# 博士論文

Transient receptor potential melastatin-2 in the rat trigeminal ganglion.

（ラット三叉神経節感覚神経細胞における TRPM2 の分布

および機能に関する研究）

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# Transient receptor potential melastatin-2 in the rat trigeminal ganglion.

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Abstract

Objective

Transient receptor potential melastatin-2 (TRPM2) is a thermosensitive, $Ca2^{+}$-permeable nonselective cation channel. Distribution of TRPM2 and its co-expression with TRPV1 and calcitonin gene-related peptide (CGRP) were examined in the trigeminal ganglion (TG) and its periphery to know function of TRPM2 in oro-facial tissues.

Material and methods

Double immunofluorescence method for TRPM2 with TRPV1 or CGRP was performed in the rat TG. Combination of retrograde tracing method and immunohistochemistry was also used to know expression of TRPM2 in TG neurons innervating the facial skin and tooth pulp. In addition, the effect of infraorbital nerve transection and complete Freund's adjuvant application into the facial skin was investigated by quantitative real-time reverse transcription-polymerase chain reaction analysis.

Results

TRPM2-immunoreactivity was expressed by 33.5% in the TG. Small to medium-sized neurons in the TG contained TRPM2-immunoreactivity. Half of TRPM2-immunoreactive TG neurons were also immunoreactive for TRPV1 (47.8%) or CGRP (53.4%). TRPM2 expression was common in cutaneous TG neurons (34.1%) but not in pulpal TG neurons (15.7%).

Distribution of TRPM2-immunoreactivity was barely affected by ION transection and CFA application into the facial skin. Expression of TRPM2 mRNA in the TG was elevated after ION injury (60% increase), but not CFA application.

Conclusions

The present study demonstrated expression of TRPM2 in TG neurons innervating peripheral tissues. These findings suggest that TRPM2 have function about nociceptive transmission from oral and facial structures. This channel may also respond to the nerve injury as an oxidative stress sensor in the injured TG.

# 1. Introduction

Transient receptor potential melastatin-2 (TRPM2) is a thermosensitive, $Ca2^{+}$-permeable nonselective cation channel. This ion channel is widely distributed in the brain, spleen, heart, liver, lung and immune cells, and can act as a metabolic and oxidative stress sensor (Knowles, Li, & Perraud,2013). In the spinal nervous system, TRPM2 is known to be associated with the pathogenesis of neuropathic and inflammatory pain (Hung & Tan, 2018; So et al., 2015). In TRPM2-knockout mice, acetic acid-induced nociceptive behavior was suppressed. And, TRPM2 deficiency also decreased paclitaxel-induced mechanical allodynia (So et al., 2015). TRPM2 is thought to contribute to transduction of nociceptive information in the spinal nervous system of these animal models. In the trigeminal nervous system, TRPM2 is also suggested to affect expression of inflammation-related cytokines. TRPM2 can regulate synthesis of interleukin 6 and chemokine in sensory neurons of the trigeminal ganglion (TG) (Chung et al., 2015). Therefore, I have a hypothesis that TRPM2 has a function to transduce nociception in TG neurons with peripheral injury. However, little is known about distribution of TRPM2 and effect of inflammation and nerve injury on its expression in the TG.

The transient receptor potential cation channel subfamily V member 1 (TRPV1) is a heat sensor > 43$^{o}$C, and also respond to protons and vanilloid compounds (Caterina et al., 1997). TRPV1 is expressed by small to medium-sized sensory neurons in the TG (Guo et al., 1999;

Ichikawa & Sugimoto 2001; Hironaka et al., 2014; Sato et al., 2018; Yajima et al., 2019). Previous studies have demonstrated that TG neurons innervating the facial skin and tooth pulp contain TRPV1 (Ichikawa & Sugimoto 2001; Shimada et al., 2016; Yajima et al., 2019). TRPV1-positive TG neurons are abundant in the facial skin and infrequent in the tooth pulp. The content of nociceptive sensors appears to be different among oro-facial structures. Recently, TRPV1 and TRPM2 have been shown to have co-operative function in the brain. In hippocampal neurons of ischemia-induced animals, apoptosis, calcium entry and oxidative stress are decreased by regulation of TRPV1 and TRPM2 activation (Akpınar et al., 2016). On the other hand, TRPV1 is co-expressed by TG neurons containing calcitonin gene-related peptide (CGRP), a marker for small and medium-sized nociceptors (Kim et al., 2018; Sato et al., 2018; Silverman & Kruger 1989; Yajima et al., 2019). CGRP is known to mediate neurogenic inflammation in the trigeminal system (Cady et al., 2011). However, relationship of TRPM2 with TRPV1 and CGRP has not been reported in the trigeminal sensory systems.

In this study, distribution of TRPM2 and its relationship to TRPV1 and CGRP were examined in the TG by a double immunofluorescence method. Expression of TRPM2 was also investigated in TG neurons which innervate the facial skin and tooth pulp. In addition, transection of infraorbital nerve (ION) or application of complete Freund's adjuvant (CFA) to the facial skin were performed to know an effect on the nerve injury and inflammation on TRPM2 expression in the TG by immunohistochemistry and quantitative real-time reverse transcription-polymerase chain

reaction (qPCR). This study may facilitate understanding contributions of TRPM2 on sensory transduction in oro-facial regions with peripheral injuries.

## 2. Materials and Methods

### *2.1. Animals*

To know distribution of TRPM2 and its co-expression with TRPV1 or CGRP, the left TGs were obtained from 4 male Wistar rats (180-250 g). Rats were deeply anesthetized by isoflurane to the level at which respiration was markedly suppressed and perfused through the left ventricle with 50 ml saline and 500 ml Zamboni fixative (Stefanini, De Martino & Zamboni, 1967). These ganglia were removed and immersed in the same fixative. Then, the materials were immersed in 20% sucrose-containing phosphate-buffered saline until sunken, sectioned at 8 μm with a cryostat (Hyrax C25, Thermo Fisher Scientific, USA).

### *2.2. Tracer application*

For demonstration of TRPM2-positive sensory neurons innervating peripheral tissues, 8 animals were used. One μl of 1% fluorogold (FG, Fluorochrome, USA) dissolved with distilled water was injected into the infraorbital skin and tooth pulp under deep anesthesia by intraperitoneal injection with a mixture of medetomidine (0.15 mg/kg), midazolam (2.0 mg/kg) and butorphanol (2.5 mg/kg). At 3 days after FG injection, the animals were reanesthetized with isoflurane, and perfusion-fixed with Zamboni fixative.

*2.3. Peripheral injury*

Transection of the unilateral ION was performed on 8 animals under deep anesthesia by intraperitoneal injection with a mixture of medetomidine (0.15 mg/kg), midazolam (2.0 mg/kg) and butorphanol (2.5 mg/kg). The left nerves were exposed through incision of the skin and subcutaneous layer, and transected using microscissors with the transected proximal stump ligated using 4-0 silk (Natsume Seisakusho Co., Ltd, Japan) to inhibit spontaneous reconnection and regeneration. Control group comprised sham-operated animals (n = 8) in which the left nerves were exposed without nerve injury. In addition, 100 µl of CFA solution (Sigma-Aldrich, USA) or saline was injected into the infraorbital skin on the left side in 16 animals. At 7 days after ION transection or CFA application, the rats were deeply anesthetized and, perfused with Zamboni fixative or decapitated without fixation for qPCR (n = 4 at each group).

*2.4. Immunohistochemistry*

Sections were reacted overnight at room temperature with a cocktail of TRPM2 antiserum and antiserum against TRPV1 or CGRP (Table 1). They were further reacted with a cocktail of appropriate secondary antibodies (Table 1). Then, the sections were observed with conventional fluorescence microscopy (ECLIPSE 80i, Nikon, Japan) or confocal laser scanning microscopy (A1+, Nikon).

*2.5. Morphometric analysis*

To analyze the distribution and morphology of sensory neurons showing immunofluorescence and FG fluorescence, optic images of the ganglia were obtained with a digital camera (Nikon). For proportion and cell size of TRPM2-, TRPV1- and CGRP-positive TG neurons, 3 sections of the ganglion were randomly selected in each of 4 animals and the average proportion of those neurons was obtained for each sample. All FG-labeled sensory neurons were analyzed in every tenth section of the TG to demonstrate expression of TRPM2 among sensory neurons innervating the facial skin and tooth pulp. The area of cell bodies of TG neurons with the distinct nuclei was measured (Lumina Vison software, Mitani Corporation, Japan).

For the specificity of rabbit TRPM2 antiserum, the antiserum was absorbed with the protein (20 μg/ml, Alomone Labs, Israel) and applied to sections of the TG. The fluorescence was abolished in the control. The specificity of antibodies against TRPV1 and CGRP has been also described previously (Atsumi et al., 2020; Sato et al., 2015; Yajima et al., 2019).

*2.6. Quantitative real-time PCR (qPCR) analysis*

The expression of TRPM2 mRNA in the TG of different treated rats (n = 4 at each group) was quantified by qPCR. After decapitation of animals, left TG was dissected and stored in RNA protect Tissue Reagent (Qiagen, Germany) at 4ºC for overnight. RNeasy Lipid Tissue Mini Kit (Qiagen) was used for RNA extraction. The cDNA was synthetized using a Transcriptor First

Strand cDNA Synthesis Kit (Roche, Switzerland) and stored at -80ºC until use (Liu et al., 2020). GAPDH was used for normalization, and relative mRNA expression was calculated by the Pfaffl method (Pfaffl, 2001). The TaqMan Gene Expression Assays were conducted for TRPM2 Rn01429410_m1 and GAPDH Rn01775763_g1 (Applied biosystems; Thermo Fisher scientific). The results of qPCR analyses were presented as average fold change ± S.D.

*2.6. Statistical analysis*

Differences between animals with sham-operation and ION-transection, or saline- and CFA-treatment were examined by Welch's t-test.

## 3. Results

### *3.1. Expression of TRPM2 and its co-expression with TRPV1 and CGRP*

TRPM2-immunoreactivity was detected in sensory neurons within the TG. The immunofluorescence was located throughout the cytoplasm of these neurons. They were scattered throughout the ganglion. One third of TG neurons were immunoreactive for TRPM2 (Table 2). Most of them had small (< 400 μm²) to medium-sized (400-800 μm²) cell bodies (Table 3). The double immunofluorescence analysis also revealed co-expression of TRPM2 with TRPV1 or CGRP (Fig. 1). In the TG, more than 30% of sensory neurons were immunoreactive for TRPV1 or CGRP (Table 2). Similar to TRPM2-immunoreactive (IR) TG neurons, TRPV1- or CGRP-IR neurons were predominantly small to medium-sized (Fig. 1g, h). And, half of TRPM2-IR neurons showed TRPV1- (mean ± S.D. = 47.8 ± 3.2%) or CGRP-immunoreactivity (53.4 ± 3.1%) in the TG. TRPM2-IR TG neurons with TRPV1- and CGRP-immunoreactivity mainly had small cell bodies in the TG (Table 3). In the sensory ganglia, satellite cells and Schwann cells were also immunoreactive for TRPM2, but immuno-negative for TRPV1 or CGRP. These cells occasionally had intense TRPM2-immunofluorescence.

*3.2. TRPM2-positive TG neurons innervating the skin and tooth pulp*

Many cell bodies of sensory neurons in the maxillary division of the TG were labeled with FG from the infraorbital skin or upper molar tooth pulp (Fig. 2a, d). The cell body size of cutaneous neurons was smaller than that of tooth pulp neurons (Fig. 2g, h). Combination of a retrograde tracing method and immunohistochemistry showed that cutaneous and pulpal TG neurons contained TRPM2-immunoreactivity (Fig. 2). TRPM2 expression among cutaneous TG neurons was more numerous among pulpal TG neurons (Table 4). On the other hand, TRPV1- and CGRP-IR TG neurons innervating the facial skin mainly had small to medium-sized cell bodies. Pulpal TG neurons with TRPV1-immunoreactivity were small to medium-sized, whereas those with CGRP-immunoreactivity had medium-sized to large cell bodies (Table 5). And, subpopulations of TRPM2-IR TG neurons innervating the skin and tooth pulp were also immunoreactive for TRPV1 (skin, 50.0%; tooth pulp, 69.6%) and CGRP (skin, 49.6%; tooth pulp, 79.3%). By the cell size analysis, expression of TRPM2 and its co-expression with TRPV1 were predominantly detected in cutaneous and pulpal TG neurons with small to medium-sized cell bodies (skin, 98.2%; tooth pulp, 94.9%). Most of cutaneous TG neurons co-expressing TRPM2 and CGRP also had small to medium-sized cell bodies (96.5%). Sensory neurons containing TRPV1 or CGRP alone were abundant in the facial skin and tooth pulp, respectively.

Expression of TRPM2 and its co-expression with CGRP was statistically significant between the facial skin and tooth pulp (Welch's t-test, $p < 0.05$).

*3.3 Effect of ION transection and CFA application on TRPM2 expression*

By immunohistochemistry, TRPM2 expression in the TG was barely affected by the ION transection or CFA application (Fig. 3). There was no significant difference on proportion of TRPM2-IR TG neurons in animals with the nerve injury (36.9 ± 3.0%), sham operation (30.6 ± 5.7%), and CFA (33.7 ± 4.3%) and saline (29.5 ± 4.5%) application (Figs. 3, 4). In addition, TRPM2-IR satellite cells and Schwann cells showed similar distributions in the TG of these animals. By qPCR analysis, however, transection of the ION changed expression of TRPM2 mRNA in the ipsilateral TG. TRPM2 mRNA expression was elevated by the nerve injury compared to the sham operation. A 60% increase was detected in the injured TG (Fig. 4). Expression of TRPM2 mRNA was similar in the ipsilateral TGs of animals with CFA and saline injection into the infraorbital skin.

## 4. Discussion

In this study, many sensory neurons with small to medium-sized cell bodies contained TRPM2-iunmunoreactivity in the TG. By a double immunofluorescence method, half of TRPM2-containing TG neurons also had TRPV1 and CGRP. TRPV1- and CGRP-containing neurons in the sensory ganglion are mainly unmyelinated (Valtschanoff et al., 2001; Silverman & Kruger, 1987; Sugimoto et al., 1997). In this study, a triple immunofluoresence method for TRPM2, TRPV1 and CGRP was not performed. It remains unclear whether TRPM2-containing TG neurons have both TRPV1 and CGRP or not. However, at least half of TRPM2-containing neurons probably have unmyelinated, respond to heat > 43°C, vanilloid compounds and proton, and use CGRP as a nociceptive transmitter or modulator.

The facial skin has nociceptors and low-threshold mechanoreceptors in the TG. However, the tooth pulp is exclusively innervated by nociceptors in the TG. By the present retrograde tracing and immunohistochemical analysis, however, TRPM2 was common in TG neurons innervating the facial skin but not in those innervating the tooth pulp. And, expression of TRPM2 among cutaneous nociceptors is likely to be much more than among pulpal nociceptors. I also demonstrated that cutaneous sensory neurons were smaller than pulpal sensory neurons in the TG. Therefore, expression of TRPM2 is likely to be correlated to the cell size of TG neurons and the variety of their receptive fields. Activation of TRPM2 and TRPV1 are known to contribute to

inflammatory and neuropathic nociception (Hung & Tan, 2018; So et al., 2015). CGRP can act as a neurotransmitter or modulator for nociception in acute and chronic inflammatory conditions. Thus, abundance of TRPM2 and its co-expression with TRPV1 and CGRP in the facial skin suggest that TRPM2 may play an important role in pathological noxious transmission from the facial skin.

By the present qPCR and immunohistochemical analysis, the treatment of CFA has little or no effect on expression of TRPM2 mRNA or TRPM2 protein in the TG. In the TG, suppression of TRPM2 attenuates induced up-regulation of interleukin 6 and chemokine (Chung et al., 2015). Peripheral inflammation probably induces activation of TRPM2 but not its mRNA or protein level in the trigeminal nervous system. CGRP is also known as a mediator of neurogenic inflammation in the trigeminal sensory system (Cady et al., 2011). CGRP can induce vasodilation and plasma extravasation through TRPV1 activation. Similar to CGRP and TRPV1, TRPM2 may be associated with neurogenic inflammation. By sensing oxidative stress, TRPM2 activation in TG neurons may be associated with neurogenic inflammation in the periphery.

In the present study, the nerve injury caused an increase for TRPM2 mRNA in the ipsilateral TG. TRPM2-imunreactivity was located to sensory neurons, satellite cells and Schwann cells in the TG of intact animals and animals with sham-operation and ION-transection. And, the distribution of TRPM2-immunoreactivity was similar in the sham-operated and ION-transected

TGs. Thus, it remains unclear which types of cell components showed increase of TRPM2 mRNA in the TG after ION transection. The increase of TRPM2 mRNA may be not so high to detect change of the protein level by the present immunohistochemical method. TRPM2 has been shown to have a neuroprotective function in the brain by responding to oxidative stress. On the other hand, oxidative stress is considered to occur in the axotomized nerve. Axotomy of the spinal nerve increases lipid peroxidation and decreases antioxidant enzyme activities in the tissue. Increase of TRPM2 mRNA may suggest that the oxidative stress sensor has a neuroprotective effect on injured sensory neurons in the TG. On the other hand, TRPV1 has been suggested to responsible to neuropathic pain in the animal model. This channel contributes the behavioral heat hypersensitivity after the spinal nerve ligation. In this study, I have demonstrated that TRPM2 and TRPV1 are co-expressed by many sensory neurons in the TG. However, it remains unclear whether increase of TRPM2 mRNA in the TG is associated with neuropathic pain or not. Further studies will be necessary to know the function of TRPM2 and its functional relationship with TRPV1 and CGRP about in the TG.

## 5. Conclusions

The present study described expression of TRPM2 in the rat TG. Small to medium-sized neurons in the TG contained TRPM2-immunoreactivity. Subpopulations of TRPM2-IR TG neurons were also immunoreactive for TRPV1 or CGRP. TRPM2 expression was common in cutaneous TG neurons but not in pulpal TG neurons. Distribution of TRPM2-immunoreactivity was barely affected by ION transection and CFA application into the facial skin. Expression of TRPM2 mRNA in the TG increased after ION injury but not CFA application. These findings suggest that TRPM2 have function about nociceptive transmission from oral and facial structures. This channel may also respond to the nerve injury as an oxidative stress sensor in the injured TG.

## Conflict of interest

The author declares that I have no conflict of interest.

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References

Akpınar, H., Nazıroğlu, M., Övey, İ. S., Çiğ, B., & Akpınar, O. (2016) The neuroprotective action of dexmedetomidine on apoptosis, calcium entry and oxidative stress in cerebral ischemia-induced rats: Contribution of TRPM2 and TRPV1 channels. Sci Rep 6:37196.

Atsumi, K., Yajima, T., Tachiya, D., Kokubun, S., Shoji, N., Sasano, T., Ichikawa, H., & Sato, T. (2020) Sensory neurons in the human jugular ganglion. Tissue Cell 64:101344.

Cady, R. J., Glenn, J. R., Smith, K. M., & Durham, P. L. (2011) Calcitonin gene-related peptide promotes cellular changes in trigeminal neurons and glia implicated in peripheral and central sensitization. Mol Pain 7:94.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., & Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

Chung, M. K., Asgar, J., Lee, J., Shim, M. S., Dumler, C., & Ro, J. Y. (2015) The role of TRPM2 in hydrogen peroxide-induced expression of inflammatory cytokine and chemokine in rat trigeminal ganglia. Neuroscience 297:160-169.

Guo, A., Vulchanova, L., Wang, J., Li, X., & Elde, R. (1999) Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 11:946–958.

Hironaka, K., Ozaki, N., Hattori, H., Nagamine, K., Nakashima, H., Ueda, M., & Sugiura, Y. (2014) Involvement of glial activation in trigeminal ganglion in a rat model of lower gingival cancer pain. Nagoya Journal of Medical Science 76:323-332.

Hung, C. Y., & Tan, C. H. (2018) TRP Channels in Nociception and Pathological Pain. Adv Exp Med Biol 1099:13-27.

Ichikawa, H., & Sugimoto, T. (2001) VR1-immunoreactive primary sensory neurons in the rat trigeminal ganglion. Brain Res 890:184-188.

Kim, Y. S., Kim, S. K., Lee, J. S., Ko, S. J., & Bae, Y. C. (2018) Expression of vesicular glutamate transporters in transient receptor potential ankyrin 1 (TRPA1)-positive neurons in the rat trigeminal ganglion. Brain Res 1690: 31-39.

Knowles, H., Li, Y., & Perraud, A. L. (2013) The TRPM2 ion channel, an oxidative stress and metabolic sensor regulating innate immunity and inflammation. Immunol Res 55:241-248.

Liu, F., Yajima, T., Wang, M., Shen, J. F., Ichikawa, H., & Sato, T. (2020) Effects of trigeminal nerve injury on the expression of galanin and its receptors in the rat trigeminal ganglion. Neuropeptides 84:102098.

Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Sato, M., Sato, T., Yajima, T., Shimazaki, K., & Ichikawa, H. (2018) The transient receptor potential cation channel subfamily V members 1 and 2, P2X purinoceptor 3 and calcitonin gene-

related peptide in sensory neurons of the rat trigeminal ganglion, innervating the periosteum, masseter muscle and facial skin. Arch Oral Biol 96:66-73.

Sato, T., Fujita, M., Shimizu, Y., Kanetaka, H., Chu, L. W., Côté, P. D., Ichikawa, H. (2015) Glial reaction in the spinal cord of the degenerating muscle mouse (Scn8a(dmu)). Neurochem Res 40:124-129.

Shimada, Y., Sato, T., Yajima, T., Fujita, M., Hashimoto, N., Shoji, N., Sasano, T., & Ichikawa, H. (2016) SCN2B in the Rat Trigeminal Ganglion and Trigeminal Sensory Nuclei. Cell Mol Neurobiol 36:1399-1408.

Silverman, J.D., & Kruger, L. (1987) An interpretation of dental innervation based upon the pattern of calcitonin gene-related peptide (CGRP)-immunoreactive thin sensory axons. Somatosens Res 5 :157-175.

Silverman, J.D., & Kruger, L. (1989) Calcitonin-gene-related-peptide-immunoreactive innervation of the rat head with emphasis on specialized sensory structures. J Comp Neurol 280: 303-330.

So, K., Haraguchi, K., Asakura, K., Isami, K., Sakimoto, S., Shirakawa, H., Mori, Y., Nakagawa, T., & Kaneko, S. (2015) Involvement of TRPM2 in a wide range of inflammatory and neuropathic pain mouse models. J Pharmacol Sci 127:237-243.

Stefanini, M., De Martino, C., & Zamboni, L. (1967) Fixation of ejaculated spermatozoa for electoron microscopy. Nature 216:173-174.

Sugimoto, T., Fujiyoshi, Y., Xiao, C., He, Y. F., & Ichikawa, H. (1997) Central projection of calcitonin gene-related peptide (CGRP)- and substance P (SP)-immunoreactive trigeminal primary neurons in the rat. J Comp Neurol 378:425-442.

Valtschanoff, J.G., Rustioni, A., Guo, A., & Hwang, S. J. (2001) Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. J Comp Neurol 436:225-235.

Yajima, T., Sato, T., Shimazaki, K., & Ichikawa, H. (2019) Transient receptor potential melastatin-3 in the rat sensory ganglia of the trigeminal, glossopharyngeal and vagus nerves. J Chem Neuroanat 96:116-125.

Table 1: List of antibodies

| Antibody | Species | Source | Catalog No. | Dilution |
|---|---|---|---|---|
| **Primary anti serum** | | | | |
| CGRP | Guinea pig | Peninsula Laboratories, USA | T-5053 | 1:1000 |
| TRPM2 | Rabbit | Alomone Labs, Israel | ACC-043 | 1:300 |
| TRPV1 | Goat | R&D systems Inc., USA | AF3066 | 1:200 |
| **Secondary antibody** | | | | |
| Fluorescein isothiocyanate-conjugated anti-goat IgG for TRPV1 | Donkey | Jackson ImmunoResearch Labs., USA | 705-096-147 | 1:100 |
| Fluorescein isothiocyanate-conjugated anti-guinea pig IgG for CGRP | Donkey | Jackson ImmunoResearch Labs., USA | 706-096-148 | 1:100 |
| Lissamine rhodamine red$^{TM}$-X-conjugated anti-rabbit IgG for TRPM2 | Donkey | Jackson ImmunoResearch Labs., USA | 711-296-152 | 1:300 |

Table 2: Proportion of sensory neurons which contain TRPM2, TRPV1 and CGRP in the TG.

| | Proportion mean ± S.D. (%) |
|---|---|
| TRPM2 | 33.5 ± 2.1 |
| TRPV1 | 35.6 ± 2.4 |
| CGRP | 31.4 ± 7.5 |
| TRPM2 & TRPV1 | 16.8 ± 1.6 |
| TRPM2 & CGRP | 17.1 ± 1.3 |

The data were obtained from 10375 TG neurons in 4 animals.

Table 3: Expression of TRPM2 and other substances among small, medium-sized and large TG neurons.

| | Mean ± S.D. (μm²) | Small (%) | Medium (%) | Large (%) |
|---|---|---|---|---|
| All TG neurons | 623.5 ± 371.5 | 34.6 | 38.9 | 26.4 |
| TRPM2 | 478.6 ± 247.8 | 46.3 | 43.3 | 10.4 |
| TRPV1 | 450.9 ± 191.6 | 43.2 | 51.3 | 5.5 |
| CGRP | 466.9 ± 264.9 | 53.9 | 35.1 | 11.0 |
| TRPM2 & TRPV1 | 432.9 ± 184.5 | 46.9 | 48.4 | 4.7 |
| TRPM2 & CGRP | 432.6 ± 184.5 | 56.4 | 37.4 | 6.2 |

The data were obtained from 10375 TG neurons in 4 animals.

Table 4: Expression of TRPM2 and other substances among TG neurons which innervate the facial skin and tooth pulp.

| | Facial skin (%) | Tooth pulp (%) |
|---|---|---|
| TRPM2 | 34.1 ± 1.6 | 15.7 ± 4.1 |
| TRPV1 | 32.3 ± 3.7 | 22.5 ± 9.2 |
| CGRP | 26.8 ± 4.1 | 47.3 ± 7.6 |
| TRPM2 & TRPV1 | 17.7 ± 3.7 | 13.4 ± 8.8 |
| TRPM2 & CGRP | 16.1 ± 3.4 | 9.6 ± 5.8 |

The data were obtained from design of a specific tissue labeled per rat, with n = 4 rats for the external ear canal and n = 4 rats for the circumvallate papilla.

Table5:

Expression of TRPM2 and other substances among small, medium-sized and large TG neurons which innervate the facial skin and tooth pulp.

| | Facial skin | | | | Tooth pulp | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± S.D. ($\mu m^2$) | Small | Medium | Large | Mean ± S.D. ($\mu m^2$) | Small | Medium | Large |
| All TG neurons | 522.4 ± 321.1 | 40.6% | 39.5% | 19.9% | 784.3 ± 369.6 | 18.8% | 36.6% | 44.7% |
| TRPM2 | 420.6 ± 194.6 | 58.6% | 37.1% | 4.3% | 516.8 ± 254.5 | 37.5% | 50.0% | 12.5% |
| TRPV1 | 425.2 ± 166.7 | 50.2% | 47.3% | 2.5% | 480.5 ± 212.5 | 35.8% | 56.7% | 7.5% |
| CGRP | 460.6 ± 246.5 | 48.1% | 39.5% | 12.4% | 734.5 ± 310.1 | 17.7% | 40.7% | 41.6% |

The data were obtained from design of a specific tissue labeled per rat, with n = 4 rats for the facial skin and n = 4 rats for the tooth pulp.

Fig. 1 Microphotographs for TRPM2 (A, C, D, F), TRPV1 (B, C) and CGRP (E, F) in the TG. Panels A-C and D-F are from the same fields of the view, respectively. Double immunofluorescence method demonstrates that some TRPM2-IR TG neurons (arrows in A, D) have TRPV1- (arrows in B, C) or CGRP-immunoreactivity (arrows in E, F). Asterisks in A-F indicate TRPM2-IR TG neurons which are free of TRPM2-, TRPV1- and CGRP-immunoreactivity. Bar = 50 μm (A). Panels A-F are at the same magnification. Histograms showing cell size distribution of TRPM2-IR TG neurons with TRPV1- (G) or CGRP-immunoreactivity (H). The data were analyzed from 4950 and 5425 TG neurons for TRPV1 and CGRP, respectively.

Fig. 2 Microphotographs for FG (A, D), TRPM2 (B, E), TRPV1 (C), CGRP (F) in the TG. Panels A-C and D-F are from the same fields of view, respectively. A TG neuron retrogradely labeled from the facial skin (arrow in A) contains both TRPM2- (arrow in B) and TRPV1-immunoreacyivity (an arrow in C). A TG neuron retrogradely labeled from the tooth pulp (arrowhead in D) contains CGRP- (arrowhead in F) but not TRPM2-immunoreactivity (arrowhead in E). Bar = 50 μm (A). Panels A-F are at the same magnification. Histograms showing cell size distribution of TRPM2-IR neurons which innervate the facial skin (G) and tooth pulp (H). The data were analyzed from 978 facial skin neurons and 309 tooth pulp neurons.

Fig. 3 Microphotographs for TRPM2 in the TG of animals with sham operation (A), ION transection (B), and saline (C) and CFA (D) treatments. Asterisks show TG neurons which are free of TRPM2-immunoreactivity. Bar = 50 μm (A). All panels are at the same magnification.

Fig. 4 Bar graphs showing expression of TRPM2 mRNA (A) and proportion of TRPM2-IR neurons (B) in the TG of animals with sham operation, ION transection, and saline and CFA treatments. Expression of TRPM2 mRNA between sham operation and ION transection was statistically significant ($p < 0.05$ Welch's t-test).

**Fig. 1**

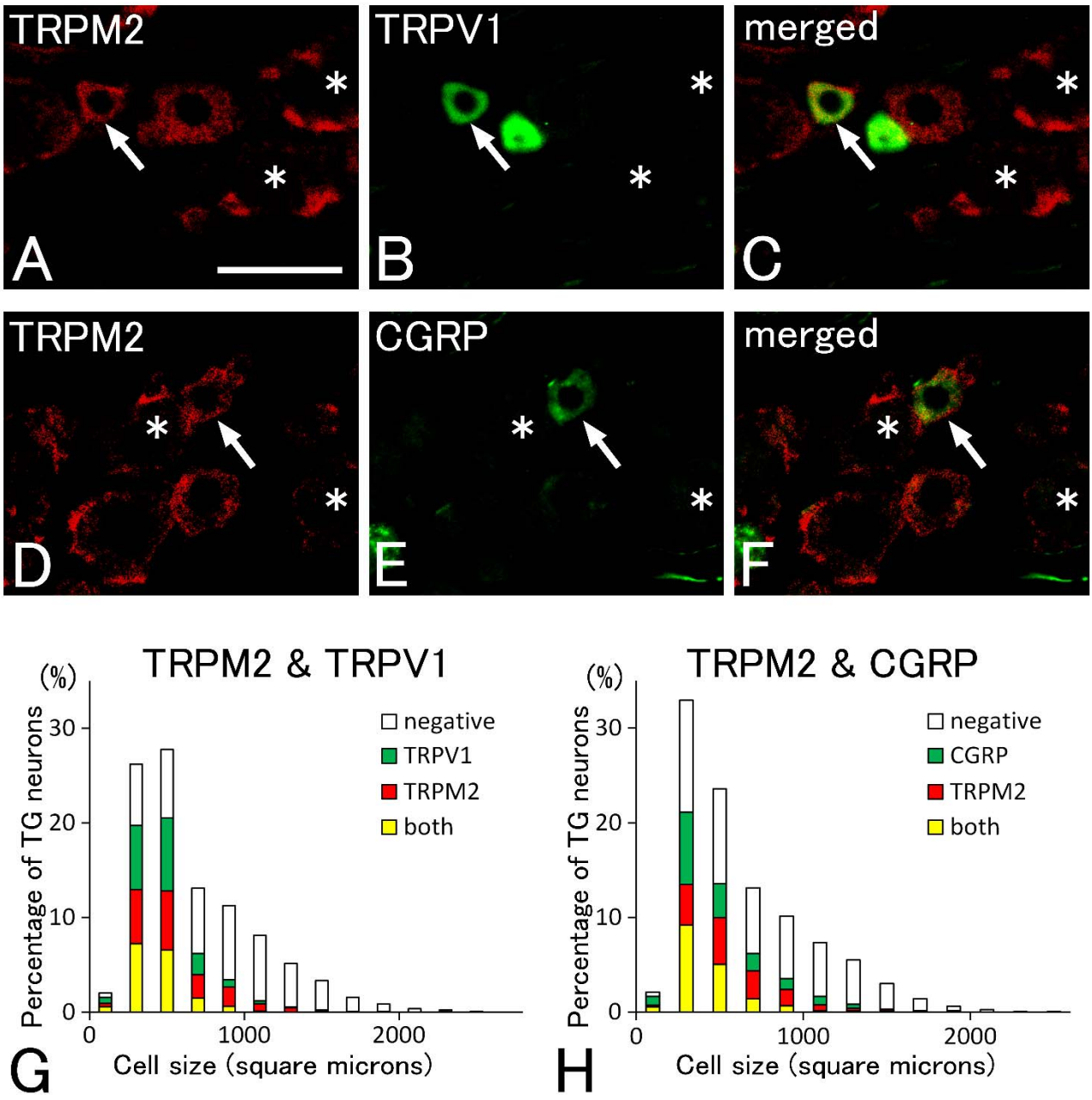

TRPM2
TRPV1
merged
A
B
C
TRPM2
CGRP
merged
D
E
F
TRPM2 & TRPV1
(%)
Percentage of TG neurons
negative
TRPV1
TRPM2
both
Cell size (square microns)
G
TRPM2 & CGRP
(%)
Percentage of TG neurons
negative
CGRP
TRPM2
both
Cell size (square microns)
H

**Fig. 2**

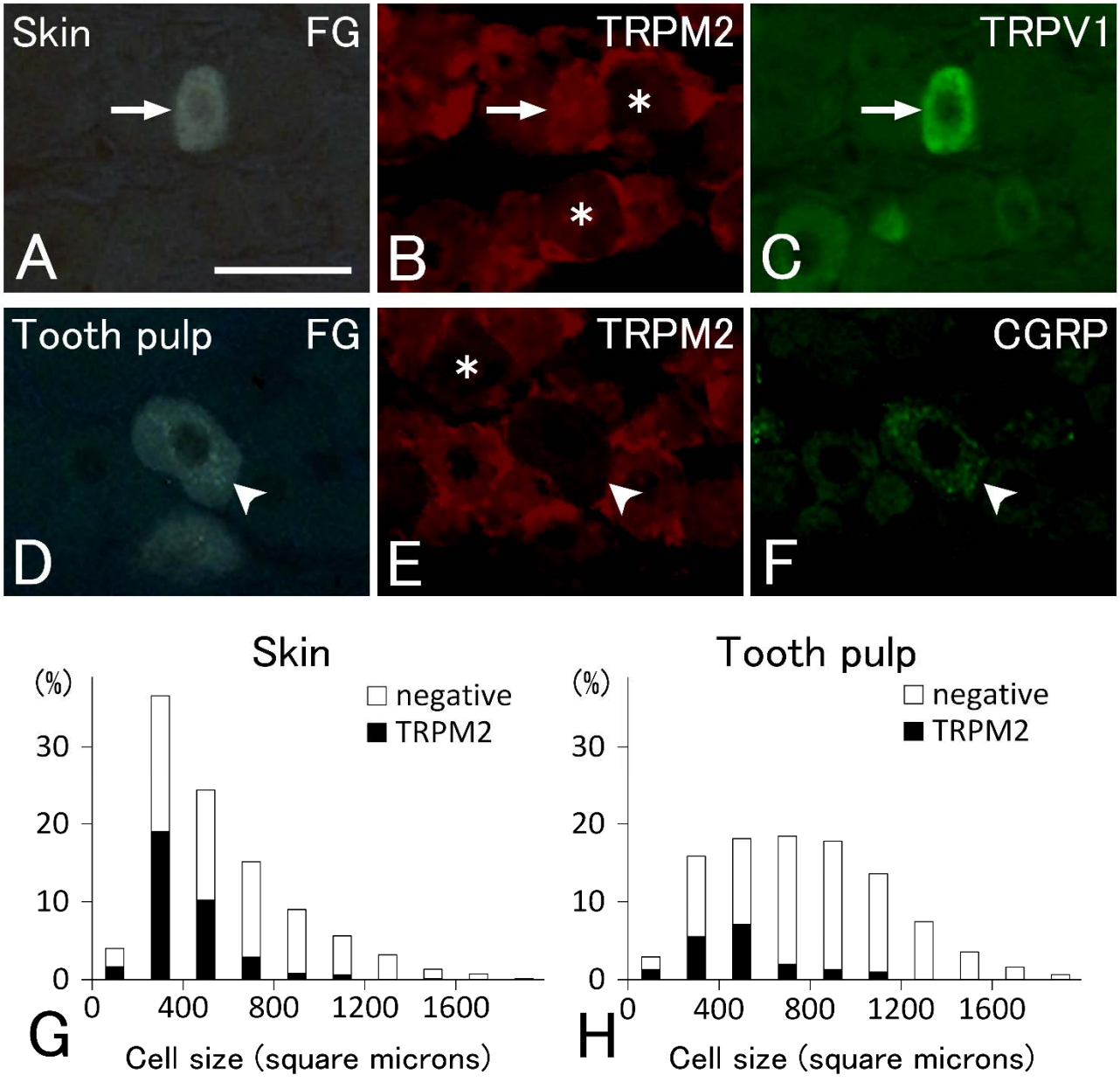

Skin
FG
A
TRPM2
B
TRPV1
C
Tooth pulp
FG
D
TRPM2
E
CGRP
F
Skin
(%)
negative
TRPM2
30
20
10
0
0
400
800
1200
1600
Cell size (square microns)
G
Tooth pulp
(%)
negative
TRPM2
30
20
10
0
0
400
800
1200
1600
Cell size (square microns)
H

**Fig. 3**

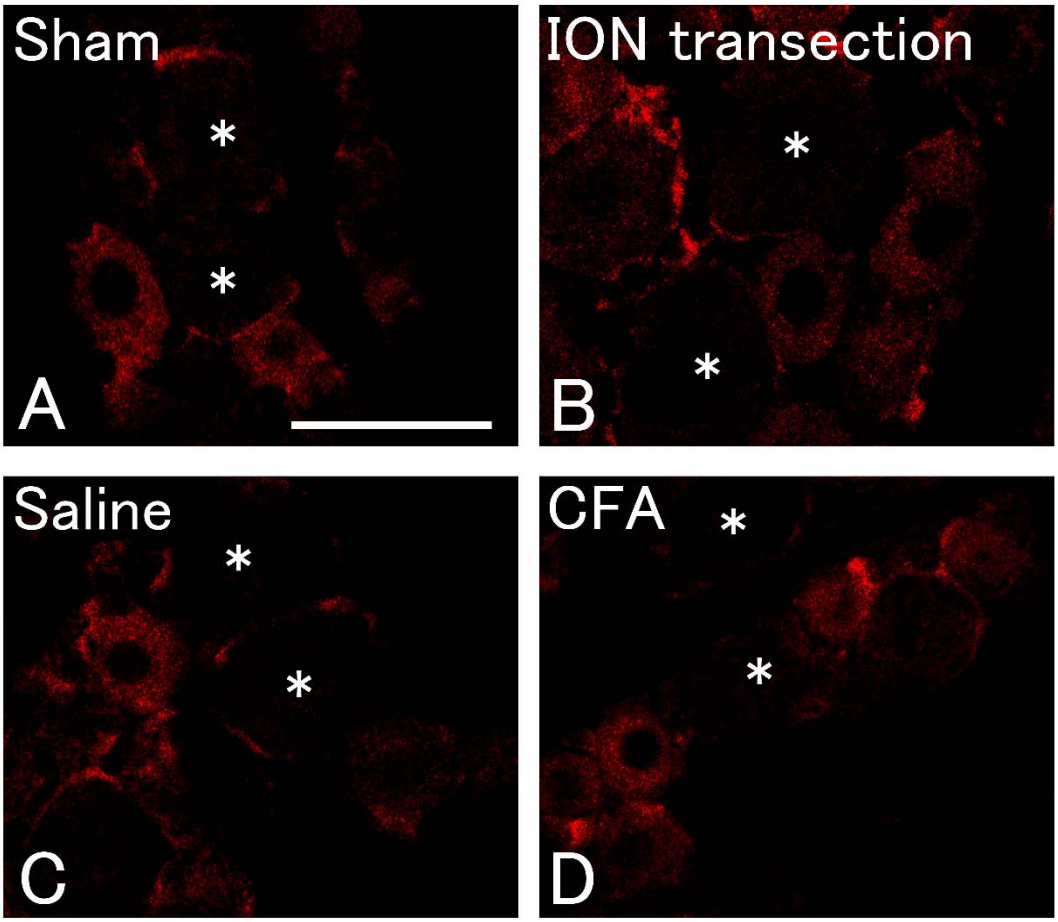
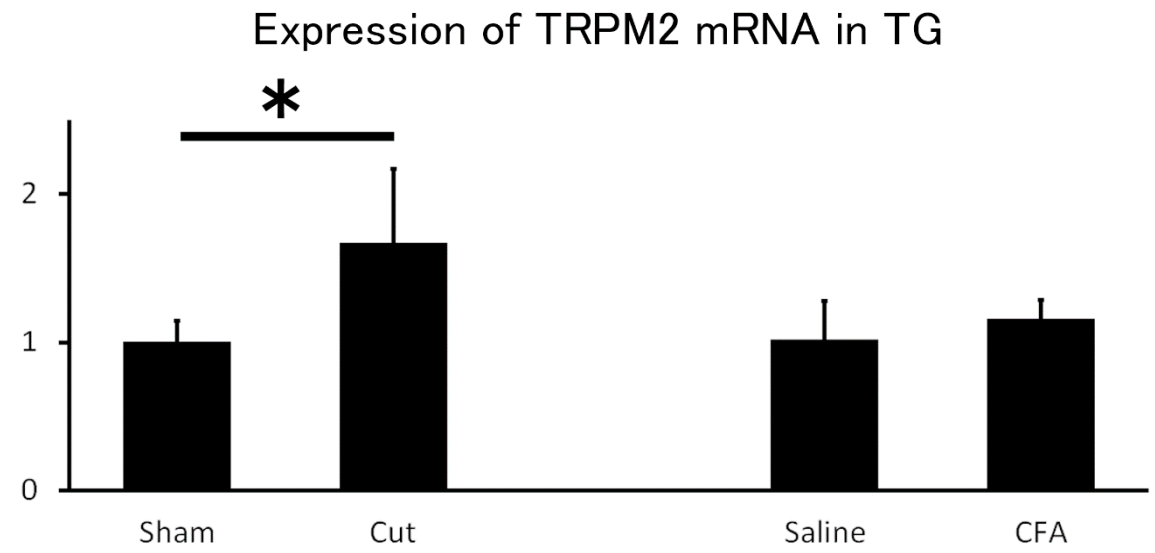
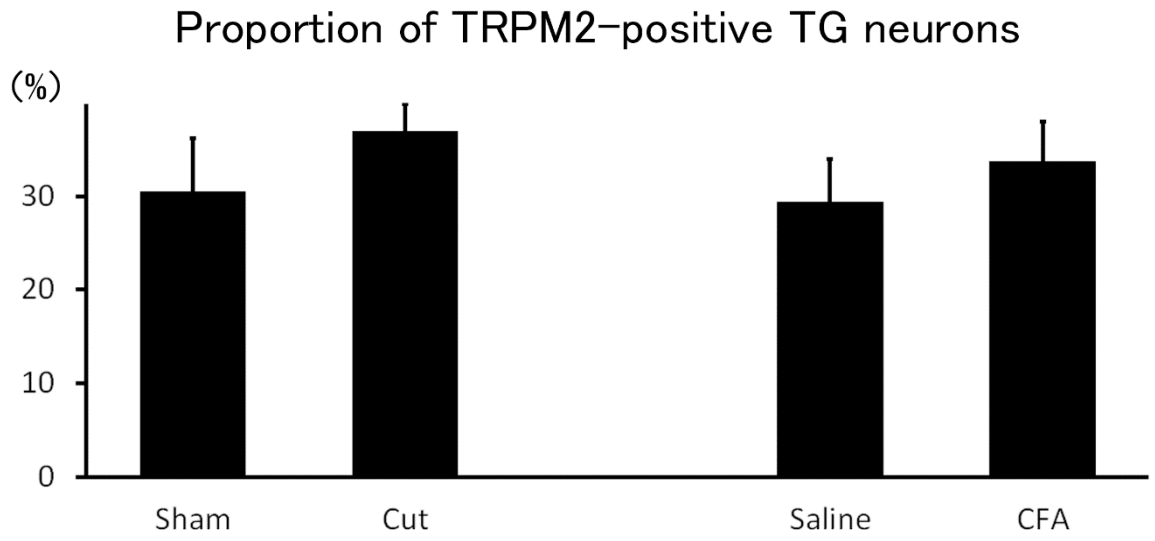


**Fig. 4**


Expression of TRPM2 mRNA in TG
*
2
1
0
Sham
Cut
Saline
CFA


Proportion of TRPM2-positive TG neurons
(%)
30
20
10
0
Sham
Cut
Saline
CFA