

as well as gustatory interaction at the peripheral receptor level. The results of Ueda et al. also suggest that one of the essential kokumi actions is based on the umami substances. The present study is the first attempt to reveal the nature of kokumi on the basis of behavioral and electrophysiological studies in animals.

C57BL/6 mice showed no particular preference for aqueous solutions of GSH compared with dw in the 2-bottle preference test at low (0.1 and 0.3 mM) and high (30 mM) concentrations, but they preferred the solutions at concentrations ranging from 1 to 10 mM. In a human sensory evaluation test, however, GSH elicited no remarkable taste except sourness in water because of its acidic nature (Ueda et al. 1997). Ueda et al. (1997) reported that the threshold for GSH in inducing the kokumi effect was 0.04% (about 0.8 mM), corresponding well with the preference threshold in mice, which occurs between 0.3 and 1 mM.

MSG evokes both sodium and umami tastes in rodents and dogs (Sato and Akaike 1965; Ninomiya and Funakoshi 1987; Kumazawa and Kurihara 1990; Yamamoto et al.. 1991; Grobe and Spector 2008). In the present study, therefore, we used MPG instead of MSG to avoid possible influences of sodium taste on the taste of glutamate. The taste solution used in the present behavioral study was mostly fixed to one concentration for each stimulus. Although this is a limitation for a complete experiment with a wide range of concentrations, we selected 0.1 M MPG and 1 mM IMP, because these stimuli were commonly used for the umami study in rodents and induced dominant synergy when a mixture containing these solutions was used (Ninomiya and Funakoshi 1987; Yamamoto et al. 1991; Sako and Yamamoto 1999), and 1mM GSH, because this concentration was near the threshold for taste effectiveness as described above for both mice and humans.

The present short-term (10 min) and long-term (48 h) 2-bottle tests showed that the umami solutions, IMP and a mixture of IMP and MPG, were preferred more in the presence of GSH. These results may not be explained simply by the addition of the preferable component of GSH to the taste of umami solutions because the addition of GSH to MPG did not increase the preference for this mixture. It is difficult to determine which component, that is, GSH, IMP, or MPG, is influenced and shows increased taste intensity when mixed. Taste quality may change depending on the combination of the 3 chemicals because the taste of IMP may not be identical to the taste of glutamate as suggested in rats (Witfall et al. 2007).

To investigate whether the enhanced preference caused by the addition of GSH was elicited by the taste effect or a postingestive effect, we conducted the short-term 2-bottle preference test in naive mice and the long-term preference test in mice with the taste nerves transected. The results of these 2 experiments suggested that the GSH effects were mainly due to the taste effect. However, we cannot exclude possible influences of postingestive factors because of the lack of a well-defined preference for IMP + GSH over IMP, whereas statistically significant, in the short-term test, and the tendency of persistence of preference even after the CT and GL were transected. Alternatively, the persistence of preference after denervation of the CT and GL may be attributed to umami responses of the greater superficial petrosal nerve innervating the palatal taste buds (S. Harada, personal communication).

The lack of an additive effect of GSH on the preference for MPG, but not for IMP, which was confirmed at different concentrations of MPG and IMP, suggests that GSH exerts its taste effect by binding to common receptor sites with MPG, but not with IMP, indicating a kind of competitive interaction between GSH and MPG. GSH is a tripeptide with glutamic acid in its chemical structure, so this part may interact with the same receptor sites as those for glutamate of MPG. The lack of synergism in the trinary mixture of GSH, MPG, and IMP might be due to the ceiling effect. The taste effectiveness of GSH under the presence of IMP might be explained by binding of GSH to possible exposed receptor sites for glutamate as a result of the action of IMP, the idea being proposed by Torii and Cagan (1980). Alternatively, as a recent molecular study (Zhang et al. 2008) suggests, IMP may trap both glutamate and/or GSH to synergistically increase their responses.

To investigate whether GSH elicits a synergistic effect at the taste receptor level, we recorded taste nerve responses of the CT and GL to umami substances, GSH, and their mixtures. Both nerves showed essentially the same response characteristics to these taste stimuli, and the noticeable findings were that the potentiation ratios for MPG + IMP and IMP + GSH, but not for MPG + GSH, exceed 1.0, suggesting that GSH elicits synergism with IMP but not with MPG. The lack of synergism between GSH and MPG is comparable to the lack of an additive effect of GSH on the preference for MPG in the present behavioral experiments. Although the additive effects of GSH on umami responses were not identical in these 2 taste nerves, the difference may not be significant because mice without either of these nerves showed essentially the same result (see Figure 5). Both nerves are important in exerting the GSH effect because the simultaneous denervation of the 2 nerves was effective in abolishing the effect.

If GSH and MPG have common binding sites in taste receptors, the taste of GSH may be similar to that of MPG. This assumption was partly proved in the present CTA experiment in which mice trained to reject MPG also rejected GSH although the degree of rejection was not so strong. However, the reverse was not the same, that is, mice trained to reject GSH did not reject any other taste stimuli including MPG and IMP as compared with the control group, indicating that the taste of GSH is unique and is independent of other tastes. Although we have to confirm these results with different concentrations of each tastant in future experiments, the results suggest that GSH also has interactions

with other taste receptors than those for the 5 taste receptors including glutamate receptors. One possibility is an interaction with the calcium-sensing receptor (CaSR) because Wang et al. (2006) identified that GSH acted as a potential ligand to the rat CaSR and showed that GSH acted as a potent enhancer of calcium-induced activation of the CaSR. Recently, San Gabriel et al. (2009) actually found CaSR in a subset of cells in circumvallate and foliate papillae, with fewer cells in the fungiform papillae, isolated from rat and mice.

It is not known whether rodents are good models for the study of kokumi because of the species difference of the umami receptor, T1R1 and T1R3, that is, this receptor responds to various amino acids including glutamate in rodents (Nelson et al. 2002), whereas it functions as a much more specific receptor, responding selectively to MSG and aspartate in humans (Li et al. 2002). We conducted the present experiments in C57BL mice to obtain any hints about the nature of kokumi, that is, enhanced taste reinforcement accompanied by thickness, continuity, and mouthfulness in a simplified experimental setup consisting of GSH as a kokumi-inducing substance and umami substances such as MPG and IMP as taste stimuli. Our results showed that GSH had a preferable taste for mice in comparison to humans who were insensitive to GSH (Ueda et al. 1997). The results also showed that the mixture of GSH with IMP, but not with MPG, was more preferred than was each component of the mixture and showed a synergistic taste nerve response to a mixture of GSH and IMP. These findings, along with the fact that umami is very important in increasing the deliciousness of food (Yamaguchi 1987, 1998), suggest that GSH enhances deliciousness induced by umami substances. A relevant conclusion is that the enhanced umami response plays at least in part an important role in the kokumi-inducing action of GSH.

Further studies are necessary to elucidate the underlying receptor mechanisms of taste characteristics with the use of other kokumi-inducing substances in different species of animals and also to elucidate the mode of interaction and integration of a range of sensory information produced in the brain regions responsible for rewarding and emotional processing during ingestive behavior.

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