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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ABSTRACT

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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Funding: This work was supported by JSPS KAKENHI Grant Number JP18K06673 and The Salt Science Research Foundation (1828).

Contribution: KN, study design, manuscript drafting; KN, SB, AI, TK, experiments performing; AO, KNa, technical assistance; KNa, manuscript editing. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: The authors declare that they have no competing interests, and all authors confirm accuracy.

Data availability: The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

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.[1-5] In rats, zinc deficiency is also reported to cause taste disorders by decreasing the number of taste buds.[6-8] 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.[9] It accumulates with glutamate in presynaptic vesicles of glutamatergic neurons owing to the action of ZnT3, a zinc transporter, and is released *via* exocytosis.[10,11] 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.[26,33,35-38] 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.[32,39-42] 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 tastant 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 dispase II (Cat. No.: 4942078; Roche Applied Science, Tokyo, Japan), 1.0 mg/mL collagenase D (Cat. No.: 1088858; Roche Applied Science), 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 Zn^{2+} 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 $CaCl_2$, 1 mM $MgCl_2$, 10 μM D-glucose, 10 mM HEPES, and 10 mM sodium pyruvate) containing 100 mM ZnAF-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.[45,46] Rats (3 per group) were intraperitoneally administered 0.1% sodium selenium (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 thiosulfate was added for 10 min, and then the tissue sections were sealed with the EUKITT mounting medium (ORSAtec, Bobingen, Germany). Images were acquired using a Moticam1000 (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-

turer's instructions. RT of total RNA from epithelial tissues was carried out at 37°C for 15 min and at 85°C for 5 s. RT-PCR was performed as nested PCR using rTaq DNA polymerase (Takara) for both the 1st and 2nd PCR. The primer sets and PCR conditions are listed in Table 1.

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, 197-0P, 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)		F	5 -TCATTGACCTGAACTACATGGTC-3	55	38	567
		R	5 -CGTTCAGCTCTGGGATGAC-3			
Kcnq1 (NM_032073)	1st	F	5 -CAGTGGATGCAGGGATGATG-3	52	25	511
		R	5 -ACATCTCGCACATCGTAGGG-3			
	2nd	F	5 -CTATGCTGCGGAGAATCCTGAC-3		40	456
		R	5 -CGTGCTTGCTGGAATTTCTTC-3			
Ntpdase2 (NM_172030)	1st	F	5 -GGGTGACTGCCAACTACCTG-3	55	15	911
		R	5 -GACTGTGAAGAGCAGCAGGAG-3			
	2nd	F	5 -CGACTCTATGGCCAGCATTACC-3		38	453
		R	5 -GCAGGTGATCTCTGTGGCTTC-3			
Plcb2 (NM_053478.1)	1st	F	5 -AAGGCATATCTGAGCCAAGG-3	52	25	535
		R	5 -TTGCAAGGTGACAGGCACTG-3			
	2nd	F	5 -GGACGATGAAACCTCAATAGCC-3		40	475
		R	5 -GGCAGCTTCTACACGTTTGC-3			
Gnat3 (NM_173139)	1st	F	5 -TAGTTCAGAGAGCAAGGAGTCAGC-3	55	15	520
		R	5 -CCGGGAATGTAGAACGTCTTG-3			
	2nd	F	5 -AAGAACTGGAGAAGAAGCTTCAGG-3		38	366
		R	5 -AGGCTTGAATTCCTGGATCG-3			
Aadc (NM_012545)	1st	F	5 -CACATCTGATCAGGCACATTCCTC-3	52	40	683
		R	5 -TTAGCCGGAAGCAGACCAAC-3			
	2nd	F	5 -AGCAATTCCTTCAGATGGCAAC-3		40	537
		R	5 -TGAGACAGCTTCACGTGCTTTC-3			
Znt3 (NM_001013243)	1st	F	5 -TGCGTCGTGTGCTTCATCTTC-3	52	40	624
		R	5 -ATCTCGAAGTGTAGGAGCTGTGG-3			
	2nd	F	5 -TGGCAGACATAGGCAGTATGATGG-3		40	500
		R	5 -GCAGATAGAGAAGAGGAAGGTGCTG-3			

Table 2. **Antibodies used for immunohistochemistry.**

Antigen	Primary antibody	Secondary Ab
ZnT3	Guinea pig anti-ZnT3 Ab (1:100; 197 004, Synaptic Systems)	Goat anti-guinea pig IgG conjugated with Alexa Fluor® 488 (1:1000; Cat. No.: A11073, Thermo Fisher Scientific)
NTPDase2	Sheep anti-NTPDase2 Ab (1:200; AF5797, R&D Systems)	Donkey anti-sheep IgG conjugated with Alexa Fluor® 594 (1:1000; Cat. No.: A11016, Thermo Fisher Scientific)
PLC-β2	Rabbit anti-PLC-β2 Ab (1:1000; sc-206, Santa Cruz Biotechnology)	Goat anti-rabbit IgG conjugated with Alexa Fluor® 546 (1:1000; Cat. No.: A11010, Thermo Fisher Scientific)
AADC	Rabbit anti-AADC Ab (1:50; BML-AZ1030-0050, Enzo)	Goat anti-rabbit IgG conjugated with Alexa Fluor® 546 (1:1000; Cat. No.: A11010, Thermo Fisher Scientific)

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 (LSM510META).

Detection of zinc using zinc-sensor cells

We established HEK293T/human transient receptor potential A1 (h*TRPA1*) 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/h*TRPA1* stable cells, the transfectants were selected with 3 mg/mL geneticin (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 Ca^{2+}-free conditions. An increase in the intracellular Ca^{2+} level was determined 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/h*TRPA1* stable cells were used as zinc-sensor cells. Intracellular Ca^{2+} levels in Fluo-4/AM-treated HEK293T/h*TRPA1* stable cells (Dojindo Lab., Kumamoto, Japan) were measured to evaluate zinc release from taste cells following taste stimulation, based on previous reports,[47] 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 (LSM510META). In preliminary experiments, we confirmed the optimum concentrations of tastants that did not activate HEK293T/h*TRPA1* stable cells. As taste stimuli, we used the recording medium (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 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 stimulus, we treated the cells with the recording medium without tastants. For the evaluation of zinc release from taste cells, HEK293T/h*TRPA1* stable cells were cultured on four-well micro-inserts (ibidi GmbH, Gräfelfing, Germany) for 24 h, washed with the recording medium, and treated with 5 μM Fluo-4/AM for 1 h at 37°C. As a condition for the absence or presence of taste cells, taste mixture was injected to Fluo-4/AM-loaded HEK293T/hTRPA1 stable cells (Supplementary Figure 3 A,B, respectively). As a condition for the presence of 100 μM MgEDTA or 100 μM ZnEDTA, taste mixture was injected to taste cell-seeded Fluo-4/AM-loaded HEK293T/hTRPA1 stable cells (Supplementary Figure 3 C,D, respectively). As a condition for no taste stimulation, the recording medium was injected to taste cell-seeded Fluo-4/AM-loaded HEK293T/hTRPA1 stable cells (Supplementary Figure 3E). Cells were washed again with the recording medium and imaged under a confocal laser microscope (LSM510 META) at an excitation wavelength of 488 nm. One minute after the start of the measurement, the taste mixture was added, and the fluorescence intensity of Fluo-4 was measured every 2 s for 6 min. The fluorescence intensity of each cell type was calculated based on the obtained image data using the LSM 510 software (Carl Zeiss, Oberkochen, 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. Fluorescent signals with a spindle or pyriform morphology were observed in the taste buds of the epithelial tissue of the circumvallate papillae (Figure 1 A,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 1 C,D). 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 located 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*),[48] nucleoside triphosphate diphosphohydrolase 2 (*Ntpdase2*),[20] phospholipase C beta-2 (*Plcb2*),[49] guanine nucleotide-binding protein, alpha transducing 3 (*Gnat3*),[34] and aromatic L-amino acid decarboxylase (*Aadc*),[50] which were used as markers of types I, II, and III; type I; type II; 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,[47] 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/h*TRPA1* 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/h*TRPA1* 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).

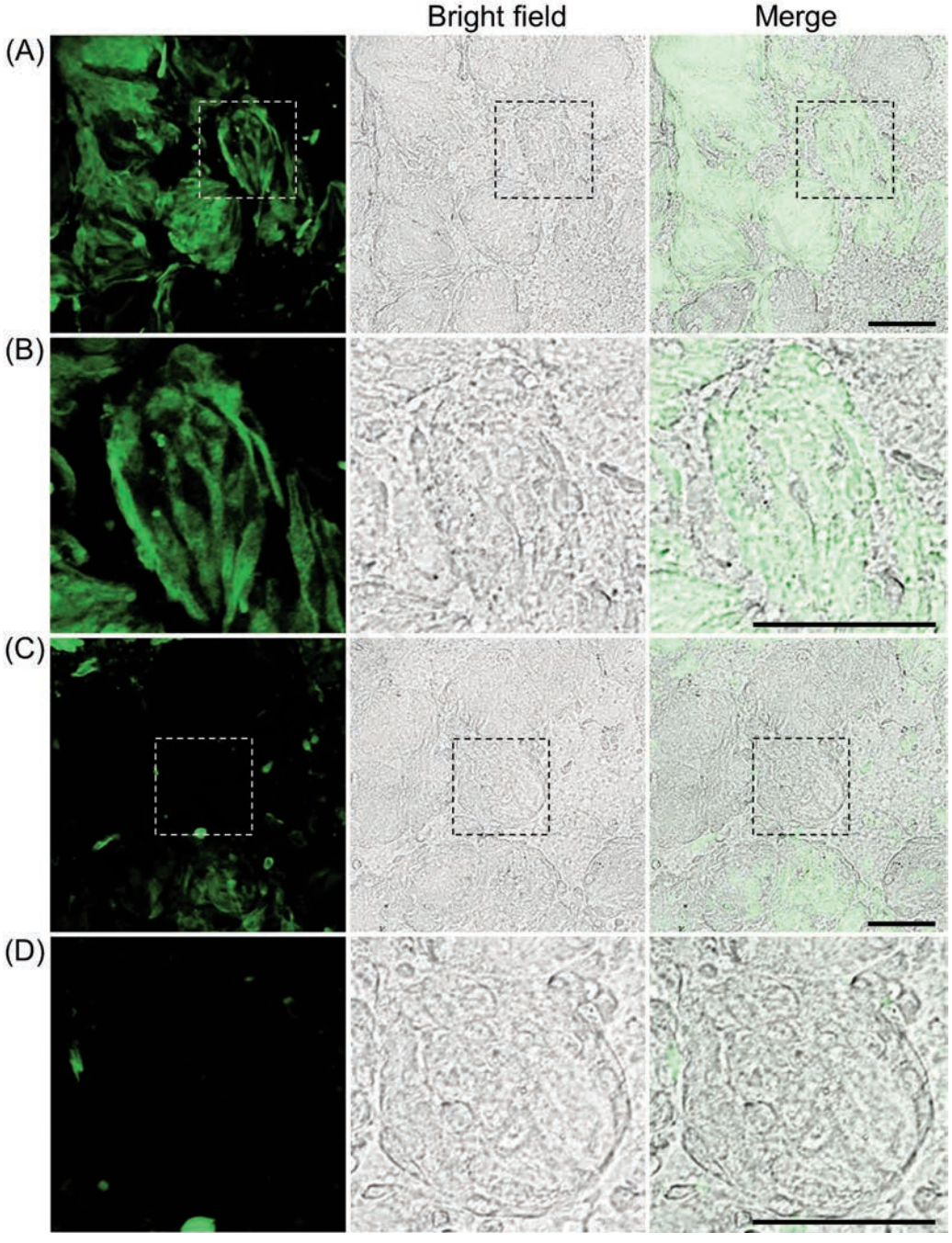

Figure 1. Fluorescence images of endogenous zinc in taste cells from the taste buds of rat circumvallate papillae. A) ZnAF-2 DA, a membrane-permeable zinc indicator, was loaded into taste bud-containing epithelial tissues at the circumvallate papillae. B) Higher magnification version of (A). C) Rats 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/h*TRPA1* 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 Ca^{2+} 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),

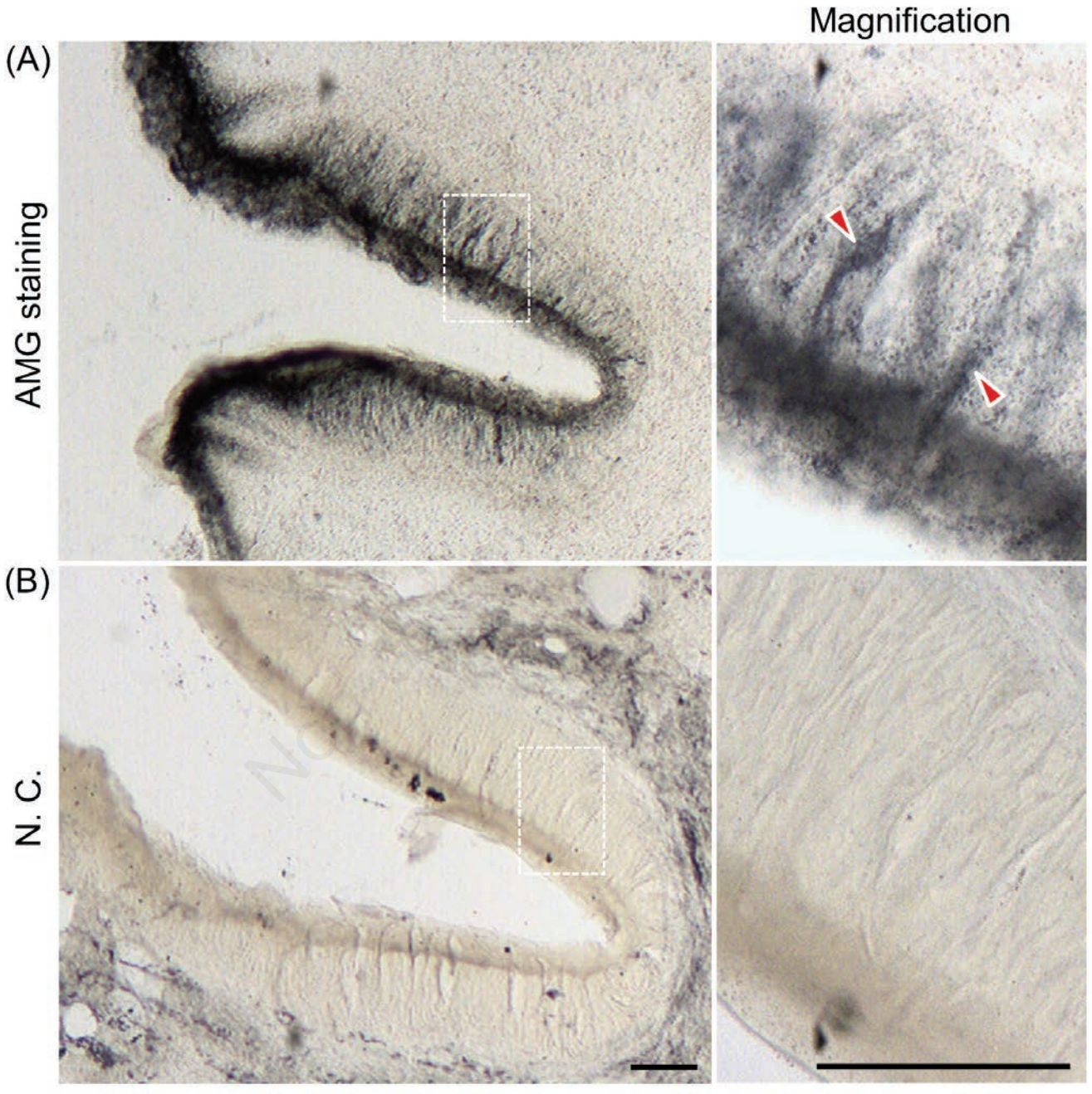

Figure 2. AMG staining in taste cells from the taste buds of rat circumvallate papillae. A) Distribution of AMG staining in circumvallate papillae is shown; the right panel shows a magnified version of the image in the dotted box; AMG staining was observed in taste cells (arrowheads). B) As a negative 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.

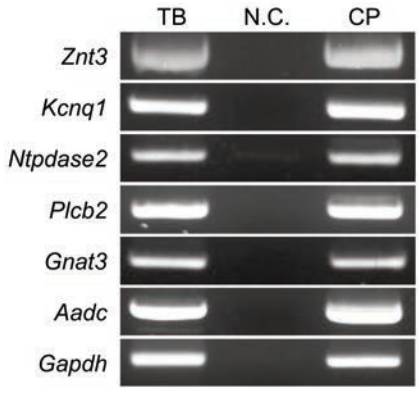

Figure 3. mRNA expression of *Znt3* in isolated taste buds. *Znt3* expression was examined using semi-quantitative RT-PCR. TB, NC, and CP indicate isolated taste buds, negative control (H_2O; template in place of the cDNA), and taste bud-containing epithelial tissues (circumvallate papillae), respectively. Data are presented as a representative image of three independent experiments. *Kcnq1, Ntpdase2, Plcb2, Gnat3*, and *Aadc* were used as taste cell markers of types I, II, and III; type I; type II; type II; and type III, respectively.

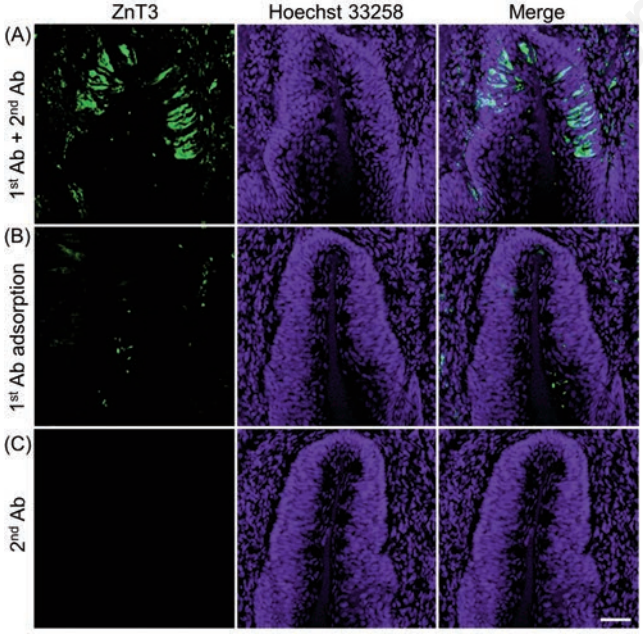

Figure 4. Immunohistochemical analysis of ZnT3 expression in the cross-sections of rat circumvallate papillae. A) Representative laser scanning microscopy images of immunohistochemistry with ZnT3 in the circumvallate papillae; the nuclei were counterstained with Hoechst 33258 (blue, 10 μg/mL). B) Immunostaining with an anti-ZnT3 antibody (1st Ab) adsorbed by the immunogen peptide containing the same epitope sequence to generate the antibody. C) The 2nd antibody was used alone as a negative control. Data are presented as a representative image of three independent experiments. Scale bar: 50 μm.

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 Ca^{2+} levels.[10,13] 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,[27,32] 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.[51] However,

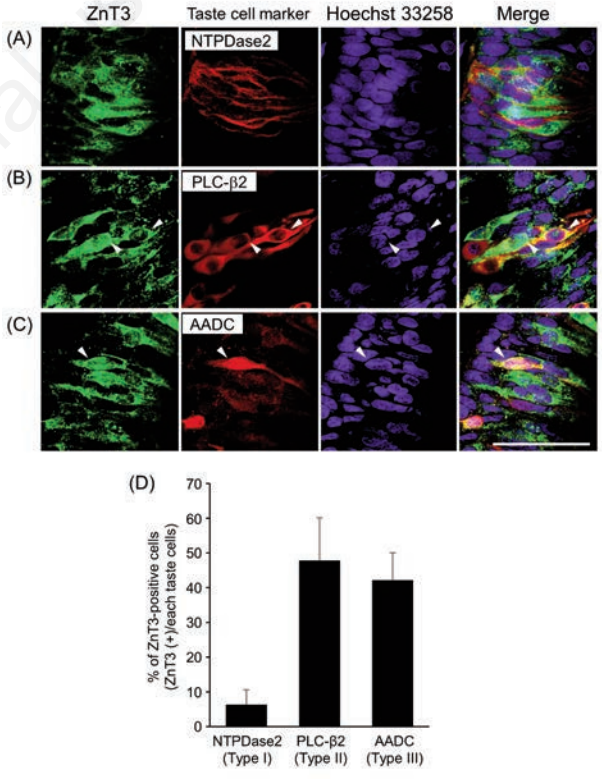

Figure 5. Immunohistochemical localization of ZnT3 and NTPDase2, PLC-β2, or AADC in cross-sections cut through the circumvallate papillae. Representative photomicrographs for double staining of ZnT3 (green) and the type I cell marker NTPDase2 (A; red), type II cell marker PLC-β2 (B; red), or type III cell marker AADC (C; red) are shown. Arrowheads show the colocalization of ZnT3 and taste cell markers in the cell bodies of taste cells. Data are presented as a typical image of three independent experiments. Scale bar: 50 μm. Immunopositive rates of ZnT3 in NTPDase2-, PLC-beta2-, and AADC-positive cells (D). Each value represents the mean ± SD (n=3).

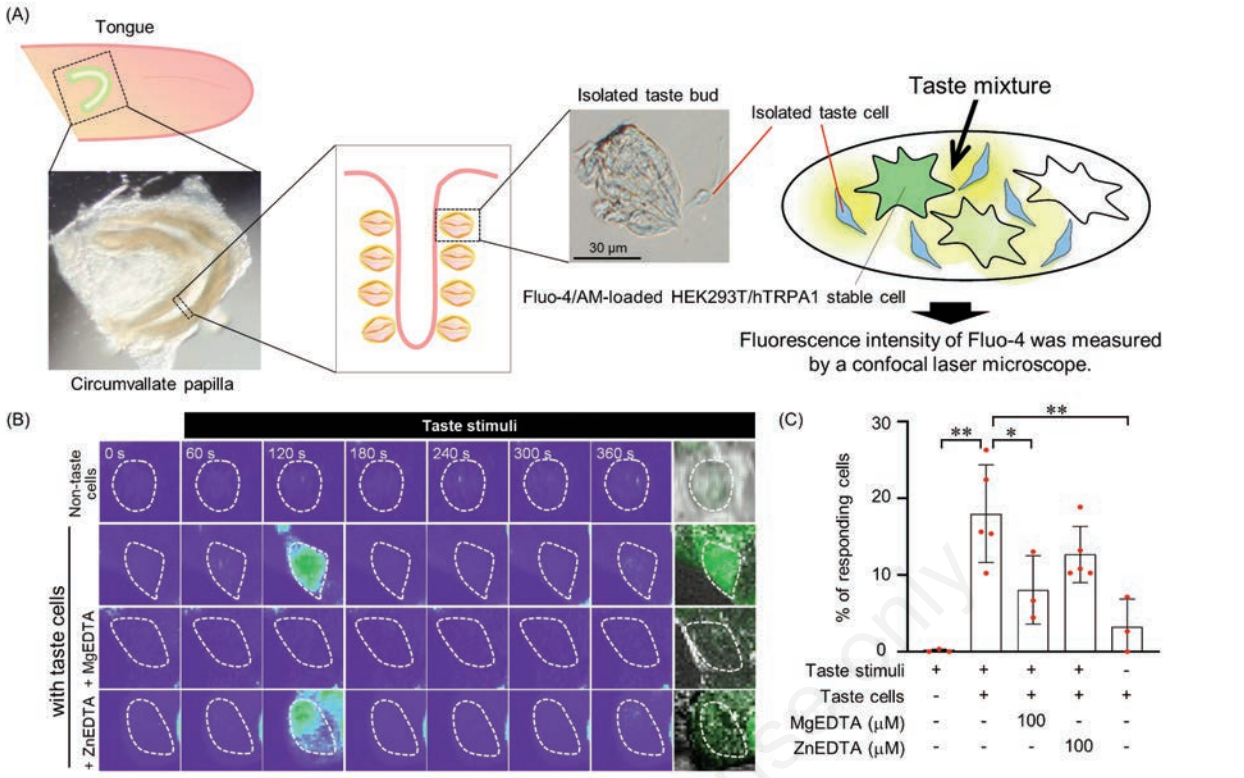

Figure 6. Zinc release from isolated taste cells upon taste stimulation. A) Schematic overview of the experiment to detect released zinc from isolated taste cells is shown. B) Representative images of Fluo-4/AM-loaded HEK293T/h*TRPA1* stable (zinc-sensitive) cells are shown on the rainbow scale; the white dotted circle shows Fluo-4/AM-loaded HEK293T/h*TRPA1* stable cells. C) Summary of the percentage of responding cells. Fluo-4/AM-loaded HEK293T/hTRPA1 stable cells were considered responders when $[Ca^{2+}]_i$ increased more than 2-fold from the basal $[Ca^{2+}]_i$ levels before taste stimuli. Each bar represents mean ± SD (n=3–5). *p<0.05; **p<0.01.

whether GPR39 receptors are expressed in gustatory nerve fibers remains to be determined.

Sour tastants induce 5-HT-release from type III taste cells,[26,33] 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.[52] 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.[31,53-58] The released ATP transmits signals by activating P2X2/P2X3 receptors on nerve terminals.[24] Uchida *et al.* reported that extracellular zinc dose-dependently inhibits TRPM5 activation,[59] 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.[12,60,61] 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.

Acknowledgments

The authors thank Ms. Saki Matsumoto, a member of the Environmental Biochemistry laboratory (Kyoto Pharmaceutical University, Kyoto, Japan), and Ms. Monami Miyazaki, a member of the Integrative Pharmaceutical Sciences laboratory (Setsunan University, Osaka, Japan), for their help and support with the experiments associated with this research. The authors thank Editage (Cactus Communications, Tokyo, Japan), a professional English proofreading service, for editing the manuscript.

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Received for publication: 16 August 2022. Accepted for publication: 28 October 2022.

European Journal of Histochemistry 2022; 66:3534
doi:10.4081/ejh.2022.3534