

## Expression of Vesicular Nucleotide Transporter in Rat Odontoblasts

Erina Ikeda<sup>1</sup>, Tetsuya Goto<sup>2</sup>, Kaori Gunjigake<sup>1</sup>, Kayoko Kuroishi<sup>1</sup>, Masae Ueda<sup>1</sup>,  
Shinji Kataoka<sup>3</sup>, Takashi Toyono<sup>3</sup>, Mitsushiro Nakatomi<sup>3</sup>, Yuji Seta<sup>3</sup>, Chiaki Kitamura<sup>4</sup>,  
Tatsuji Nishihara<sup>5</sup> and Tatsuo Kawamoto<sup>1</sup>

<sup>1</sup>Division of Orofacial Functions and Orthodontics, Kyushu Dental University, <sup>2</sup>Department of Oral Anatomy and Cell Biology, Kagoshima University, 8–35–1, Sakuragaoka, Kagoshima 890–8544, Japan, <sup>3</sup>Division of Anatomy, Kyushu Dental University, <sup>4</sup>Division of Endodontics and Restorative Dentistry, Kyushu Dental University and <sup>5</sup>Division of Infections and Molecular Biology, Kyushu Dental University, 2–6–1, Manazuru, Kokurakita-ku, Kitakyushu 803–8580, Japan

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Several theories have been proposed regarding pain transmission mechanisms in tooth. However, the exact signaling mechanism from odontoblasts to pulp nerves remains to be clarified. Recently, ATP-associated pain transmission has been reported, but it is unclear whether ATP is involved in tooth pain transmission. In the present study, we focused on the vesicular nucleotide transporter (VNUT), a transporter of ATP into vesicles, and examined whether VNUT was involved in ATP release from odontoblasts. We examined the expression of VNUT in rat pulp by RT-PCR and immunostaining. ATP release from cultured odontoblast-like cells with heat stimulation was evaluated using ATP luciferase methods. VNUT was expressed in pulp tissue, and the distribution of VNUT-immunopositive vesicles was confirmed in odontoblasts. In odontoblasts, some VNUT-immunopositive vesicles were colocalized with membrane fusion proteins. Additionally P2X<sub>3</sub>, an ATP receptor, immunopositive axons were distributed between odontoblasts. The ATP release by thermal stimulation from odontoblast-like cells was inhibited by the addition of siRNA for VNUT. These findings suggest that cytosolic ATP is transported by VNUT and that the ATP in the vesicles is then released from odontoblasts to ATP receptors on axons. ATP vesicle transport in odontoblasts seems to be a key mechanism for signal transduction from odontoblasts to axons in the pulp.

**Key words:** VNUT, ATP, pain, odontoblast, pain transmission

### I. Introduction

In addition to the specific role of odontoblasts in dentin formation, odontoblasts located in the outermost cellular layer of dental pulp also function as sensory receptor cells in pain transmission in teeth [22]. Previously, several hypotheses have been proposed regarding the mechanism(s) of dental nociception [4, 18], such as the neural theory [30], hydrodynamic theory [2, 19], and odontoblast transducer theory. However, the signaling mechanism from

odontoblasts to pulp nerves has remained unclear.

Recent studies have suggested that transient receptor potential (TRP) channels in odontoblasts or pulp nerve endings around odontoblasts are involved in the development of dental pain [6, 7, 31]. However, the exact nature of the signaling between odontoblasts and axons in the pulp near odontoblasts remains unknown.

Although adenosine triphosphate (ATP) was originally characterized as a molecule for intracellular energy transfer, more recently ATP has been found to play roles in intracellular and extracellular signaling, including as a neurotransmitter [3, 10, 32]. For extracellular signaling, ATP is released from cells through channels or gap junctions [8, 17, 33]. Furthermore, some reports have indicated the

Correspondence to: Tetsuya Goto, D.D.S., Ph.D., Department of Oral Anatomy and Cell Biology, Kagoshima University, 8–35–1 Sakuragaoka, Kagoshima 890–8544, Japan. E-mail: tgoto@dent.kagoshima-u.ac.jp

involvement of ATP in signaling from odontoblasts to pulp nerves [5, 28]. However, the mechanism of ATP release from odontoblasts remains controversial.

Recently, vesicular nucleotide transporter (VNUT) was identified as an ATP transporter from the cytoplasm into vesicles for subsequent ATP secretion in purinergic signal transmission [27]. Since then, VNUT has been found in the taste cells of taste buds [14], in microglia [12], and in dorsal root ganglion neurons [24]. There is also evidence that P2X<sub>3</sub> receptors, which are ATP receptors, are present in the nerve fibers of the odontoblast layer near dental pulp [1, 15, 26], and that NTPDase (an ATP-degrading enzyme) is expressed in dental nerve fibers around the odontoblast layer [20]. Thus, ATP signaling would be involved in pain transmission in teeth, but it is unclear whether or how ATP is released from odontoblasts.

In the present study, to investigate the involvement of VNUT in ATP signal transduction between odontoblasts and pulp nerves, we first examined the expression and the localization of VNUT in odontoblasts *in vivo* and *in vitro*. We then confirmed the involvement of VNUT in the release of ATP from odontoblast-like cells under heat stress. Our data contribute to understanding ATP signal transduction in pain transmission in teeth.

## II. Materials and Methods

### *Animals*

Male Sprague-Dawley (SD) rats weighing 150–200 g were used. Five male rats were habituated to the testing paradigms for 7 days before data collection. Animals had free access to food and water during experiments and were maintained in a temperature-controlled room (23°C) with a 12/12-hr light/dark cycle. The experimental protocol was reviewed and approved by the Kyushu Dental University Animal Care Committee.

### *Tissue preparation*

Rats were anesthetized with diethylether and perfused transcardially with 4% paraformaldehyde in 0.2 M phosphate buffer (PFA, pH 7.4) containing 0.2% picric acid [9]. Maxillary bones, including upper molars, were then removed, post-fixed with PFA overnight, decalcified with EDTA for 2 weeks and then dehydrated through ascending graded sucrose solutions (10, 20 and 30%). Maxillary bones were rapidly frozen, cut into 6- $\mu$ m-thick serial sections with a cryostat (Leica Instruments, Wetzlar, Germany), and mounted on gelatin/chrome alum-coated glass slides [13].

### *Cell culture*

A rat clonal odontoblast-like cell line, KN-3, cloned by Noguchi *et al.* [25], was seeded in 35-mm culture dishes ( $3 \times 10^5$  cells/dish) and cultured with minimal essential medium, Eagle's alpha modification (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS)

(Cellgro; Mediatech Inc., Herndon, VA, USA), 100 mg/mL streptomycin (Sigma-Aldrich), and 100 U/mL penicillin (Sigma-Aldrich) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Odontoblastic phenotype of the cells was confirmed by the expression of nestin, a marker of odontoblasts.

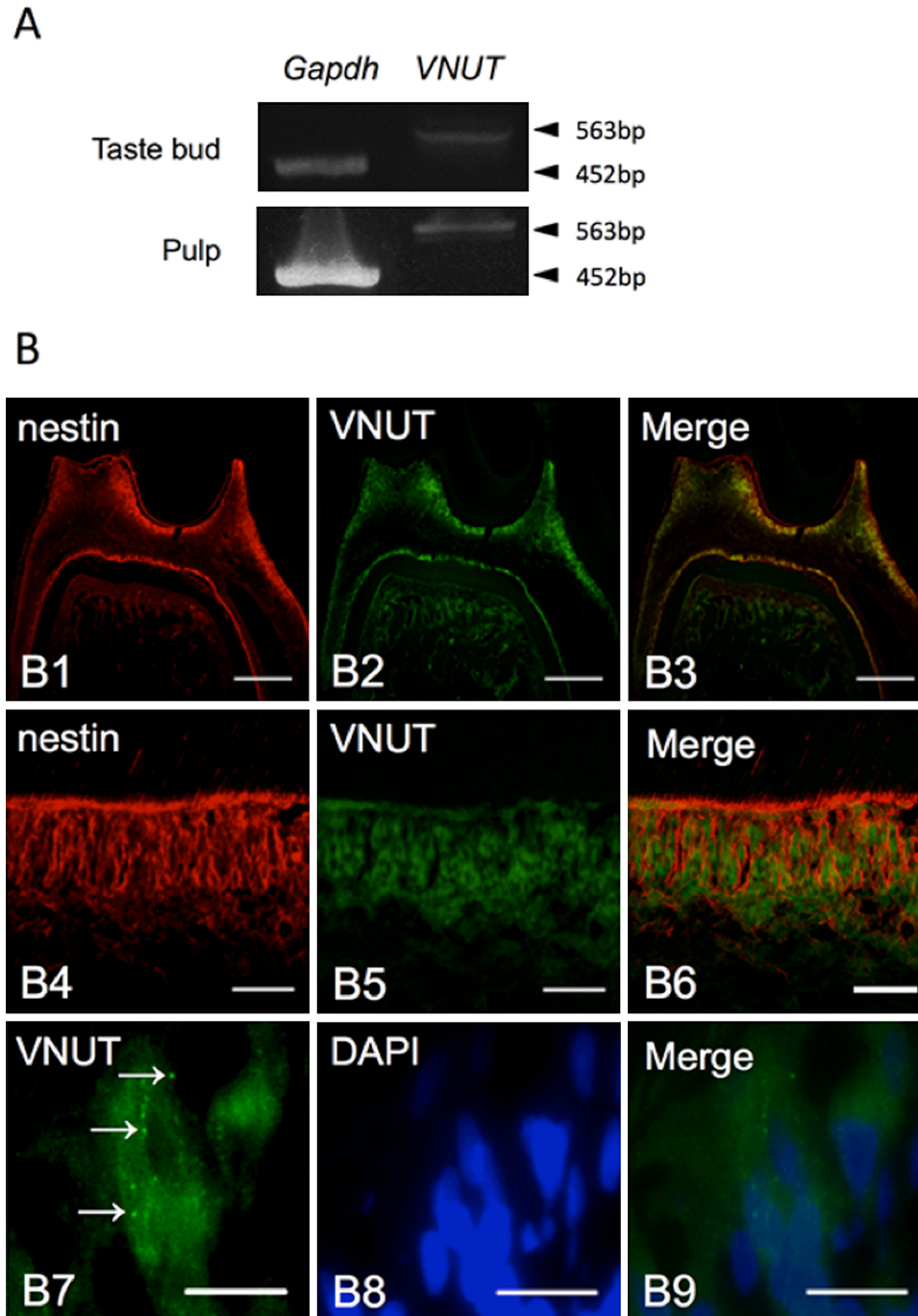
### *Immunohistochemistry*

Antibody against rat VNUT was generated in rabbits using synthetic peptides corresponding to residues 5–19, RSSLMQPIPEETRKT. Specificity of the VNUT antibody in odontoblasts was confirmed by its preabsorption with the peptide (data not shown). Double labeling for VNUT with nestin (an odontoblast marker) or Snap25 (a marker of membrane fusion proteins), and double labeling for neurofilament 200 (NF200, a marker of nerve fibers) with P2X<sub>3</sub> (an ATP receptor) were performed. First, the sections were pre-treated with 0.1% Triton X-100 for 10 min at room temperature and pre-incubated with Blocking One (1:20; Nacalai, Tesque Inc., Kyoto, Japan) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 20 min at 37°C. The sections were then incubated with mouse monoclonal antibodies against nestin (1:100; Millipore, Billerica, MA, USA), rabbit polyclonal antibodies against Snap25 (1:500; Sigma-Aldrich), or rabbit polyclonal antibodies against NF200 (1:1000; Sigma-Aldrich) for 2 hr at 37°C. After rinsing with 0.1 M PBS, the sections were incubated with goat anti-mouse IgG 568 (1:400; Invitrogen, Carlsbad, CA, USA) or goat anti-rabbit IgG 488 (1:400; Invitrogen) for 2 hr at 37°C. After rinsing again with 0.1 M PBS, the sections were incubated with rabbit polyclonal antibodies against VNUT (1:100) or guinea pig polyclonal antibodies against P2X<sub>3</sub> (1:250; Neuromics, Edina, MN, USA) for 2 hr at 37°C, washed in PBS, and incubated with goat anti-rabbit IgG 488 (1:400; Invitrogen) or goat anti-guinea pig IgG 546 (1:400; Invitrogen) for 2 hr at 37°C. After rinsing again with 0.1 M PBS, the sections were incubated with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) for 5 min at room temperature. Negative control sections were incubated with secondary antibody in buffer alone. Finally, the sections were washed in 0.1 M PBS and covered with coverslips. Samples were examined under a fluorescence microscope (Keyence, Osaka, Japan).

### *Reverse transcription-polymerase chain reaction (RT-PCR) analysis*

#### *1. Tissue preparation*

Maxillary first molars and taste buds were obtained from the SD rats. The teeth were separated, and the pulps were isolated. Total RNA was extracted from freshly excised pulps and taste buds using RNAqueous (Ambion; Life Technologies, Austin, TX, USA) according to the manufacturer's protocol. cDNA was synthesized from 2.0  $\mu$ g of total RNA in 30  $\mu$ L of reaction buffer composed of 500  $\mu$ M dNTPs, 20 U ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U Superscript II reverse transcriptase (Invitrogen). Cycling conditions were 94°C for 60



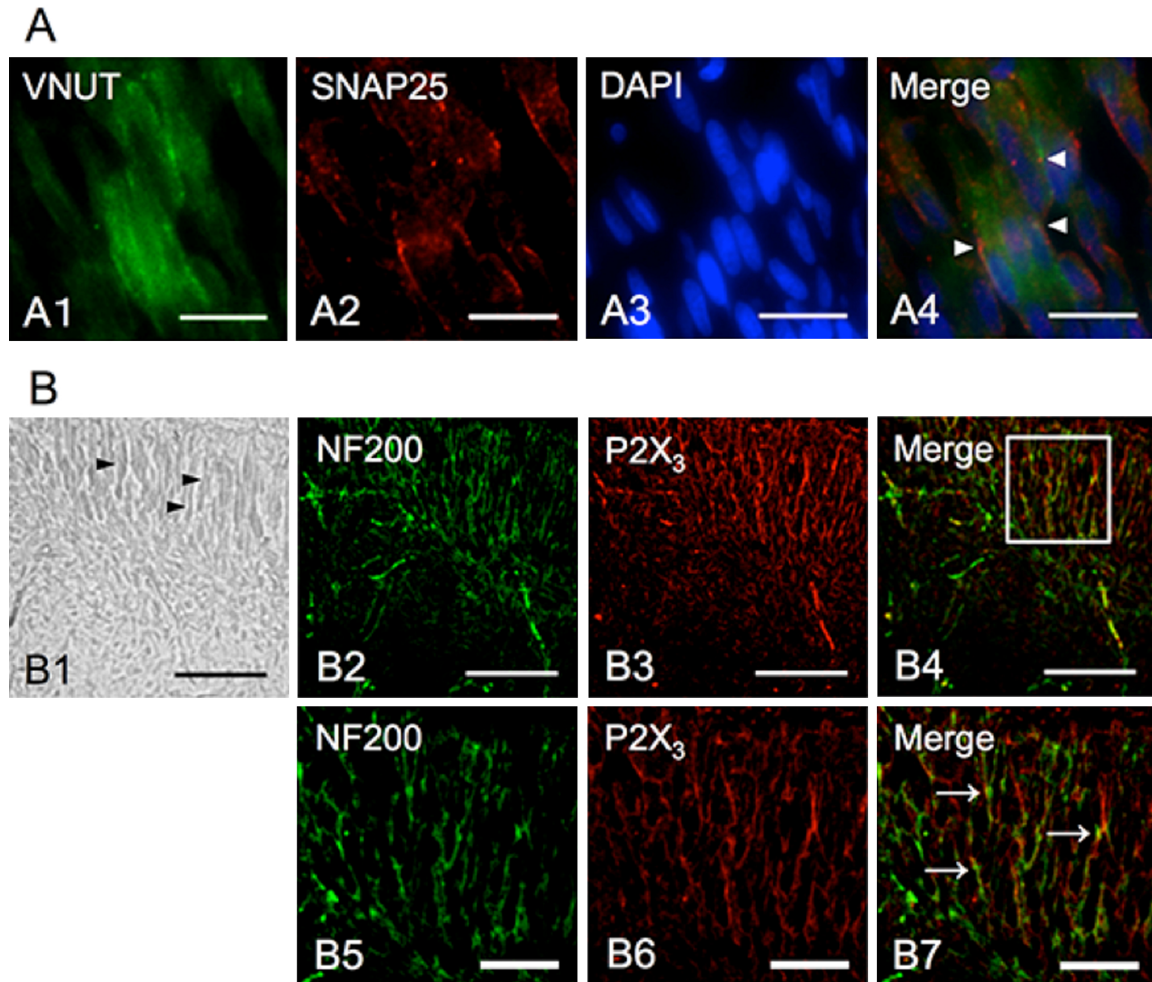
**Fig. 1.** VNUT expression in rat pulp. (A) *VNUT* mRNA expression in pulp and taste buds was confirmed by RT-PCR using primers specific to VNUT. (B) Immunofluorescence staining for VNUT (B2, B5, and B7) and nestin (B1 and B4) or DAPI (B8) in odontoblasts from rat tooth tissue. B3, B6, and B9 are merged images. Arrows indicate vesicle-like expression of VNUT (B7). Bars=200  $\mu$ m (B1–3), 50  $\mu$ m (B4–6), 20  $\mu$ m (B7–9).

sec, 60°C for 60 sec, and 72°C for 60 sec for 42 cycles. The primers used to amplify *VNUT* were 5'-AGAGTGCAGGAGAGCGAGAG-3' and 5'-GTGGTGTGACCCAGACACAG-3', giving a PCR product of 563 bp. PCR products were subjected to 2% agarose gel electrophoresis with ethidium bromide staining and visualized under ultraviolet light illumination. The expression of *Gapdh* was used as an internal control.

## 2. Cell culture and heat stress

KN-3 cells were seeded in 35-mm culture dishes ( $3 \times 10^5$  cells/dish) and cultured with minimal essential medium, Eagle's alpha modification containing 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. After 24 hr, KN-3 cells were exposed to heat stress at 43°C for 45 min. For rapid heat stress, culture medium was changed to





**Fig. 2.** Immunohistochemical localization of Snap25 and P2X<sub>3</sub> in rat odontoblasts and pulp nerve fibers. (A) Immunofluorescence staining of VNUT (A1), Snap25 (A2), and DAPI (A3) in rat odontoblasts. A4 is merged image. Arrowheads indicate the co-localization of VNUT and Snap25. (B) B1 is a bright-field image at low magnification of rat pulp, and the arrowheads indicate the location of odontoblasts. Immunofluorescence image for NF200 (B2 and B5) and P2X<sub>3</sub> (B3 and B6) in odontoblasts from rat tooth tissue. B2–4 are immunofluorescence images in the same region as B1. B2–4 are high magnification images. P2X<sub>3</sub> receptor expression on pulp nerve fibers was confirmed (arrows in B7). Bars=20  $\mu$ m (A1–4), 100  $\mu$ m (B1–4), 50  $\mu$ m (B5–6).

medium preheated at 43°C, and culture dishes were placed in an incubator preheated to 43°C, followed by incubation for 45 min at 43°C [23]. As a control, non-heat-treated KN-3 cells were cultured in medium containing 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37°C. Total RNA was extracted from non-heat-treated and heat-stressed cells using RNAqueous at 1, 3, and 6 hr after heat stress. The following primers were used: for *Hsp25* (Heat shock protein 25), 5'-GCAGGATGAACATGGCTACATCTC-3' and 5'-TGGTGATCTCCGCTGATTGTG-3'; for *VNUT*, 5'-AGAGTGCAGGAGAGCGAGAG-3' and 5'-GTGGTGTGACCCAGACACAG-3', giving a PCR product of 279 bp (for *Hsp25*) and 563 bp (for *VNUT*). Each cycle consisted of a denaturation step at 94.0°C for 60 sec, an annealing step (*Hsp25*, 68°C for 60 sec for 38 cycles, *VNUT*, 60°C for 60 sec for 42 cycles), and an extension step at 72°C for 60 sec. The expression of *Gapdh* was used as an internal control.

#### *siRNA and transfection*

*VNUT* siRNA (ON-TARGET plus siRNA, GE Healthcare Japan, Tokyo, Japan) and negative control siRNA (GE Healthcare Japan) were used. At 24 hr before transfection, KN-3 cells were plated in 2.5 mL complete growth medium per well in a six-well plate. The density of KN-3 cells was  $3.0 \times 10^5$  cells/well. After the cells were incubated overnight, siRNAs were transfected using the TransIT-X2 Dynamic Delivery System (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. At 24 hr after transfection, RT-PCR was then performed to assess the effects of the siRNA.

#### *Measurement of ATP release*

At 24 hr after transfection of siRNA, KN-3 cells were exposed to heat stress at 43°C for 45 min. Just after that 50  $\mu$ L of the supernatant was collected. Similarly, 50  $\mu$ L of the supernatant was collected with the medium of the control,

non-heat-treated KN-3 cells. ATP concentrations in the culture medium samples were assayed with ATP assay reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol with an ATP standard curve (10 pM to 10  $\mu$ M) [16]. Duplicate measurements were performed for each sample, and the average concentration was taken from five wells.

#### Statistical analysis

One-way analysis of variance (ANOVA) followed by individual *post hoc* comparisons (Scheffé) was used to assess the significance of differences.

### III. Results

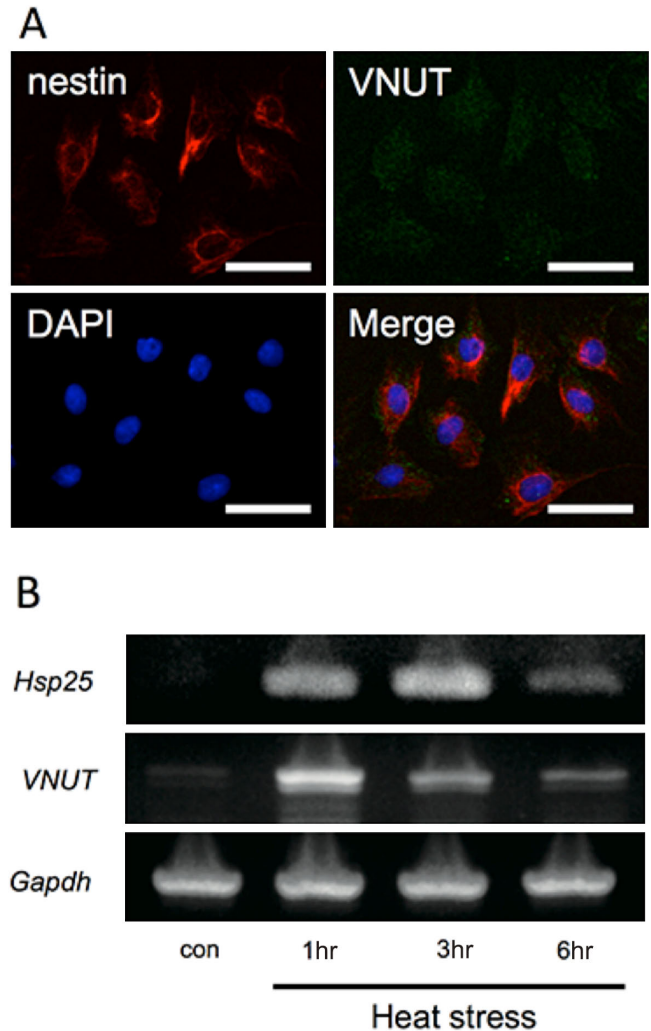
#### *In vivo* studies

The expression of *VNUT* mRNA in rat pulp tissue, including odontoblasts, was examined initially by RT-PCR. The pulpal tissue including odontoblasts expressed *VNUT* mRNA of the same size as the taste buds (Fig. 1A). To investigate which cell type(s) expressed VNUT in pulpal tissue, we performed immunofluorescence staining using a rabbit polyclonal antibody against VNUT. As shown in Figure 1B (1–6), strong immunopositive reaction was found in odontoblasts that were immunopositive for nestin, an odontoblast marker. Additionally, the expression of vesicle-like VNUT was confirmed in an odontoblast at high magnification (Fig. 1B (7–9)). Sections that were incubated in the absence of primary antibody showed no specific staining (data not shown).

To confirm the involvement of VNUT in vesicle transport in odontoblasts, double immunofluorescence staining for VNUT and Snap25, a membrane fusion protein, was performed. Some immunopositive reactions for VNUT were colocalized with the immunopositive reactions for Snap25 at the periphery of odontoblasts (Fig. 2A (1–4)). Furthermore, to indicate the distribution of ATP receptors close to odontoblasts, we performed double staining for NF200, a neuron-specific neurofilament protein, and P2X<sub>3</sub>, a receptor for ATP. We found that most of the pulp nerves were immunoreactive with anti-NF200 antibodies, including many fine neurons running between odontoblastic cells. The immunopositive reactions for P2X<sub>3</sub> receptors were distributed on the NF200 immunopositive axons between odontoblasts. These findings may possibly indicate that ATP vesicle transport had mediated the signal transduction from odontoblasts to axons in the pulp (Fig. 2B (1–7)).

#### *In vitro* studies

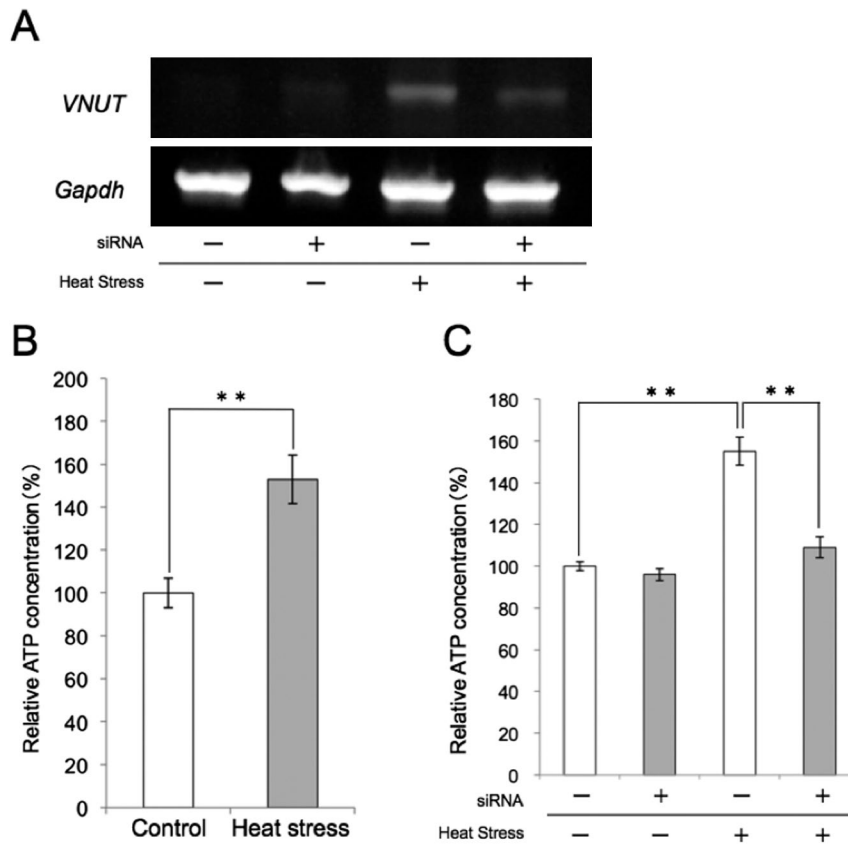
The odontoblastic phenotype in cultured KN-3 cells was confirmed by expression of the odontoblast marker nestin by immunofluorescence staining. The expression of VNUT in KN-3 cells was also confirmed (Fig. 3A). RT-PCR was performed to investigate the time-dependent changes in the expression of *VNUT* mRNA, compared with the expression of *Hsp25* mRNA, in heat treatment in KN-3



**Fig. 3.** Immunohistochemical staining in KN-3 cells and Reverse-transcriptase PCR analysis for the expression of *Hsp25* and *VNUT* in heat-treated KN-3 cells. **(A)** Immunofluorescence staining for VNUT and nestin and DAPI and merged in KN-3 cells. **(B)** Expression of *Hsp25* was observed in KN-3 cells at 1, 3, and 6 hr after heat treatment. *VNUT* was most strongly expressed at 1 hr after heat treatment, subsequent to which the expression decreased gradually.

odontoblastic cells. After KN-3 cells were exposed to heat stress at 43°C for 45 min, *Hsp25* mRNA increased gradually from 1 to 3 hr after heat stress, whereas *VNUT* mRNA was expressed at the highest level at 1 hr after heat stress and then decreased (Fig. 3B).

Finally, we examined the effects of *VNUT* siRNA interference on ATP release in KN-3 cells with and without heat stress. As indicated in Figure 4A, *VNUT* siRNA interference decreased *VNUT* mRNA expression after heat stress. RT-PCR was performed to confirm the effect after transfection. ATP release from KN-3 cells was then examined by an ATP luciferase assay. ATP release from KN-3 cells after heat treatment increased significantly compared with ATP release from non-heat-treated KN-3 cells (Fig. 4B). The increased ATP release after heat stress was



**Fig. 4.** ATP release by KN-3 cells after heat stress. (A) The expression of *VNUT* in non-heat-treated or heat-stressed KN-3 cells after siRNA or non-siRNA KN-3 cells. The expression of *VNUT* was strongest in heat-stressed KN-3 cells after non-siRNA treatment. (B) ATP release by heat-stressed KN-3 cells was significantly greater than the control (not heat treated). The values represent means±S.E. \*\*  $p < 0.05$ . (C) The effect of heat stress under siRNA on ATP release by KN-3 cells. The increased ATP release after heat stress was decreased significantly by *VNUT* siRNA interference. The values represent means±S.E. \*\* $p < 0.05$ .

decreased significantly by *VNUT* siRNA interference. There was no significant difference in ATP release between heat-stressed and non-heat-treated KN-3 cells after *VNUT* siRNA interference (Fig. 4C).

#### IV. Discussion

Although the mechanism of pain transmission from odontoblasts to pulpal nerves remains to be clarified, in the present study we propose a possible mechanism for ATP signal transduction via *VNUT* in odontoblasts. Among the vesicle transporters, *VNUT* is a relatively recently discovered transporter that actively transports cytoplasmic ATP into vesicles [27]. If *VNUT* is present in odontoblasts, it may be involved in signal transduction from non-nerve cells to axons, as it is in taste buds [14]. In the present study, *VNUT* mRNA was detected in pulpal tissue, and then odontoblasts were shown to be immunopositive for *VNUT*. In a high-magnification view, ATP-immunopositive vesicle-like structures were detected in odontoblasts. This localization of *VNUT* in odontoblasts suggests the involvement of *VNUT* in ATP release from odontoblasts to pulpal axons. There are many reports that have shown the expres-

sion and function of TRP channels in odontoblasts [5, 6, 29, 31]. Taken together with these previous reports, this suggests that odontoblasts could receive stimulation by TRP channels on their membrane, activate *VNUT*, and release ATP to ATP receptors on axons in the pulp.

To confirm vesicle transport, we examined the colocalization of *VNUT*-positive vesicles with Snap25, a membrane fusion protein. The expression of Snap25 in dental pulp has been identified in previous studies [11]. In the present study, some *VNUT*-immunopositive vesicles colocalized with Snap25, close to the plasma membrane of odontoblasts. Because the colocalization of *VNUT* immunopositive vesicles and Snap25 suggested the direction of vesicle secretion, it was conjectured that ATP is being released in bilateral directions.

Furthermore, the  $P2X_3$  receptor, an ATP receptor, was demonstrated on the axons in the pulp mainly between odontoblasts (Fig. 2B). This is consistent with previous reports indicating the presence of ATP receptors in human dental pulp [1] and the expression of the ecto-ATPase NTPDase2 in dental pulp [20]. Not only the localization of *VNUT* but also the distribution of Snap25 in odontoblasts and the  $P2X_3$  receptor suggest an ATP-mediated signal



transduction from odontoblasts to ATP receptors on axons in the pulp.

Next, we examined the functional role of VNUT for ATP release under thermal stimulation in odontoblast-like (KN-3) cells *in vitro*. After thermal stimulation, there was a rapid increase in *VNUT* mRNA expression, compared with the expression of *Hsp25* mRNA. After KN-3 cells were exposed to heat stress at 43°C for 45 min, *Hsp25* mRNA increased gradually from 1 to 3 hr after heat treatment, whereas *VNUT* mRNA was expressed at the highest level at 1 hr after heat treatment and then decreased. It was conjectured that the rapid increase in *VNUT* mRNA expression was due to its role in sensory signal transduction. Additionally, in *VNUT* siRNA cells, *VNUT* gene expression was barely increased compared with the case in cells that received the negative control siRNA. Odontoblasts have been suggested to contribute to thermal responses in the tooth via expression of TRP channels [5, 6, 31]. Although it remains to be determined which TRP channel(s) are associated with the activation or expression of VNUT in odontoblasts, our findings indicate that VNUT is involved in the thermal response of odontoblasts. Because there are some candidate molecules for ATP release in odontoblasts, we confirmed the role of VNUT in ATP release in odontoblasts using siRNA. Consequently, extracellular ATP release by thermal stimulation of odontoblast-like cells increased significantly compared with the control, and the increased ATP release by thermal stimulation was decreased significantly, to control levels, by adding siRNA. In a recent study, it is reported that pannexin 3 is involved in the release of ATP by external stimulus in the pulp [21]. However, our findings indicate that ATP release with thermal stimulation is related to the expression of VNUT. Further study is needed to clarify which TRP channel is involved in the expression of VNUT and how TRP channel signaling leads to the expression or activation of VNUT in odontoblasts.

In conclusion, the present study demonstrated that cytosolic ATP is transported by VNUT and that ATP in vesicles is released from odontoblasts to ATP receptors on axons. ATP vesicle transport in odontoblasts would seem to be a key mechanism for signal transduction from odontoblasts to axons in the pulp. From our findings, we propose a model of thermal pain signal transduction via a TRP-VNUT ATP release pathway, and suggest that VNUT may be a target molecule for the treatment of dentine hypersensitivity.

## V. Acknowledgments

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