Differential Expression of Capsaicin-, Menthol-, and Mustard Oil-Sensitive Receptors in Naive Rat Geniculate Ganglion Neurons

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Abstract

The roles of capsaicin, menthol, and mustard oils and their receptors in geniculate ganglion (GG) neurons still remain to be elucidated. These receptors belong to the transient receptor potential (TRP) family. Capsaicin-, menthol-, and mustard oil-sensitive receptors are TRPV1, TRPM8, and TRPA1, respectively. The present study aimed to investigate the expression of TRPV1, TRPM8, and TRPA1 in naive rat GG neurons. Furthermore, we examined whether these TRP-expressing GG neurons are myelinated A-fiber or unmyelinated C-fiber neurons. Firstly, using reverse transcription–polymerase chain reaction, TRPV1 mRNA and TRPA1 mRNA were distinctly detected in the naive GG. TRPM8 mRNA was faintly detected. Secondly, using in situ hybridization, TRPV1 mRNA– or TRPA1 mRNA–labeled neurons (signal/noise ratio ≥ 10) were observed in 15–20% of GG neurons. Few neurons were labeled by TRPM8 mRNA. Thirdly, neurofilament 200 (NF200) protein, a marker of mylinated A-fiber neurons, was detected in 57% of naive GG neurons. Coexpression of TRPV1 mRNA or TRPA1 mRNA with NF200 was detected in 10% of GG neurons. The present study confirmed the expression of the TRP receptors in the naive GG. The possible roles of TRP receptors in naive GG neurons in somatosensory or gustatory function were suggested.

Key words: geniculate ganglion, NF200, TRPA1, TRPM8, TRPV1

Introduction

Capsaicin, menthol, and mustard oils are naturally occurring compounds used in the food and pharmaceutical industries as active ingredients (Ecclecs, 2000; Umezu et al., 2001). They elicit a hot (burning) or cold/cool sensation by acting at the peripheral nerve endings. Their actions are mediated by receptors of the transient receptor potential (TRP) family. The TRP family is a superfamily of membrane proteins that act as ligand-gated cation channels and may interact with intracellular signaling systems (Clapham et al., 2001; Minke and Cook, 2002). Some members of the TRP family are sensitive to physical and chemical stimuli, including light, mechanical force, temperature, odors, nerve growth factor, pheromones, pH, osmolarity, and metabolic stress (Minke and Cook, 2002). Recently, thermosensitive TRP ion channels have been reported (Clapham, 2003; Jordt et al., 2003; Moran et al., 2004; Tominaga and Caterina, 2004; Talaveral et al., 2005). They include TRPV1 (renamed from VR1), TRPV2 (renamed from VRL1), TRPV3, TRPV4, TRPM5, TRPM8 (also known as CMR1), and TRPA1 (renamed from ANKTM1). TRPV1 is sensitive to capsaicin, noxious heat (>43°C), and protons (Caterina et al., 1997). TRPM8 is sensitive to menthol and cooling (<25–28°C) (McKemy et al., 2002; Peier et al., 2002). TRPA1 is activated by mustard oil and noxious cold stimuli (<17°C) (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004). These TRP receptors (TRPV1, TRPM8, and TRPA1) are abundantly expressed on a subpopulation of the primary afferent neurons in the trigeminal ganglion (TG) and dorsal root ganglion (DRG) and may induce somatosensory signals to the central nervous system.

With regards to the gustatory system, a previous study has reported that capsaicin and menthol stimulate the gustatory neurons and induce the taste sensation of bitterness (Green and Schullery, 2003). The TRPV1 variant was identified as an amiloride-insensitive salt receptor (Lyall et al., 2004). An electrophysiological study indicated that menthol excited the rodent chorda tympani (CT) nerve (Lundy and Contreras, 1993). On the other hand, previous studies found that neither capsaicin nor menthol had the effect on the gustatory system. Neither TRPV1- nor TRPM8-positive fibers were expressed

in the taste buds of the fungiform papillae and foliate papillae (Ishida *et al.*, 2002; Kido *et al.*, 2003; Abe *et al.*, 2005). Capsaicin is thought to be an irritant to somatosensory neurons that does not affect the gustatory neurons (Hettinger and Frank, 1992). Any effects of mustard oil on the gustatory system are still unclear. The expression of these TRP receptors in gustatory neurons has remained unknown, particularly in the geniculate ganglion (GG), which has two gustatory branches, the CT nerve and the great superficial petrosal nerve.

The purpose of the present study was to investigate the precise expression of TRPV1, TPRM8, and TRPA1 in GG neurons. Furthermore, we examined whether these TRP receptors are present in myelinated A-fiber or unmyelinated C-fiber neurons. From the view point of TRP expression, we discuss the possible roles of these receptors in GG neurons, comparing previous findings about their expression in somatosensory TG and DRG neurons.

Material and Methods

Experimental animals

Naive male Sprague–Dawley rats (200–250 g) were used. The word "naive" in the present study means "normal," "native," "nonoperated," "nontreated," "noncultured," and "nonadministered." All animal experiments conformed to the regulations of Hyogo College of Medicine Committee on Animal Research and were carried out in accordance with the guidelines of National Institutes of Health on animal care.

Reverse transcription–polymerase chain reaction

The rats were killed by decapitation under deep ether anesthesia. The bilateral GGs and lumbar DRGs were removed and rapidly frozen with powdered dry ice and stored at −80°C until used. The extractions of total RNAs of GGs and DRGs were carried out using the RNA extraction reagent, ISOGEN (Nippon Gene, Tokyo, Japan). Briefly, the samples were homogenized in 1 ml of ISOGEN reagent, mixed with 200 µl of chloroform, and centrifuged at 4°C for 15 min and 12 000 ×g. The supernatant was mixed with the same volume of isopropyl alcohol and centrifuged again under the same conditions. The precipitate was washed in 70% ethanol and air dried. The RNA extracts were quantified by measuring optical density at 260 nm. Samples of 5 µg of total RNA were mixed with 25 µl reverse transcriptase mixture to the final concentration of 200 units M-MLV reverse transcriptase (Promega, Madison, WI), 20 units RNase inhibitor (Promega), 0.8 µM deoxynucleoside triphosphate (dNTP), and 1 µg oligo-dT primer in 1× reaction buffer (pH 7.5) (Promega). Reverse transcription (RT) was carried out at 37°C for 60 min, following inactivation at 65°C for 10 min. The forward and reverse primers specific for TRPV1, TRPM8, and TRPA1 were designed as shown in Table 1.

The polymerase chain reaction (PCR) was performed in a 50-µl solution of PCR buffer (PerkinElmer, Wellesley, MA), 0.2 mM dNTP, and 1.25 units AmpliTaq (PerkinElmer) with a pair of 50 pmol primers for each gene on a DNA Thermal Cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). The PCR conditions were 30 cycles in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 35 cycles in TRPV1, TRPM8, and TRPA1 at 94°C for 15 s, 57°C for 15 s, and 72°C for 45 s. The resulting PCR products were electrophoresed through a 1.25% agarose gel containing ethidium bromide and visualized with UV illumination. Each pair of forward and reverse primers presented a single band with the expected size.

Tissue preparation for immunohistochemistry and *in situ* hybridization histochemistry

The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). After that, we made sure under a microscope that there was no infection in their middle ears. Then, the rats were perfused transcardially with 1% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4), followed by 4% paraformaldehyde in 0.1 M PB. The bilateral GGs were dissected out, postfixed in the same fixative for 24 h, and cryoprotected in 0.1 M PB containing 20% sucrose for another 24 h. The tissue samples were frozen with powdered dry ice and were sectioned with a cryostat. The GGs were cut into 12-µm thick sections that were subsequently thaw mounted onto Vectabond (Vector Laboratories, Inc., Burlingame, CA) -coated slides and stored at −80°C until ready for use.

Immunohistochemistry

The GG sections were processed for immunohistochemistry (IHC) of TRPV1, TRPM8, and neurofilament 200 (NF200), with a procedure used in our previous study (Noguchi *et al.*, 1995). The polyclonal primary antibodies for TRPV1 (1:400; Oncogene, San Diego, CA) and TRPM8 (1:100; a kind gift from Dr M. Tominaga, Okazaki Institute for Integrative Bioscience, Okazaki, Japan) were used. The monoclonal primary antibody for NF200 (1:50000; Sigma, St Louis, MO) was used.

The proportion of TRPV1-, TRPM8-, and NF200-immunoreactive (ir) GG neurons was determined by counting the neuronal profiles that showed the distinctive labeling in the

Table 1 Sequence locations of primers used in the present study

Gene	GenBank accession number	Primers	
		Forward	Reverse
TRPV1	AF029310	149–168	486–505
TRPM8	AY072788	264–283	837–818
TRPA1	AY496961	302–321	788–769

GG sections. In each rat, four to six sections of the GG were selected randomly, and 2000–3000 neuronal profiles were counted. An average percentage of TRPV1-ir, TRPM8-ir, and NF200-ir neurons, relative to the total number of neurons, was obtained for each animal across the different tissue sections. Then the mean ± SD (%) across animals was determined. Only neurons with clearly visible nuclei were counted. Because a stereological approach was not used in the present study, quantification of the data may represent a biased estimate of the actual number of cells and neurons. An assistant, who was unaware of the treatment group of the tissue sections, performed all countings.

In situ hybridization histochemistry

The GG sections, prepared using the same processes as for the IHC, were used for the *in situ* hybridization histochemistry (ISHH). The rat TRPV1, TRPM8, and TRPA1 cRNA probe corresponding to the nucleotides of 149–486, 264–837, and 302–788, respectively, was prepared (Kobayashi *et al.*, 2005). The procedure for ISHH was basically the same as that used in previous studies (Yamanaka *et al.*, 1999). For the double labeling of the protein (NF200 and TRPV1) and the mRNA (TRPV1 and TRPA1), the protein was first labeled immunohistochemically using the Avidin-biotin-peroxidase (ABC) method, and then the mRNA was labeled using ISHH with a radioisotope-labeled probe according to the procedure of our previous study (Tsuzuki *et al.*, 2001).

Measurements of the density of silver grains over randomly selected tissue profiles were performed using the NIH Image program by a blinded assistant, where only neuronal profiles that contained nuclei were used for quantification. At a magnification of 200× and with bright-field illumination, upper and lower thresholds of gray-level density were set such that only silver grains were accurately discriminated from the background in the outlined cell or tissue profile and read by the computer pixel-by-pixel. Subsequently, the area of discriminated pixels was measured and divided by the area of the outlined profile, giving a grain density for each cell or tissue profile. To reduce the risk of biased sampling of the data owing to varying emulsion thickness, we used a signal/noise (S/N) ratio for each cell in each tissue section. The S/N ratio of an individual neuron and its cross-sectioned area, which was computed from the outlined profile, was plotted. Based on this scattergram, neurons with a grain density of 10-fold the background level or higher (S/N ratio ≥ 10) were considered as positively labeled for TRPV1, TRPA1, and TRPM8 mRNAs. At least 250 neurons from each GG of each rat were measured. The number of neuronal profiles counted in each GG divided the number of positively labeled GG neurons. The size of neurons was characterized in the same way as our previous study: small- (<600 μm^2), medium- (600–1200 μm^2), and large-sized (> 1200 μm^2) neurons, according to their cross-sectional area (Kobayashi *et al.*, 2005).

Results

Differential expression of TRPV1, TRPA1, and TRPM8 mRNAs in the naive GG

Expression of TRPV1, TRPA1, and TRPM8 mRNAs in the GG was examined using RT–PCR (Figure 1). The mRNA expression of these receptors in the DRG was also examined as a control. All mRNA bands for the TRP (TRPV1, TRPA1, and TRPM8) in the DRG were clearly detected at their proper positions; 356, 486, and 573 bp, respectively. A positive control of GAPDH mRNA was detected in both the GG and DRG. The bands for the TRPV1 mRNA and TRPA1 mRNA in the GG were abundantly detected at their proper positions. In contrast, TRPM8 mRNA in the GG was faintly detected at its proper position (573 bp). TRPM8 mRNA expressed to a small degree in the GG. Here is the first evidence of the expression of the TRP mRNA in the naive rat GG.

TRPV1 expression in naive GG neurons

The localization and size distribution of TRPV1 in the GG neurons were characterized (Figure 2). A number of TRPV1-ir neurons were observed in the GG at a lower magnification using dark-field microphotography (Figure 2A). The percentage of TRPV1-ir neurons was 19.6 ± 1.5% of the overall GG neuronal profiles (n = 4). TRPV1 mRNA–labeled neurons were also detected using ISHH (Figure 2B). We calculated the S/N ratio of TRPV1 mRNA–labeled neurons in the GG using a computerized image analysis system (NIH image). The neurons with S/N ≥ 10 were considered as positively labeled (Figure 2C). The mean percentage of TRPV1 mRNA–labeled neurons in the GG was 21.5 ± 2.1%. The scatter graph also shows that the TRPV1 mRNA–labeled neurons were predominantly expressed in small-sized neurons (<600 μm^2). These results were similar to those from the TG and the DRG (Kobayashi *et al.*, 2005).

TRPM8 expression in naive GG neurons

Neither TRPM8-ir nor TRPM8 mRNA–labeled neurons were clearly observed in the GG neurons (Figure 3A,B). Few TRPM8 mRNA–labeled neurons with S/N ≥ 10 (0.06 ± 0.05%, n = 4) were detected (Figure 3C). TRPM8 mRNA seemed to be absent from the GG neurons.
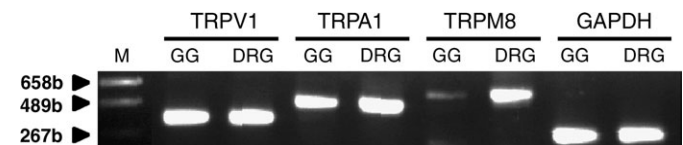

Figure 1 Differential expression of TRPV1, TRPA1, and TRPM8 mRNAs in the naive GG. Using RT–PCR, TRPV1 and TRPA1 mRNAs were detected at 356 and 486 bp, respectively. TRPM8 mRNA was faintly detected at 573 bp in the GG, whereas it was distinctly observed in a positive control of the DRG.
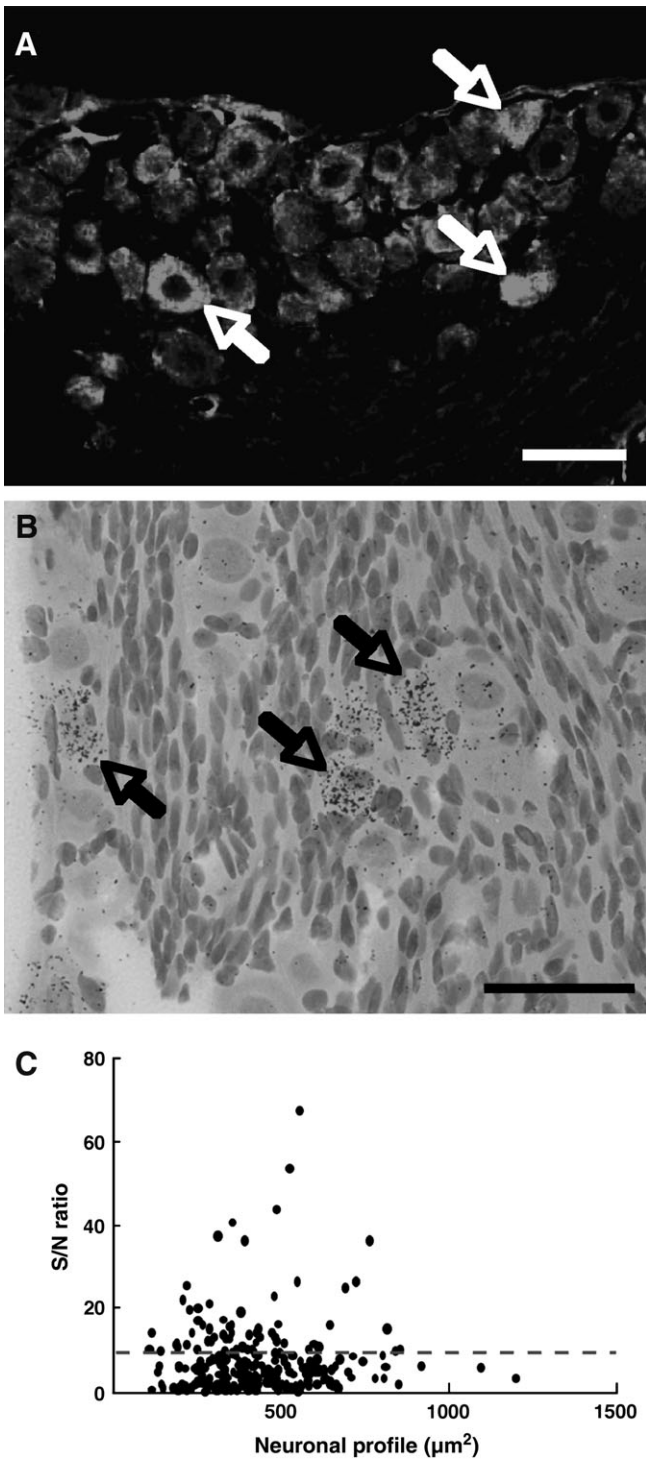
Figure 2 TRPV1 expression in the naive GG neurons. **(A)** TRPV1-ir neurons (arrows) at lower magnification in a dark field (bar: 50 μm). **(B)** TRPV1 mRNA–labeled neurons (arrows) at a higher magnification in a bright field (bar: 50 μm). **(C)** The scatter graph shows the relationship between S/N ratio of TRPV1 mRNA–labeled neurons (S/N ≥ 10) and size distribution.
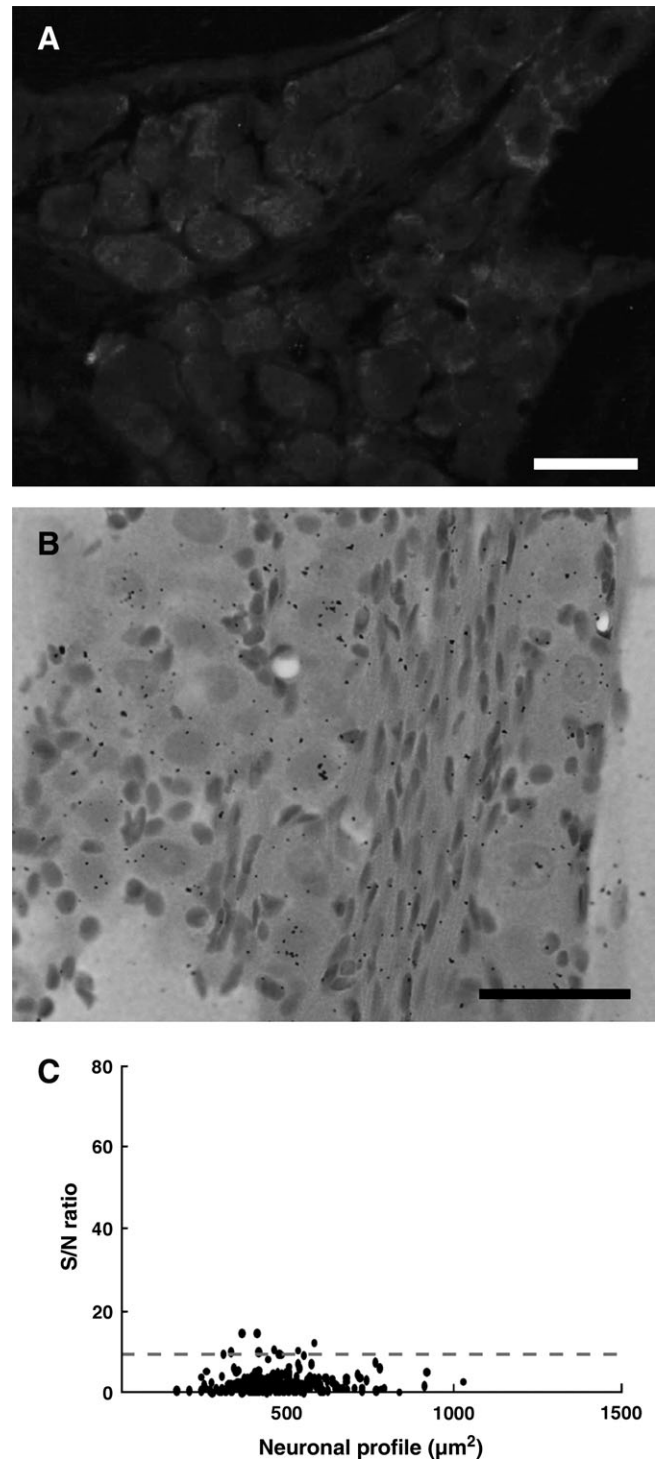
Figure 3 TRPM8 expression in the naive GG neurons. **(A)** TRPM8-ir neurons were not detected (bar: 50 μm). **(B)** TRPM8 mRNA–labeled neurons were not distinct (bar: 50 μm). **(C)** The scatter graph shows that few TRPM8 mRNA–labeled neurons (S/N ≥ 10) were detected.

TRPA1 expression in naive GG neurons

The localization and size distribution of TRPA1 in the GG neurons were characterized (Figure 4). The percentage of TRPA1 mRNA–labeled neurons with S/N $\geq$ 10 was 13.6 $\pm$ 2.4% in the GG (n = 4). The scatter graph shows that the TRPA1 mRNA–labeled neurons were predominantly expressed in small-sized neurons (Figure 4B). These results are also similar to the above results of the TRPV1 mRNA expression in the GG. Furthermore, we examined the coexpression of TRPA1 mRNA and TRPV1-ir in the GG neurons (Figure 4C). TRPA1 mRNA heavily colocalized with TRPV1-ir in the GG neurons (93.2 $\pm$ 7.3%, n = 4).

Distribution of TRPV1 mRNA and TRPA1 mRNA in myelinated A-fiber GG neurons

To clarify the percentage of myelinated and unmyelinated GG neurons, we first observed the expression of NF200 protein as a marker of myelinated A-fiber neurons. In the GG, NF200-ir profiles were 57.0 $\pm$ 6.5% of the total GG neurons (n = 4). This finding indicates that at least 57% of naive GG neurons were myelinated A-fiber neurons.

Next, we aimed to characterize the TRP-expressing GG neurons among the myelinated A-fiber neurons. We examined the coexpression of TRP mRNA (TRPV1 mRNA and TRPA1 mRNA) with NF200-ir (Figure 5). The percentage of the coexpressing neurons is presented in Table 2. The data indicate that around 10% of the TRPV1 mRNA– or TRPA1 mRNA–positive neurons were myelinated A-fiber neurons. Inversely, around 10% of the myelinated A-fiber neurons had the TRPV1 and TRPA1 receptors. These results indicate that TRPV1 mRNA and TRPA1 mRNA were predominantly expressed in the unmyelinated C-fiber GG neurons.

Discussion

The present study demonstrated in naive rat GG neurons the following new findings: (1) the TRPV1 and TRPA1 were abundantly expressed, but there was little expression of TRPM8, (2) most TRPA1-expressing GG neurons coexpressed TRPV1, and (3) a small population of TRPV1- and TRPA1-expressing neurons coexpressed NF200.

We have previously demonstrated the expression of TRPV1, TRPM8, and TRPA1 mRNAs in DRG and TG neurons (Kobayashi *et al.*, 2005; Obata *et al.*, 2005; Katsura *et al.*, 2006). In the present study, $\approx$20% of naive GG neurons had TRPV1 receptors, $\approx$15% of the neurons had TRPA1 receptors, and TRPM8 expression was observed in only a few neurons. Each percentage of GG neurons with these receptors, particularly TRPM8, was lower than those of TG and DRG neurons. The regulation of TRP expression seems to differ among primary afferent neurons, somatic sensory neurons (e.g., TG and DRG) and GG neurons.

With regards to the gustatory system, a previous study reported that capsaicin and menthol stimulate the gustatory
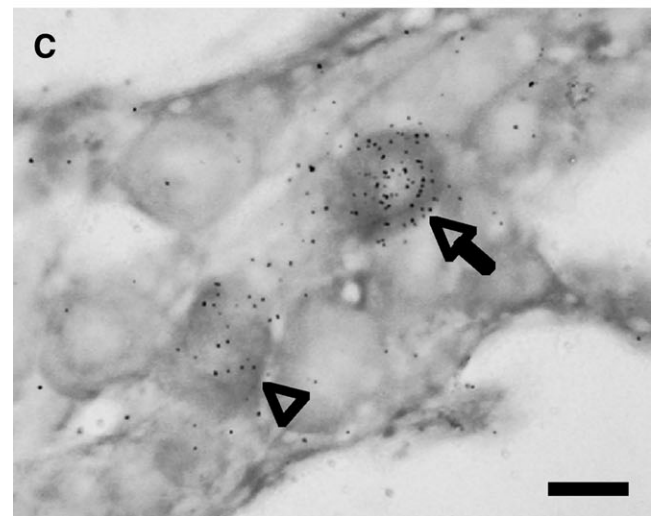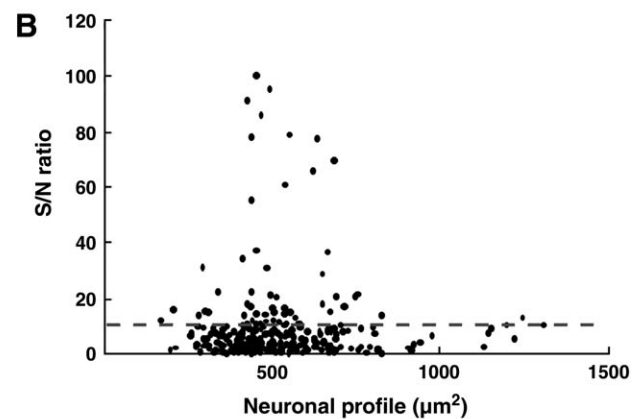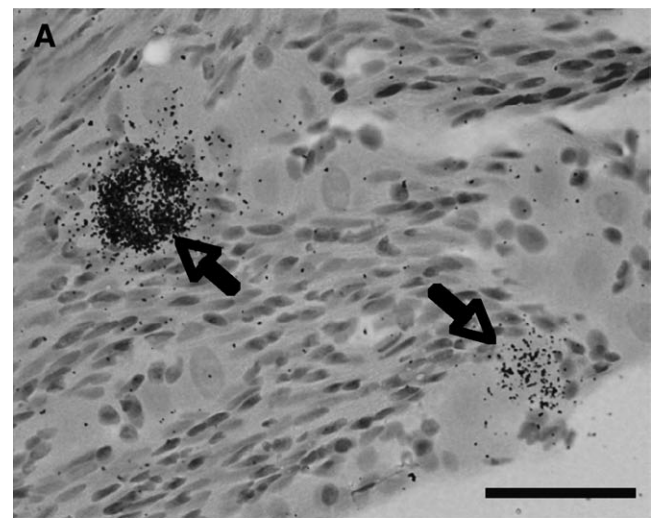

Figure 4 TRPA1 expression in the naive GG neurons. **(A)** TRPA1 mRNA–labeled neurons (arrows) at higher magnification in a bright field (bar: 50 μm). **(B)** The scatter graph shows the S/N ratio of TRPA1 mRNA–labeled neurons (S/N $\geq$ 10) and their size distribution. **(C)** Coexpression of TRPA1 mRNA with TRPV1-ir (bar: 20 μm). Arrows show the double-labeled neurons with TRPA1 mRNA and TRPV1-ir. Arrowheads show neurons with TRPV1-ir alone.
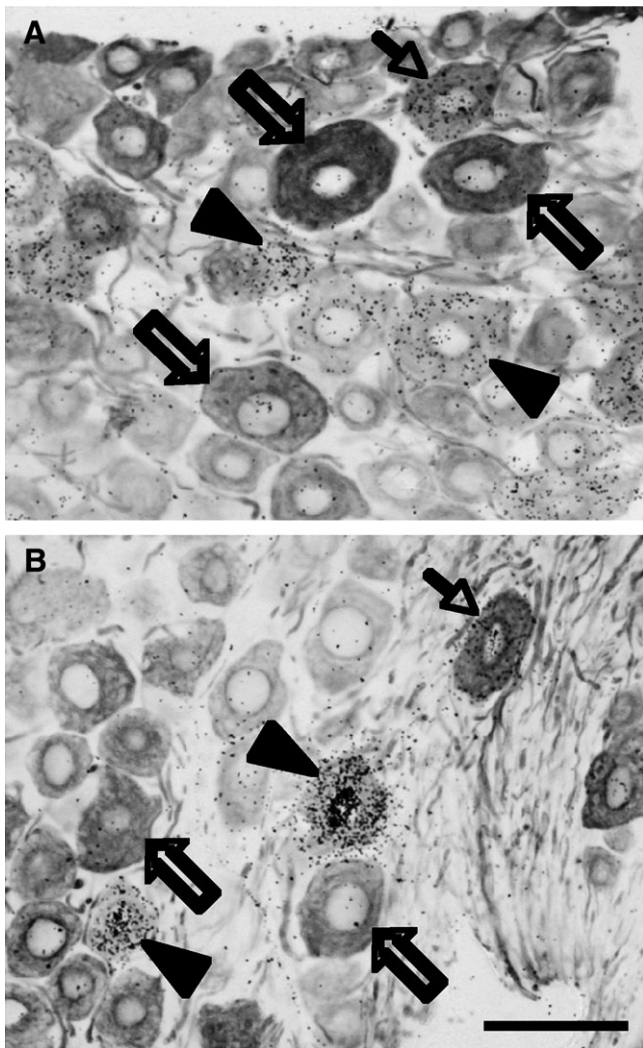
Figure 5 Myelinated A-fiber neurons in the naive GG. **(A)** Coexpression of TRPV1 mRNA and NF200-ir. Solid arrows show the double-labeled neurons with TRPV1 mRNA and NF200-ir. Open arrows show the neurons with NF200-ir alone. Arrowheads show the neurons with TRPV1 mRNA alone. **(B)** Coexpression of TRPA1 mRNA and NF200-ir. Solid arrows show the double-labeled neurons with TRPA1 mRNA and NF200-ir. Open arrows show the neurons with NF200-ir alone. Arrowheads show the neurons with TRPA1 mRNA alone. The scale bar (50 μm) is common in A and B.

Table 2 Percentage of the neurofilament-positive GG neurons that express TRP receptors mRNA

TRPV1 mRNA–positive neurons in NF200-ir neurons	12.5 ± 2.1%
TRPA1 mRNA–positive neurons in NF200-ir neurons	9.7 ± 1.9%
NF200-ir neurons in TRPV1 mRNA–positive neurons	8.5 ± 2.3%
NF200-ir neurons in TRPA1 mRNA–positive neurons	10.5 ± 1.2%

Each percentage shown is the mean ± SD (n = 4).

neurons and induce the taste sensation of bitterness (Green and Schullery, 2003). The results indicate that capsaicin, menthol, and their receptors (TRPV1 and TRPM8) have effects on the gustatory system. TRPV1 was detected in the GG neurons. This finding suggests that TRPV1 is associated with gustation. For the TRPM8 expression, we failed to demonstrate the distinct expression of the TRPM8 receptor in the naive GG neurons. Our morphological data using IHC and ISHH seem to indicate that GG neurons did not have TRPM8 receptors and did not have gustatory functional roles. If this is true, our data turn out to be consistent with the report that TRPM8-positive fibers were not expressed in the taste buds of the fungiform papillae and foliate papillae that CT innervates (Abe *et al.*, 2005). In contrast, our RT–PCR data (Figure 1) indicate that the GG has TRPM8 mRNA, albeit at a low level. The gustatory nerve response to sweet compounds mediated by TRPM5 was markedly enhanced between 15°C and 35°C (Talavera *et al.*, 2005). TRPM8 might also elicit the gustatory function within a specific range of temperature. Therefore, we could also consider that TRPM8 in the GG might have some effects on the gustatory system. The small amount of TRPM8 in the GG is one of the explanations why the effects of menthol on the CT territory are weak (Green and Schullery, 2003). Date regarding TRPA1 receptors in the gustatory system has not been reported previously. The present study has shown the possible effects of the TRPA1 receptors on the gustatory system. Experiments using other approaches are required in order to provide evidence of the gustatory functional roles of TRP receptors.

The GG neurons also project within the somatosensory nerve, which innervates the skin at the inner surface of the ear pinna via the posterior auricular nerve and causes neuralgia (Boudreau *et al.*, 1977). The percentage of the population in the GG neurons is still unknown. We found that most TRPA1 receptor-expressing neurons (≈90%) had TRPV1 receptors. This finding was similar to our previous data from the TG and DRG. The TRPV1 and TRPA1 might also have a significant role in the somatosensory system in the GG neurons.

In addition, capsaicin and menthol also elicit the sensation of burning (Green and Schullery, 2003). TRPV1 and TRPM8 might act as thermosensors. Not only these receptors (TRPV1 and TPRM8) but also TRPA1 and the other TRP receptors that are sensitive to temperature might act in this capacity.

In the present study, NF200 was expressed in 57% of GG neurons. These data indicate that 57% of GG neurons are myelinated A-fiber neurons. This result is consistent with reports that the major populations of GG neurons (50–70%) were myelinated A-fiber neurons (Farbman and Hellekant, 1978; Ylikoski *et al.*, 1983). Similar to the DRG neurons, TRPV1 mRNA and TRPA1 mRNA were expressed predominantly in small neurons that are mainly unmyelinated C-fiber neurons (Kobayashi *et al.*, 2005).

A minor population of the TRP-expressing GG neurons (≈10%) was present in myelinated A-fiber GG neurons.

Important effects of neurotrophic factors on the regulation of TRP receptors have been proposed. Trk A is coexpressed with TRPV1, TRPM8, and TRPA1 in TG and DRG neurons (Kobayashi *et al.*, 2005). The upregulation of TRPV1 and TRPA1 expression is dependent on nerve growth factor (NGF) in the damaged peripheral nerve (Fukuoka *et al.*, 2002; Obata *et al.*, 2005). TRPM8 is also dependent on NGF in somatosensory neurons (Kobayashi *et al.*, 2005). In the gustatory system, neurotrophic factors and their receptors, especially trk B, are thought to be important (Cho and Farbman, 1999). In future, we will investigate the influence of neurotrophic factors on the TRP expression in GG neurons.

Since we did not focus on glossopharyngeal neurons in the present study, we cannot discuss the whole gustatory function. Furthermore, the explanation of functional mechanisms in the gustatory system was difficult using only our morphological data. Other approaches are required in order to explain completely the gustatory function. We still need to find the experimental methods to confirm the effect of the receptors and their ligands on the gustatory system. In the present study, we conclude that the TRPV1 and TRPA1 receptors might play roles in the sensory system of the CT territory, and TRPM8 receptors might have weak influences in the CT territory.

Acknowledgements

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