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

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Abstract

In recent years, the concept of "kokumi" has garnered significant attention in gustatory physiology and food science. Kokumi refers to the enhanced and more delicious state of food flavor. However, the underlying neuroscientific mechanisms remain largely unexplored. Our previous research demonstrated that ornithine (L-ornithine), abundantly found in shijimi clams, enhances taste preferences. This study aims to build on these findings and investigate the mechanisms behind kokumi. In a two-bottle preference test in rats, the addition of ornithine, at a concentration without specific taste, enhanced the preference for solutions of umami, sweetness, fatty taste, saltiness, and bitterness, with monosodium glutamate intake showing the most significant increase. A mixture of umami and ornithine induced synergistically large responses in the chorda tympani nerve, which transmits taste information from the anterior part of the tongue. This enhancement of preference and the increase in taste nerve response were abolished by antagonists of the G-protein-coupled receptor family C group 6 subtype A (GPRC6A). Immunohistochemical experiments indicated that GPRC6A is expressed in a subset of type II taste cells in the fungiform papillae. These results provide new insights into flavor enhancement mechanisms, suggesting that ornithine is a newly identified kokumi substance and GPRC6A is a novel kokumi receptor.

Introduction

Enjoying meals together and savoring delicious food are fundamental pleasures of daily life, essential for both physical and mental well-being. The perception of deliciousness requires a harmonious integration of various sensory experiences, including the five basic tastes, the taste of fat, spices, aroma, texture, temperature, sight, and sound. These sensory inputs are transmitted to the brain via sensory nerves, where they are analyzed for sensory modalities and qualitative information (1,2). Beyond these cognitive aspects, the brain further processes these inputs to evoke emotional responses, which significantly contribute to the perception of deliciousness or unpleasantness (3-5). Therefore, the sensory information from substances entering the oral cavity influences both cognition and emotion in the brain.

Conversely, there are substances and their receptors that, although having minimal direct taste effects and not sending clear information to the brain, can modify taste information at the peripheral level and thereby control the intensity of deliciousness. Recent research on taste modification has focused on “kokumi” substances and kokumi receptors (6-8). According to Ohsu et al. (9), kokumi substances do not have a taste of their own but enhance the thickness (or richness) of food, increasing its mouthfeel and prolonging its aftertaste. However, the concept of kokumi is deeply rooted in Japanese culinary culture (10,11), and a global consensus on its essence has yet to be established.

Ueda et al. (12) conducted the first scientific study on kokumi. They initially demonstrated that adding garlic extract to Chinese soup or curry soup enhances three elements—thickness, mouthfulness, and continuity—without affecting the aroma. Additionally, they found that these three elements are amplified when garlic extract is added to an umami solution, which is a mixture of monosodium glutamate (MSG) and inosine monophosphate (IMP). They termed this enhanced state of the three elements as kokumi (or kokumi flavor). The term "kokumi" is derived from the Japanese word "koku." In everyday language, Japanese people use "koku" to describe a taste that is strong, rich, and delicious. Since "koku" is a conceptual term, Ueda et al. introduced the elements of thickness, mouthfulness, and continuity for scientific clarity, and named the state where these elements are enhanced as kokumi.

Ueda et al. (13,14) further reported that glutathione and sulfur-containing components found in garlic and onion enhance the three elements in soups and umami solutions, thus contributing to kokumi. Subsequently, Ohsu et al. (9) investigated the binding abilities of γ-glutamyl peptides to the calcium-sensing receptor (CaSR) and revealed through sensory evaluations of chicken consommé and umami solutions that those with stronger binding abilities produce a more pronounced kokumi effect. They also found that the kokumi effect disappears with the antagonist of CaSR and demonstrated that γ-Glu-Val-Gly is the strongest kokumi substance, being ten times more potent than γ-Glu-Cys-Gly (glutathione). Thus, it is proposed that CaSR is the receptor (kokumi receptor) responsible for producing kokumi. CaSR was found to exist in taste bud cells in rodents (15,16). Since then, numerous studies have been conducted on CaSR agonists as kokumi substances (17-25), although neuroscientific research on the mechanism of kokumi induction remains limited and underdeveloped (26-29).

One might wonder, "Aren't there any other kokumi receptors besides CaSR?" A clue can be found in Japanese food culture. Many Japanese people have long observed that adding shijimi clams (Corbicula japonica) to miso soup enhances its kokumi flavor, making it more delicious. This suggests the possibility that shijimi clams contain unknown kokumi substances. Among various substances released from shijimi clams (30), our previous study (31) focused on ornithine, a non-protein amino acid abundantly present in these clams (30,32). Behavioral experiments, electrophysiological nerve response recordings, and immunohistochemical studies in mice suggested that the G-protein-coupled receptor family C group 6 subtype A (GPRC6A) is responsible for the action of ornithine, given that ornithine has a high affinity for this receptor (33-35). Since this is the only study proposing GPRC6A as a potential kokumi receptor other than CaSR, further research is needed to confirm this hypothesis.

Neuroscientific research concerning kokumi has been reported for glutathione (36) in mice and for γ-Glu-Val-Gly in mice (27) and rats (29). However, for ornithine, only one study has been conducted in mice (31). Therefore, this study aims to investigate the taste effects of ornithine in rats and compare and verify the results with previous reports. Although it remains unclear whether rodents

are suitable model animals for kokumi research, if the expression of kokumi flavor is fundamental to the enjoyment of delicious food, there should be basic mechanisms common to both humans and rodents, even if the mechanisms of kokumi expression are less developed in rodents. Elucidating the neuroscientific mechanisms could potentially shed light on the elusive nature of kokumi in humans.

Results

First, we confirmed through human sensory tests that adding ornithine to miso soup enhances the three elements of kokumi: thickness, mouthfulness, and continuity (Fig. S1).

Two-bottle preference tests

To investigate whether ornithine has a preferable taste, we conducted a preference test comparing distilled water (DW) with aqueous solutions containing various concentrations of ornithine (Fig. 1A). A two-way ANOVA (solution × concentration) revealed significant main effects of solution [$F(1,50) = 33.93$, $P < 0.0001$], no main effects 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 mM and 30 mM ornithine was significantly greater ($P < 0.01$) than that of plain water, 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 MSG (Fig. 1B). A two-way ANOVA (solution × concentration) revealed significant main effects 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 [$F(4,50) = 0.899$, $P > 0.05$]. Post hoc Bonferroni analyses indicated that the addition of ornithine at concentrations ranging from 1 mM to 30 mM significantly increased intake ($P < 0.001$) over that of MSG alone. Based on these results, we used a 1 mM concentration to examine the additive effects of ornithine in subsequent experiments.

We examined the intake of each of the eight taste solutions at four different concentrations, both alone and with 1 mM ornithine (Fig. 2). Bonferroni's multiple

comparisons analysis between different concentrations of solutions with and without ornithine revealed that MSG and MPG at all four concentrations were statistically significantly ($P < 0.05$, 0.01, or 0.001) more preferred with ornithine than without (Fig. 2B and C). This was also observed for IMP (Fig. 2A), Intralipos (Fig. 2D), and sucrose (Fig. 2E) at two concentrations, and for NaCl (Fig. 2F) at one concentration. However, citric acid showed no difference regardless of whether 1 mM ornithine was present. QHCl was also preferred when ornithine was added at three concentrations, suggesting that the aversive taste of QHCl was attenuated by ornithine. Notably, the additive effects of ornithine differed among the umami solutions: ornithine increased the preference more effectively for MSG and MPG than for IMP.

To determine if the preference enhancement by ornithine was caused by an intra-oral event rather than post-oral consequences, a two-bottle brief (10 min) exposure preference test was conducted. We confirmed that the intake of water with 1 mM ornithine was not different ($P > 0.05$, paired t-test, two-tailed) from that of plain water (Fig. 3A), but the intake of 0.03 M MSG was significantly ($P < 0.01$) increased by the addition of ornithine (Fig. 3B). This increased preference was maintained in the presence of calindol, a GPRC6A antagonist, in both solutions at a 60 µM concentration (Fig. 3C), but disappeared at a concentration of 300 µM (Fig. 3D). Similarly, another GPRC6A antagonist, EGCG, had no effect at a concentration of 30 µM (Fig. 3E) but abolished the enhanced preference at 100 µM (Fig. 3F).

Effects of chorda tympani (CT) transection

To determine how taste information from the anterior part of the tongue influences the enhancement of preference by ornithine, we compared intake before and after transection of the CT in the two-bottle long-term preference test. As shown in Fig. 4A and 4C, the mixture of 1 mM ornithine and 0.03 M MSG was significantly ($P < 0.01$, paired t-test, two-tailed) more ingested than plain MSG before both sham and experimental treatments. Under the sham control operation, the same enhanced preference was essentially reproduced (Fig. 4B). However, actual transection of the CT abolished the increased preference induced by ornithine supplementation (Fig. 4D).

Taste nerve recording

Aqueous solutions of ornithine used in the present behavioral experiments induced very small CT responses but increased dose-dependently, as shown by sample recordings (Fig. 5A) and a graph (Fig. 5D). One-way ANOVA revealed significant differences among the values shown in Fig. 5D [$F(4,15) = 14.660$, $P < 0.001$]. Post hoc Bonferroni tests indicated that the response to 30 mM ornithine was significantly larger ($P < 0.01$) than the responses to other concentrations of ornithine. The response to ornithine at the concentration of 1 mM, which was used in this study, was negligibly small compared to the standard response to NH_4Cl. However, when ornithine was added at this concentration, the response to 0.03 M MSG increased significantly ($P < 0.05$) compared to plain MSG (Fig. 5E). Essentially the same additive effect of ornithine was observed when the solution was prepared with 0.1 mM amiloride, a sodium channel blocker, with a similar increase (Fig. 5E), suggesting that glutamate, rather than sodium ions, is responsible for this increased response. Sample recordings for these responses are shown in Fig. 5B. One-way ANOVA revealed significant differences among the 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 sample recordings (Fig. 5C) and a graphical representation (Fig. 5F), the MSG response was increased by the addition of ornithine ($P < 0.01$); however, this increase was no longer observed when calindol was added to the mixture of MSG and ornithine. One-way ANOVA revealed significant differences among the values shown in Fig. 5F [$F(2,9) = 4.690$, $P < 0.05$]. Other GPRC6A antagonists, such as NPS-2143 and EGCG, showed similar effects as calindol (data not shown).

Immunohistochemical localization of GPRC6A in rat taste cells

To determine whether GPRC6A protein is expressed in taste cells of rat fungiform, foliate and circumvallate taste buds, immunohistochemical studies were performed. In the fungiform papillae, a significant number of spindle-shaped taste cells exhibited intense GPRC6A-immunoreactivity (Fig. 6A). In the foliate and circumvallate papillae, GPRC6A-immunopositive taste cells were barely detectable (Fig. 6B, C). However, the cells were likely to constitute less than 1% of the total taste cell population in the respective taste papillae.

These results demonstrated that GPRC6A proteins were preferentially located in subpopulations of fungiform taste cells in the rat.

Since rat taste cells can be divided into several cell types, we examined the colocalization of GPRC6A-immunoreactivity and cell type-specific markers in the fungiform papilla by immunohistochemistry. It was found that some, but not all, GPRC6A-immunopositive cells also exhibited immunoreactivity for IP3R3, a marker of the majority of the type II cell population (Fig. 7A), whereas α-gustducin, another marker of a subset of type II cells, was unlikely to be colocalized with GPRC6A in single taste cells (Fig. 7B). As for type III cell markers, neither 5-HT nor SNAP25 were likely to be colocalized with GPRC6A in single taste cells (Fig. 7C, D). These results suggest that GPRC6A protein is preferentially localized in a subpopulation of IP3R3-expressing taste cells in the rat fungiform papillae, and further suggest that GPRC6A may exert taste-modifying activity in at least some type II cells in the fungiform papillae.

Discussion

In this study using rats, we demonstrated through a 24-hour two-bottle preference test that the addition of ornithine at a concentration of 1 mM, which itself does not exhibit taste preference, to solutions of umami substances, Intralipos (representing fat taste), and sucrose (sweet taste) increased the intake of each solution, thereby enhancing their palatability. Similar increases in intake were observed in a short-term two-bottle preference test using MSG, indicating that this effect is due to taste sensation in the oral cavity rather than post-ingestive effects. The disappearance of the palatability-enhancing effect of ornithine on MSG with the application of a GPRC6A antagonist and the transection of the chorda tympani nerve, which innervates the taste buds in the anterior part of the tongue, suggests that GPRC6A, expressed in taste cells in the anterior part of the tongue, is involved in this effect. Supporting this, the MSG response of the chorda tympani nerve was increased with the addition of ornithine, and this increase was abolished by the action of a GPRC6A antagonist. Immunohistochemical staining showed that GPRC6A predominantly appears in the fungiform papillae of the anterior tongue, rather than in the foliate or circumvallate papillae of the posterior tongue. Despite several differences noted below, these results align well with our previous findings using mice (31). These

findings strongly suggest that ornithine is a kokumi substance and that GPRC6A is a candidate for the kokumi receptor. They provide neuroscientifically important basic knowledge for interpreting the sensory evaluation results (see Supplementary Fig. 1) indicating that adding ornithine to miso soup enhances the three elements of kokumi: thickness, continuity, and mouthfulness.

When comparing the results obtained using rats in this study with those from our previous study using mice (31), several similarities and differences can be noted. In both studies, the concentration of ornithine used for supplementation was the same (1 mM). The addition of ornithine increased the preference for solutions of basic tastes, such as umami, sweet, fat, salty, and bitter tastes, with a more pronounced enhancement of preference for MSG over IMP among the umami substances. However, while a weak enhancement of citric acid preference was observed in mice, no such effect was noted in rats. The effects of the GPRC6A antagonist and the chorda tympani nerve responses were consistent between the two species. A significant species difference was observed in the expression of GPRC6A; in mice, it is expressed throughout the tongue, particularly in the posterior part, whereas in rats, it is predominantly localized to the anterior part of the tongue. Another major difference is that while mice avoid high concentrations of ornithine, rats show a preference for it.

Regarding the phenomenon of increased preference for umami substances such as IMP, MSG, and MPG with the addition of ornithine, it is natural to interpret this as ornithine enhancing the umami responses. However, the possibility that umami substances enhance the response to ornithine cannot be ignored. Ornithine may stimulate at least two receptors, GPRC6A and the conventional amino acid receptor, T1R1/T1R3 heterodimer (37), depending on its concentration. At the low concentration (1 mM) used in the present study, GPRC6A may be exclusively stimulated, whereas higher concentrations may also stimulate the T1R1/T1R3 receptor, thereby transmitting the taste information of ornithine. Umami substances may promote ornithine's binding to these receptors. For example, Tanase et al. (38) showed that the taste intensity of ornithine increased in the presence of IMP in humans. Similarly, Ueda et al. (14) and Dunkel et al. (39) showed that the threshold for the taste of glutathione and glutamyl peptides, agonists of CaSR, was lowered in umami solutions. In rats, ornithine is a favorable taste, and its palatability increases with

concentration (see Fig. 1A). In a mixture of ornithine and MSG, MSG may enhance the response to ornithine, thereby increasing its palatability. This concept also applies to the explanation of the mixed effects of ornithine and MSG in mice. In mice, the taste of ornithine itself becomes disliked as the concentration increases (31). Therefore, while the addition of ornithine increases palatability at concentrations below 3 mM, it ceases to be palatable at concentrations of 10 mM and 30 mM. The taste information sent by ornithine as its concentration increases, whether it is favorable or unpleasant, is likely mediated by the conventional amino acid receptor (T1R1/T1R3) rather than GPRC6A. Thus, umami substances facilitate ornithine to stimulate both the kokumi receptor and amino acid receptor in a concentration-dependent manner. Umami substances and ornithine interact to produce a synergistic enhancement effect.

In experiments with rats (present study) and mice (31), the addition of ornithine to solutions of favorable tastes such as umami, sweet, and fat increased their palatability. This can be explained by the enhancement of responses to these tastes by ornithine, as evidenced by the increased chorda tympani nerve responses. Conversely, the increased palatability of the aversive bitter taste in both species suggests that ornithine suppresses the bitter response. Although the addition of ornithine to quinine in the chorda tympani nerve response in mice resulted in a slight decrease in response, this difference was not statistically significant. There are reports showing that ornithine decreases bitter taste sensation in humans (40,41), and arginine, which has a structure highly similar to ornithine, also suppresses the bitterness of quinine (42), but the mechanism is not well understood. Recently, we conducted experiments in rats using gallate as an agonist for GPRC6A (43). Similar to ornithine, gallate increases the palatability of MSG and quinine, but this effect is abolished by EGCG, an antagonist of GPRC6A (Fig. S2). Interestingly, this suggests that GPRC6A agonists may enhance responses to favorable tastes like umami and sweet, while inhibiting responses to bitter tastes, leading to an overall enhancement of food deliciousness.

The CaSR agonist γ-Glu-Val-Gly similarly enhanced the preference for umami, sweet, and fat tastes, but it showed no effect on the preference for salty, sour, or bitter tastes in rats (29). Additionally, while ornithine increased the preference for

MSG more than IMP, γ-Glu-Val-Gly increased the preference for IMP more than MSG. Unlike ornithine, the preference-enhancing effect of γ-Glu-Val-Gly was not affected by the severance of the chorda tympani nerve, likely because CaSR is expressed in both the anterior and posterior parts of the tongue (16). The results of the present study, along with our previous animal experiments, clearly demonstrate that binding of the kokumi substances to the kokumi receptors triggers the expression of kokumi. However, the mechanism by which this binding enhances the responses to tastes like umami, fat, and sweet remains unknown. Considering that GPRC6A is co-expressed in type II taste cells expressing IP3R3, as shown in this study, it is plausible that some of the type II cells are 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 in this study. While this study did not examine the co-expression of GPRC6A with the T1R or T2R families, Maruyama et al. (27) reported that in mice, CaSR does not co-express with receptors for umami, sweet, or other tastes. It is possible that some form of intercellular communication within taste buds affects these tastes, but a more detailed mechanism awaits future research. It is also interesting to consider whether GPRC6A and CaSR co-exist in the same taste cells; however, in our ongoing experiments, we have not identified any taste cells where these are co-expressed (Fig. S3). Besides the above discussion, it is noteworthy that kokumi-active γ-glutamyl peptides show a stronger affinity for the T1R1-MSG receptor complex than for the T1R2-sucrose receptor complex, as revealed by molecular modeling approaches (44) This suggests that these peptides exhibit a higher umami-enhancing effect without the participation of kokumi receptors.

While many candidate kokumi substances have been reported (7,17,18,21-25,28), we are 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 most strongly enhance umami. They also enhance sweet and fat tastes, and to a lesser extent, salty taste. The essence of kokumi expression is to enhance tastes associated with deliciousness or to bring out tastes that were not previously perceived (8). When each taste is enhanced and recognized collectively, the complexity of flavor arises, described as having thickness or richness. During this process, more taste cells in the oral cavity are

stimulated more strongly, and this information is sent to the brain. As a result, the brain perceives this as information coming from the entire mouth, leading to the sensation of mouthfulness. Regarding the enhancement of aftertaste (lingeringness), it is sensory physiologically common knowledge that a strong stimulus causes a large response that takes longer to return to baseline, thereby inducing a longer-lasting sensation. Enhancing umami, a taste known for its lingering quality (45,46), further strengthens this characteristic. The prolonged aftertaste of umami compared to other tastes 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 larger and more specific umami responses from the posterior rather than anterior part of the tongue in rodents (47,48) and in primates (46, 49). Moreover, MSG binds deeply within the Venus flytrap domain of the umami receptor (50), making it harder to dislodge.

In conclusion, when kokumi substances are present in complex materials containing umami substances, their interaction primarily enhances umami, followed by sweet and fat tastes, and to a lesser extent, salty taste and possibly other tastes. This enhancement is the core of richness, while the sensations of mouthfulness and lingeringness are secondary phenomena that accompany it. Since ornithine is also abundant in cheese (51) and mushrooms such as shimeji and maitake (52,53), we can enjoy the kokumi flavor in cuisines that incorporate these ingredients.

Materials and methods

Animals

Male Wistar rats (8 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The rats were housed individually in home cages within a temperature (25°C) and humidity (60%)-controlled room, following a 12:12 hour light/dark cycle with lights on at 6:00 am. The tests were conducted during the light cycle. The animals had free access to food (CLEA Rodent Diet CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water, except during brief-access tests described below, where food access was partially restricted. All animal care and experimental procedures adhered to the guidelines established by the National Institutes of

Health. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Kio University (Protocol No. H28-01).

Behavioral experiment: two-bottle preference tests

Each rat was trained to drink distilled water (DW) from a stainless-steel spout connected to a plastic bottle. After a 1-week training period, a preference test was conducted. The two-bottle preference test involved simultaneously presenting two bottles with stainless-steel spouts to each cage. Each spout, designed to minimize dripping with an internal ball, had an inner diameter of 6 mm and was separated by 5 cm from the center of each spout. The two bottles contained the same taste stimulus (or DW), but one was mixed with ornithine (L-ornithine, Kanto Chemical, Tokyo, Japan). In the long-term test, the positions of 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 test, the two bottles, with spouts separated by 1 cm, were presented to the animals for 10 minutes after an overnight water deprivation. After the test, the animals had free access to water until 6:00 pm, after which the water was removed for the subsequent overnight period. The next day, the left and right bottles were switched, and the 10-minute test session was repeated. The bottles with fluid were weighed before and after testing to measure intake volume. The total intake volume over 48 hours or 20 minutes was divided by 2 to obtain the intake volume per day or per 10 minutes in the long-term and short-term tests, respectively.

We used six basic taste stimuli: sucrose (Kanto Chemical), NaCl (Kanto Chemical), citric acid (Kanto Chemical), quinine hydrochloride (QHCl, Kanto Chemical), MSG (Kanto Chemical), and Intralipos (a parenteral stable soybean oil emulsion, Otsuka Pharmaceutical Factory, Tokyo, Japan). In addition to MSG, monopotassium glutamate (MPG, supplied by Ajinomoto Co.) and IMP (supplied by Ajinomoto Co.) were used as umami substances. The taste stimuli were dissolved in DW at various concentrations. We presented a range of concentrations of individual tastants to the same group of animals, starting from the lowest concentration. Ornithine was typically added at a concentration of 1 mM, with other concentrations used as needed. The Na-channel blocker amiloride (Sigma-Aldrich Co., Tokyo, Japan) was used to reduce the sodium

responses to MSG.

For antagonists of GPRC6A, we used NPS-2143 (Chemscene, USA), calindol (Cayman Chemical, USA), and epigallocatechin gallate (EGCG, Tokyo Chemical Industry, Tokyo, Japan). NPS-2143 and calindol were dissolved in 99.5% ethanol at a concentration of 0.1% and then diluted to each concentration with DW. EGCG was directly prepared in DW.

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

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

Transection of the chorda tympani (CT)

Eight naïve rats were randomly divided into two groups: a transection group and a sham operation group (4 rats in each). They underwent the long-term two-bottle preference test as described above, with the stimulus being 0.03 M MSG with and without 1 mM ornithine. The animals were then anesthetized via

intraperitoneal injection of a combination anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). For 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 the operation was ceased just before the removal of the ear ossicles for the sham operation group. 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 same long-term two-bottle preference test using the same taste stimuli. The mean preference of the groups was compared. After the experiment, the transection was confirmed by microscopic verification of the loss of taste buds on the tongue.

Taste nerve recordings

Five naïve rats were deeply anesthetized using the procedure described above. The rats were tracheotomized and secured in a head holder. The left CT nerve was exposed using a lateral approach (54) and excised as it exited the tympanic bulla, then dissected away from the underlying tissue. The nerve was placed onto a platinum wire recording electrode (0.1mm diameter), while an indifferent electrode was placed in contact with a nearby exposed tissue. The responses were filtered using a band-pass filter with cutoff frequencies from 40 Hz to 3 kHz and visualized on an oscilloscope (VC11, Nihon Kohden, Tokyo, Japan). Responses were fed to a digitally controlled summator (55). The number of discharges was summed over 500 ms epochs with 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/sp4; AD Instruments, Bella Vista, NSW, Australia) for quantitative analyses. Each taste stimulus (3 mL) was applied to the anterior dorsal tongue for 30 seconds, followed by a distilled water rinse 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 averaged values of repeated trials in individual animals.

Immunohistochemistry

Male Wistar rats (8-12 weeks of age) 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 (56), rats were injected with 5-HTP (5-hydroxy-l-tryptophan) at the concentration of 80 mg/kg body weight 1 h prior to anesthesia. Their tongues were dissected out, soaked in 20% sucrose/0.01 M phosphate-buffered saline (PBS) overnight at 4°C, embedded in OCT compound (Sakura Finetechnical, Japan), and then sectioned (20 mm thick) using a cryostat. The sections were immersed in 0.01 M PBS containing 0.2% Triton X-100, and subsequently incubated overnight at 4°C with the rabbit anti-GPRC6A (1:50; orb385435; Biorbyt) antibody in combination with either the goat anti-IP3R3 (inositol 1,4,5-trisphosphate receptor type III) (1:100; NB100-2545; NOVUS), -a-gustducin (1:500; LSB4942; LSBio), -5-HT (5-hydroxytryptamine, serotonin) (1:100; ab66047; Abcam), or -SNAP-25 (synaptosomal-associated protein 25 kDa) (1:100; ab31281; Abcam) antibody diluted in the same Triton X-100-containing solution. This was followed by Alexa 594™-conjugated anti-rabbit IgG (1:500; A-21207; Thermo Fisher Scientific) and 488™-conjugated anti-goat IgG (1:500; A-11055; Thermo Fisher Scientific) secondary antibodies. After washing, the sections were cover-slipped with Fluormount (Diagnostic BioSystems), and then imaged using a Nikon A1Rs confocal laser scanning microscope (Nikon, Japan). The specificity of the anti-GPRC6A antibody was verified as previously described (31) (data not shown). IP3R3 is a marker protein of the majority of type II taste cell population (57). Gustducin, 5-HT and SNAP-25 are markers of a subset of type II, type III and type III taste cells, respectively (31,56,57).

Data analysis

Data are presented as mean ± SEM. A Shapiro-Wilk test was performed to confirm that the samples had a normal distribution. The Student's *t*-test (paired, two-tailed) was used to assess statistical differences between two groups. For analyzing more than three groups, we used a one-way ANOVA, or a repeated measures two-way ANOVA with Bonferroni post-hoc tests for statistical comparisons. P values < 0.05 were considered statistically significant. These statistical analyses were performed using IBM SPSS Statistics (ver. 25). Statistical significance was set at $P < 0.05$.

Data availability

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

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Declaration of interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1

Additive effects of different concentrations of ornithine (Orn).

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

Figure 2

Additive effects of 1 mM ornithine (Orn) on fluid intake for four different taste solutions.
Fluid intake (mean ± SEM; n = 8) with and without Orn is shown for IMP **(A)**, MSG **(B)**, MPG **(C)**, Intralipos **(D)**, sucrose **(E)**, NaCl **(F)**, citric acid **(G)**, and quinine hydrochloride (QHCl) **(H)**. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

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

(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.

Figure 4

Effects of bilateral chorda tympani transection (xCT) and control sham operation (Sham OP) on the intake of 0.03 M MSG with and without 1 mM ornithine (Orn).
Fluid intake was compared before **(A, C)** and after **(B, D)** the operations. Each value represents the mean ± SEM; n = 4. **P < 0.01.

Figure 5

Sample recordings of chorda tympani (CT) responses and quantitative representation of the mean magnitude of CT responses.
(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, a sodium channel blocker. **(C)** Nerve responses to MSG, MSG with Orn, and MSG with Orn in 300 μM calindol. **(D, E, F)** Graphical representations of the mean magnitude of CT responses corresponding to A, B, 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.

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 papilla. **(B, C)** GPRC6A-immunopositive taste cells were barely detectable in the foliate **(B)** and 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 right and middle panels. Scale bars: 50 μm.

Figure 7

Immunohistochemical analysis of colocalization of GPRC6A-immunoreactivity 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 for the majority of the type II cell population. Arrow indicates a GPRC6A/IP3R3 double-positive taste cell. **(B)** α-Gustducin, another marker for a subset of type II cells, was unlikely to be colocalized with GPRC6A in single taste cells. **(C, D)** Neither 5-HT **(C)** nor SNAP25 **(D)**, both are specific markers for type III taste cells, were likely to be colocalized with GPRC6A in single taste cells. Scale bars, 10 μm.

Supplemental Information

Supplemental information includes 3 figures (Figs. 1-3).

Figure S1:

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

The addition of ornithine at three concentrations (1, 3, and 10 mM, labeled as T-1, T-2, and T-3, respectively) increased these three attributes in a dose-dependent manner. Palatability also improved in a dose-dependent manner. All values for kokumi attributes and palatability were set to 0 for the control miso soup without ornithine (C). Each value represents the mean ± SEM (n = 17).

Materials and methods

We recruited a total of 17 female participants (age range, 21-28 years) from Kio University, including students and staff members. Based on responses obtained from a pre-experiment questionnaire, we confirmed that none of the participants had any sensory abnormalities, eating disorders, mental disorders, or were taking any medications that could potentially affect their sense of taste. We instructed all participants not to eat or drink anything for one hour prior to the start of the experiment. We provided a detailed explanation of the experiment's purpose, safety measures, and protection of personal information. After obtaining their understanding and consent, we collected written informed consent from each participant. This study received approval from the Kio University ethics committee (No. R2-31), and all experiments were conducted in adherence to the principles outlined in the Declaration of Helsinki.

Miso soup with a 0.7% salt concentration was prepared by dissolving commercial miso paste (Tokujyo; Takeya-Miso Co., Ltd., Suwa, Japan) in hot tap water (control soup, C). Three test soups were then prepared by dissolving L-ornithine in the control soup at three concentrations (1, 3, and 10 mM, labeled as T-1, T-2, and T-3, respectively). An aliquot of 30 mL of each test soup was randomly served in a paper cup along with a control soup as a pair. The intensities of the thickness of taste, mouthfulness, and continuity were evaluated

according to the procedure of Ohsu et al. (9). Thickness was expressed as increased taste intensity at 5 seconds after tasting. Continuity was expressed as taste intensity at 20 seconds after tasting. Mouthfulness was expressed as the reinforcement of the taste sensation throughout the mouth, not just on the tongue. The participants evaluated 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 5-point rating scale ranging from -2 (apparently bad) to +2 (apparently good). All the values of the three kokumi attributes and palatability were set at 0 for the control soup.

Figure S2

Long-term exposure (one day) two-bottle preference test for different liquids with and without 1mM gallate (ethyl gallate, Kanto Chemical, Toko, Japan) and effects of 100 μM epigallocatechin gallate (EGCG), an antagonist of GPRC6A.

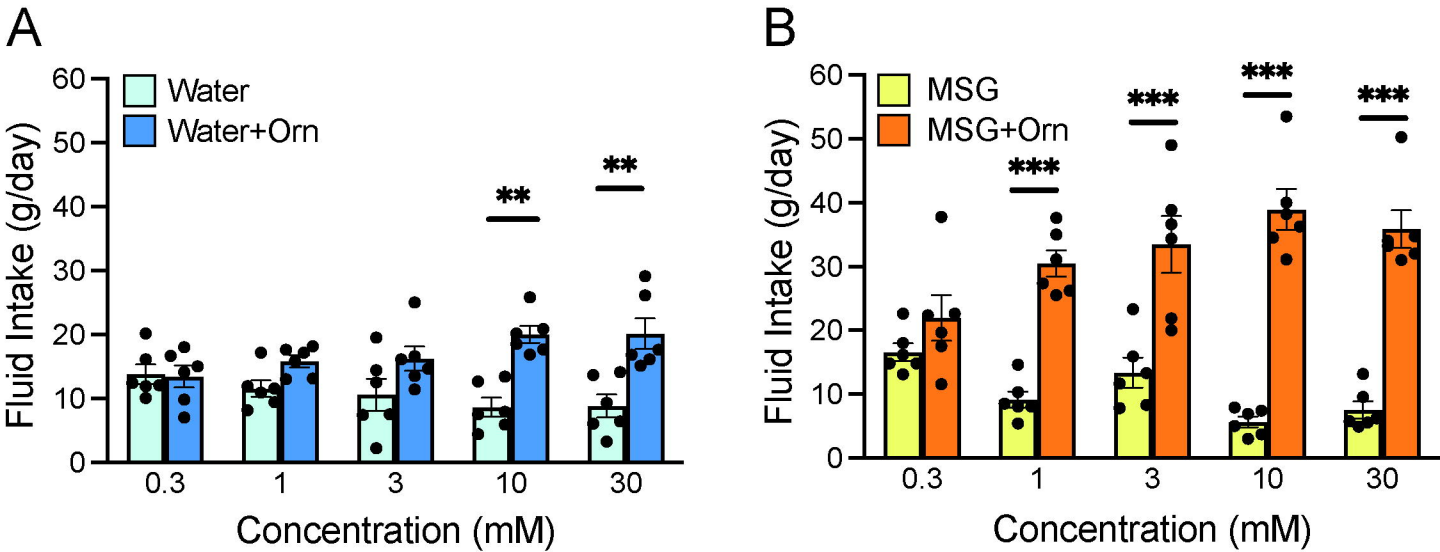
(A) Intake of water (W) with and without gallate (G), **(B)** Intake of 0.03M MSG (M) with and without gallate. **(C)** Comparison between MSG + EGCG and MSG + gallate + EGCG, **(D)** Intake of 0.01mM quinine-HCl (Q) with and without gallate, **(E)** Comparison between Q + EGCG and Q + gallate + EGCG. 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 the preference for MSG and QHCl and an antagonist of GPRC6A eliminates these preferences.

Figure S3

Immunohistochemical localization of GPRC6A and CaSR (calcium-sensing receptor) in rat fungiform taste papillae.

GPRC6A and CaSR are basically expressed in distinct subpopulations of fungiform taste cells. Nomarski image of the tissue section examined is shown in the rightmost panel. Scale bar, 10 μm. The procedures for immunohistochemistry were essentially the same as those described in the

Materials and methods section. For CaSR staining, the mouse anti-CaSR antibody (1:100; ab19347, Abcam) was used.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Water+Orn
Fluid Intake (g/day)
60
50
40
30
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0.3
1
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10
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Concentration (mM)
B
MSG
MSG+Orn
Fluid Intake (g/day)
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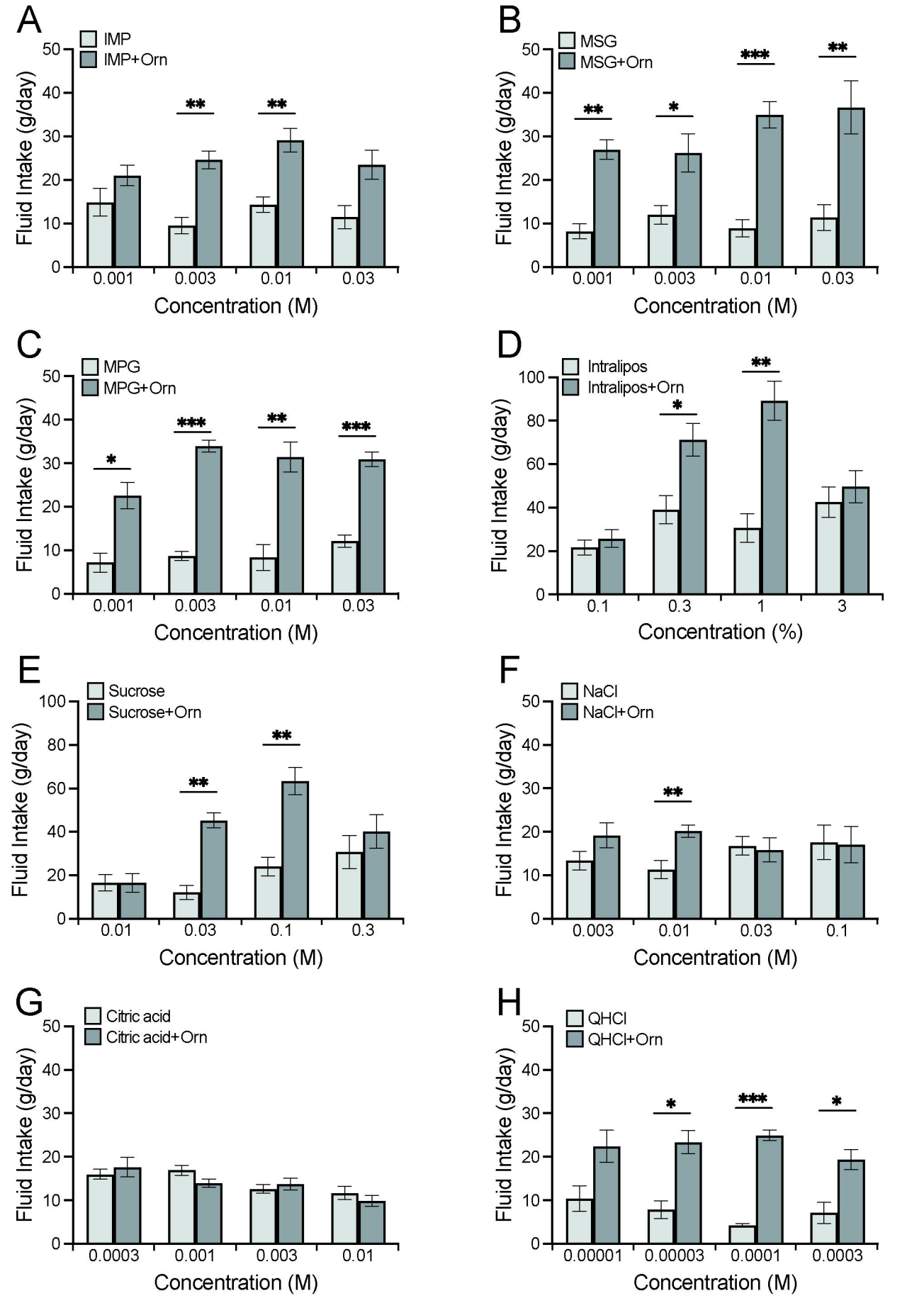
0.3
1
3
10
30
Concentration (mM)


A
IMP
IMP+Orn
Fluid Intake (g/day)
Concentration (M)
0.001
0.003
0.01
0.03
B
MSG
MSG+Orn
Fluid Intake (g/day)
Concentration (M)
C
MPG
MPG+Orn
Fluid Intake (g/day)
Concentration (M)
D
Intralipos
Intralipos+Orn
Fluid Intake (g/day)
Concentration (%)
0.1
0.3
1
3
E
Sucrose
Sucrose+Orn
Fluid Intake (g/day)
Concentration (M)
0.01
0.03
0.1
0.3
F
NaCl
NaCl+Orn
Fluid Intake (g/day)
Concentration (M)
0.003
0.01
0.03
0.1
G
Citric acid
Citric acid+Orn
Fluid Intake (g/day)
Concentration (M)
0.0003
0.001
0.003
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H
QHCl
QHCl+Orn
Fluid Intake (g/day)
Concentration (M)
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0.00003
0.0001
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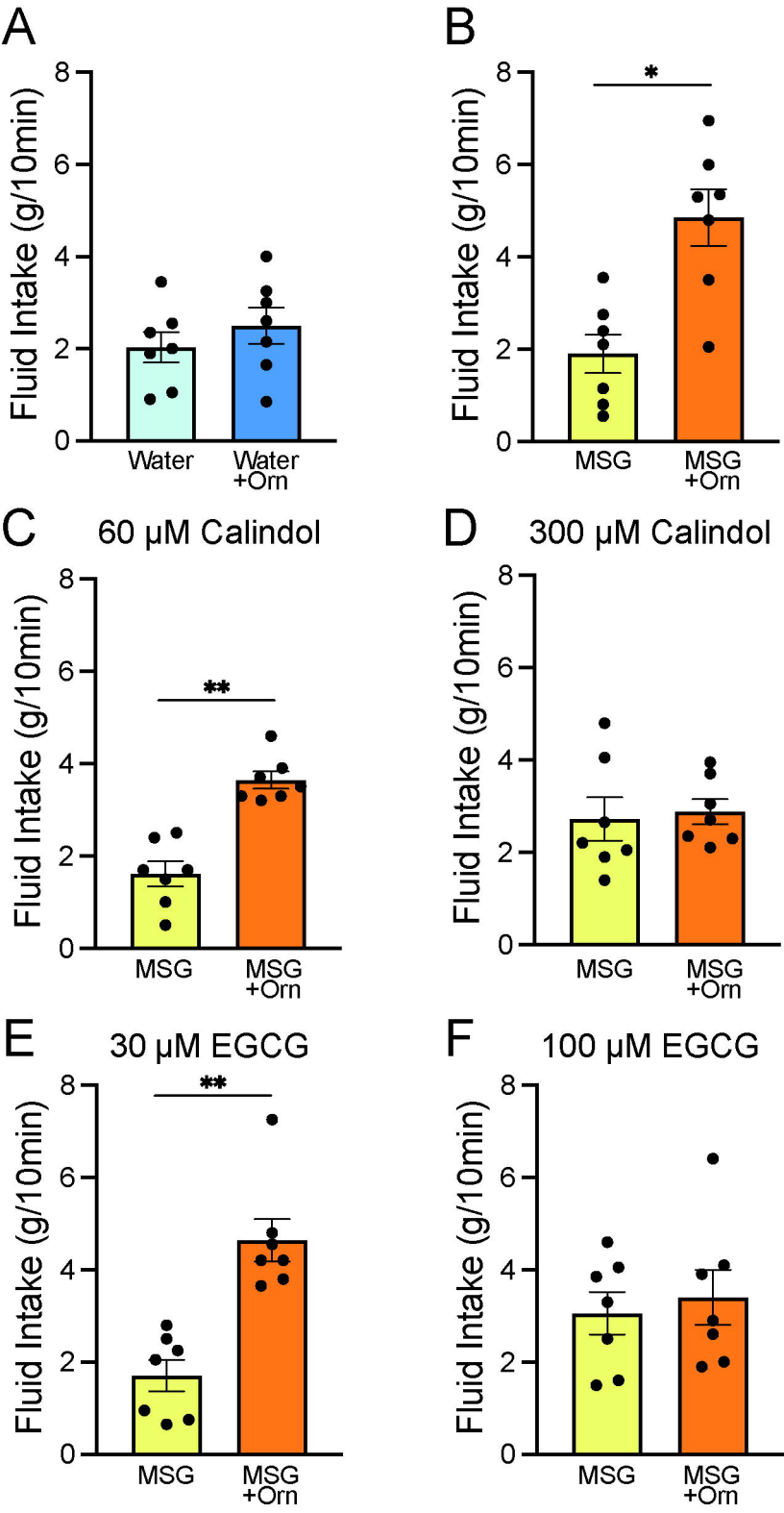
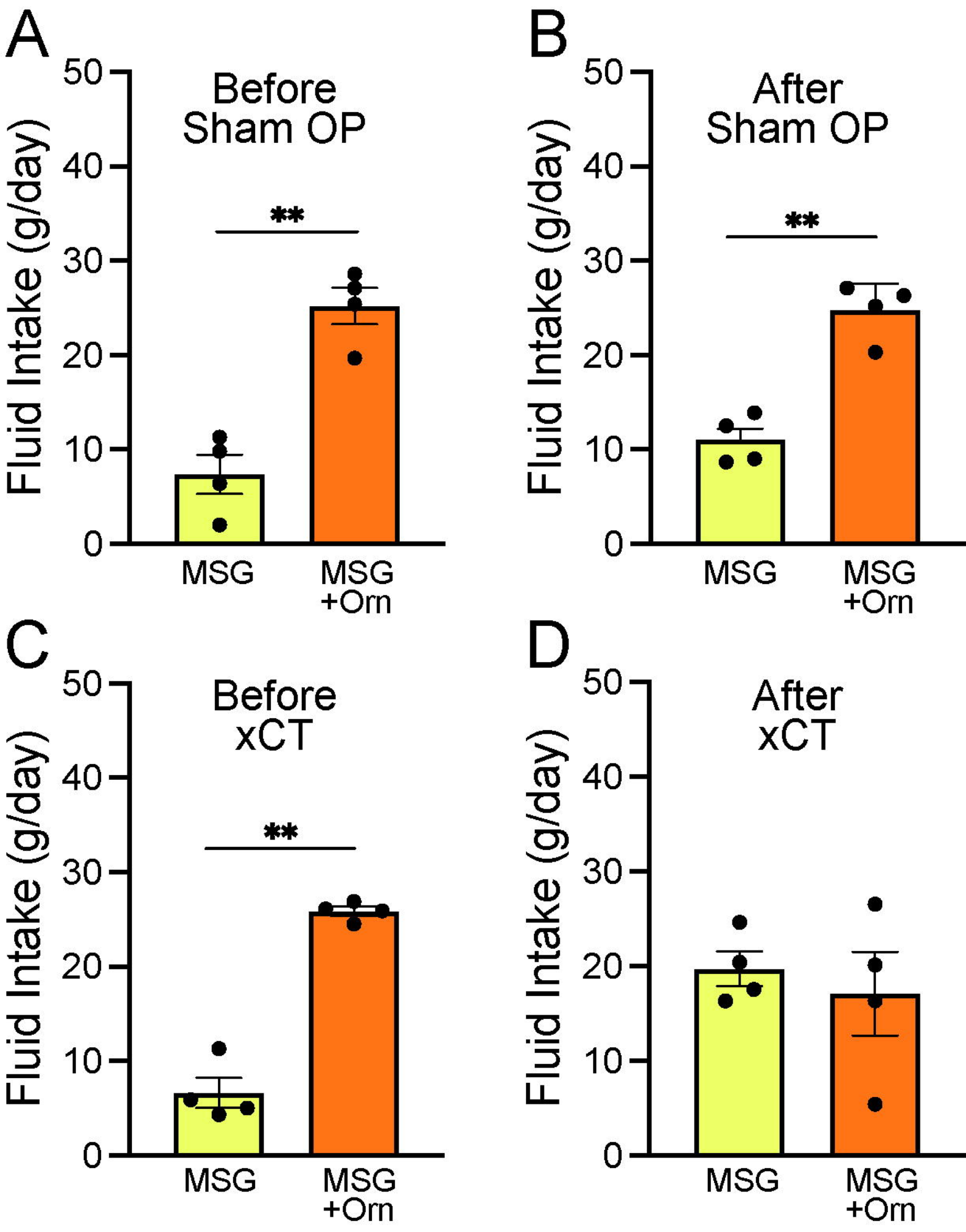
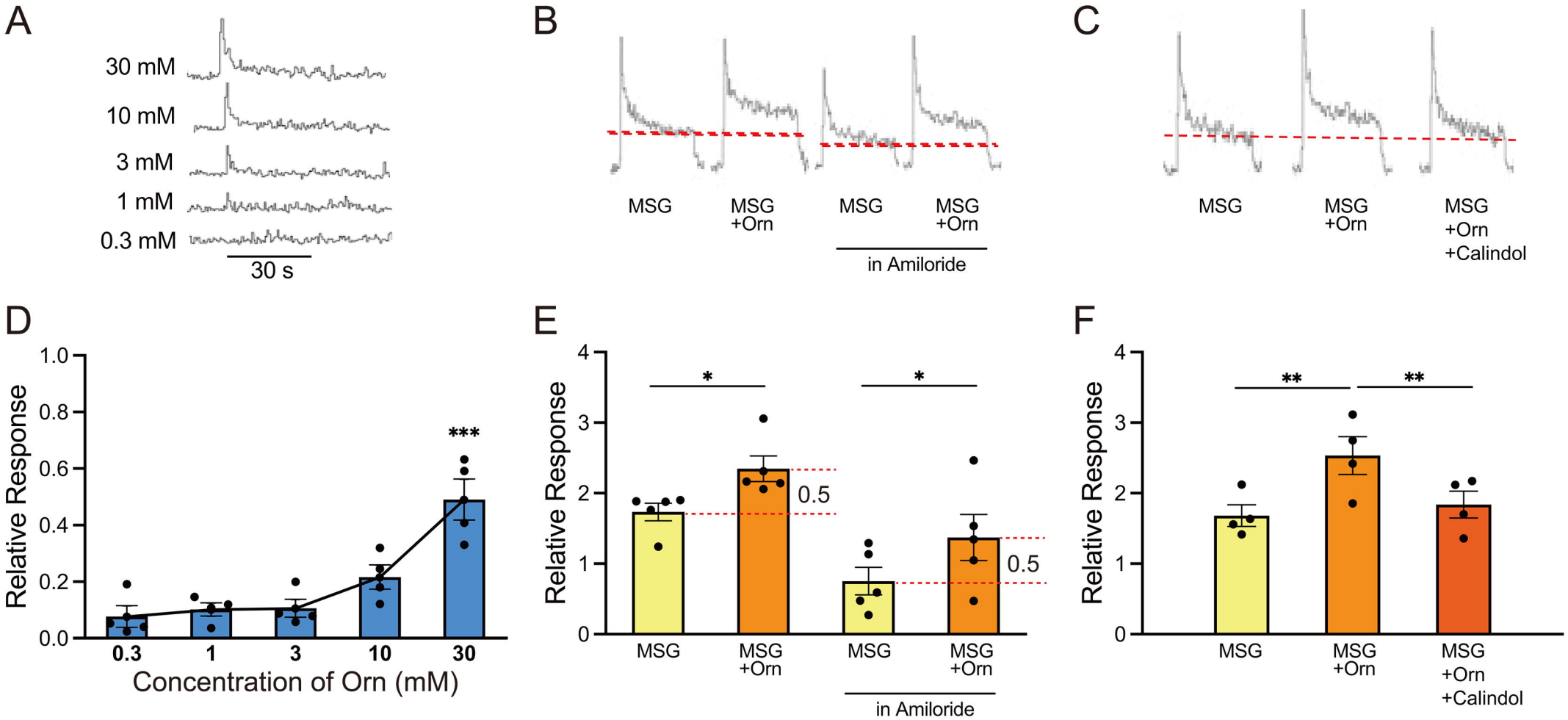
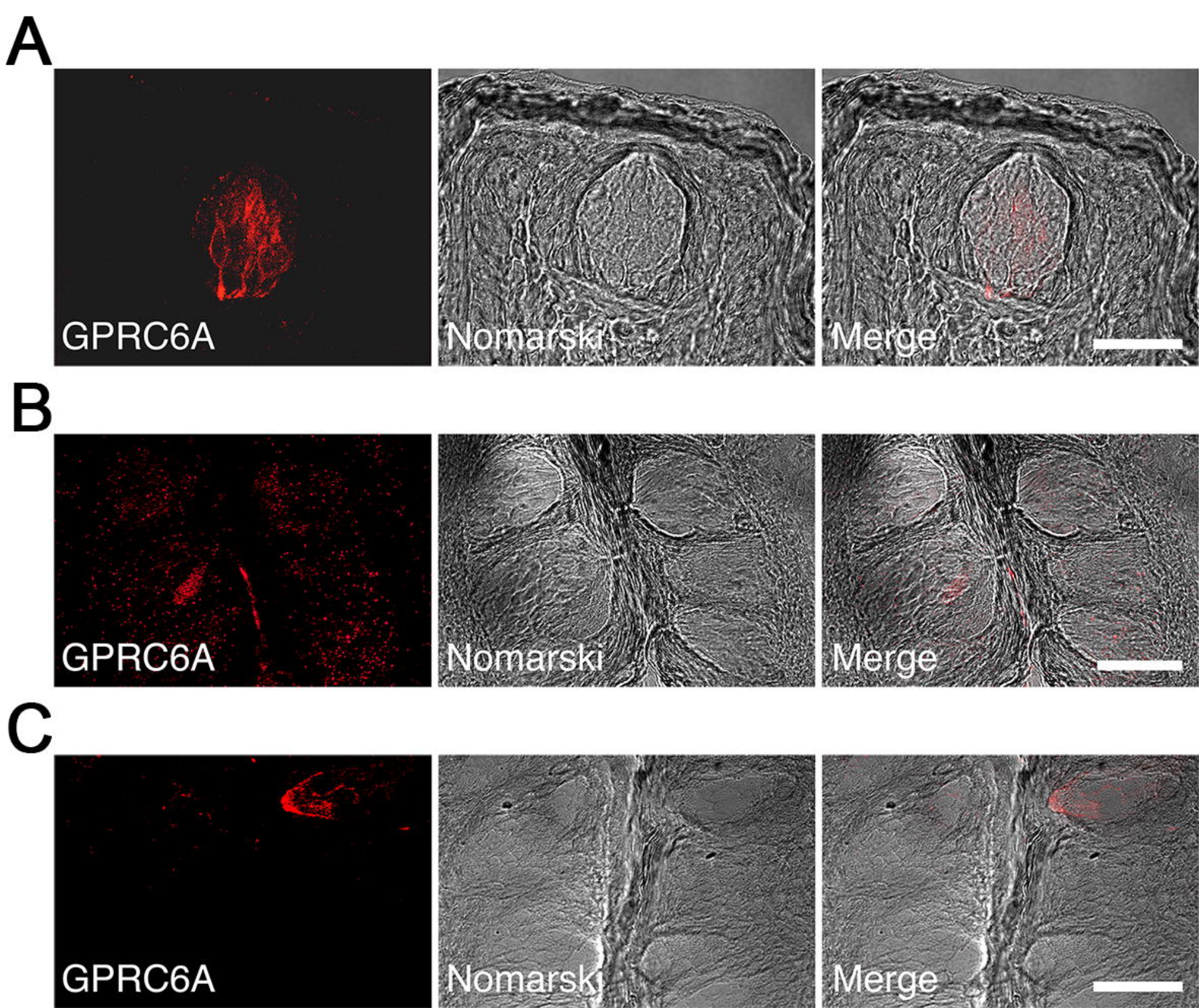
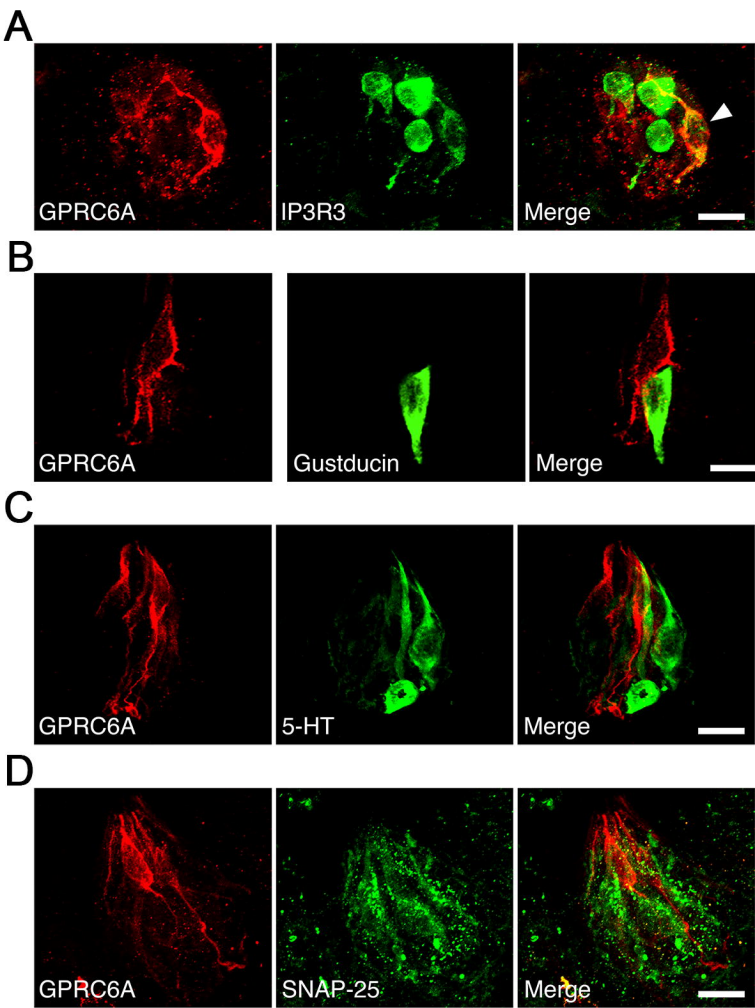

A
Fluid Intake (g/10min)
0
2
4
6
8
Water
Water +Orn
B
*
MSG
MSG +Orn
C
60 μM Calindol
**
D
300 μM Calindol
E
30 μM EGCG
**
F
100 μM EGCG


A
Before
Sham OP
Fluid Intake (g/day)
50
40
30
20
10
0
**
MSG
MSG
+Orn
B
After
Sham OP
Fluid Intake (g/day)
50
40
30
20
10
0
**
MSG
MSG
+Orn
C
Before
xCT
Fluid Intake (g/day)
50
40
30
20
10
0
**
MSG
MSG
+Orn
D
After
xCT
Fluid Intake (g/day)
50
40
30
20
10
0
MSG
MSG
+Orn


A
30 mM
10 mM
3 mM
1 mM
0.3 mM
30 s
B
MSG
MSG +Orn
MSG
MSG +Orn
in Amiloride
C
MSG
MSG +Orn
MSG +Orn +Calindol
D
Relative Response
1.0
0.8
0.6
0.4
0.2
0.0
***
0.3
1
3
10
30
Concentration of Orn (mM)
E
Relative Response
4
3
2
1
0
*
*
0.5
0.5
MSG
MSG +Orn
MSG
MSG +Orn
in Amiloride
F
Relative Response
4
3
2
1
0
**
**
MSG
MSG +Orn
MSG +Orn +Calindol


A
GPRC6A
Nomarski
Merge
B
GPRC6A
Nomarski
Merge
C
GPRC6A
Nomarski
Merge


A
GPRC6A
IP3R3
Merge
B
GPRC6A
Gustducin
Merge
C
GPRC6A
5-HT
Merge
D
GPRC6A
SNAP-25
Merge