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GPRC6A as a novel kokumi receptor responsible for enhanced taste preferences by ornithine

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eLife Assessment

In this **valuable** study, the authors used rats to determine the receptor for a food-related perception (kokumi) that has been characterized in humans. They employ a combination of behavioral, electrophysiological, and immunohistochemical results to provide **solid** support for their conclusion that ornithine-mediated kokumi effects are mediated by the GPRC6A receptor. They complemented the rat data with some human psychophysical data. The results are intriguing, but there are some deficiencies.

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Abstract

The concept of kokumi, which refers to an enhanced and more delicious flavor of food, has recently generated considerable interest in food science. However, kokumi has not been well studied in gustatory physiology, and the underlying neuroscientific mechanisms remain largely unexplored. Our previous research demonstrated that ornithine (L-ornithine), which is abundant in shijimi clams, enhanced taste preferences in mice. The present study aimed to build on these findings and investigate the mechanisms responsible for kokumi in rats. In two-bottle preference tests, the addition of ornithine, at a low concentration that did not increase the favorability of this substance alone, enhanced the preferences of animals for umami, sweet, fatty, salty, and bitter solutions, with the intake of monosodium glutamate showing the most significant increase. Additionally, a mixture of umami and ornithine synergistically induced significant responses in the chorda tympani nerve, which transmits taste information to the brain from the anterior part of the tongue. The observed preference enhancement and increase in taste-nerve response were abolished by antagonists of the G-protein-coupled receptor family C group 6 subtype A (GPRC6A). Furthermore, immunohistochemical analysis indicated that GPRC6A was expressed in a subset of type II taste cells in rat fungiform papillae. These results provide new insights into flavor-enhancement mechanisms, confirming that ornithine is a kokumi substance and GPRC6A is a novel kokumi receptor.

Introduction

Enjoying delicious food is a fundamental pleasure in daily life and contributes toward both physical and mental well-being. The perception of deliciousness requires the harmonious integration of various sensory experiences, including the five basic tastes (sweet, salty, sour, bitter, and umami), the taste of fat, spices, aroma, texture, temperature, sight, and sound. These inputs are transmitted to the brain via sensory nerves, where sensory modalities and qualitative information are analyzed (1,2). Beyond these cognitive aspects, the brain further processes these sensations to evoke emotional responses, which significantly contribute to the perception of tastiness or unpleasantness (3-5). Therefore, sensory information from substances entering the oral and nasal cavities influences both cognition and emotions.

Conversely, despite having minimal direct taste effects and a lack of clear information for the brain to process, some substances and their receptors can modify taste information at the peripheral level, thereby controlling palatability. Specifically, recent research on taste modification has focused on "kokumi" substances and receptors (6-8). Kokumi is a Japanese word that denotes enhanced tastiness of food comprising complex ingredients. According to Ohsu et al. (9), when kokumi substances such as glutathione or γ-Glu-Val-Gly are added to chicken consommé at low concentrations that do not produce a specific taste of their own, they bind to the calcium-sensing receptor (CaSR) to induce kokumi by enhancing the "thickness," "mouthfulness," and "continuity" of the dish.

These three attributes were originally introduced by Ueda et al. (10), who translated Japanese terms describing the profound characteristics of kokumi, which are deeply rooted in Japanese culinary culture (8,11,12). However, these simply translated terms have caused global misunderstanding and confusion, because they sound like somatosensory rather than gustatory descriptions. Therefore, to clarify that kokumi attributes are inherently gustatory, in the present study we use the terms "intensity of whole complex tastes (rich flavor with complex tastes)" instead of thickness, "mouthfulness (spread of taste and flavor throughout the oral cavity)," and "persistence of taste (lingering flavor)" instead of continuity.

Several studies have been conducted on CaSR agonists as candidate kokumi substances (13-21), although neuroscientific research on the mechanism of kokumi induction remains limited and underdeveloped (22-25). In contrast, our previous study in mice (26) focused on ornithine, a non-protein amino acid, as a potential kokumi substance and suggested that the G-protein-coupled receptor family C group 6 subtype A (GPRC6A) is responsible for its action, given that ornithine has a high affinity for this receptor (27-29). As this is the only published study to propose GPRC6A as a potential kokumi receptor other than CaSR, this hypothesis needs to be confirmed.

Neuroscientific studies have reported glutathione (30) in mice and γ-Glu-Val-Gly in mice (23) and rats (25) as kokumi substances. However, to our best knowledge, only our prior study in mice has examined ornithine as a kokumi candidate (26). Therefore, the current study aimed to investigate the effects of ornithine on sweet, salty, sour, bitter, umami, and fatty taste enhancement in rats and to clarify the involvement of the GPRC6A receptor. Whether rodents are suitable models for kokumi research is unclear; however, if the expression of kokumi flavor is fundamental to the enjoyment of delicious food, there should be basic mechanisms common to humans and rodents, even if a similar human perception of kokumi is difficult to detect in rats. Elucidating these neuroscientific mechanisms may shed light on the elusive nature of kokumi in humans.

Results

First, we used human sensory tests to confirm that adding ornithine to miso soup enhances the three elements of kokumi, namely the intensity of whole complex tastes, mouthfulness, and persistence of taste (see Fig. S1 with the supplemental materials and methods).

Two-bottle preference tests in rats

1) Additive effects of ornithine at different concentrations

To investigate whether ornithine has a favorable taste, we conducted a preference test by comparing distilled water (DW) with aqueous solutions containing various ornithine concentrations (**Fig. 1A**). Two-way analysis of variance (ANOVA; solution × concentration) revealed a significant main effect of solution [$F(1, 50) = 33.93$, $P < 0.0001$], no main effect of concentration [$F(4, 50) = 0.145$, $P > 0.05$], and a significant solution-concentration interaction [$F(4, 50) = 4.070$, $P < 0.01$]. Post hoc Bonferroni tests showed that the intake of 10 and 30 mM ornithine was significantly greater ($P < 0.01$) than that of plain DW, indicating a preference for higher concentrations of ornithine. Next, we examined the additive effect of ornithine at the same concentrations on the preference for 0.03 M monosodium glutamate (MSG; **Fig. 1B**). Two-way ANOVA revealed a significant main effect of solution [$F(1, 50) = 175.41$, $P < 0.0001$] and a solution-concentration interaction [$F(4, 50) = 8.371$, $P < 0.0001$], with no main effect of concentration [$F(4, 50) = 0.899$, $P > 0.05$]. Subsequent Bonferroni analyses indicated that the addition of ornithine at concentrations ranging from 1–30 mM resulted in significantly greater intake ($P < 0.001$) than that for MSG alone. Based on these results, we used 1 mM ornithine to examine its additive effects in subsequent experiments.

2) Additive effects of 1 mM ornithine on intake of different tastants

We examined the intake of eight taste solutions at four concentrations, both alone and in combination with 1 mM ornithine (**Fig. 2**). The eight solutions were sweet (sucrose), salty (NaCl), sour (citric acid), bitter (quinine hydrochloride [QHCl]) umami (MSG, monopotassium glutamate [MPG], or inosine monophosphate [IMP]), and fatty (Intralipos). Bonferroni's multiple-comparison analysis revealed that MSG and MPG at all four concentrations were significantly preferred when combined with ornithine ($P < 0.05$, 0.01, or 0.001; **Fig. 2B and C**). This was also observed for IMP (**Fig. 2A**), Intralipos (**Fig. 2D**), and sucrose (**Fig. 2E**) at two concentrations, as well as NaCl (**Fig. 2F**) at one concentration. However, the rats showed no difference in preference for citric acid regardless of the addition of 1 mM ornithine. In contrast, QHCl with ornithine was preferred at three of the four concentrations, suggesting that the aversive taste of QHCl was attenuated by ornithine. Notably, the effects of ornithine differed among the umami solutions; ornithine more effectively increased the preference for MSG and MPG than for IMP.

3) Additive effects of ornithine in brief-exposure tests with and without antagonists

To determine whether the enhancement of preference induced by ornithine was caused by an intra-oral event rather than by post-oral consequences, a brief-exposure (or short-term, 10-minute) two-bottle preference test was conducted. We confirmed that the intake of DW with 1 mM ornithine did not differ from that of plain DW ($P > 0.05$; **Fig. 3A**). However, the intake of 0.03 M MSG was significantly increased ($P < 0.01$) by the addition of 1 mM ornithine (**Fig. 3B**). This preference was maintained in the presence of 60 μM calindol, a GPRC6A antagonist (**Fig. 3C**),

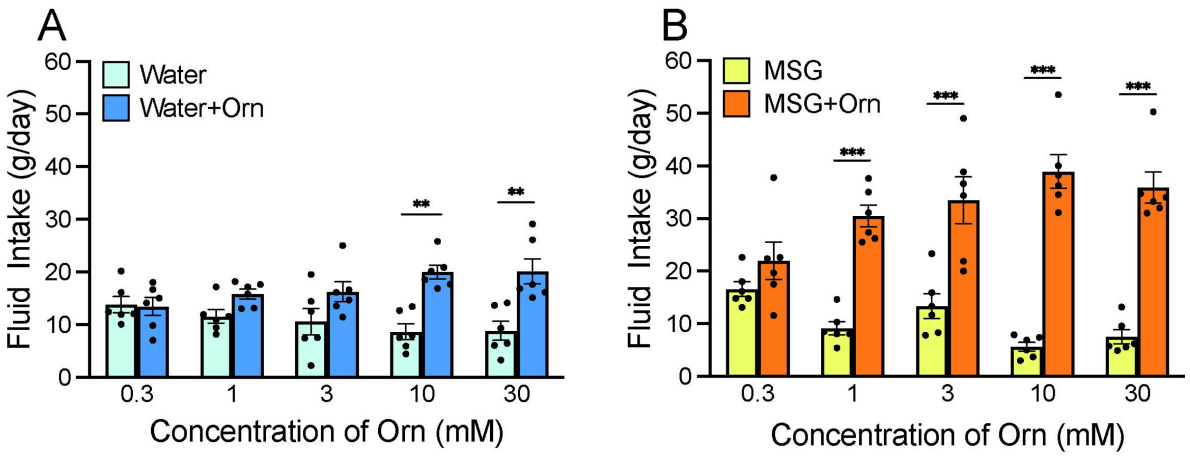


Figure 1

Additive effects of ornithine (Orn) at different concentrations.

(A) Water intake with and without five Orn concentrations. **(B)** Intake of 0.03 M MSG with and without Orn. Each value represents the mean ± SEM; n = 6. **P < 0.01, ***P < 0.001 (Bonferroni correction). MSG, monosodium glutamate; SEM, standard error of the mean

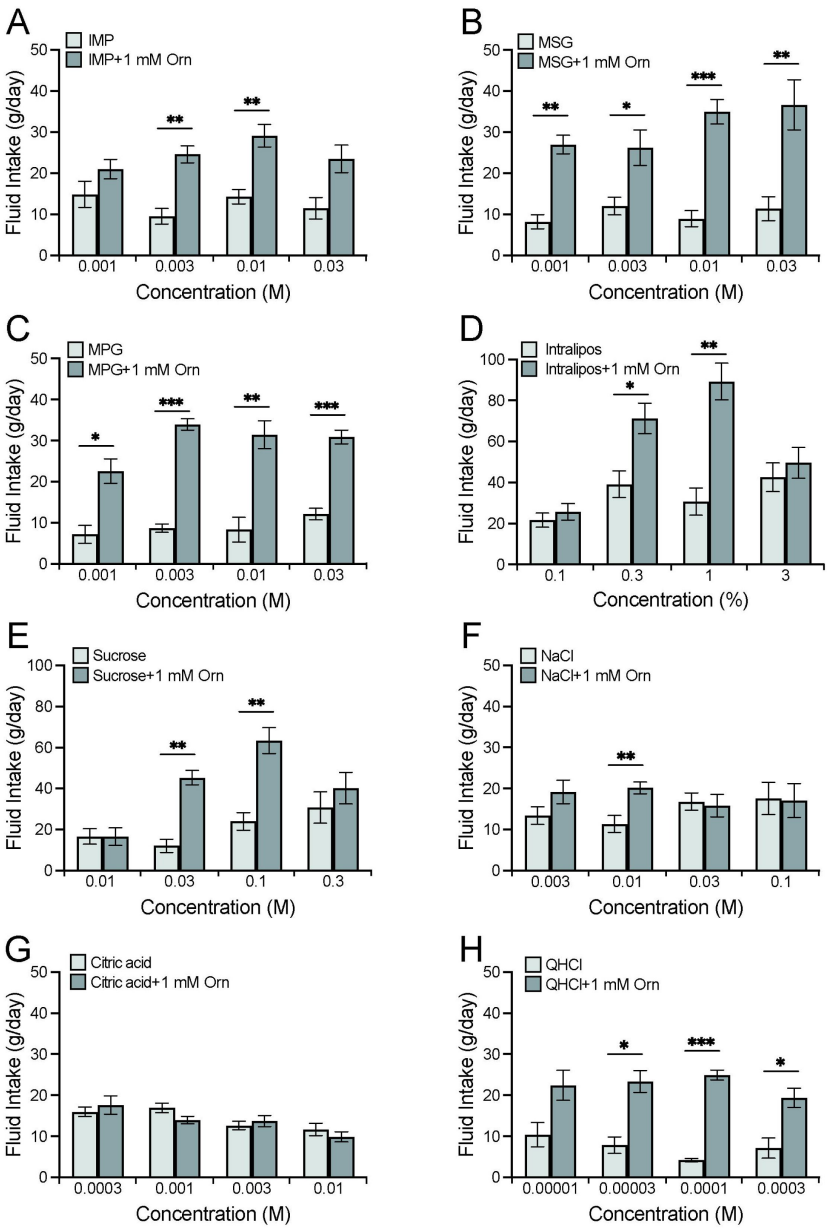


Figure 2

Additive effects of 1 mM ornithine (Orn) on fluid intake for eight different taste solutions.

Fluid intake with and without Orn is shown for IMP **(A)**, MSG **(B)**, MPG **(C)**, Intralipos **(D)**, sucrose **(E)**, NaCl **(F)**, citric acid **(G)**, and QHCl **(H)**. Each value represents the mean ± SEM; n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 (Bonferroni correction). IMP, inosine monophosphate; MSG, monosodium glutamate; MPG, monopotassium phosphate; QHCl, quinine hydrochloride; SEM, standard error of the mean

but disappeared at a calindol concentration of 300 μM (**Fig. 3D**). Similarly, the GPRC6A antagonist epigallocatechin gallate (EGCG) had no effect at a concentration of 30 μM (**Fig. 3E**) but abolished the ornithine-enhanced preference at 100 μM (**Fig. 3F**).

4) Additive effects of ornithine after chorda tympani transection

To determine how taste information from the anterior part of the tongue influences ornithine-induced taste preferences, we compared intake during the long-term two-bottle preference test before and after transection of the chorda tympani (CT). As shown in **Fig. 4A and C**, the combination of 1 mM ornithine and 0.03 M MSG was significantly more ingested than MSG alone ($P < 0.01$, paired *t*-test, two-tailed) before both sham and experimental treatments. The same preference was observed following the sham-control operation (**Fig. 4B**). However, in the experimental group, CT transection abolished the increased favorability induced by ornithine supplementation (**Fig. 4D**).

Taste-nerve recording in rats

The aqueous solutions of ornithine used in the behavioral experiments induced small CT responses, which increased in a dose-dependent manner, as shown by sample recordings (**Fig. 5A**) and a graphical representation of mean response magnitudes (**Fig. 5D**). One-way ANOVA revealed significant differences among the responses at different ornithine concentrations [$F(4, 15) = 14.660$, $P < 0.001$]. Additionally, the response to 30 mM ornithine survived the Bonferroni correction for multiple testing, which indicated that this response was significantly higher ($P < 0.01$) than those at lower concentrations. The response to 1 mM ornithine, which was used in the behavioral experiments, was negligible compared to the standard response to NH_4Cl. However, when 1 mM ornithine was added to 0.03 M MSG, the response increased significantly ($P < 0.05$) compared with that for plain MSG (**Fig. 5E**). A similar additive effect of ornithine was observed when the solution was prepared with 0.1 mM amiloride, a sodium-channel blocker (**Fig. 5B and E**), suggesting that glutamate, rather than sodium ions, was responsible for this increased response. One-way ANOVA revealed significant differences among the relative response values shown in **Fig. 5E** [$F(3, 16) = 9.174$, $P < 0.001$]. Finally, we examined the effect of calindol on the increased response to MSG with ornithine. As shown by the sample recordings (**Fig. 5C**) and graphical representation (**Fig. 5F**), the increased response to MSG with the addition of ornithine ($P < 0.01$) was no longer observed when calindol was added. One-way ANOVA revealed significant differences among these responses visualized in **Fig. 5F** [$F(2, 9) = 4.690$, $P < 0.05$]. EGCG, another GPRC6A antagonist, showed effects similar to those of calindol (data not shown).

Immunohistochemical localization of GPRC6A in rat taste cells

Immunohistochemical analyses were performed to determine whether GPRC6A was expressed in the taste cells of rat fungiform, foliate, and circumvallate taste buds. In the fungiform papillae, a small number of spindle-shaped taste cells exhibited GPRC6A-immunoreactivity (**Fig. 6A**). In the foliate and circumvallate papillae, GPRC6A-immunopositive taste cells were barely detectable, and GPRC6A-expressing cells were likely to constitute less than 1% of the total taste cell population in the respective taste papillae (**Fig. 6B, C**). These results demonstrated that GPRC6A was preferentially located in subpopulations of fungiform taste cells in the rat.

Since rat taste cells comprise several cell types, we examined the colocalization of GPRC6A and cell type-specific markers in the fungiform papillae. A small subpopulation of GPRC6A-immunopositive cells was found to be immunoreactive for IP3R3, a marker for the majority of the type II cell population (**Fig. 7A**). Approximately 11% of GPRC6A-positive cells overlapped with IP3R3 (9 double-positive cells/80 GPRC6A-positive cells), while approximately 8.3% of IP3R3-positive cells expressed GPRC6A (9 double-positive /109 IP3R3-positive cells). In contrast, GPRC6A-positive cells were unlikely to colocalize with a-gustducin, another marker for a subset of type II

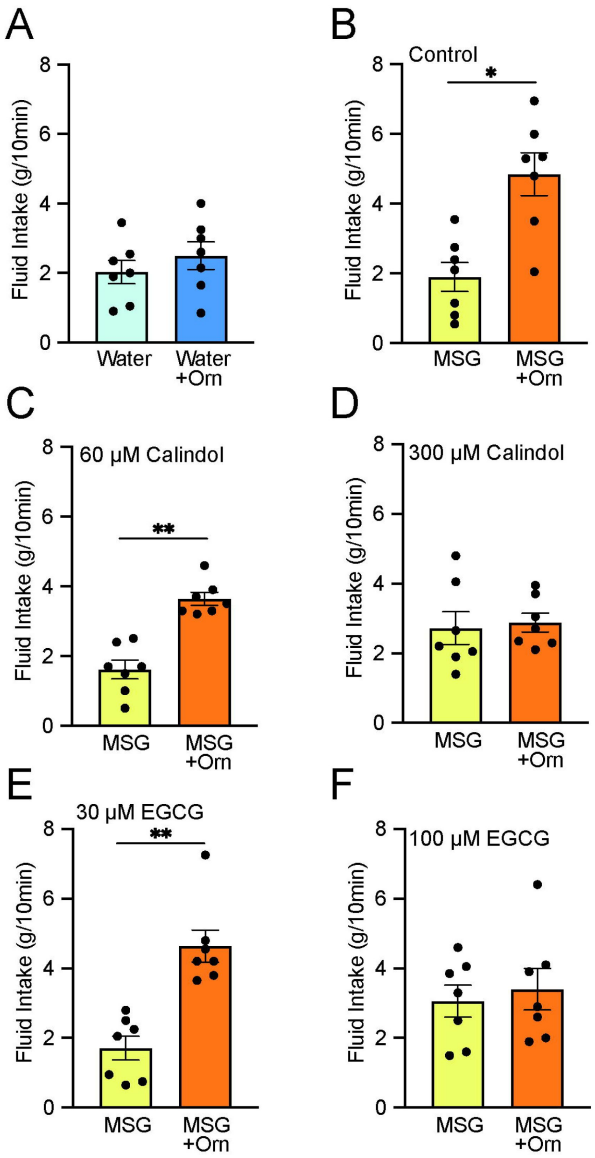


Figure 3

Brief-exposure (10-min) two-bottle preference test results for solutions with and without 1 mM ornithine (Orn) and effects of GPRC6A antagonists calindol and EGCG.

(A) Intake of water with and without Orn. **(B)** Intake of 0.03 M MSG with and without Orn. **(C)** Intake of MSG with and without Orn in 60 μM calindol. **(D)** Intake of MSG with and without Orn in 300 μM calindol. **(E)** Intake of MSG with and without Orn in 30 μM EGCG. **(F)** Intake of MSG with and without Orn in 100 μM EGCG. Each value represents the mean ± SEM; n = 7. *P < 0.05, **P < 0.01 (paired *t*-test, two-tailed). MSG, monosodium glutamate; EGCG, epigallocatechin gallate; GPRC6A, G-protein-coupled receptor family C group 6 subtype A; SEM, standard error of the mean

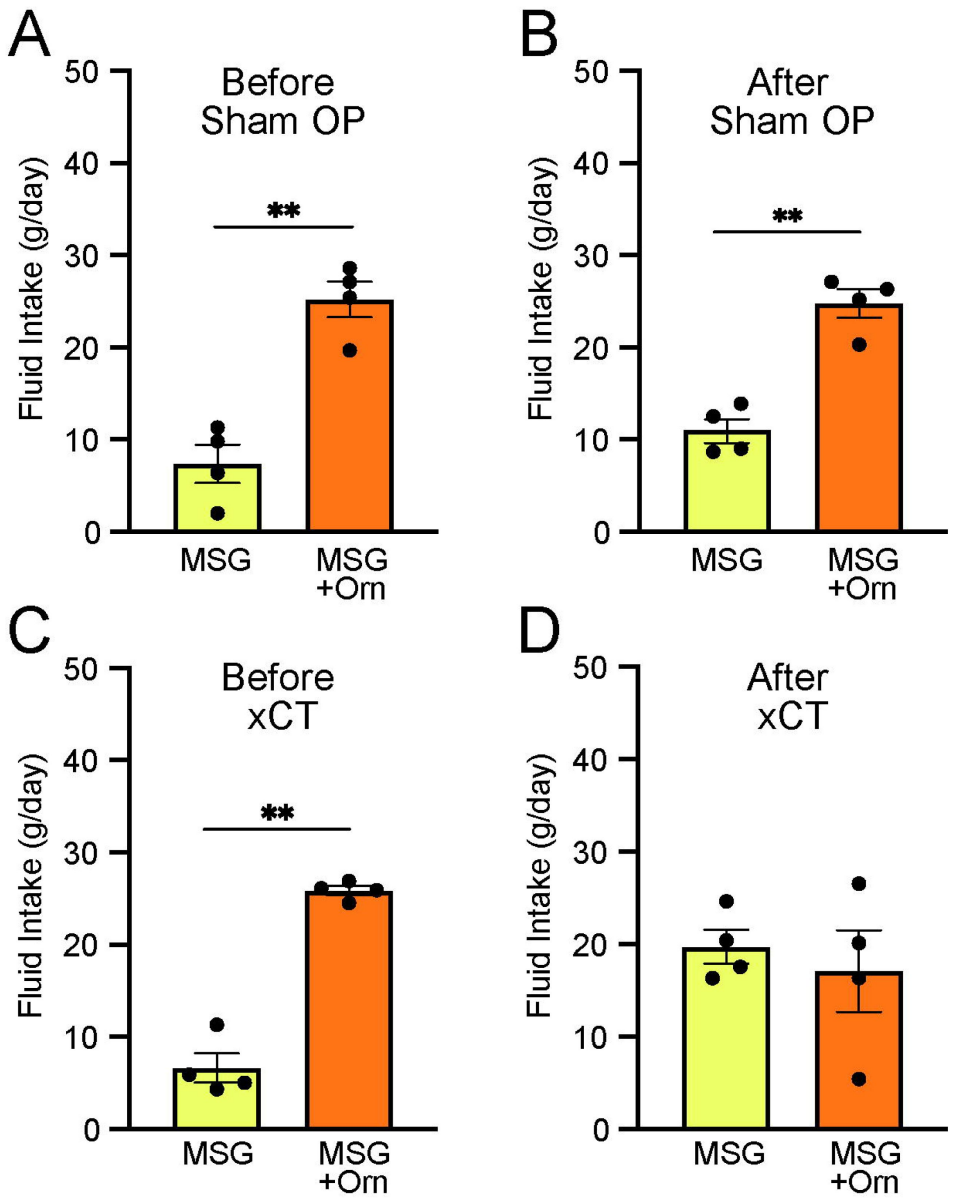


Figure 4

Effects of bilateral chorda tympani transection (xCT) and control sham operations (Sham OP) on intake of 0.03 M MSG with and without 1 mM ornithine (Orn).

Fluid intake before **(A, C)** and after **(B, D)** the operations. Each value represents the mean ± SEM; n = 4. **P < 0.01 (paired *t*-test, two-tailed). MSG, monosodium glutamate; SEM, standard error of the mean

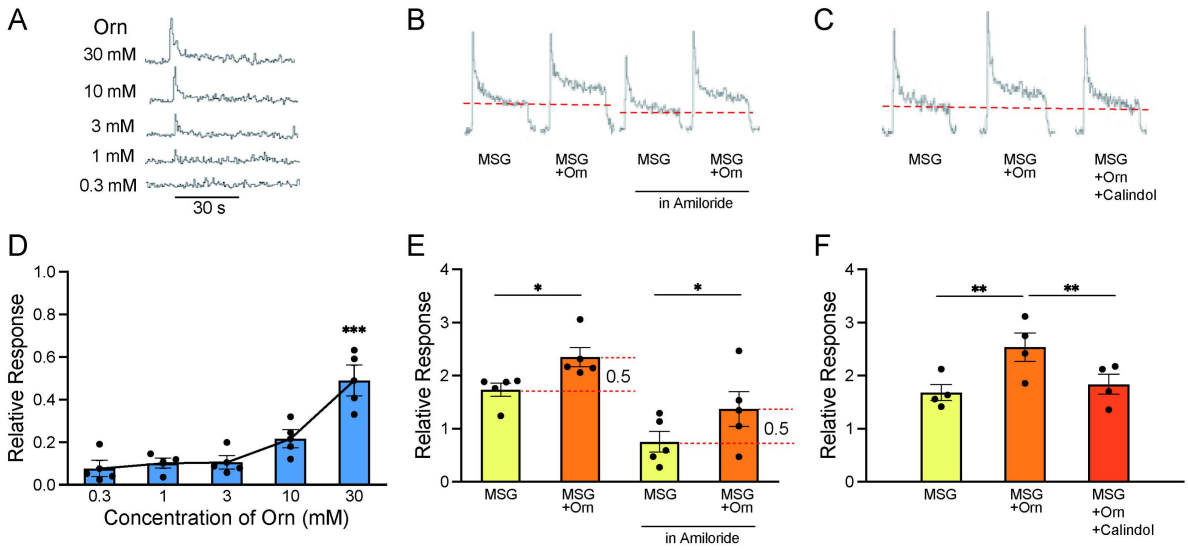


Figure 5

Sample recordings of chorda tympani (CT) responses and quantitative representations of mean response magnitudes.

(A) Nerve responses to five concentrations of ornithine (Orn). **(B)** Nerve responses to 0.03 M MSG with and without 1 mM Orn in water and in 0.01 mM amiloride (sodium-channel blocker). **(C)** Nerve responses to MSG, MSG with Orn, and MSG with Orn in 300 μM calindol. **(D-F)** Graphical representations of the mean magnitudes of CT responses corresponding to A, B, and C, respectively. Each value represents the mean ± SEM normalized to the response to 0.1 M NH_4Cl = 1.0; n = 4 or 5. *P < 0.05, **P < 0.01, ***P < 0.001 (D and F, Bonferroni correction; E, two-tailed paired *t*-test). MSG, monosodium glutamate; SEM, standard error of the mean

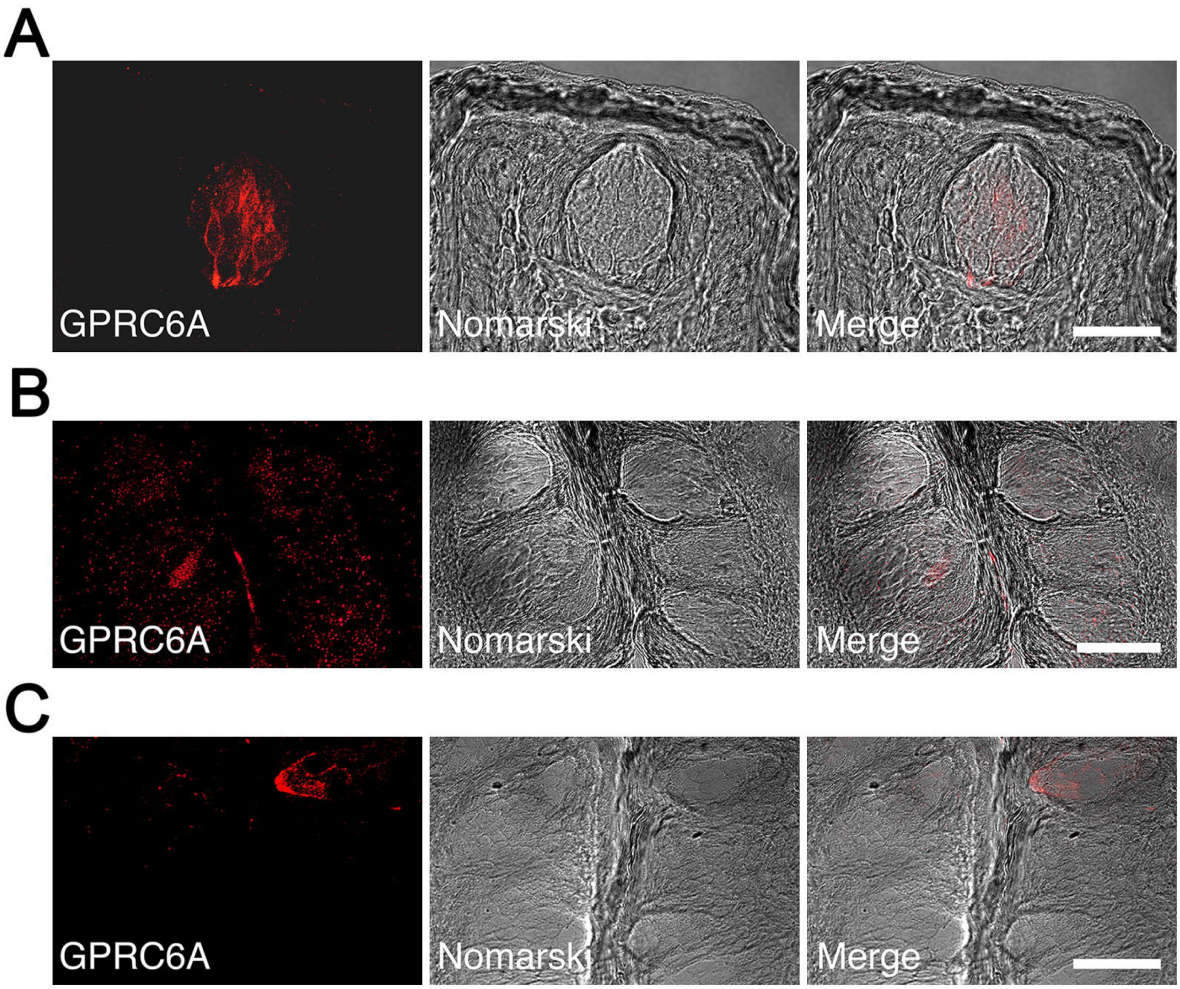


Figure 6

Immunohistochemical localization of GPRC6A in rat taste papillae.

(A) A significant number of spindle-shaped taste cells exhibited intense GPRC6A immunoreactivity in the fungiform papillae. (B, C) GPRC6A-immunopositive taste cells were barely detectable in the foliate (B) or circumvallate (C) papillae. Left panels show GPRC6A in red, middle panels show Nomarski images of the left panels, and right panels show merged images of respective left and middle panels. Scale bars, 50 μm. GPRC6A, G-protein-coupled receptor family C group 6 subtype A

cells, in single taste cells (0 double-positive cell/93 GPRC6A-positive cells) (**Fig. 7B**). Regarding type III cell markers, GPRC6A-positive cells were unlikely to colocalize with 5-hydroxytryptamine (serotonin, 5-HT) in single taste cells (0 double-positive cell/75 GPRC6A-positive cells) (**Fig. 7C**). As synaptosomal-associated protein 25 kDa (SNAP-25) labels not only type III cells but also the dense network of intragemmal nerve fibers (31), it is difficult to detect SNAP-25-expressing taste cells surrounded by intense SNAP-25-immunoreactivity of the nerve fibers. However, as far as we investigated, no SNAP-25-expressing cells were found among GPRC6A-positive cells examined (0 double-positive cell/104 GPRC6A-positive cells) (**Fig. 7D**). These results indicate that GPRC6A is preferentially localized in a subpopulation of IP3R3-expressing taste cells in rat fungiform papillae, and suggest that GPRC6A exerts taste-modifying activity in at least some type II cells in the papillae.

Discussion

In this study, we used a 24-hour two-bottle preference test in rats to demonstrate that the addition of 1 mM ornithine (which itself did not exhibit any taste preference) to tastants comprising umami substances, Intralipos (representing fatty taste), sucrose (sweetness), NaCl (saltiness), and QHCl (bitterness) increased the intake of each solution, suggesting enhanced palatability, especially to umami stimuli. Similar increases in ingestion were observed in a short-term two-bottle preference test using MSG supplemented with ornithine, indicating that this effect was due to the taste sensation in the oral cavity rather than post-ingestive effects. Furthermore, the disappearance of ornithine's effect on MSG following the application of GPRC6A antagonists and transection of the CT nerve, which innervates taste buds in the anterior tongue, suggests that GPRC6A, which was expressed in the taste cells in this area, is involved in this effect. Supporting this finding, the response of the CT nerve to MSG increased with ornithine supplementation, and the action of a GPRC6A antagonist abolished this change. In addition, immunohistochemical staining showed that GPRC6A predominantly appeared in the fungiform papillae of the anterior tongue rather than in the foliate or circumvallate papillae of the posterior tongue. Despite the differences described below, these results align with our previous findings in mice (26). Together with those from our human study (Fig. S1), these results confirm that ornithine is a potential kokumi substance and that GPRC6A is a candidate kokumi receptor. These insights provide basic neuroscientific knowledge for interpreting the observation in humans that adding ornithine to miso soup enhances the three elements of kokumi: intensity of whole complex tastes, mouthfulness, and persistence of taste.

Several similarities and differences are apparent when comparing the results obtained with those of our previous study in mice (26). In both studies, the same concentration of ornithine was used for supplementation (1 mM). The addition of ornithine increased the favorability of basic tastes including umami, sweet, fatty, salty, and bitter tastes, with a more pronounced preference for MSG than IMP among the umami substances. However, while a weak increase in citric acid preference was observed in mice, no such effect was identified in rats. The effects of GPRC6A antagonists and the CT-nerve responses were consistent between the two species. However, the expression of GPRC6A differed considerably; in mice, this protein was expressed throughout the tongue, particularly in the posterior part, whereas in rats, it was predominantly localized to the anterior region of the tongue. Finally, another major difference is that while mice avoided high concentrations of ornithine, rats seemed to prefer them.

Regarding the increased preference for the umami substances IMP, MSG, and MPG when supplemented with ornithine, one may naturally interpret this as an ornithine-enhanced umami response. However, the possibility that umami substances enhance the response to ornithine should be considered. Depending on its concentration, ornithine may stimulate at least three receptors, namely GPRC6A, CaSR (32,33), and the conventional amino acid receptor, the T1R1/T1R3 heterodimer (34). At the low concentration of 1 mM used in the present study,

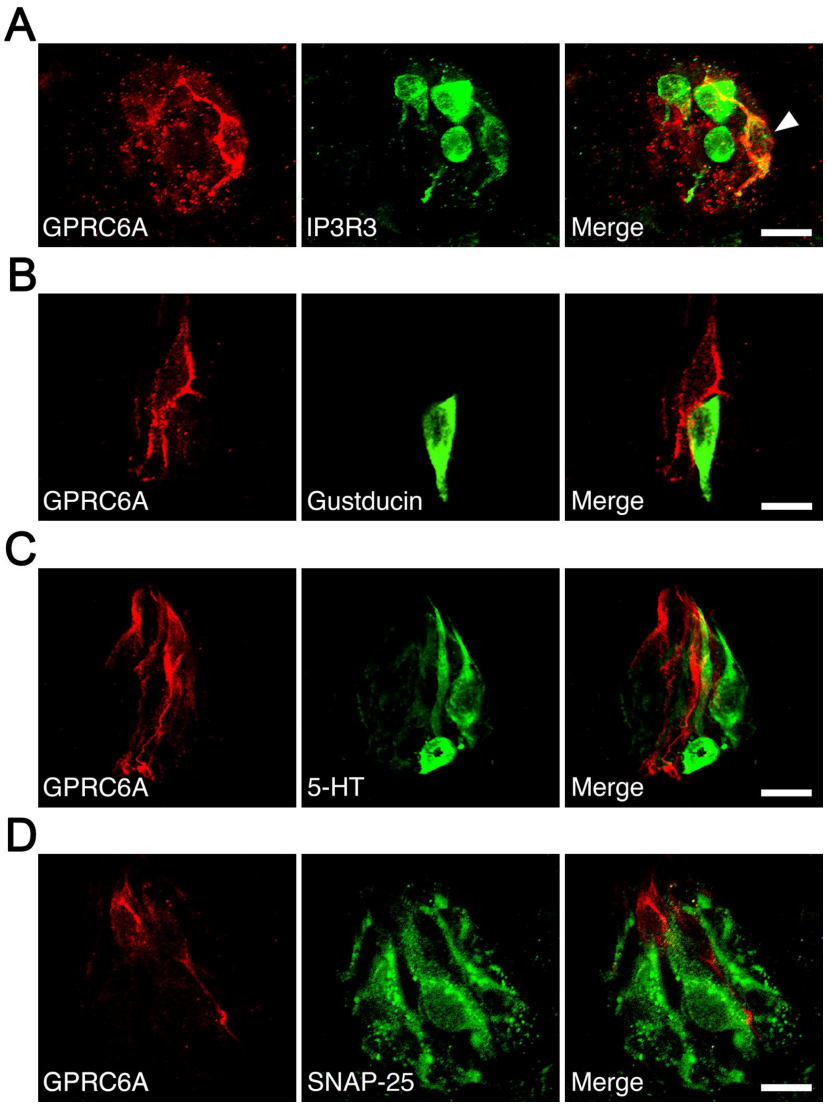


Figure 7

Immunohistochemical analysis of colocalization of GPRC6A and cell type-specific markers in taste cells of rat fungiform papilla taste buds.

(A) Some but not all GPRC6A-immunopositive cells exhibited immunoreactivity for IP3R3, a marker of most type II cells. White arrow indicates GPRC6A/IP3R3 double-positive taste cell. (B) α-Gustducin, another marker of a subset of type II cells, was unlikely to colocalize with GPRC6A in single taste cells. (C, D) Neither 5-HT (C) nor SNAP25 (D), specific markers of type III taste cells, were likely to colocalize with GPRC6A in single taste cells. Scale bars, 10 μm. GPRC6A, G-protein-coupled receptor family C group 6 subtype A; IP3R3, inositol 1,4,5-trisphosphate receptor type 3; 5-HT, 5-hydroxytryptamine; SNAP25, synaptosomal-associated protein 25 kDa

GPRC6A may be exclusively stimulated, whereas higher concentrations may also stimulate T1R1/T1R3, thereby transmitting ornithine taste information via this receptor. Compared with GPRC6A, CaSR may have limited to no involvement, since EGCG, a GPRC6A-specific antagonist, significantly abolished the increased preference induced by ornithine. Umami substances may promote the binding of ornithine to these receptors. For example, Tanase et al. (35) showed that the taste intensity of ornithine increased in the presence of IMP in humans. Similarly, Ueda et al. (36) and Dunkel et al. (37) reported that the taste thresholds for glutathione and glutamyl peptides, agonists of CaSR, were reduced for umami solutions. In rats, ornithine has a favorable taste, and its palatability increased with its concentration (**Fig. 1A**).

When added to ornithine, MSG may enhance the response to ornithine, thereby increasing its palatability. This concept also explains the mixed effects of ornithine and MSG observed in mice. The taste of ornithine itself was increasingly disliked as its concentration was raised (26). Therefore, although the addition of ornithine at concentrations below 3 mM increased palatability, its favorability ceased at concentrations of 10 and 30 mM. Taste information sent by ornithine as its concentration increases, whether pleasant or unpleasant, is likely mediated by the conventional amino acid receptor T1R1/T1R3 rather than GPRC6A. Thus, umami substances may facilitate ornithine stimulation of both kokumi and amino acid receptors in a concentration-dependent manner. Ornithine and umami substances interact to produce synergistic effects on palatability (8).

In our experiments with rats (present study) and mice (26), adding ornithine to favorable-tasting solutions such as umami, sweet, and fatty solutions increased their tastiness. This can be explained by the enhanced electrophysiological responses to these tastes induced by ornithine, as evidenced by increased CT-nerve responses for MSG in rats and mice and for other tastes in mice. Conversely, the increased palatability of the aversive bitter taste in both species suggests that ornithine suppresses the response to bitterness. Although the addition of ornithine to quinine slightly decreased the CT-nerve response in mice, this difference was not statistically significant. Reports have shown that ornithine (38,39), and arginine (40), which is structurally highly similar to ornithine, both decrease the bitter taste sensation in humans; however, the underlying mechanism is not well understood. We conducted additional experiments in rats using gallate (Fig. S2), which has been reported as an agonist of GPRC6A (40). Similar to ornithine, gallate increased the palatability of MSG and quinine, although this effect was abolished by EGCG. Notably, this suggests that GPRC6A agonists may augment responses to favorable tastes such as umami and sweetness, while inhibiting responses to bitter tastes, leading to an overall enhancement of deliciousness.

The CaSR agonist γ-Glu-Val-Gly similarly enhanced the preference for umami, sweet, and fatty tastes in rats, but showed no effect on the favorability of salty, sour, or bitter tastes (25). Additionally, while ornithine increased the preference for MSG more than that for IMP, γ-Glu-Val-Gly had the opposite effect. Unlike those of ornithine, the effects of γ-Glu-Val-Gly were not altered by severance of the CT nerve, likely because CaSR is expressed in both the anterior and posterior parts of the tongue (42). The results of the present study, along with those of our previous animal experiments, clearly demonstrate that the binding of kokumi substances to their receptors triggers the expression of kokumi.

However, the mechanism by which this binding enhances the response to umami, fatty, sweet, and other tastes remains unknown. Considering our observation that GPRC6A was co-expressed in type II taste cells positive for IP3R3, some of these cells may be affected by GPRC6A activation. However, it is difficult to explain why co-expression with α-gustducin, which binds to taste receptors in type II cells, was not observed. While we did not examine the co-expression of GPRC6A with the T1R or T2R families, Maruyama et al. (23) reported that CaSR is not co-expressed with receptors for umami, sweet, or other tastes in mice. Some form of intercellular communication within taste buds that affects these tastes is possible, although the detailed

mechanism requires further research (43). Whether GPRC6A and CaSR colocalize to the same taste cells should also be considered; however, we have not yet identified any taste cells in which these proteins are co-expressed in our ongoing experiments (Fig. S3). In addition to the above, it is noteworthy that kokumi-active γ-glutamyl peptides have a stronger affinity for the T1R1-MSG receptor complex than for the T1R2-sucrose receptor complex, as demonstrated by molecular modeling approaches (44). This suggests that these peptides exhibit a greater umami-enhancing effect without the involvement of kokumi-specific receptors.

While many candidate kokumi substances have been reported (7,13,14,17-21,22), we are currently exploring the underlying neuroscientific mechanisms using CaSR agonists such as glutathione and γ-Glu-Val-Gly, and GPRC6A agonists such as ornithine and gallate. A common finding in our animal experiments is that these substances enhanced umami to a greater extent than other tastes. They also enhanced sweet and fatty tastes, and to a lesser extent, saltiness. The essence of kokumi expression is the enrichment of tastes associated with deliciousness or the drawing out of newly perceived tastes (8). When tastes are individually enhanced and collectively recognized, the "intensity of whole complex tastes (or rich flavor with complex tastes)" associated with kokumi may arise. During this process, additional taste cells in the oral cavity are more strongly stimulated, and this information is sent to the brain. As a result, the brain perceives this input as coming from the entire mouth, leading to the sensation of "mouthfulness." Regarding the "persistence of taste (lingering flavor)," it is common sensory physiological knowledge that a strong stimulus causes a large response that takes more time to return to baseline, thereby inducing a longer-lasting sensation. Using a kokumi substance to enhance umami, a taste known for its mouthfulness and lingering quality (8,45,46), further intensifies these characteristics. The comparatively prolonged aftertaste of umami is due to the dominance of umami receptors in taste buds located in the grooves of the circumvallate and foliate papillae at the back of the tongue, where umami substances are less likely to be washed away. This is evidenced by stronger and more specific umami responses from the posterior rather than the anterior part of the tongue in mice (47,48) and primates (46,49). Moreover, MSG binds deeply within the Venus flytrap domain of the umami receptor (50), making it difficult to dislodge.

In conclusion, when kokumi substances are present in complex foods containing umami substances, their interaction primarily enriches the umami taste, followed by sweet and fatty tastes, and to a lesser extent salty and possibly other tastes. This enhancement is at the core of richness, which is accompanied by the secondary sensations of mouthfulness and persistence of taste. Since ornithine is abundant in foods such as shijimi clams (*Corbicula japonica*) (51,52), cheese (53), and shimeji and maitake mushrooms (54,55), we can enjoy the kokumi-enhanced flavor of cuisines that incorporate these ingredients.

Materials and methods

Animals

Eight-week-old male Wistar rats were obtained from Japan SLC (Shizuoka, Japan). The rats were individually housed in home cages within a temperature-(25 °C) and humidity-controlled (60%) room. A 12:12 hour light/dark cycle was followed, with lights on at 6:00 am and experiments conducted during the light cycle. The animals had free access to food (CE-2 Rodent Diet; CLEA Japan, Inc., Tokyo, Japan) and tap water, except during the brief tests described below in which food access was partially restricted. All animal care and experimental procedures were performed in accordance with the National Institutes of Health guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Kio University (protocol no. H28-01).

Behavioral experiment: two-bottle preference tests

Each rat was trained to drink DW from a stainless-steel spout connected to a plastic bottle. After a 1-week training period, short- and long-term two-bottle preference tests were conducted, in which two bottles were simultaneously presented to each cage. Each stainless-steel spout, designed to minimize dripping with an internal ball, had an inner diameter of 6 mm and was separated from the center of each spout by 5 cm. Each bottle contained the same taste stimulus (or DW), although one of the two was mixed with ornithine (L-ornithine; Kanto Chemical, Tokyo, Japan).

In the long-term test, the two bottles were switched after 24 hours of the 48-hour test session to account for any potential positional preference. In the short-term (or brief exposure) test, the two bottles with spouts 1 cm apart were presented to the animals for 10 minutes after an overnight water deprivation. Thereafter, the animals had free access to water until 6:00 pm, after which the water was removed overnight. The next day, the bottles were switched and the 10-minute test session was repeated. The bottles containing the fluids were weighed before and after testing to measure the intake volume. The total intake volume over 48 hours or 20 minutes was divided by 2 to obtain the intake volume per day or 10 minutes for the long- and short-term tests, respectively.

Six basic taste stimuli were used: sucrose, NaCl, citric acid, QHCl, MSG (all Kanto Chemical), and Intralipos (a parenteral-stable soybean oil emulsion; Otsuka Pharmaceutical Factory, Tokyo, Japan). In addition to MSG, MPG and IMP (both Ajinomoto, Tokyo, Japan) were also used as umami substances. The taste stimuli were dissolved in DW at various concentrations. A range of concentrations of individual tastants was presented to the same group of animals, starting with the lowest concentration. Ornithine was typically added at a concentration of 1 mM, with other concentrations used as required. A sodium-channel blocker, amiloride (Sigma-Aldrich, Tokyo, Japan), was used to reduce the sodium response to MSG.

For GPRC6A antagonists, we used NPS-2143 (ChemScene, Monmouth Junction, NJ, USA), calindol (Cayman Chemical, Ann Arbor, MI, USA), and EGCG (Tokyo Chemical Industry, Tokyo, Japan). NPS-2143 and calindol were dissolved in 99.5% ethanol at a concentration of 0.1% and diluted to various concentrations using DW. EGCG was prepared in DW.

A total of 89 rats was divided into two groups of 6, three groups of 7, and seven groups of 8 for the taste-preference tests. The first two groups were used for long-term exposure tests of: 1) DW vs. DW with different ornithine concentrations, and 2) 0.03 M MSG vs. 0.03 M MSG with different ornithine concentrations, respectively. The next three groups were used for respective brief-exposure tests of: 1) DW vs. DW with 1 mM ornithine and 0.03 M MSG vs. 0.03 M MSG with 1 mM ornithine, 2) MSG vs. MSG with ornithine, both containing 0.002 or 0.005% calindol, and 3) MSG vs. MSG with ornithine, both containing 30 or 100 μM EGCG. The final seven groups underwent long-term tests. Six of these were used to assess different concentrations of IMP, MSG, MPG, sucrose, Intralipos, and NaCl with and without 1 mM ornithine, respectively. The last group was used to test both citric acid and QHCl with and without 1 mM ornithine.

When examining the range of ornithine and tastant concentrations, we presented the solutions to the animals starting with the lowest concentration. If two different tastants were tested in one group, as in the first of the brief-exposure tests and the long-term test for citric acid and QHCl, the order of presentation was counterbalanced. Statistical analyses were performed within groups or between taste stimuli with and without ornithine, but not across different groups or taste stimuli.

Transection of the CT nerve

Eight naïve rats were randomly divided into transection and sham-operation groups (four rats each). They underwent the long-term two-bottle preference test as described above, with the bottles containing 0.03 M MSG with and without 1 mM ornithine. Thereafter, the animals were

anesthetized via intraperitoneal injection of a combination anesthetic (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol). In the transection group, the ear ossicles through which the CT innervates the taste buds on the anterior part of the tongue were removed bilaterally, whereas in the sham-operation group, the operation was stopped immediately before the removal of ear ossicles. Postoperatively, the rats were injected with penicillin G sodium (100 mg/kg) to prevent infection. After 6 days of recovery, the same rats were subjected to the long-term two-bottle preference test using the same taste stimuli. The mean group preferences were then compared. After the experiment, transection was confirmed by microscopic verification of the loss of taste buds on the tongue.

Taste-nerve recordings

Five naïve rats were deeply anesthetized as described above. Each rat was tracheotomized and secured in a head-holder. The left CT nerve was exposed using a lateral approach (56), excised as it exited the tympanic bulla, and dissected from the underlying tissue. The nerve was placed onto a platinum-wire recording electrode (0.1 mm diameter), while an indifferent electrode was placed in contact with a nearby exposed tissue. The responses were processed using a bandpass filter with cutoff frequencies ranging from 40 Hz to 3 kHz and visualized using an oscilloscope (VC11; Nihon Kohden, Tokyo, Japan). The responses were fed to a digitally controlled summator (57). The number of discharges was summed over 500-ms epochs using a spike counter (DSE-345; DIA Medical System, Tokyo, Japan) to derive summated responses. The data were stored on a PC, and the total spikes over the entire 30-second stimulus period were counted using the PowerLab system (PowerLab/4SP; ADInstruments, Bella Vista, NSW, Australia) for quantitative analyses.

Each taste stimulus (3 mL) was applied to the anterior dorsal tongue for 30 seconds, followed by rinsing with DW for at least 60 seconds. The response to each stimulus was expressed relative to the magnitude of responses to 0.1 M NH_4Cl. The analyses of CT results were based on the average values from at least 3 repeated trials in individual animals.

Immunohistochemistry

Male Wistar rats (8-12 weeks old) were deeply anesthetized with isoflurane and transcardially perfused with saline followed by 2% paraformaldehyde in 0.1 M phosphate buffer. To label serotonin-accumulating type III taste cells (58), rats were injected with 5-hydroxy-l-tryptophan at 80 mg/kg body weight 1 hour before anesthesia. Their tongues were dissected out, soaked in 20% sucrose/0.01 M phosphate-buffered saline (PBS) overnight at 4 °C, embedded in optimal-cutting-temperature compound (Sakura Finetek, Tokyo, Japan), and cut into 20-μm thick sections using a cryostat. The sections were immersed in 0.01 M PBS containing 0.2% Triton X-100 and incubated overnight at 4 °C with rabbit anti-GPRC6A antibody (1:50; orb385435; Biorbyt, Cambridge, UK) in combination with either goat anti-IP3R3 (1:100; NB100-2545; Novus, Centennial, CO, USA), anti-α-gustducin (1:500; LSB4942; LSBio, Seattle, WA, USA), anti-5-HT (1:100; ab66047; Abcam, Cambridge, UK), or anti-SNAP25 antibody (1:100; ab31281; Abcam) diluted in the same Triton X-100-containing solution. This was followed by Alexa Fluor 594™-conjugated anti-rabbit immunoglobulin G (IgG; 1:500; A-21207; Thermo Fisher Scientific, Waltham, MA, USA) and 488™-conjugated anti-goat IgG secondary antibodies (1:500; A-11055; Thermo Fisher Scientific). After washing, the sections were cover-slipped with Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA) and imaged using a Nikon A1Rs confocal laser scanning microscope (Nikon, Tokyo, Japan). The specificity of the anti-GPRC6A antibody was verified as previously described (26) (data not shown). IP3R3 is a marker protein in most type II taste cell populations (59). Further, α-gustducin, 5-HT, and SNAP25 are markers of a subset of types II, III, and III taste cells, respectively (26,58,59).

Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM). A Shapiro–Wilk test was performed to confirm that the data were normally distributed. Student's *t*-tests (paired, two-tailed) were used to assess statistical differences between two groups. Additionally, to analyze more than three groups, we used a one-way ANOVA or repeated-measures two-way ANOVA with post hoc Bonferroni tests to account for multiple comparisons. All statistical analyses were conducted in SPSS Statistics Version 25.0 (IBM, Armonk, NY, USA). Statistical significance was set at $P < 0.05$, except when the Bonferroni correction was used.

Data availability

All data are available in the manuscript and Supplementary Information or are available upon request from the corresponding author.

Supplemental information

Figure S1

Effects of ornithine (L-ornithine, Orn) supplementation of low-sodium (0.7%) miso soup on three kokumi attributes in humans: intensity, mouthfulness, and persistence of taste.

The addition of three concentrations of Orn (1, 3, and 10 mM, labeled T-1, T-2, and T-3, respectively) increased these three attributes along with palatability in a dose-dependent manner. All values for the kokumi attributes and palatability were set to 0 for the control miso soup without Orn (C). Each value represents the mean ± SEM; n = 22. *P < 0.05, **P < 0.01, ***P < 0.001 (paired *t*-test, two-tailed, comparison between control and test stimuli). SEM, standard error of the mean

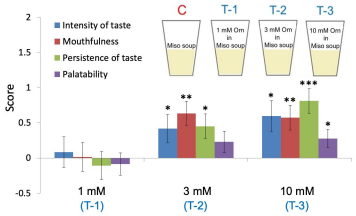
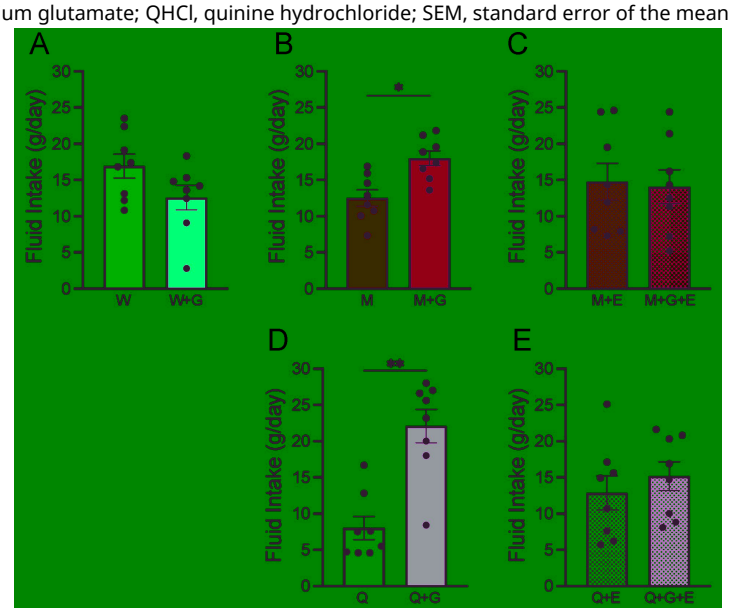


Figure S2

Long-term exposure (1-day) two-bottle preference test results for solutions with and without 1mM ethyl gallate (Kanto Chemical, Tokyo, Japan), a GPRC6A agonist, and effects of 100 μM EGCG, a GPRC6A antagonist.

(A) Intake of water (W) with and without gallate (G). **(B)** Intake of 0.03 M MSG (M) with and without gallate. **(C)** Intake of MSG and EGCG (E) with and without gallate. **(D)** Intake of 0.01 mM QHCl (Q) with and without gallate. **(E)** Intake of QHCl and EGCG with and without gallate. Each value represents the mean ± SEM; n = 8. *P < 0.05, **P < 0.01 (paired *t*-test, two-tailed). These results show that gallate itself is not palatable; however, it increases preference for MSG and QHCl, and a GPRC6A antagonist eliminates these preferences. GPRC6A, G-protein-coupled receptor family C group 6 subtype A; EGCG, epigallocatechin gallate; MSG, monosodium glutamate; QHCl, quinine hydrochloride; SEM, standard error of the mean



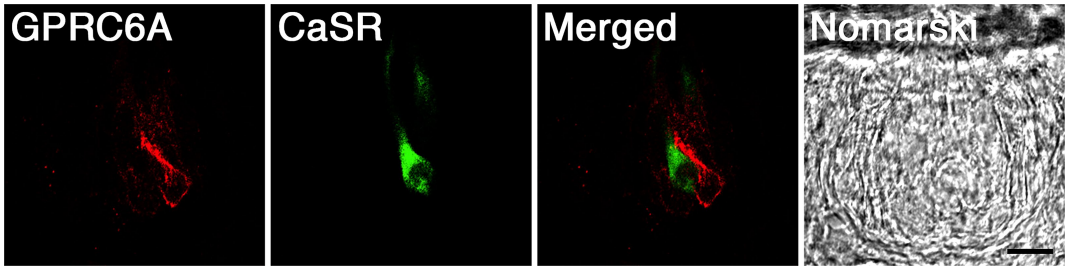


Figure S3

Immunohistochemical colocalization of GPRC6A and CaSR in rat fungiform taste papillae.

GPRC6A and CaSR are expressed in distinct subpopulations of fungiform taste cells. The right panel shows the Nomarski image of the examined tissue section. Scale bar, 10 μm. Immunohistochemical procedures were the same as those described in the Materials and methods section of the article. The mouse anti-CaSR antibody (1:100; ab19347; Abcam, Cambridge, UK) was used for CaSR staining. GPRC6A, G-protein-coupled receptor family C group 6 subtype A; CaSR, calcium-sensing receptor

Materials and methods: human sensory testing

We recruited 22 participants (19 women and three men, aged 21-28 years) from Kio University who were not affiliated with our laboratory, including students and staff members. All participants passed a screening test based on taste sensitivity. According to the responses obtained from a pre-experimental questionnaire, we confirmed that none of the participants had any sensory abnormalities, eating disorders, or mental disorders, or were taking any medications that may potentially affect their sense of taste. All participants were instructed not to eat or drink anything for 1 hour prior to the start of the experiment. We provided them with a detailed explanation of the experimental procedures, including safety measures and personal data protection, without revealing the specific goals of the study. Thereafter, each participant provided written informed consent. This study was approved by the Ethics Committee of Kio University (approval no. H30-10), and all experiments adhered to the principles of the Declaration of Helsinki.

Miso soup with 0.7% salt was prepared by dissolving commercial miso paste (Tokujyo; Takeya Miso Co., Suwa, Japan) in hot tap water (control soup). Subsequently, three test soups were prepared by dissolving 1, 3, or 10 mM L-ornithine in the control soup. An aliquot of 30 mL of each test soup was randomly served in a paper cup along with a cup of control soup. Intensity of taste, mouthfulness, and persistence of taste were evaluated according to Ohsu et al. (9). Intensity was expressed as the increased taste intensity 5 seconds after tasting. Persistence was defined as lingering taste intensity 20 seconds after tasting. Finally, mouthfulness was described as the reinforcement of taste sensations throughout the mouth, not just on the tongue. The participants evaluated the three attributes of the test soups on a 5-point rating scale ranging from -2 (apparently suppressed) to +2 (apparently strong). Additionally, the palatability of each sample was evaluated on a similar scale ranging from -2 (apparently bad) to +2 (apparently good). All the values for the three kokumi attributes and palatability were set to 0 for the control soup.

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Author contributions

Conceptualization: TY

Data curation: TY, SU

Investigation: TY, HM, NK, YS, SU

Formal analysis: TY, KU, C I-Y, SU

Writing-original draft: TY, SU

Writing-review and editing: TY

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Editors

Reviewer #1 (Public review):

Summary:

This paper contains what could be described as a "classic" approach towards evaluating a novel taste stimuli in an animal model, including standard behavioral tests (some with nerve transections), taste nerve physiology, and immunocytochemistry of taste cells of the tongue. The stimulus being tested is ornithine, from a class of stimuli called "kokumi" (in terms of human taste); these kokumi stimuli appear to enhance other canonical tastes, increasing what are essentially hedonic attributes of other stimuli. The mechanism for ornithine detection is thought to be GPRC6A receptors expressed in taste cells. The authors showed evidence for this in an earlier paper with mice; this paper evaluates ornithine taste in a rat model, and comes to a similar conclusion, albeit with some small differences between the two rodent species.

Strengths:

The data show effects of ornithine on taste/intake in laboratory rats: In two-bottle and briefer intake tests, adding ornithine results in higher intake of most, but all not all stimuli tested. Bilateral chorda tympani (CT) nerve cuts or the addition of GPRC6A antagonists decreased or eliminated these effects. Ornithine also evoked responses by itself in the CT nerve, but mainly at higher concentrations; at lower concentrations it potentiated the response to monosodium glutamate. Finally, immunocytochemistry of taste cell expression indicated that GPRC6A was expressed predominantly in the anterior tongue, and co-localized (to a small extent) with only IP3R3, indicative of expression in a subset of type II taste receptor cells.

Weaknesses:

As the authors are aware, it is difficult to assess a complex human taste with complex attributes, such as kokumi, in an animal model. In these experiments they attempt to uncover mechanistic insights about how ornithine potentiates other stimuli by using a variety of established experimental approaches in rats. They partially succeed by finding evidence that GPRC6A may mediate effects of ornithine when it is used at lower concentrations. In the revision they have scaled back their interpretations accordingly. A supplementary experiment measuring certain aspects of the effects of ornithine added to Miso soup in human subjects is included for the express purpose of establishing that the kokumi sensation of a complex solution is enhanced by ornithine; however, they do not use any such complex solutions in the rat studies. Moreover, the sample size of the human experiment is (still) small - it really doesn't belong in the same manuscript with the rat studies.

https://doi.org/10.7554/eLife.101629.2.sa3

Reviewer #2 (Public review):

Summary:

The authors used rats to determine the receptor for a food-related perception (kokumi) that has been characterized in humans. They employ a combination of behavioral, electrophysiological, and immunohistochemical results to support their conclusion that ornithine-mediated kokumi effects are mediated by the GPRC6A receptor. They complemented the rat data with some human psychophysical data. I find the results intriguing, but believe that the authors overinterpret their data.

Strengths:

The authors provide compelling evidence that ornithine enhances the palatability of several chemical stimuli (i.e., IMP, MSG, MPG, Intralipos, sucrose, NaCl, quinine). Ornithine also increases CT nerve responses to MSG. Additionally, the authors provide evidence that the effects of ornithine are mediated by GPRC6A, a G-protein-coupled receptor family C group 6 subtype A, and that this receptor is expressed primarily in fungiform taste buds. Taken together, these results indicate that ornithine enhances the palatability of multiple taste stimuli in rats and that the enhancement is mediated, at least in part, within fungiform taste buds. This is an important finding that could stand on its own. The question of whether ornithine produces these effects by eliciting kokumi-like perceptions (see below) should be presented as speculation in the Discussion section.

Weaknesses:

I am still unconvinced that the measurements in rats reflect the "kokumi" taste percept described in humans. The authors conducted long-term preference tests, 10-min avidity tests and whole chorda tympani (CT) nerve recordings. None of these procedures specifically model features of "kokumi" perception in humans, which (according to the authors) include increasing "intensity of whole complex tastes (rich flavor with complex tastes), mouthfulness (spread of taste and flavor throughout the oral cavity), and persistence of taste (lingering flavor)." While it may be possible to develop behavioral assays in rats (or mice) that effectively model kokumi taste perception in humans, the authors have not made any effort to do so. As a result, I do not think that the rat data provide support for the main conclusion of the study--that "ornithine is a kokumi substance and GPRC6A is a novel kokumi receptor."

Why are the authors hypothesizing that the primary impacts of ornithine are on the peripheral taste system? While the CT recordings provide support for peripheral taste enhancement, they do not rule out the possibility of additional central enhancement. Indeed, based on the definition of human kokumi described above, it is likely that the effects of kokumi stimuli in humans are mediated at least in part by the central flavor system.

The authors include (in the supplemental data section) a pilot study that examined the impact of ornithine on variety of subjective measures of flavor perception in humans. The presence of this pilot study within the larger rat study does not really mice sense. While I agree with the authors that there is value in conducting parallel tests in both humans and rodents, I think that this can only be done effectively when the measurements in both species are the same. For this reason, I recommend that the human data be published in a separate article.

The authors indicated on several occasions (e.g., see Abstract) that ornithine produced "synergistic" effects on the CT nerve response to chemical stimuli. "Synergy" is used to describe a situation where two stimuli produce an effect that is greater than the sum of the response to each stimulus alone (i.e., 2 + 2 = 5). As far as I can tell, the CT recordings in Fig. 3 do not reflect a synergism.

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Reviewer #3 (Public review):

Summary:

In this study the authors set out to investigate whether GPRC6A mediates kokumi taste initiated by the amino acid L-ornithine. They used Wistar rats, a standard laboratory strain, as the primary model and also performed an informative taste test in humans, in which miso soup was supplemented with various concentrations of L-ornithine. The findings are valuable and overall the evidence is solid. L-Ornithine should be considered to be a useful test substance in future studies of kokumi taste and the class C G protein coupled receptor known as GPRC6A (C6A) along with its homolog, the calcium-sensing receptor (CaSR) should be considered candidate mediators of kokumi taste. The researchers confirmed in rats their previous work on Ornithine and C6A in mice (Mizuta et al Nutrients 2021).

Strengths:

The overall experimental design is solid based on two bottle preference tests in rats. After determining the optimal concentration for L-Ornithine (1 mM) in the presence of MSG, it was added to various tastants including: inosine 5'-monophosphate; monosodium glutamate (MSG); mono-potassium glutamate (MPG); intralipos (a soybean oil emulsion); sucrose; sodium chloride (NaCl; salt); citric acid (sour) and quinine hydrochloride (bitter). Robust effects of ornithine were observed in the cases of IMP, MSG, MPG and sucrose; and little or no effects were observed in the cases of sodium chloride, citric acid; quinine HCl. The researchers then focused on the preference for Ornithine-containing MSG solutions. Inclusion of the C6A inhibitors Calindol (0.3 mM but not 0.06 mM) or the gallate derivative EGCG (0.1 mM but not 0.03 mM) eliminated the preference for solutions that contained Ornithine in addition to MSG. The researchers next performed transections of the chord tympani nerves (with sham operation controls) in anesthetized rats to identify a role of the chorda tympani branches of the facial nerves (cranial nerve VII) in the preference for Ornithine-containing MSG solutions. This finding implicates the anterior half-two thirds of the tongue in ornithine-induced kokumi taste. They then used electrical recordings from intact chorda tympani nerves in anesthetized rats to demonstrate that ornithine enhanced MSG-induced responses following the application of tastants to the anterior surface of the tongue. They went on to show that this enhanced response was insensitive to amiloride, selected to inhibit 'salt tastant' responses mediated by the epithelial Na+ channel, but eliminated by Calindol. Finally they performed immunohistochemistry on sections of rat tongue demonstrating C6A positive spindle-shaped cells in fungiform papillae that partially overlapped in its distribution with the IP3 type-3 receptor, used as a marker of Type-II cells, but not with (i) gustducin, the G protein partner of Tas1 receptors (T1Rs), used as a marker of a subset of type-II cells; or (ii) 5-HT (serotonin) and Synaptosome-associated protein 25 kDa (SNAP-25) used as markers of Type-III cells.

At least two other receptors in addition to C6A might mediate taste responses to ornithine: (i) the CaSR, which binds and responds to multiple L-amino acids (Conigrave et al, PNAS 2000), and which has been previously reported to mediate kokumi taste (Ohsu et al., JBC 2010) as well as responses to Ornithine (Shin et al., Cell Signaling 2020); and (ii) T1R1/T1R3 heterodimers which also respond to L-amino acids and exhibit enhanced responses to IMP (Nelson et al., Nature 2001). These alternatives are appropriately discussed and, taken together, the experimental results favor the authors' interpretation that C6A mediates the Ornithine responses. The authors provide preliminary data in Suppl. 3 for the possibility of co-expression of C6A with the CaSR.

Weaknesses:

The authors point out that animal models pose some difficulties of interpretation in studies of taste and raise the possibility in the Discussion that umami substances may enhance the taste response to ornithine (Line 271, Page 9).

One issue that is not addressed, and could be usefully addressed in the Discussion, relates to the potential effects of kokumi substances on the threshold concentrations of key tastants such as glutamate. Thus, an extension of taste distribution to additional areas of the mouth (previously referred to as 'mouthfulness') and persistence of taste/flavor responses (previously referred to as 'continuity') could arise from a reduction in the threshold concentrations of umami and other substances that evoke taste responses.

The status of one of the compounds used as an inhibitor of C6A, the gallate derivative EGCG, as a potential inhibitor of the CaSR or T1R1/T1R3 is unknown. It would have been helpful to show that a specific inhibitor of the CaSR failed to block the ornithine response.

It would have been helpful to include a positive control kokumi substance in the two bottle preference experiment (e.g., one of the known gamma glutamyl peptides such as gamma-glu-Val-Gly or glutathione), to compare the relative potencies of the control kokumi compound and Ornithine, and to compare the sensitivities of the two responses to C6A and CaSR inhibitors.

https://doi.org/10.7554/eLife.101629.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This paper contains what could be described as a "classic" approach towards evaluating a novel taste stimuli in an animal model, including standard behavioral tests (some with nerve transections), taste nerve physiology, and immunocytochemistry of the tongue. The stimulus being tested is ornithine, from a class of stimuli called "kokumi", which are stimuli that enhance other canonical tastes, increasing essentially the hedonic attributes of these other stimuli; the mechanism for ornithine detection is thought to be GPRC6A receptors expressed in taste cells. The authors showed evidence for this in an earlier paper with mice; this paper evaluates ornithine taste in a rat model.*
>
> *Strengths:*
>
> *The data show the effects of ornithine on taste: in two-bottle and briefer intake tests, adding ornithine results in a higher intake of most, but not all, stimuli tests. Bilateral nerve cuts or the addition of GPRC6A antagonists decrease this effect. Small effects of ornithine are shown in whole-nerve recordings.*
>
> *Weaknesses:*
>
> *The conclusion seems to be that the authors have found evidence for ornithine acting as a taste modifier through the GPRC6A receptor expressed on the anterior tongue. It is hard to separate their conclusions from the possibility that any effects are additive rather than modulatory. Animals did prefer ornithine to water when presented by itself. Additionally, the authors refer to evidence that ornithine is activating the T1R1-T1R3 amino acid taste receptor, possibly at higher concentrations than they use for most of the study, although this seems speculative. It is striking that the largest effects on taste*

> *are found with the other amino acid (umami) stimuli, leading to the possibility that these are largely synergistic effects taking place at the tas1r receptor heterodimer.*

We would like to thank Reviewer #1 for the valuable comments. Our basis for considering ornithine as a taste modifier stems from our observation that a low concentration of ornithine (1 mM), which does not elicit a preference on its own, enhances the preference for umami substances, sucrose, and soybean oil through the activation of the GPRC6A receptor. Notably, this receptor is not typically considered a taste receptor. The reviewer suggested that the enhancement of umami taste might be due to potentiation occurring at the TAS1R receptor heterodimer. However, we propose that a different mechanism may be at play, as an antagonist of GPRC6A almost completely abolished this enhancement. In the revised manuscript, we will endeavor to provide additional information on the role of ornithine as a taste modifier acting through the GPRC6A receptor.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors used rats to determine the receptor for a food-related perception (kokumi) that has been characterized in humans. They employ a combination of behavioral, electrophysiological, and immunohistochemical results to support their conclusion that ornithine-mediated kokumi effects are mediated by the GPRC6A receptor. They complemented the rat data with some human psychophysical data. I find the results intriguing, but believe that the authors overinterpret their data.*
>
> *Strengths:*
>
> *The authors examined a new and exciting taste enhancer (ornithine). They used a variety of experimental approaches in rats to document the impact of ornithine on taste preference and peripheral taste nerve recordings. Further, they provided evidence pointing to a potential receptor for ornithine.*
>
> *Weaknesses:*
>
> *The authors have not established that the rat is an appropriate model system for studying kokumi. Their measurements do not provide insight into any of the established effects of kokumi on human flavor perception. The small study on humans is difficult to compare to the rat study because the authors made completely different types of measurements. Thus, I think that the authors need to substantially scale back the scope of their interpretations. These weaknesses diminish the likely impact of the work on the field of flavor perception.*

We would like to thank Reviewer #2 for the valuable comments and suggestions. Regarding the question of whether the rat is an appropriate model system for studying kokumi, we have chosen this species for several reasons: it is readily available as a conventional experimental model for gustatory research; the calcium-sensing receptor (CaSR), known as the kokumi receptor, is expressed in taste bud cells; and prior research has demonstrated the use of rats in kokumi studies involving gamma Glu-Val-Gly (Yamamoto and Mizuta, Chem. Senses, 2022).

We acknowledge that fundamentally different types of measurements were conducted in the human psychophysical study and the rat study. Kokumi can indeed be assessed and expressed in humans; however, we do not currently have the means to confirm that animals experience kokumi in the same way that humans do. Therefore, human studies are necessary to evaluate kokumi, a conceptual term denoting enhanced flavor, while animal studies are needed to explore the potential underlying mechanisms of kokumi. We believe that a combination of both human and animal studies is essential, as is the case with research on

sugars. While sugars are known to elicit sweetness, it is unclear whether animals perceive sweetness identically to humans, even though they exhibit a strong preference for sugars. In the revised manuscript, we will incorporate additional information to address the comments raised by the reviewer. We will also carefully review and revise our previous statements to ensure accuracy and clarity.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In this study, the authors set out to investigate whether GPRC6A mediates kokumi taste initiated by the amino acid L-ornithine. They used Wistar rats, a standard laboratory strain, as the primary model and also performed an informative taste test in humans, in which miso soup was supplemented with various concentrations of L-ornithine. The findings are valuable and overall the evidence is solid. L-Ornithine should be considered to be a useful test substance in future studies of kokumi taste and the class C G protein-coupled receptor known as GPRC6A (C6A) along with its homolog, the calcium-sensing receptor (CaSR) should be considered candidate mediators of kokumi taste.*
>
> *Strengths:*
>
> *The overall experimental design is solid based on two bottle preference tests in rats. After determining the optimal concentration for L-Ornithine (1 mM) in the presence of MSG, it was added to various tastants, including inosine 5'-monophosphate; monosodium glutamate (MSG); mono-potassium glutamate (MPG); intralipos (a soybean oil emulsion); sucrose; sodium chloride (NaCl); citric acid and quinine hydrochloride. Robust effects of ornithine were observed in the cases of IMP, MSG, MPG, and sucrose, and little or no effects were observed in the cases of sodium chloride, citric acid, and quinine HCl. The researchers then focused on the preference for Ornithine-containing MSG solutions. The inclusion of the C6A inhibitors Calindol (0.3 mM but not 0.06 mM) or the gallate derivative EGCG (0.1 mM but not 0.03 mM) eliminated the preference for solutions that contained Ornithine in addition to MSG. The researchers next performed transections of the chord tympani nerves (with sham operation controls) in anesthetized rats to identify the role of the chorda tympani branches of the facial nerves (cranial nerve VII) in the preference for Ornithine-containing MSG solutions. This finding implicates the anterior half-two thirds of the tongue in ornithine-induced kokumi taste. They then used electrical recordings from intact chorda tympani nerves in anesthetized rats to demonstrate that ornithine enhanced MSG-induced responses following the application of tastants to the anterior surface of the tongue. They went on to show that this enhanced response was insensitive to amiloride, selected to inhibit 'salt tastant' responses mediated by the epithelial Na+ channel, but eliminated by Calindol. Finally, they performed immunohistochemistry on sections of rat tongue demonstrating C6A positive spindle-shaped cells in fungiform papillae that partially overlapped in its distribution with the IP3 type-3 receptor, used as a marker of Type-II cells, but not with (i) gustducin, the G protein partner of Tas1 receptors (T1Rs), used as a marker of a subset of type-II cells; or (ii) 5-HT (serotonin) and Synaptosome-associated protein 25 kDa (SNAP-25) used as markers of Type-III cells.*
>
> *Weaknesses:*
>
> *The researchers undertook what turned out to be largely confirmatory studies in rats with respect to their previously published work on Ornithine and C6A in mice (Mizuta et al Nutrients 2021).*
>
> *The authors point out that animal models pose some difficulties of interpretation in studies of taste and raise the possibility in the Discussion that umami substances may*

enhance the taste response to ornithine (Line 271, Page 9). They miss an opportunity to outline the experimental results from the study that favor their preferred interpretation that ornithine is a taste enhancer rather than a tastant.

At least two other receptors in addition to C6A might mediate taste responses to ornithine: (i) the CaSR, which binds and responds to multiple L-amino acids (Conigrave et al, PNAS 2000), and which has been previously reported to mediate kokumi taste (Ohsu et al., JBC 2010) as well as responses to Ornithine (Shin et al., Cell Signaling 2020); and (ii) T1R1/T1R3 heterodimers which also respond to L-amino acids and exhibit enhanced responses to IMP (Nelson et al., Nature 2001). While the experimental results as a whole favor the authors' interpretation that C6A mediates the Ornithine responses, they do not make clear either the nature of the 'receptor identification problem' in the Introduction or the way in which they approached that problem in the Results and Discussion sections. It would be helpful to show that a specific inhibitor of the CaSR failed to block the ornithine response. In addition, while they showed that C6A-positive cells were clearly distinct from gustducin-positive, and thus T1R-positive cells, they missed an opportunity to clearly differentiate C6A-expressing taste cells and CaSR-expressing taste cells in the rat tongue sections.

It would have been helpful to include a positive control kokumi substance in the two-bottle preference experiment (e.g., one of the known gamma-glutamyl peptides such as gamma-glu-Val-Gly or glutathione), to compare the relative potencies of the control kokumi compound and Ornithine, and to compare the sensitivities of the two responses to C6A and CaSR inhibitors.

The results demonstrate that enhancement of the chorda tympani nerve response to MSG occurs at substantially greater Ornithine concentrations (10 and 30 mM) than were required to observe differences in the two bottle preference experiments (1.0 mM; Figure 2). The discrepancy requires careful discussion and if necessary further experiments using the two-bottle preference format.

We would like to thank Reviewer #3 for the valuable comments and helpful suggestions. We propose that ornithine has two stimulatory actions: one acting on GPRC6A, particularly at lower concentrations, and another on amino acid receptors such as T1R1/T1R3 at higher concentrations. Consequently, ornithine is not preferable at lower concentrations but becomes preferable at higher concentrations. For our study on kokumi, we used a low concentration (1 mM) of ornithine. The possibility mentioned in the Discussion that 'the umami substances may enhance the taste response to ornithine' is entirely speculative. We will reconsider including this description in the revised version. As the reviewer suggested, in addition to GPRC6A, ornithine may bind to CaSR and/or T1R1/T1R3 heterodimers. However, we believe that ornithine mainly binds to GPRC6A, as a specific inhibitor of this receptor almost completely abolished the enhanced response to umami substances, and our immunohistochemical study indicated that GPRC6A-expressing taste cells are distinct from CaSR-expressing taste cells (see Supplemental Fig. 3). We conducted essentially the same experiments using gamma-Glu-Val-Gly in Wistar rats (Yamamoto and Mizuta, Chem. Senses, 2022) and compared the results in the Discussion. The reviewer may have misunderstood the chorda tympani results: we added the same concentration (1 mM) used in the two-bottle preference test to MSG (Fig. 5-B). Fig. 5-A shows nerve responses to five concentrations of plain ornithine. In the revised manuscript, we will strive to provide more precise information reflecting the reviewer's comments.

***Recommendations for the authors**:*

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) The behavioral effects found with the CPRC6A antagonists are not entirely convincing, as the antagonist is seemingly just mixed up in the solution with the stimuli. There are no control experiments demonstrating that the antagonists do not have a taste themselves.*

We mixed the antagonists into both liquids used in the two-bottle preference test to eliminate any potential taste effects of the antagonists themselves. In the electrophysiological experiments, the antagonist was incorporated into the solution after confirming that it did not elicit any appreciable response in the taste nerve.

> *(2) The effects of ornithine found with quinine did not have a satisfying explanation - if there is some taste cell-taste cell modulation that accounts for the taste enhancement, why is the quinine less aversive? Why is it not enhanced like the other compounds?*

The effects of ornithine on quinine responses remain difficult to explain. A previous study (Tokuyama et al., Chem Pharm Bull, 2006) proposed that ornithine prevents bitter substances from binding to bitter receptors, although this hypothesis lacks definitive evidence. In the present study, our findings suggest that the binding of quinine to bitter receptors is essential, as another agonist, gallate, also enhanced the preference for quinine, but this effect was abolished by EGCG, a GPRC6A antagonist (see Supplemental Fig. 2).

> *(3) Unless I am missing something, there appears to be no quantitative analysis of the immunocytochemical data, just assertions.*

We have made quantitative analyses in the revised text, and the following sentences have been added: “Approximately 11% of GPRC6A-positive cells overlapped with IP3R3 (9 double-positive cells/80 GPRC6A-positive cells), while approximately 8.3% of IP3R3-positive cells expressed GPRC6A (9 double-positive /109 IP3R3-positive cells). In addition, GPRC6A-positive cells were unlikely to colocalize with a-gustducin, another marker for a subset of type II cells, in single taste cells (0 double-positive cell/93 GPRC6A-positive cells). Regarding type III cell markers, GPRC6A-positive cells were unlikely to colocalize with 5-HT in single taste cells (0 double-positive cell/75 GPRC6A-positive cells).”

> *(4) The hallmarks of Kokumi taste include descriptors such as "thickness", and "mouthfeel", which sound like potential somatosensory attributes. Perhaps the authors should consider this possibility for at least some of the effects found.*

The term kokumi, a Japanese word, refers to a phenomenon in which the flavor of complexly composed food is enhanced through certain processes, making them more delicious. To date, kokumi has been described using the representative terms *thickness*, *mouthfulness*, and *continuity*, originally introduced in the first paper on kokumi by Ueda et al. (1990). However, these terms are derived from Japanese and may not fully convey the nuances of the original language when translated into these simple English words. In particular, *thickness* is often interpreted as referring to physical properties such as viscosity or somatosensory sensations. Since kokumi inherently lacks somatosensory elements, this revised paper adopts alternative terms and explanations for the three components of kokumi to prevent misunderstanding and confusion.

Therefore, to clarify that kokumi attributes are inherently gustatory, *thickness* is replaced with *intensity of whole complex tastes* (*rich flavor with complex tastes*), emphasizing the synergistic effects of a variety of tastes rather than the mere enhancement of a single flavor. *Mouthfulness* is clarified as not referring to *mouthfeel* (the tactile sensation a food gives in the

mouth) but rather as *spread of taste and flavor throughout the oral cavity*, describing how the flavor fills the mouth. *Continuity* is replaced with *persistence of taste* (*lingering flavor*).

> *(5) I don't think the human experiment (S1) belongs to the paper, even as a supplementary bit of data. It's only 17 subjects, they are all female, and we don't know anything about how they were selected, even though it states they are all students/staff at Kio. Were any of them lab members? Were they aware of the goals of the experiment? Could simply increasing the amount of solute in the soup make it seem thicker? This (sparse) data seems to have been shoehorned into the paper without enough detail/justification.*

Despite the reviewer's suggestion, we would like to include the human experiment because the rationale of the present study is to confirm, through a human sensory test, that the kokumi of a complex solution (in this case, miso soup) is enhanced by the addition of ornithine. This is followed by basic animal experiments to investigate the underlying mechanisms. Therefore, this human study serves an important role.

The total number of participants increased to 22 (19 women and three men) following an additional experiment with 5 new participants. New results have been shown in Supplemental Figure 1 with statistical analyses. The rewritten parts are as follows:

We recruited 22 participants (19 women and three men, aged 21-28 years) from Kio University who were not affiliated with our laboratory, including students and staff members. All participants passed a screening test based on taste sensitivity. According to the responses obtained from a pre-experimental questionnaire, we confirmed that none of the participants had any sensory abnormalities, eating disorders, or mental disorders, or were taking any medications that may potentially affect their sense of taste. All participants were instructed not to eat or drink anything for 1 hour prior to the start of the experiment. We provided them with a detailed explanation of the experimental procedures, including safety measures and personal data protection, without revealing the specific goals of the study.

> *(6) The introduction could be more concise - for example, when describing Kokumi stimuli such as ornithine and its possible receptors, the authors do not need to add the detail about how this stimulus was deduced from adding clams to the soup. Details like this can be reserved for the discussion.*

Thank you for this comment. We have tried to shorten the Introduction.

> *(7) Line 86: awkward phrasing - this doesn't need to be a rhetorical question.*

We have deleted the sentence.

> *(8) Supplementary Figure 1: The labels on the figure say "Miso soup in 1 mM Orn" when the Orn is dissolved into the soup.*

Thank you for pointing out our mistake. We have changed the description, such as "1 mM Orn in miso soup".

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Major concerns*
>
> *(1) The impact of "kokumi" taste ligands on food perception appears to be profound in humans. This observation is fascinating because it implies that molecules like ornithine impact a variety of flavor perceptions, some of which are non-gustatory in nature (e.g.,*

> *spread, mouthfulness and harmony). What remains unclear is whether "kokumi" ligands produce analogous sensations in rodents. If they don't, then rodents are an inappropriate model system for studying the impact of kokumi on flavor perceptions. The authors fail to address this key issue, and uncritically assume that kokumi ligands produce sensations like thickness, mouthfulness, and continuity in rodents. For this reason, the authors' reference to GPRC6A as a kokumi receptor is inappropriate.*

Thank you very much for the valuable comments. The term kokumi refers to a phenomenon in which the flavor of complexly composed foods is enhanced through certain processes, making them more delicious. It is an important concept in the field of food science, which studies how to make prepared dishes more enjoyable. Kokumi is also considered a higher-order, profound cognitive function evaluated by humans who experience a wide variety of foods. However, it is unclear whether animals, particularly experimental animals, can perceive kokumi in the same way humans do.

To date, kokumi has been described using the representative terms *thickness*, *mouthfulness*, and *continuity*, originally introduced in the first paper on kokumi by Ueda et al. (1990). However, these terms are derived from Japanese and may not fully convey the nuances of the original language when translated into these simple English words. In particular, *thickness* is often interpreted as referring to physical properties such as viscosity or somatosensory sensations. Since kokumi inherently lacks somatosensory elements, this revised paper adopts alternative terms and explanations for the three components of kokumi to prevent misunderstanding and confusion.

Therefore, to clarify that kokumi attributes are inherently gustatory, *thickness* is replaced with *intensity of whole complex tastes* (*rich flavor with complex tastes*), emphasizing the synergistic effects of a variety of tastes rather than the mere enhancement of a single flavor. *Mouthfulness* is clarified as not referring to *mouthfeel* (the tactile sensation a food gives in the mouth) but rather as *spread of taste and flavor throughout the oral cavity*, describing how the flavor fills the mouth. *Continuity* is replaced with *persistence of taste* (*lingering flavor*).

Rodents are thought to possess basic taste functions similar to humans, such as the expression of taste receptors, including kokumi receptors, in taste cells. Regardless of whether rodents can perceive kokumi, findings from studies on rodents may provide insights into aspects of the kokumi concept as experienced by humans.

Indeed, the results of this study indicate that ornithine enhances umami, sweetness, fat taste, and saltiness, leading to the enhancement of complex flavors—referred to as *intensity of whole taste*. The activation of various taste cells, resulting in the enhancement of multiple tastes, may contribute to the sensation of flavors spreading throughout the oral cavity. Furthermore, the strong enhancement of MSG and MPG suggests that glutamate contributes to the *mouthfulness* and *persistence of taste* characteristic of kokumi.

> *(2) A related concern is that the authors did not make any measurements that model kokumi sensations documented in the literature. For example, they would need to develop behavioral/electrophysiological measurements that reflect the known effects of kokumi ligands on flavor perception (i.e., increases in intensity, spread, continuity, richness, harmony, and punch). For example, ornithine is thought to produce more "punch" (i.e., a more rapid rise in intensity). This could be manifested as a more rapid rise in peripheral taste response or a more rapid fMRI response in the taste cortex. Alternatively, ornithine is thought to increase "continuity" (i.e., make the taste response more persistent). This response would presumably be manifested as a peripheral taste response that adapts more slowly or a more persistent fMRI response. As it stands, the authors have documented that ornithine increases (i) the preference of rats for some*

> *chemical stimuli, but not others; and (ii) the response of the CT nerve to some but not all taste stimuli.*

In animal experiments, it is challenging to examine each attribute of kokumi. The *increase of complex tastes* can be investigated through behavioral experiments and neural activity recordings. However, phenomena such as *spread* or *harmony*, which arise from profound human judgments, are difficult to validate in animal studies.

While it was possible to examine *persistence* through neural responses to tastants, all stimuli were rinsed at 30 seconds after onset of stimulation, so the exact duration of persistence was not investigated. However, since the MSG response was enhanced approximately 1.5 times with the addition of ornithine, it is strongly suggested that the duration might also have been prolonged.

Regarding *punch*, no differences were observed in the neural responses when ornithine was added, likely because the phasic response already had a rapid onset.

In the context of fMRI studies, there has been a report that adding glutathione to mixtures of umami and salt solutions increases responses (Goto et al. Chem Senses, 2016). However, research specifically examining the attributes of kokumi has not yet been reported.

> *(3) The quality of the SNAP-25 immunohistochemistry is poor (see Figure 7D), with lots of seemingly nonspecific staining in and outside the taste bud.*

The quality of the SNAP-25 is not poor. It is known that SNAP-25 labels not only type III cells but also the dense network of intragemmal nerve fibers (Tizzano et al., Immunohistochemical Analysis of Human Vallate Taste Buds. Chem Senses.40:655-60, 2015). Therefore, lots of seemingly nonspecific staining is due to intense SNAP-25-immunoreactivity of the nerve fibers.

> *(4) The authors need to drastically scale back the scope of their conclusions. What they can say is that ornithine appears to enhance the taste responses of rats to a variety of taste stimuli and that this effect appears to be mediated by the GPRC6A receptor. They cannot use their data to address kokumi effects in humans, as they have not attempted to model any of these effects. Given the known problems with pharmacological blocking agents (e.g., nonspecificity), the authors would significantly strengthen their case if they could generate similar results in a GPRC6A knockout mouse.*

Our research approach begins with confirming in humans that the addition of ornithine to complex foods (such as miso soup) induces kokumi. Based on this confirmation, we conduct fundamental studies using animal models to investigate the peripheral taste mechanisms underlying the expression of kokumi.

It is possible that the key to kokumi expression lies in the enhancement of desirable tastes (particularly umami) and the suppression of unpleasant tastes. Moving forward, we will deepen our fundamental research on the action of ornithine mediated through GPRC6A, including studies using knockout mice.

> *(5) The introduction is too long. Much of the discussion of kokumi perception in humans should either be removed or shortened considerably.*

Following the reviewer's suggestion, the introduction has been shortened.

> *(6) I recommend that the authors break up the Methods and Results sections into different experiments. This would enable the authors to provide separate rationales for*

> *each procedure. For instance, the authors conducted a variety of different behavioral procedures (e.g., long- and short-term preference tests, and preference tests with and without GPRC6A receptor antagonists).*

Rather than following the reviewer's suggestion, we have added subheadings to describe the purpose of each experiment. This approach would help readers better understand the experimental flow, as each experiment is relatively straightforward.

> *(7) The inclusion of the human data is odd for two reasons. First, the measurements used to assess the impact of ornithine on flavor perception in humans were totally different than those used in rats. This makes it impossible to compare the human and rat datasets. Second, the human study was rather limited in scope, had small effect sizes, and had a lot of individual variation. For these reasons, the human data are not terribly helpful. I recommend that the authors remove the human data from this paper, and publish them as part of a more extensive study on humans.*

Despite the reviewer's suggestion, we would like to include the human experiment because the rationale of the present study is to confirm, through a human sensory test, that the kokumi of a complex solution (in this case, miso soup) is enhanced by the addition of ornithine. This is followed by basic animal experiments to investigate the underlying mechanisms. Therefore, this human study serves an important role. The considerable variation in the scores suggests that evaluating the three kokumi attributes is challenging and likely influenced by differences in judgment criteria among participants.

The total number of participants increased to 22 (19 women and three men) following an additional experiment with 5 new participants. New results have been shown in Supplemental Figure 1 with statistical analyses. The rewritten parts are as follows:

We recruited 22 participants (19 women and three men, aged 21-28 years) from Kio University who were not affiliated with our laboratory, including students and staff members. All participants passed a screening test based on taste sensitivity. According to the responses obtained from a pre-experimental questionnaire, we confirmed that none of the participants had any sensory abnormalities, eating disorders, or mental disorders, or were taking any medications that may potentially affect their sense of taste. All participants were instructed not to eat or drink anything for 1 hour prior to the start of the experiment. We provided them with a detailed explanation of the experimental procedures, including safety measures and personal data protection, without revealing the specific goals of the study.

> *(8) While the use of English is generally good, there are many instances where the English is a bit awkward. I recommend that the authors ask a native English speaker to edit the text.*

Thank you for this comment. The text has been edited by a native English speaker.

> *Minor concerns*
>
> *(1) Lines 13-14: The authors state that "the concept of 'kokumi' has garnered significant attention in gustatory physiology and food science." This is an exaggeration. Kokumi has generated considerable interest in food science but has yet to generate much interest in gustatory physiology.*

We have rewritten this part: "The concept of "kokumi" has generated considerable interest in food science but kokumi has not been well studied in gustatory physiology."

> *(2) Line 20: The use of "specific taste" is unclear in this context. The authors indicate (in Figure 5A) that 1 mM ornithine generates a CT nerve response. They also reveal (in Figure 1A) that rats do not prefer 1 mM ornithine over water. The results from a preference test do not provide insight into whether a solution can be tasted; they merely demonstrate a lack of preference for that solution. Based on these data, the authors cannot infer that 1 mM ornithine cannot be tasted.*

We agree with the reviewer's comment. Ornithine at 1 mM concentration may have a weak taste because this solution elicited a small neural response (Fig. 5-A). We have rewritten the text: "… at a concentration without preference for this solution."

> *(3) Line 44: Sensory information from foods enters the oral and the nasal cavity.*

The nasal cavity has been added.

> *(5) Lines 59: The terms "thickness", "mouthfulness" and "continuity" are not intuitive in English, and may reflect, at least in part, a failure in translation. The word thickness implies a tactile sensation (e.g., owing to high viscosity), but the authors use it to indicate a flavor that is more intense and onsets more quickly. The word mouthfulness is supposed to indicate that a flavor is experienced throughout the oral cavity. The problem here is that this happens with all tastants, independent of the presence of substances like ornithine. Indeed, taste buds occur in a limited portion of the oral epithelium, but we nevertheless experience tastes throughout the oral cavity, owing to a phenomenon called tactile referral (see the following reference: Todrank and Bartoshuk, 1991, A taste illusion: taste sensation localized by touch" Physiology & Behavior 50:1027-1031). The word continuity does not imply that the taste is long-lasting or persistent.*

These three attributes were originally introduced by Ueda et al. (1990), who translated Japanese terms describing the profound characteristics of kokumi, which are deeply rooted in Japanese culinary culture. However, these simply translated terms have caused global misunderstanding and confusion, because they sound like somatosensory rather than gustatory descriptions. Therefore, to clarify that kokumi attributes are inherently gustatory, in the revised version we use the terms "intensity of whole complex tastes (rich flavor with complex tastes)" instead of thickness, "mouthfulness (spread of taste and flavor throughout the oral cavity)," and "persistence of taste (lingering flavor)" instead of continuity.

The results of this study indicate that ornithine enhances umami, sweetness, fat taste, and saltiness, leading to the enhancement of complex flavors—referred to as *intensity of whole taste*. The activation of various taste cells, resulting in the enhancement of multiple tastes, may contribute to the sensation of flavors spreading throughout the oral cavity. Furthermore, the strong enhancement of MSG and MPG suggests that glutamate contributes to the *mouthfulness* and *persistence of taste* characteristic of kokumi.

> *(6) Figure legends: The authors provide results of statistical comparisons in several of the figures. They need to explain what statistical procedures were performed. As it stands, it is impossible to interpret the asterisks provided.*

We have explained statistical procedures in each Figure legend.

> *(7) I did not see any reference to the sources of funding or any mention of potential conflicts of interest.*

We have added the following information:

Funding: JSPS KAKENHI Grant Numbers JP17K00935 (to TY) and JP22K11803(to KU).

Declaration of interests: The authors declare that they have no competing interests.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) I suggest that the authors increase their level of interest in glutathione and gamma-glutamyl peptides. This might include an appropriate gamma-glutamyl control substance in the two-bottle preference study (see Public Review). It might also include more careful attention to the work that identified glutathione as an activator of the CaSR (Wang et al., JBC 2006) and the nature of its binding site on the CaSR which overlaps with its site for L-amino acids (Broadhead et al., JBC 2011). This latter article also identified S-methyl glutathione, in which the free-SH group is blocked, as a high-potency activator of the CaSR. It would be expected to show comparable potency to gamma-glu-Val-Gly in assays of kokumi taste.*

We have appropriately referenced glutathione and gamma-Glu-Val-Gly, potent agonists of CaSR, where necessary. In our previous study (Yamamoto and Mizuta, Chem Senses, 2022), we examined the additive effects of these substances on basic taste stimuli in rodents, and the results were compared in greater detail with those obtained from the addition of ornithine in the present study. We have also discussed the potential binding of ornithine to other receptors, including CaSR and T1R1/T1R3 heterodimers.

> *(2) Figures:*
>
> *-None of the figures were labelled with their Figure numbers. I have inferred the Figure numbers from the legends and their positions in the pdf.*

We are sorry for this inconvenience.

> *- The labelling of Figure 1 and Figure 2 are problematic. In Figure 1 it should be made clear that the horizontal axes refer to the Ornithine concentration. In Figure 2 it should be made clear that the horizontal axes refer to the tastant concentrations (MSG, IMP, etc) and that the Ornithine concentrations were fixed at either zero or 1.0 mM.*

We are sorry for the lack of information about the horizontal axes. We have explained the horizontal axes in figure legends in Figs. 1 and 2. The labelling of both figures has also been modified to make this clear.

> *- Figure 3B: 'Control' should appear at the top of this panel since the panels that follow all refer to it.*

Following the reviewer's suggestion, we have added 'Control' at the top of Figure 3B.

> *- Figure 5A. Provide a label for the test substance, presumably Ornithine.*

Yes, we have added 'Ornithine'.

> *- Figure 7 would be strengthened by the inclusion of immunohistochemistry analyses of the CaSR.*

We are sorry that we did not analyze immunohistochemistry for the CaSR because a previous study precisely had analyzed the CaSR expression on taste cells in rats. We have analyzed co-expression of GPRC6A and CaSR (see Supplemental Figure 3).

> *(3) Other Matters:*
>
> *- Line 38: list the five basic taste modalities here.*

Yes, we have included the five basic taste modalities here.

> *- Line 107: 'even if ... kokumi ... is less developed in rodents' - if there is evidence that kokumi is less developed in rodents it should be cited here.*

We cannot cite any references here because no studies have compared the perception of kokumi between humans and rodents.

> *- Line 308: 'recently we conducted experiments in rats using gallate ...' - the authors appear to imply that they performed the research in Reference 43, however, I was unable to find an overlap between the two lists of authors.*

We are not doing a similar study as the research in Reference 43 (40 in the revised paper). Following the result that gallate is an agonist of GPRC6A as shown by Reference 43, we were interested in doing similar behavioral experiments using gallate instead of ornithine.

The sentences have been rewritten to avoid misunderstanding.

> *- Line 506: the sections are said to be 20 mm thick - should this read 20 micrometers?*

Thank you. We have changed to 20 micrometers.

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