Expression profile of the zinc transporter ZnT3 in taste cells of rat circumvallate papillae and its role in zinc release, a potential mechanism for taste stimulation

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Zinc is an essential trace element, and its deficiency causes taste dysfunction. Zinc accumulates in zinc transporter (ZnT)3-expressing presynaptic vesicles in hippocampal neurons and acts as a neurotransmitter in the central nervous system. However, the distribution of zinc and its role as a signal transmitter in taste buds remain unknown. Therefore, we examined the distribution of zinc and expression profiles of ZnT3 in taste cells and evaluated zinc release from isolated taste cells upon taste stimuli. Taste cells with a spindle or pyriform morphology were revealed by staining with the fluorescent zinc dye ZnAF-2DA and autometallography in the taste buds of rat circumvallate papillae. Znt3 mRNA levels were detected in isolated taste buds. ZnT3-immunoreactivity was found in phospholipase-β2-immunopositive type II taste cells and aromatic amino acid decarboxylase-immunopositive type III cells but not in nucleoside triphosphate diphosphohydrolase 2-immunopositive type I cells. Moreover, we examined zinc release from taste cells using human transient receptor potential A1-overexpressing HEK293 as zinc-sensor cells. These cells exhibited a clear response to isolated taste cells exposed to taste stimuli. However, pretreatment with magnesium-ethylenediaminetetraacetic acid, an extracellular zinc chelator - but not with zinc-ethylenediaminetetraacetic acid, used as a negative control - significantly decreased the response ratio of zinc-sensor cells. These findings suggest that taste cells release zinc to the intercellular area in response to taste stimuli and that zinc may affect signaling within taste buds.

Key words: zinc; zinc transporter; taste cell; taste bud; circumvallate papilla; lingual epithelium; taste signaling.

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Introduction

Zinc plays an important role in the sense of taste, and its deficiency causes taste disorders in humans. Zinc treatment has been reported as effective for some taste disorders.1,3 In rats, zinc deficiency is also reported to cause taste disorders by decreasing the number of taste buds.4-6 These results indicate that zinc is essential for the differentiation and proliferation of taste cells. However, the functional role of zinc in taste signaling remains unclear.

Zinc is abundant in the hippocampus of the central nervous system.8 It accumulates with glutamate in presynaptic vesicles of glutamatergic neurons owing to the action of ZnT3, a zinc transporter, and is released via exocytosis.9-11 Zinc is also an allosteric modulator of the P2X2 and N-methyl-D-aspartic acid (NMDA) receptors, which are ATP and glutamate receptors, respectively.12-16 Therefore, zinc is vital to the regulation of signal transduction in the central nervous system.

Taste buds are composed of taste cells, which are classified into types I–IV based on their morphology.17-19 Type I taste cells are glia-like supporting cells involved in the maintenance of taste buds.20-22 Type II taste cells have taste receptors for sweet, bitter, and umami tastants;23-25 ATP,24 5-HT,26 glutamate,27 epinephrine,28 GABA,29 acetylcholine,30 and other compounds act as signaling molecules in taste buds. Among these, ATP is a particularly vital signaling molecule released by type II taste cells in response to sweet, bitter, and umami stimuli to transmit information to type III taste cells or sensory nerve terminals.24,26,31-34 Type III taste cells have the sour receptor OTOP1 for sour tastants, which evoke 5-HT-release from type III taste cells in response to acid stimulation.35-37,39-40 Interestingly, type III taste cells, but not type II cells, store 5-HT in vesicles located at synaptic sites and form typical chemical synapses with afferent nerve fibers.31,38-42 However, whether zinc directly plays a role in the taste signaling of taste buds or acts as a modulator of ATP signaling remains unclear. To address this knowledge gap, we aimed to clarify the presence and localization/expression profile of ZnT3 in the taste buds of rat circumvallate papillae and whether taste cells release zinc upon taste stimulation.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats (total 43 rats, 6-week-old, 200–300 g body weight; Japan SLC, Hamamatsu, Japan) were randomly divided and housed in cages (3–4 rats per cage) with ad libitum access to food and water under a 12-h/12-h light/dark cycle in a controlled environment of approximately 55% relative humidity at 23°C. Rats were fed a normal diet (Oriental Yeast Co., Tokyo, Japan). The sample sizes were 3–5, the minimum number required to perform a significance test. None of the rats were excluded from the analysis. All experiments were performed strictly according to the ARRIVE guidelines as well as the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University and Setsunan University.

The study protocol was authorized by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (reference number: 16-12-005) and Setsunan University (reference number: K19-24).

Detachment of epithelial tissues in circumvallate papillae and isolation of taste buds

SD rats (3 rats per group) were transcardially perfused with saline under deep isoflurane anesthesia. Epithelial tissues containing circumvallate papillae were detached from the tongue by injecting an enzyme mixture comprising 2.5 mg/mL collagenase D, 2.5 mg/mL dispase II, and 1.0 mg/mL trypsin inhibitor (Cat. No.: T9128; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 20°C, and then the epithelial tissues were treated with RNAlater® solution (Sigma-Aldrich) at −20°C until reverse transcription (RT) within 30 min of collection, as reported previously.43 To isolate taste buds, detached epithelial tissues of circumvallate papillae were treated with the enzyme mixture at 37°C for 10 min, and the dispersed taste buds were collected using a glass capillary (World Precision Ins., Sarasota, FL, USA) under an inverted microscope (CKX41; Olympus, Tokyo, Japan).

Live imaging of endogenous zinc in epithelial tissues containing taste buds

SD rats (3 rats per group) were perfused with saline under deep isoflurane anesthesia, and epithelial tissues, including taste buds, were removed from the tongue by treatment with 1.0 mg/mL collagenase D, 2.5 mg/mL dispase II, and 1.0 mg/mL trypsin inhibitor. As negative controls, rats received 0.7 g/kg diethyldithiocarbamate (DEDTC; Sigma-Aldrich) intraperitoneally 80 min before perfusion with saline. DEDTC was used as a chelating agent for Zn2+ and other group IIb metal ions.44 Subsequently, the detached epithelial tissues were treated with Tyrode’s solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 μM d-glucose, 10 mM HEPES, and 10 mM sodium pyruvate) containing 100 mM ZnNaF-2 DA (Enzo Life Sciences, Farmingdale, NY, USA) and 0.04% Pluronic F-127 for 90 min at 23°C, followed by washing in Tyrode’s solution. Fluorescence images were taken using an LSM510 META confocal laser microscope (Carl Zeiss, Jena, Germany).

Autometallography

Autometallography (AMG) was performed as described previously.35,46 Rats (3 per group) were intraperitoneally administered 0.1% sodium selenite (20 mg/kg body weight). After 90 min, the rats were perfused with saline and 3% glutaraldehyde in 0.1 M phosphate buffer under deep anesthesia with isoflurane. The tongue tissues containing circumvallate papillae were post-fixed with 3% glutaraldehyde in a 0.1-M phosphate buffer for 2 h at 4°C and immersed overnight in a 30% sucrose solution at 4°C. Frozen sections (40-μm-thick) were prepared using a cryostat (CM1850; Leica, Wetzlar, Germany) and placed on glass slides (Matsunami Glass, Osaka, Japan). DEDTC was administered intraperitoneally 1 h before the administration of 0.1% sodium selenite as a negative control. Subsequently, tissue sections were incubated in the AMG developer for 3 h at 26°C. The AMG developer contained 30% gum arabic colloid, citrate buffer, 5.7% hydroquinone solution, and 0.73% silver lactate solution. After reaction with the AMG developer, 5% sodium thioulate was added for 10 min, and then the tissue sections were sealed with the EUKITT mounting medium (ORS/Atec, Böblingen, Germany). Images were acquired using a Motomicam1000 (Shimadzu, Kyoto, Japan).

RT-PCR analysis

cDNA was obtained from isolated taste buds using the CellAmp Whole Transcriptome Amplification Kit (Real Time) ver. 2 (Takara, Kusatsu, Japan) according to the manufacturer’s instructions. Total RNA from epithelial tissues containing taste buds was extracted and reverse-transcribed using the NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) and the PrimeScript RT reagent kit with gDNA Eraser (Takara) according to the manufac-
Immunohistochemical analysis

Rats (3 rats per group) were perfused transcardially with saline and 4% paraformaldehyde (Wako, Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4) containing 0.2% picric acid (Wako) under 2% isoflurane deep anesthesia. Tongues containing circumvallate papillae were sectioned at 40 μm using a cryostat (CM1850; Leica). Free-floating sections were incubated with primary antibodies for 3 days at 4°C, followed by incubation for 1 day at 4°C with secondary antibodies, as shown in Table 2. Thereafter, the floating cross-sections were exposed to the guinea pig anti-ZnT3 antibody (1:100) and subsequently washed with PBS. Next, the sections were treated with the secondary antibody Alexa Fluor 488-labeled anti-guinea pig IgG (green, 1:1000). The nuclei were counterstained with Hoechst 33258 (blue, 10 μg/mL). A negative control test was performed by omitting the primary antibodies (data not shown). The anti-ZnT3 antibody for the adsorption test was adsorbed by the immunogen peptide (40 μg peptide per 3 μL antibody, 197-OP, Synaptic Systems, Germany) containing the same epitope sequence to generate the antibody. The cryosections were then treated with the adsorbed antibodies, followed by washing with PBS, and then Alexa Fluor 488-labeled anti-guinea pig IgG (green, 1:1000) was applied as the secondary antibody.

Table 1. Primers and conditions for RT-PCR.

| Gene (Accession No.) | Primer pair | Primer sequences | Annealing temperature (°C) | Cycle number (cycles) | Product size (bp) |
|----------------------|-------------|------------------|----------------------------|----------------------|-----------------|
| Gapdh (NM_017008.4) | 1st         | F 5'-TCATTGACCTGCTCTACTACCTG-3 | 55                        | 38                   | 567 (NM_017008.4) |
|                      |             | R 5'-CGTTCAAGCTCTGCTGAGATGAC-3 |                           |                      |                 |
| Kenq1 (NM_032073)   | 1st         | F 5'-CAGTTGATGACCGGGGATGATG-3 | 52                        | 25                   | 511             |
|                      |             | R 5'-ACATCTGCACTGATGGAGGGG-3   |                           |                      |                 |
|                      |             | R 5'-CTATGCTCAGGAGAACTCTGAC-3 |                           |                      |                 |
|                      |             | R 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
|                      | 2nd         | F 5'-AAGGCCATATCTGGAGCAAGAAGG-3 | 55                        | 15                   | 911             |
|                      |             | R 5'-TCGGAGCTTCTACACGTTTG-3    |                           |                      |                 |
| Ntpdase2 (NM_172030)| 1st         | F 5'-GGGTGACTGCCAACTACCTG-3    | 52                        | 35                   | 624             |
|                      |             | R 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | R 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | R 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
| Plcb2 (NM_053478.1) | 1st         | F 5'-TGCGTCGTGTGCTTCATCTTC-3   | 52                        | 15                   | 511             |
|                      |             | R 5'-CACATCTGATCAGGCACATTCCTC-3 |                           |                      |                 |
|                      |             | R 5'-AGCAATTCCTTCAGATGGCAAC-3  |                           |                      |                 |
|                      |             | R 5'-AGGAGTTGATCTCTGCTGAG-3    |                           |                      |                 |
|                      | 2nd         | F 5'-TGGCAGACATAGGCAGTATGAG-3  | 40                        | 500                  |                 |
|                      |             | F 5'-CGTTCAGCTCTGGGATGAC-3     |                           |                      |                 |
|                      |             | F 5'-TCATTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CGTTCAAGCTCTGCTGAGATGAC-3 |                           |                      |                 |
|                      |             | F 5'-CAGTTGATGACCGGGGATGATG-3  |                           |                      |                 |
|                      |             | F 5'-ACATCTGCACTGATGGAGGGG-3   |                           |                      |                 |
|                      |             | F 5'-CTATGCTCAGGAGAACTCTGAC-3  |                           |                      |                 |
|                      |             | F 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
|                      |             | F 5'-AAGGCCATATCTGGAGCAAGAAGG-3 |                           |                      |                 |
|                      |             | F 5'-TCGGAGCTTCTACACGTTTG-3    |                           |                      |                 |
|                      |             | F 5'-GGGTGACTGCCAACTACCTG-3    |                           |                      |                 |
|                      |             | F 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
|                      | 2nd         | F 5'-TGGCAGACATAGGCAGTATGAG-3  | 40                        | 500                  |                 |
|                      |             | F 5'-CGTTCAGCTCTGGGATGAC-3     |                           |                      |                 |
|                      |             | F 5'-TCATTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CGTTCAAGCTCTGCTGAGATGAC-3 |                           |                      |                 |
|                      |             | F 5'-CAGTTGATGACCGGGGATGATG-3  |                           |                      |                 |
|                      |             | F 5'-ACATCTGCACTGATGGAGGGG-3   |                           |                      |                 |
|                      |             | F 5'-CTATGCTCAGGAGAACTCTGAC-3  |                           |                      |                 |
|                      |             | F 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
|                      |             | F 5'-AAGGCCATATCTGGAGCAAGAAGG-3 |                           |                      |                 |
|                      |             | F 5'-TCGGAGCTTCTACACGTTTG-3    |                           |                      |                 |
|                      |             | F 5'-GGGTGACTGCCAACTACCTG-3    |                           |                      |                 |
|                      |             | F 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CAGTTGACCTGCTCTACTACCTG-3 |                           |                      |                 |
|                      |             | F 5'-CCGGGAATGTGAAACGTCTTG-3   |                           |                      |                 |
|                      |             | F 5'-AAGGCCATATCTGGAGCAAGAAGG-3 |                           |                      |                 |
|                      |             | F 5'-TCGGAGCTTCTACACGTTTG-3    |                           |                      |                 |

Table 2. Antibodies used for immunohistochemistry.

| Antigen | Primary antibody | Secondary Ab |
|---------|------------------|--------------|
| ZnT3    | Guinea pig anti-ZnT3 Ab (1:100; Cat. No.: A11073, Thermo Fisher Scientific) | Goat anti-guinea pig IgG conjugated with Alexa Fluor® 488 (1:1000; Cat. No.: A11073, Thermo Fisher Scientific) |
| NTPDase2| Sheep anti-NTPDase2 Ab (1:1000; Cat. No.: A11073, Thermo Fisher Scientific) | Donkey anti-sheep IgG conjugated with Alexa Fluor® 594 (1:1000; Cat. No.: A11073, Thermo Fisher Scientific) |
| PLC-p2  | Rabbit anti-PLC-p2 Ab (1:1000; sc-206, Santa Cruz Biotechnology) | Goat anti-rabbit IgG conjugated with Alexa Fluor® 546 (1:1000; Cat. No.: A11073, Thermo Fisher Scientific) |
| AADC    | Rabbit anti-AADC Ab (1:50; Cat. No.: A11073, Thermo Fisher Scientific) | Goat anti-rabbit IgG conjugated with Alexa Fluor® 546 (1:1000; Cat. No.: A11073, Thermo Fisher Scientific) |

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The sections were mounted on glass slides and sealed using a Prolong® antifade kit (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence micrographs were obtained using a confocal laser microscope (LSM510 META).

**Detection of zinc using zinc-sensor cells**

We established HEK293T/human transient receptor potential A1 (hTRPA1) stable cells, which overexpressed hTRPA1, as zinc-sensor cells to validate zinc detection (Supplementary Figures 1 and 2). Briefly, the hTRPA1-pF5A vector was generated as follows: an hTRPA1-pF1K vector (pF1KB7348, Product ID FXC07217; Kazusa DNA Res. Inst., Kisarazu, Japan) was digested using SgfI and PmeI (New England Biolabs, Ipswich, MA, USA), and the digested fragment was ligated into the pF5A CMV-neo Flexi vector, which was digested using SgfI and PmeI according to the ligation high ver. 2 manual (TOYOBO, Osaka, Japan). HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA). Transfection of the hTRPA1-pF5A vector into cells was performed using Lipofectamine 2000 (Thermo Fisher Scientific). To establish HEK293T/hTRPA1 stable cells, the transfectants were selected with 3 mg/mL genetin (Thermo Fisher Scientific). The functionality of TRPA1 in stable cells was evaluated by the responses to AITC, a TRPA1 agonist, HC-030031, a TRPA1 antagonist, and Ca2+-free conditions. An increase in the intracellular Ca2+ level was measured using the Fura-2/AM ratio (ex. 340 nm/380 nm, em. 510 nm) with a microplate reader (Wallac 1420; PerkinElmer, Waltham, MA, USA).

HEK293T/hTRPA1 stable cells were used as zinc-sensor cells. Intracellular Ca2+ levels in Fluo-4/AM-treated HEK293T/hTRPA1 stable cells (Dojindo Lab., Kumamoto, Japan) were measured to evaluate zinc release from taste cells following taste stimulation, based on previous reports, demonstrating hTRPA1 activation by zinc. Fluo-4/AM was adopted to distinguish between zinc-sensor cells and isolated taste cells using a confocal laser microscope (LSM510 META). In preliminary experiments, we confirmed the optimum concentrations of tastants that did not activate HEK293T/hTRPA1 stable cells. As taste stimuli, we used the recording medium (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 13.8 mM glucose) containing sweet (2 mM sucrose and 2 mM saccharin sodium), umami (2 mM monosodium glutamate), and bitter (2 μM quinine hydrochloride) tastants. For non-taste stimuli, we treated the cells with the recording medium without tastants. For the evaluation of zinc release from taste cells, HEK293T/hTRPA1 stable cells were cultured on four-well micro-inserts (ibidi GmbH, Gräfelfing, Germany). Furthermore, fluorescence intensity-time curves were constructed using the average fluorescence intensity before adding the taste mixture as 1.0.

**Statistical analysis**

Data are shown as the mean ± SD. Comparisons between two or more groups were performed using one-way ANOVA followed by the Dunnett’s multiple comparison test with the Prism software (version 8; GraphPad Inc., La Jolla, CA, USA). Differences with a p<0.05 were considered statistically significant.

**Results**

**Detection of zinc-derived signals in taste cells of rat circumvallate papillae**

We investigated the localization of endogenous zinc in the taste buds of the circumvallate papillae using ZnAF-2 DA, a zinc-specific fluorescent probe. Fluorescence signals with a guanine or pyrimidin morphology were observed in the taste buds of the epithelial tissue of the circumvallate papillae (Figure 1A, B). By contrast, the fluorescence signal derived from ZnAF-2 DA was clearly attenuated in the taste buds of the circumvallate papillary epithelium pretreated with DEDTC, a zinc and heavy metal ion chelator (Figure 1C). AMG staining revealed the distribution of endogenous heavy metals in the circumvallate papillae epithelium. Partial staining was observed in cells with a spindle or pyriform morphology (Figure 2A). By contrast, AMG staining was not detected in the circumvallate papillary epithelial tissues pretreated with DEDTC (Figure 2B). These results indicate that zinc was localized in the taste cells of taste buds.

**Expression of zinc transporter ZnT3 in type II and III taste cells of rat circumvallate papillae**

Isolated taste buds were collected from rat circumvallate papillae for semi-quantitative RT-PCR analysis. Isolated taste cells and circumvallate papillae were positive for taste cell markers, namely potassium voltage-gated channel, KQT-like subfamily, member 1 (Kcnq1), phospholipase C beta-2 (Plcb2), phospholipase C beta-2 (Plcb2), guanine nucleotide-binding protein, alpha transducing 3 (Gnat3), and aromatic L-amino acid decarboxylase (Aadc), which were used as markers of types I, II, and III; type I; type II; and type III, respectively. Znt3 mRNA was expressed in the isolated taste buds of rat circumvallate papillae (Figure 3).

ZnT3-immunoreactivity was detected in the taste buds of circumvallate papillae using immunohistochemistry; ZnT3-immunopositive cells showed a spindle or pyriform morphology (Figure 4). By contrast, the antigen pre-adsorbed group and negative controls without the anti-ZnT3 antibody did not exhibit fluorescence signals (Figure 4 B, C), demonstrating the specificity of the anti-ZnT3 antibody. These results indicate that ZnT3 is expressed in the taste buds of rat circumvallate papillae.

The localization of ZnT3 in taste buds was assessed using co-immunofluorescence staining with taste cell markers. ZnT3-immunoreactivity partly overlapped with the PLC-β2-signals detected in taste cells with spindle or pyriform morphology (arrowheads in Figure 5B and Supplementary Figure 4B). ZnT3-immunoreactivity was detected in a part of AADC-positive type III cells, with a spindle morphology (arrowheads in Figure 5C and Supplementary Figure 4C), whereas ZnT3-immunoreactivity was not detected in NTPDase 2 type I taste cell marker-positive taste cells (Figure 5A and Supplementary Figure 4A). As shown in Figure 5D, ZnT3-positive cells were observed in type I, II, and III...
taste cells (6.3±1.2%, 47.8±15.4%, and 42.2±15.6%, respectively). These results suggest that ZnT3 is expressed in type II and III taste cells rather than in type I taste cells.

Zinc release from isolated taste cells by taste stimuli

TRPA1 is reportedly activated upon Zn\(^{2+}\) influx through TRPA1 and binding of Zn\(^{2+}\) to its intracellular domain, suggesting that TRPA1-expressing cells are able to detect extracellular zinc. Thus, TRPA1-expressing cells were validated as extracellular zinc-sensor cells (Supplementary Figures 1 and 2). First, in HEK293T/hTRPA1 stable cells, intracellular Ca\(^{2+}\) levels were increased upon treatment with AITC, a TRPA1 agonist. The increase was inhibited by pretreatment with HC-030031, a TRPA1 antagonist, or in Ca\(^{2+}\)-free conditions (Supplementary Figure 1). Next, zinc treatment increased the intracellular Ca\(^{2+}\) levels in HEK293T/hTRPA1 stable cells compared with those in HEK296T cells (mock), whereas Ca\(^{2+}\)-free conditions completely abolished the increase in intracellular Ca\(^{2+}\) levels (Supplementary Figure 2).

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**Figure 1.** Fluorescence images of endogenous zinc in taste cells from the taste buds of rat circumvallate papillae. A) ZnAF-2 DA, a membrane-permeable zinc indicator, was loaded into taste bud-containing epithelial tissues at the circumvallate papillae. B) Higher magnification version of (A). C) Rats were pretreated with DEDTC, a zinc chelator, 80 min before perfusion with saline solution, and then taste bud-containing epithelial tissues were treated with ZnAF-2 DA. D) Higher-magnification version of (C). Data are presented as a representative image of at least three independent experiments. Scale bar: 50 μm.
These results indicate that HEK293T/hTRPA1 stable cells are permeable to zinc without affecting the channel functionality of TRPA1 and are useful zinc-sensor cells for evaluating extracellular zinc levels. We examined whether zinc was released from isolated taste cells by treating zinc-sensor cells with taste mixtures (Figure 6A). When isolated taste cells were treated with a taste mixture, the percentage of zinc-responding cells exhibiting greater than 2-fold increases in intracellular Ca²⁺ level compared with basal levels was 18.00±6.36% (mean ± SD, n=5; Figure 6 B,C; Supplementary Figure 3B). With 100 µM magnesium-ethylenediaminetetraacetic acid (MgEDTA), used as an extracellular zinc chelator, the percentage of zinc-responding cells decreased significantly (8.05±4.46%; mean ± SD, n=3; Figure 6 B,C; Supplementary Figure 3C), whereas 100 µM zinc-ethylenediaminetetraacetic acid (ZnEDTA), which has no zinc-chelating ability, did not affect the response (12.68±3.67%; mean ± SD, n=5; Figure 6 B,C; Supplementary Figure 3D). By contrast, the percentages of zinc-responding cells under conditions of taste mixture treatment alone in the absence of taste cells and non-taste stimulus in the presence of taste cells were 0.11±0.19% and 3.26±3.61% (mean ± SD, n=3),

Figure 2. AMG staining in taste cells from the taste buds of rat circumvallate papillae. A) Distribution of AMG staining in circumvallate papillae is shown; the right panel shows a magnified version of the image in the dotted box; AMG staining was observed in taste cells (arrowheads). B) As a negative control (NC), rats were pretreated with DEDTC 1 h before injecting sodium selenite solution to chelate heavy metals; the right panel shows a magnified version of the image in the dotted box. Data are presented as a representative image of at least three independent experiments. Scale bar: 50 µm
respectively (Figure 6 B,C; Supplementary Figure 3 A,E). Thus, these results suggest that taste cells treated with tastants release zinc into the extracellular space.

**Discussion**

In this study, we sought to elucidate the role of zinc in taste buds. Our key findings are as follows: i) zinc accumulates in the taste cells of rat circumvallate papillae; ii) ZnT3 is expressed in type II and type III taste cells; iii) treating isolated taste cells with tastants induces the activation of hTRPA1, expressed by HEK293T cells; however, this activation is blocked by pretreatment with an extracellular zinc chelator. Thus, we suggest that type II and type III taste cells release zinc into the extracellular space upon stimulation with tastants. In addition, zinc may function as a transmitter or modulator of taste signaling in taste cells.

In the hippocampus, ZnT3, expressed in presynaptic vesicles of glutamatergic neurons, accumulates zinc in presynaptic vesicles. Upon stimulation, as a neurotransmitter, zinc is released into the synaptic cleft through depolarization-induced exocytosis owing to an increase in intracellular Ca²⁺ levels. In the circumvallate papillae, we found that type III taste cells expressed ZnT3 (Figure 5). Because type III taste cells play crucial roles in synaptic transmission via exocytosis of synaptic vesicles, zinc may accumulate in vesicles of ZnT3-positive taste cells and be released via exocytosis as a signaling molecule. Additionally, zinc released from synaptic vesicles activates GPR39, which is expressed in the hippocampus and is an ionic zinc-sensing receptor. However,
whether GPR39 receptors are expressed in gustatory nerve fibers remains to be determined.

Sour tastants induce 5-HT-release from type III taste cells, whereas sweet and bitter tastants indirectly cause 5-HT-release via actions of type III taste cells by ATP release from type II taste cells. In this study, the taste mixture containing sweet, bitter, and umami tastants evoked zinc release from taste cells, and activation of zinc-sensor cells was abolished by treatment with a zinc chelator (Figure 6). Thus, zinc and 5-HT may be released from type III taste cells via taste mixture-evoked ATP release from type II taste cells.

We also found that taste cells released zinc in response to taste stimuli (Figure 6). In taste buds, transient receptor potential melastatin 5 (TRPM5) is expressed in type II taste cells. It participates in regulating ATP release following taste stimuli, such as sweet, bitter, and umami tastants. The released ATP transmits signals by activating P2X2/P2X3 receptors on nerve terminals. Uchida et al. reported that extracellular zinc dose-dependently inhibits TRPM5 activation, suggesting that zinc released from taste cells upon taste stimuli might regulate taste cell depolarization by inhibiting the channel activity of TRPM5. By contrast, zinc is an allosteric modulator that enhances ATP responses via P2X2 receptors. Therefore, it is considered that zinc released from taste cells upon taste stimulation may play a critical role in fine-tuning taste signaling by regulating ATP release and response in taste buds. More detailed investigations are warranted to decipher the relationship with other signaling molecules - not only with ATP - and clarify the roles of zinc in the regulation of taste signaling.

Overall, our findings indicate that ZnT3, expressed by type II and III taste cells, may mediate zinc accumulation in presynaptic vesicles, and taste stimuli-induced zinc release from taste cells may function as a transmitter or modulator for taste signaling. Further experiments using taste cells lacking ZnT3 would clarify the mechanism underlying fine-tuning of taste signaling through zinc released into the extracellular space of taste buds.

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References

1. Atkin-Thor E, Goddard BW, O’Nion J, Stephen RL, Kolff WJ. Hypogeusia and zinc depletion in chronic dialysis patients. Am J Clin Nutr 1978;31:1948-51.
2. Heyneman CA. Zinc deficiency and taste disorders. Ann Pharmacother 1996;30:186-7.

3. Mahajan SK, Frasad AS, Lambujon J, Abbasi AA, Briggs WA, McDonald FD. Improvement of uremic hypoguesia by zinc: a double-blind study. Am J Clin Nutr 1980;33:1517-21.

4. Weismann K, Christensen E, Dreyer V. Zinc supplementation in alcoholic cirrhosis. A double-blind clinical trial. Acta Med Scand 1979;205:361-6.

5. Hewlings S, Kalman D. A Review of zinc-l-carnosine and its positive effects on oral mucositis, taste disorders, and gastrointestinal disorders. Nutrients 2020;12:665.

6. Chau HC, Chien CL, Huang HL, Lu KS. Effects of zinc deficiency on the vallate papillae and taste buds in rats. J Formos Med Assoc 2001;100:326-35.

7. Hamano H, Yoshinaga K, Eta R, Emori Y, Kawasaki D, Iino Y, et al. Effect of polaprezinc on taste disorders in zinc-deficient rats. Biofactors 2006;28:185-93.

8. Jakinovich W, Jr., Osborn DW. Zinc nutrition and salt preference in rats. Am J Physiol 1981;241:R233-9.

9. Perez-Clausell J, Danscher G. Intravesicular localization of zinc in rat telencephalic boutons. A histochemical study. Brain Res 1985;337:91-8.

10. Frederickson CJ, Moncrieff DW. Zinc-containing neurons. Biol Signals 1994;3:127-39.

11. Haug FM. Electron microscopical localization of the zinc in hippocampal mossy fibre synapses by a modified sulfide silver procedure. Histochemistry 1967;8:355-68.

12. Ma B, Ruan HZ, Burnstock G, Dunn PM. Differential expression of P2X receptor on neurons from different parasympathetic ganglia. Neuropharmacology 2005;48:766-77.

13. Takeda A, Sakurada N, Ando M, Kanno S, Oku N. Facilitation of zinc influx via AMPA/kainate receptor activation in the hippocampus. Neurochem In. 2009;55:376-82.

14. Amico-Ruvio SA, Murthy SE, Smith TP, Popescu GK. Zinc effects on NMDA receptor gating kinetics. Biophys J 2011;100:1910-8.

15. Qian J, Noebels JL. Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. J Physiol 2005;566:747-58.

16. Westbrook GL, Mayer ML. Micromolar concentrations of Zn2+ antagonize NMDA and GABA responses of hippocampal neurons. Nature 1987;328:640-3.

17. Farbman AI. Fine structure of the taste bud. J Ultrastruct Res 1965;12:328-50.

18. Takeda M, Hoshino T. Fine structure of taste buds in the rat. Arch Histol Jpn 1975;37:395-413.

19. Yang R, Dzowoy YK, Wilson CE, Russell RL, Kidd GJ, Salcedo E, et al. Three-dimensional reconstructions of mouse circumvallate taste buds using serial blockface scanning electron microscopy: I. Cell types and the apical region of the taste bud. J Comp Neurol 2020;528:756-71.

20. Bartel DL, Sullivan SL, Lavoie EG, Sevigny J, Finger TE. Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds. J Comp Neurol 2006;497:1-12.

21. Dvoryanchikov G, Sinclair MS, Perea-Martinez I, Wang T, Chaudhari N. Inward rectifier channel, ROMK, is localized to the apical tips of glial-like cells in mouse taste buds. J Comp Neurol 2009;517:1-14.

22. Lawton DM, Furness DN, Lindemann B, Hackney CM. Localization of the glutamate-aspartate transporter, GLAST, in rat taste buds. Eur J Neurosci 2000;12:1363-71.

23. Chaudhari N, Roper SD. The cell biology of taste. J Cell Biol 2010;190:285-96.

24. Finger TE, Danilova V, Barrows J, Bartel DL, Vigers AJ, Stone L, et al. ATP signaling is crucial for communication from taste buds to gustatory nerves. Science 2005;310:1495-9.

25. Romanov RA, Rogachevskaya OA, Bystrova MF, Jiang P, Margolskee RF, Kolesnikov SS. Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J 2007;26:657-67.

26. Huang YJ, Maruyama Y, Lu KS, Pereira E, Plonsky I, Baur JE, et al. Mouse taste buds use serotonin as a neurotransmitter. J Neurosci 2005;25:843-7.

27. Vandenbeuch A, Zorec R, Kinnamon SC. Capacitance measurements of regulated exocytosis in mouse taste cells. J Neurosci 2010;30:14695-701.

28. Herness S, Zhao FL, Kaya N, Lu SG, Shen T, Sun XD. Adrenergic signalling between rat taste receptor cells. J Physiol 2002;543:601-14.

29. Dvoryanchikov G, Huang YA, Barro-Soria R, Chaudhari N, Roper SD. GABA, its receptors, and GABAergic inhibition in mouse taste buds. J Neurosci 2011;31:5782-91.

30. Ogura T. Acetylcholine increases intracellular Ca2+ in taste cells via activation of muscarinic receptors. J Neurophysiol 2002;87:2643-9.

31. Taruno A, Vingldeux V, Ohmoto M, Ma Z, Dvoryanchikov G, Li A, et al. CALHM1 ion channel mediates purinergic neurotransmission of sweet, bitter and umami tastes. Nature 2013;495:223-6.

32. DelFazio RA, Dvoryanchikov G, Maruyama Y, Kim JW, Pereira E, Roper SD, et al. Separate populations of receptor cells and presynaptic cells in mouse taste buds. J Neurosci 2006;26:3971-80.

33. Huang YA, Dando R, Roper SD. Autocrine and paracrine roles for ATP and serotonin in mouse taste buds. J Neurosci 2009;29:13909-18.

34. Yang R, Tabata S, Crowley HH, Margolskee RF, Kinnamon JC. Ultrastructural localization of gustducin immunoreactivity in microvilli of type II taste cells in the rat. J Comp Neurol 2006;425:139-51.

35. Liman ER, Kinnamon SC. Sour taste: receptors, cells and circuits. Curr Opin Physiol 2021;20:8-15.

36. Wu Y, Cooper AJ, Teng B, Chang RB, Artiga DJ, Turner HN, et al. An evolutionarily conserved gene family encodes proton-selective ion channels. Science 2018;359:1047-50.

37. Zhang J, Jin H, Zhang W, Ding C, O’Keeffe S, Ye M, et al. Sour sensing from the tongue to the brain. Cell 2019;179:392-402.e15.

38. Teng B, Wilson CE, Tu YH, Joshi NR, Kinnamon SC, Liman ER. Cellular and neural responses to sour stimuli require the proton channel otop1. Curr Biol 2019;29:3647-56.e5.

39. Kinnamon JC, Taylor BJ, Delay RJ, Roper SD. Ultrastructure of mouse vallate taste buds. I. Taste cells and their associated synapses. J Comp Neurol 1985;235:48-60.

40. Fujimoto S, Ueda H, Kagawa H. Immunocytochemistry on the localization of 5-hydroxytryptamine in monkey and rabbit taste buds. Acta Anat (Basel) 1987;128:400-3.

41. Yang R, Dzowoy YK, Wilson CE, Russell RL, Kidd GJ, Salcedo E, et al. Three-dimensional reconstructions of mouse circumvallate taste buds using serial blockface scanning electron microscopy: I. Cell types and the apical region of the taste bud. J Comp Neurol 2020;528:756-71.

42. Bartel DL, Sullivan SL, Lavoie EG, Sevigny J, Finger TE. Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds. J Comp Neurol 2006;497:1-12.

43. Nishida K, Dohi Y, Yamanaka Y, Miyata A, Tsukamoto K, Yabu M, et al. Expression of adenosine A2b receptor in rat type 9.5, and serotonin. J Comp Neurol 2001;440:97-108.

44. Danscher G, Haug FM, Fredens K. Effect of diethyldithiocarbamate (DEDTC) on sulphide silver stained boutons.
Reversible blocking of Timm’s sulphide silver stain for “heavy” metals in DEDTC treated rats (light microscopy). Exp Brain Res 1973;16:521-32.

45. Danscher G. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. Histochemistry 1981;71:1-16.

46. Danscher G, Stoltenberg M. Zinc-specific autometallographic in vivo selenium methods: tracing of zinc-enriched (ZEN) terminals, ZEN pathways, and pools of zinc ions in a multitude of other ZEN cells. J Histochem Cytochem 2005;53:141-53.

47. Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A. Zinc activates damage-sensing TRPA1 ion channels. Nat Chem Biol 2009;5:183-90.

48. Ohmoto M, Matsumoto I, Misaka T, Abe K. Taste receptor cells express voltage-dependent potassium channels in a cell age-specific manner. Chem Senses 2006;31:739-46.

49. Clapp TR, Yang R, Stoick CL, Kinnamon SC, Kinnamon JC. Morphologic characterization of rat taste receptor cells that express components of the phospholipase C signaling pathway. J Comp Neurol 2004;468:311-21.

50. Seto Y, Kataoka S, Toyono T, Toyoshima K. Immunohistochemical localization of aromatic L-amino acid decarboxylase in mouse taste buds and developing taste papillae. Histochem Cell Biol 2007;127:415-22.

51. Besser L, Chorin E, Sekler I, Silverman WF, Atkin S, Russell JT, et al. Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus. J Neurosci 2009;29:2890-901.

52. Huang YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc Natl Acad Sci USA 2007;104:6436-41.

53. Huang YA, Roper SD. Intracellular Ca(2+) and TRPM5-mediated membrane depolarization produce ATP secretion from taste receptor cells. J Physiol 2010;588:2343-50.

54. Murata Y, Yasuo T, Yoshida R, Obata K, Yanagawa Y, Margolskee RF, et al. Action potential-enhanced ATP release from taste cells through hemichannels. J Neurophysiol 2010;104:896-901.

55. Perez CA, Huang L, Rong M, Kozak JA, Preuss AK, Zhang H, et al. A transient receptor potential channel expressed in taste receptor cells. Nat Neurosci 2002;5:1169-76.

56. Liu D, Liman ER. Intracellular Ca2+ and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. Proc Natl Acad Sci USA 2003;100:15160-5.

57. Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, et al. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell 2003;112:293-301.

58. Zhang Z, Zhao Z, Margolskee R, Liman E. The transduction channel TRPM5 is gated by intracellular calcium in taste cells. J Neurosci 2007;27:5777-86.

59. Uchida K, Tominaga M. Extracellular zinc ion regulates transient receptor potential melastatin 5 (TRPM5) channel activation through its interaction with a pore loop domain. J Biol Chem 2013;288:25950-5.

60. Wildman SS, King BF, Burnstock G. Zn2+ modulation of ATP-responses at recombinant P2X2 receptors and its dependence on extracellular pH. Br J Pharmacol 1998;125:1214-20.

61. Xiong K, Peoples RW, Montgomery JP, Chiang Y, Stewart RR, Weight FF, et al. Differential modulation by copper and zinc of P2X2 and P2X4 receptor function. J Neurophysiol 1999;81:2088-94.