# Evaluation of TRPM8 and TRPA1 participation in dental pulp sensitivity to cold stimulation

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Conflict of interest: none declared

**Abstract**

Sensory neurons innervating the dental pulp have unique morphological and functional characteristics compared to neurons innervating other tissues. Stimulation of dental pulp afferents whatever the modality or intensity of the stimulus, even light mechanical stimulation that would not activate nociceptors in other tissues, produces an intense pain. These specific sensory characteristics could involve receptors of the Transient Receptor Potential channels (TRP) family. In this study, we evaluated 1) the expression of TRPA1 and TRPM8 receptors in trigeminal ganglion neurons innervating the dental pulp compared to sensory neurons innervating the oral mucosa or the skin of the face, and 2) the involvement of these receptors in dental pulp sensitivity to cold stimulation. We showed a similar expression of TRPM8 and CGRP in sensory neurons innervating the dental pulp, the skin or the oral mucosa. However, the proportion of neurons expressing TRPA1 was higher in the neuronal population innervating the mucosa (43%) than in the neuronal population innervating the dental pulp (19%) or the skin (24%). Moreover, neurons innervating the dental pulp had a higher proportion of large neurons (24%) compared to neurons innervating the skin (8%) or the mucosa (10%). The evaluation of the relation between TRPM8, TRPA1 and cold stimulation in trigeminal ganglion neuron culture, showed that a significant proportion of neurons innervating the skin (10%) or the mucosa (37%) were sensitive to cold stimulation but insensitive to TRPM8 and TRPA1 activation, indicating that perception of stimuli would be in part independent to TRPA1 and TRPM8 receptors. Similarly, the application of a cold stimulation on the tooth induced an overexpression of cFos in the trigeminal nucleus that was not prevented by administration of a TRPA1 antagonist or the genetic deletion of TRPM8. However, the pretreatment with the local anesthetic carbocaine abolish the cold-induced cFos overexpression. In conclusion, the unique sensory characteristics of the dental pulp would be independent to TRPA1 and TRPM8 expression and functionality.

**Key words :** TRPA1, TRPM8, cold perception, dental pulp.

## 1. Introduction

Dental pup is a unique tissue, highly innervated that has specific sensory carateristiscs compared to other tissue. In the pulp, although most peripheral nerve endings appear to be small unmyelinated C and small myelinated A delta nociceptors, they originate form large diameter myelinated fibers in the trigeminal ganglion (Paik et al., 2009; Gibbs et al., 2011). These sensory afferents have characteristics of low threshold mechanosensor that express for a part of them markers of nociceptors such as TrkA, CGRP and TRPV1 (Cadden et al., 1983; Ichikawa et al.,1995; Yang et al., 2006; Hermanstyne et al., 2008; Gibbs et al., 2011). Further, stimulation of dental pulp afferents whatever the modality or intensity of the stimulus, even light mechanical stimulation that would not activate nociceptors in other tissues, produces an intense pain (Dababneh et al., 1999).

The Transient Receptor Potential (TRP) channels, involved in the detection of thermal and mechanical stimuli (Julius, 2013), could be involved in the specific sensory characteristics of the dental pulp. Among these receptors, TRPA1 and TRPM8 are expressed in dental pulp afferents and are particularly important in the detection of cold stimulation (Park et al., 2006; Kim et al., 2012; Julius, 2013). TRPA1 receptor is expressed in peptidergic C fibers and activated by temperature below 17°C (Story et al., 2003). Previous data showed that pharmacological blockade and genetic deletion of TRPA1 prevented cold hypersensitivity in inflammatory and neuropathic conditions (Kwan et al., 2006; Nassini et al., 2014). In contrast to TRPA1, TRPM8, is expressed in a specific non peptidergic sensory neuron subpopulation that do not express IB4 and NF200, and is activated by temperature below 25°C (Peier et al., 2002; Story et al., 2003). The participation of TRPM8 in cold sensitivity is not clearly understood, while TRPM8 was suggested to be involved in cool perception, data showed that TRPM8 participate also to noxious cold sensation (Knowlton et al., 2013; Patel et al., 2014).

Cold sensitivity is an important symptom in tooth pain and the specific physiological characterictics of pulp sensitivity would be critical to understand pain mechanism and improve diagnosis in pulp inflammation. This study aims at determining the characteristics of TRPM8 and TRPA1 expression in sensory neurons innervating the dental pulp compared to neurons innervating the skin of the face or the oral mucosa. We also evaluated the participation of TRPA1 and TRPM8 in dental pulp sensitivity to cold stimulation.

## 2 Materials and Methods

### 2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the New

York University College of Dentistry and followed the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on 10-12 weeks old female C57Bl6 and TRPM8 knock-out (*Trpm8*$^{tm1Apat}$-GFP-tagged) mice purchased from Jackson Laboratory. Animals were housed 2-5/cage in an environment with controlled 12h/12h light/dark cycles and had free access to food and water.

**2.2 Sensory neuron retrolabeling**

Mice were anaesthetized with 2% isoflurane and intradermally injected with 10µl of Fluorogold (2.5% in saline) or 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, 2.5% in ethanol) in either in the skin of the cheek or in the oral mucosa to label the respective innervating neurons. Dental pulp innervated neurons retrolabeling was performed in mice anaesthetized by an intraperitoneal injection of ketamine-xylazine (100-10 mg/kg). The enamel of the first molar in each side of the jaw was removed with a ¼ round dental bur at low speed to expose dentinal tubules without exposing the dental pulp to the oral environment. Ten microliters of Fluorogold or DiI was applied three times with a 5-min interval. At the end of each procedure mice were treated once a day for three days with carprophen (5 mg/kg, i.p.) to avoid the development of a local inflammation that could affect protein expression in sensory neurons.

**2.3 TRPA1 and CGRP immunostaining**

Seven days after sensory neurons retrolabeling with Fluorogold, *Trpm8*$^{tm1Apat}$-GFP-tagged and wild type mice were euthanized and perfused intracardially with 10 ml of phosphate-buffered saline (PBS 1X) followed by 30 ml of 10% formaline in PBS. The trigeminal ganglia were removed, post-fixed for 30 min in 10% formaline and cryoprotected overnight in PBS with 30% sucrose. Trigeminal ganglia were cut (16 µm thick sections) with a Cryostat (−20°C) and collected on superfrost glass slides. Sections were washed for 30 min in PBS, followed by 1h incubation in a blocking solution containing 0.3% Triton X100 and 5% normal goat serum in PBS. Sections were then incubated at 4°C for 3 days with rabbit anti-TRPA1 antibody (1:10000; AB58844; abcam). After a 30-min wash in the blocking solution, samples were incubated for 2h with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (1:700; Life Technology). Then, sections were washed in blocking solution for 30 min and incubated at 4°C overnight with mouse anti-CGRP antibody (1:8000; C7113, Sigma). The following day, sections were washed for 30 min in PBS and successively incubated for 1h in the blocking solution and 2h with the secondary antibody Alexa Fluor 546-conjugated goat anti-mouse (1:700; Life Technology). After a final 30 min wash, sections were coverslipped. TRPM8 expressing neurons were stained green in *Trpm8*$^{tm1Apat}$-GFP-tagged mice. Photo-micrographs of trigeminal ganglia were collected with a fluorescent inverted microscope (Nikon Eclipse Ti). A quantitative analysis was performed using the NIH Image J analysis Software. Cell body of Fluorogold retrolabeled neurons was delineated, then, after background substraction, neurons stained for more than 50% of the cell body area were

considered immunoreactive and used for the analysis.

### 2.4 Trigeminal ganglion neuron culture

Trigeminal ganglia from DiI retrolabeled C57Bl6 mice were collected in F12 culture media and cut in ten pieces each. Then, they were incubated for 20 min with papain 40 units/ml in Hank's Balanced Salt Solution (HBSS) without calcium. After 2 min centrifugation at 180g the supernatant was removed, and trigeminal ganglia incubated for 20 min in collagenase-dispase solution (3.33-4.66 mg/ml in HBSS without calcium). After 4 min centrifugation at 400g the supernatant was removed. Neurons were resuspended in F12 culture media and centrifuged for 6 min at 460g to remove the excess of collagenase-dispase solution. Then, neurons were suspended in F12 media supplemented with 5% Fetal Bovine Serum, mechanically dissociated with a P200 pipet and seeded into poly-D-Lysine-coated 25 mm round glass coverslips. After 3h incubation at 37°C in a humidified atmosphere containing 5% $CO_2$, neuronal culture was processed for calcium imaging experiment.

### 2.5 Single-cell calcium imaging

Trigeminal ganglion neurons cultured on round glass coverslips were washed for 10 min in HBSS and loaded in the dark with the fluorescent calcium indicator Fura-2-acetoxy-methyl ester (Fura2, 5 µM) for 45 min at room temperature. Then, coverslips were washed for 10 min in HBSS and mounted in a microscope chamber. Loaded cells were excited successively (2 Hz) for Fura2 at 340 and 380 nm for 200 ms and emitted fluorescence was monitored at 510 nm using a charged coupled device sensor camera coupled to an inverted Nikon Eclipse Ti microscope. Fluorescence intensities from single cells excited at the two wavelengths were recorded separately, corrected for the background and the fluorescence ratio (F340/F380) was calculated using the software NIS Elements-AR version 4.0. All neurons were treated with the TRPA1 agonist acyl-isothiocyanate (AITC, 250µM), the TRPM8 agonist menthol (250µM) and ice cooled HBSS. At the end of each experiment KCl (75mM) was applied to identify healthy neurons. Only neurons retrolabeled with DiI that responded to KCl were used for the analysis.

### 2.6 Cold stimulation and cFos immunostaining

C57Bl6 mice pretreated with either vehicle, the TRPA1 antagonist HC030031 (dissolved in DMSO, 100mg/kg, i.p.) or carbocaine (3% in saline, 10µl injected periodontally) and TRPM8 knock-out mice were anaesthetized with ketamine-xylazine. The jaw was opened and a unilateral cold stimulation was applied on the first molar using a small piece of cotton cooled with frigident. The stimulation was repeated 15 times over a 30-min period. Three hours after the beginning the stimulation mice were perfused intracardially with formaline, then the brainstem was collected, post-fixed and cryoprotected as described in part 2.6. Thirty micrometers thick sections of the brainstem were cut in a cryostat (-20°C) and collected in containers filled with PBS. Floating

sections were incubated 1h in a blocking solution containing 0.3% Triton X100 and 5% normal goat serum in PBS and then incubated overnight with rabbit anti-cFos antibody (1/30000, PC38, Calbiochem). After 30 min wash with the blocking solution, sections were incubated for 1h with the secondary antibody biotinylated goat anti-rabbit (1/700; Vector). After a 30-min wash with PBS, the sections were successively incubated for 30 min in 0.3% $H_2O_2$ in PBS and for 1h with the avidin-biotinylated-horseradish-peroxidase (ABC Vectastain kit Elite, Vector). Then they were washed 10 min with PBS and stained black/gray with 3,3′-Diaminobenzidine (DAB kit, Vector). After a final 30 min wash in PBS, sections were mounted on superfrost glass slides and coverslipped.

### 2.7 Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 software. Immunostaining of TRPM8, TRPA1 and CGRP, and calcium imaging data are expressed as percentage of immunoreactive or responsive neurons relative to the number of Fluorogold/DiI positive neurons and were analysed with Chi-square test. Results of cFos immunostaining are expressed as a mean+S.E.M of the number immunoreactive neurons/section/animal and were analyzed with a 2-way ANOVA, followed by Bonferroni post hoc test. The significance level was set at $p<0.05$.

## 3 Results

### 3.1 Comparison of TRPM8 and TRPA1 expression in sensory neurons innervating the facial skin, the oral mucosa or the dental pulp

The dental pulp has unique sensory characteristic that would be dependent to TRP channels. We addressed this question comparing the expression of the cold sensitive receptors TRPM8 and TRPA1 in sensory neurons innervating the dental pulp, the skin of the cheek or the oral mucosa (Fig 1, 2). TRPM8 receptors were expressed in 5.7% of neurons innervating the dental pulp but no difference was shown when compared to TRPM8 expression in neurons innervating the facial skin and the oral mucosa (3.3% and 6.7% of TRPM8 immunoreactive neurons respectively, Fig 3A). Similarly, CGRP, a marker of small peptidergic nociceptors, was expressed in the same proportion sensory neurons whatever the innervating tissue (skin: 17.8%, mucosa: 14.3%, and dental pulp: 14.9%; Fig 3C). Interestingly, TRPA1 was expressed in a higher proportion of neurons innervating the mucosa than neurons innervating the dental pulp (43.0% versus 18.9%, $p=0.0008$, Chi-square test; Fig 3B) or the skin (43.0% versus 24.6%, $p<0.0001$, Chi-square test; Fig 3B). Moreover, we showed that a lower, but not statistically significant, proportion of neurons innervating the dental pulp co-expressed CGRP and TRPA1 compared to neurons innervating the skin (3.7% versus 13.7%, $p=0.091$, Chi-square test; Fig 3D) or the mucosa (3.7% versus 12.7%, $p=0.15$, Chi-square test, Fig 3D). To characterized further neurons innervating the dental pulp we evaluated the size distribution of neurons (Fluorogold positive) innervating the dental pulp the skin or the mucosa, and neurons expressing TRPA1 and CGRP that innervate each tissue. The proportion of large neurons

(>1200µm2) is significantly higher in the neuronal population innervating the dental pulp (24.1% of large neurons) than in the neuronal population innervating the skin (8.7% of large neurons, p<0.0001, Chi-square test; Fig 3E) or the mucosa (10.7% of large neurons, p=0.0002, Chi-square test, Fig 3E). The comparison of the size distribution of CGRP or TRPA1 immunoreactive neurons showed no difference between neuronal populations innervating the dental pulp, the skin or the mucosa (Fig 3F, 3G).

### 3.2 Relation between the sensitivity to Menthol, AITC and cold stimulation in sensory neurons innervating the facial skin and the oral mucosa

To clarify the participation of TRPM8 and TRPA1 receptor in the detection of cold stimulation, we evaluated neuronal activity in response to ice cooled HBSS, the TRPM8 agonist menthol and the TRPA1 agonist AITC in neurons innervating the oral mucosa or the skin. Because neurons innervating teeth are only a low proportion of trigeminal sensory neurons and a significant number of neurons is lost during the cell culture procedure we were not able to detect DiI retrolabeled sensory neurons innervating the dental pulp.

A similar proportion of neurons innervating the skin or the mucosa responded to menthol (3.0% and 3.1% respectively) or AITC (56.0% and 46.7% respectively, p=0.10, Chi-square test) and no neurons responded to both menthol and AITC (Fig 4C). A detailed analysis comparing neurons responding to cold stimulation and menthol showed that all neurons responding to menthol were also sensitive to cold stimulation, but a large proportion of neurons were cold sensitive and menthol insensitive (Fig 4D). Moreover, compared to the neuronal population innervating the skin, the neuronal population innervating the mucosa had a higher proportion of cold sensitive neurons (25.7% and 57.9% respectively, p<0.0001, Chi-square test; Fig 4D). The analysis of neuronal sensitivity to AITC and cold HBSS showed that a subpopulation of cold sensitive neurons were AITC insensitive (10.1% and 37% of neurons innervating the skin or the mucosa respectively; Fig 4E). Conversely, a significant proportion of neurons innervating the skin (15.5%) or the mucosa (21%) were AITC sensitive and cold insensitive, suggesting that TRPA1 is only partially involve in cold perception (Fig 4E).

### 3.3 TRPM8 and TRPA1 receptors are independent to dental pulp sensitivity to cold stimulation

The application of a unilateral cold stimulation on the first molar induced neuronal activation detected through the increase of cFos expression in the trigeminal nucleus (Fig 5A, 5B). In the caudal and the interpolaris part of the trigeminal nucleus the number of cFos imunoreactive neurons was not different in the ipsilateral versus contralateral side. However, in the transition zone between the caudal and the interpolaris part of the trigeminal nucleus, the number of cFos immunoreactive neurons was significantly higher in the ipsilateral compared to the contralateral side (34.8+/-5.1 and 10.5+/-3.0 respectively; 2-way ANOVA; side: p=0.0018, F=10.6, DF=1; region:

p=<0.0001, F=16.28, DF=2; interaction: p=0.0002, F=9.59, DF=2; Bonferroni's test, transition zone ipsi versus contra p<0.001; Fig 5C).

The involvement of TRPM8 and TRPA1 receptors in dental pulp sensitivity to cold stimulation was evaluated using TRPM8 KO mice and the TRPA1 antagonist, HC030031. In TRPM8 KO mice, cold stimulation increased the number of cFos immunoreactive neurons in the ipsilateral trigeminal nucleus transition zone (ipsi: 9.8+/-3.4 versus contra: 3.4+/-1.1) but the increase was not different to the number of cFos immunoreactive neurons in wild type mice (TRPM8 KO ipsi: 9.8+/-3.4 neurons versus WT ipsi: 8.4+/-2.1 neurons; 2-way ANOVA; side: p=0.014, F=7.26, DF=1; genotype: p=0.99, F=0.00001, DF=1; interaction: p=0.45, F=0.59, DF=1; Fig 5D). Similarly, TRPA1 receptors blockade with HC030031 did not prevent cold-induced upregulation of cFos in the ipsilateral compared to the contralateral trigeminal nucleus (2-way ANOVA; side: p=0.008, F=7.99, DF=1; treatment: p=0.54, F=0.37, DF=1; interaction: p=0.76, F=0.096, DF=1; Fig 5D). Whereas TRPA1 antagonist and genetic deletion of TRPM8 did not affect cFos upregulation, the administration carbocaine reduced the cold-induced increase of the number of cFos immunoreactive neurons in the trigeminal nucleus transition zone (2-way ANOVA; side: p<0.0001, F=65.4, DF=1; treatment: p<0.0001, F=57.3, DF=1; interaction: p<0.0001, F=33, DF=1; Bonferroni's test, carbocaine ipsi 5.1+/-1.5 neurons versus vehicle ipsi 19.3+/-1.1 neurons, p<0.001; Fig 5F).

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**Figure caption**

**Figure 1:** Expression of TRPM8 and CGRP in neurons innervating the facial skin, the oral mucosa or the dental pulp. Trigeminal sensory neurons innervating either the facial skin (A, B, C), the oral mucosa (D, E, F) or the dental pulp (G, H, I) were retrolabeled with Fluorogold (yellow) in *Trpm8*$^{tm1Apat}$-GFP-tagged mice. Seven days later trigeminal ganglia were collected and immunostained for CGRP (red). Neurons expressing TRPM8 are stained green. Arrowheads indicate fluorogold retrolabeled neurons. Scale bar : 50µm.

**Figure 2:** Expression of TRPA1 and CGRP in neurons innervating the facial skin, the oral mucosa or the dental pulp. Trigeminal sensory neurons innervating either the facial skin (A, B, C), the oral

mucosa (D, E, F) or the dental pulp (G, H, I) were retrolabeled with Fluorogold (yellow). Seven days later trigeminal ganglia were collected and immunostained for CGRP (red) and TRPA1 (green). Arrowheads indicate fluorogold retrolabeled neurons. Scale bar: 100µm

**Figure 3:** Quantitative analysis of TRPM8, TRPA1 and CGRP expression in trigeminal sensory neurons innervating the facial skin, the oral mucosa or the dental pulp. Proportion of Fluorogold retrolabeled trigeminal neurons that express, A) TRPM8, B) TRPA1, C) CGRP, and D) both TRPA1 and CGRP. E), proportion of small, medium and large neurons in the neuronal population (retrolabeled with Fluorogold) innervating either the skin, the mucosa or the dental pulp. F) and G), proportion of small (<600µm$^2$), medium (600-1200µm$^2$) and large neurons (>1200µm$^2$) expressing CGRP or TRPA1 in the neuronal population innervating either the skin, the mucosa or the dental pulp. *** $p<0.001$, Chi-square test, n=106-410 neurons.

**Figure 4:** Effects of AITC, cold stimulation and menthol on sensory neurons innervating the facial skin or the oral mucosa. A) and B), each trace represent intracellular calcium levels in one neuron (retrolabeled with DiI) innervating the facial skin or the oral mucosa that was treated with AITC (250µM), cold HBSS buffer, menthol (250µM), and KCl (75mM). Horizontal grey bars indicate the duration of each treatment. Bar-graphs show the proportion of neurons responding to, C) AITC or menthol, D) menthol or cold HBSS buffer, E) AITC and/or cold HBSS buffer. Note that no neurons were responsive to both AITC and menthol. *** $p<0.001$, Chi-square test, n=66-162 neurons.

**Figure 5:** Modulation of cold-induced cFos upregulation in the transition zone of the trigeminal nucleus. Representative photo-micrographs of cFos immunoreactive neurons in A) contralateral and B) ipsilateral transition zone of the trigeminal nucleus of mice which underwent unilateral cold stimulation of the 1$^{st}$ molar. C), quantitative analysis of cFos expression in the caudal part, the transition zone and the interpolaris part of the trigeminal nucleus. Effects of genetic deletion of TRPM8 (D), TRPA1 antagonist (E) and Cabocaine (F) on cold-induced upregulation of cFos in the trigeminal nucleus transition zone. Data are expressed as mean + S.E.M. of the number of neurons/section/animal. *** $p<0.001$, Bonferroni's test, n=5-13/group.

**Figure 1**

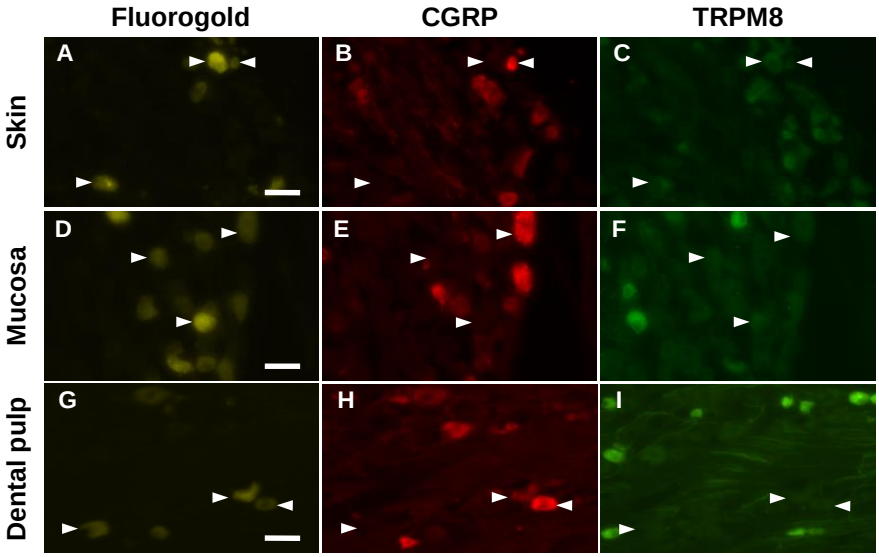


**Figure 2**

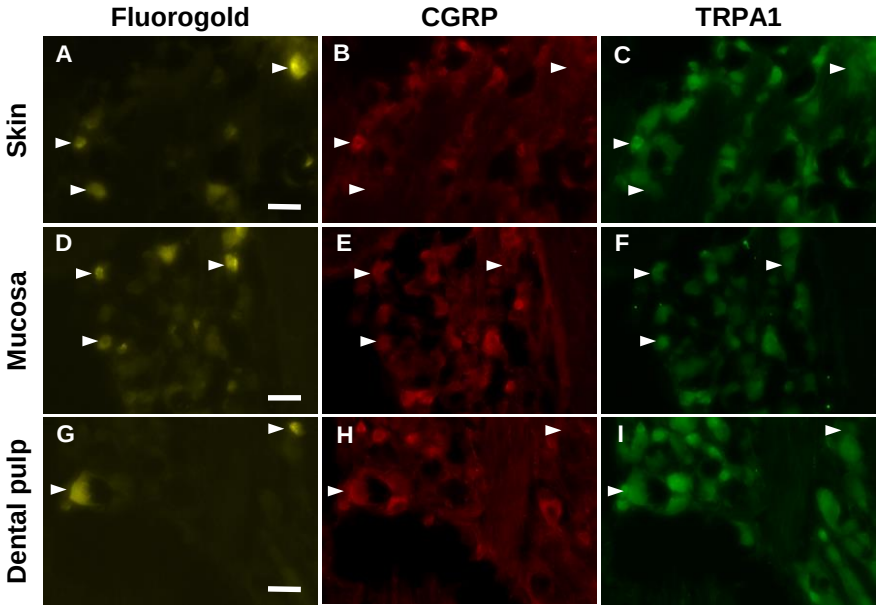


**Figure 3**

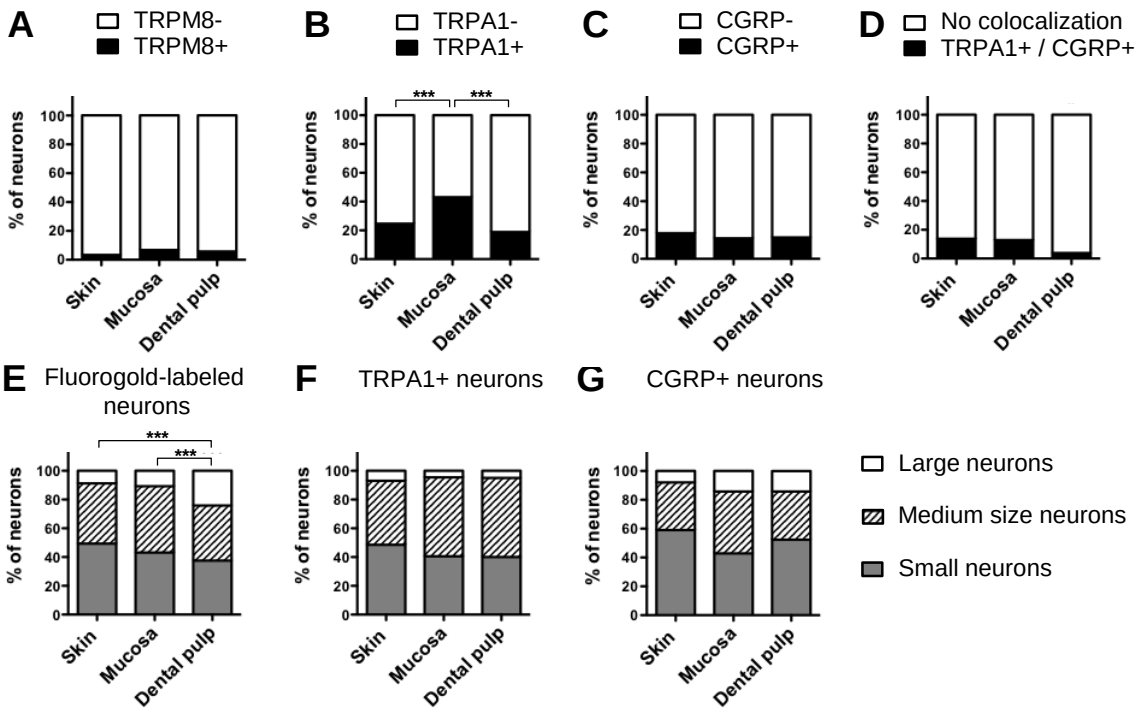
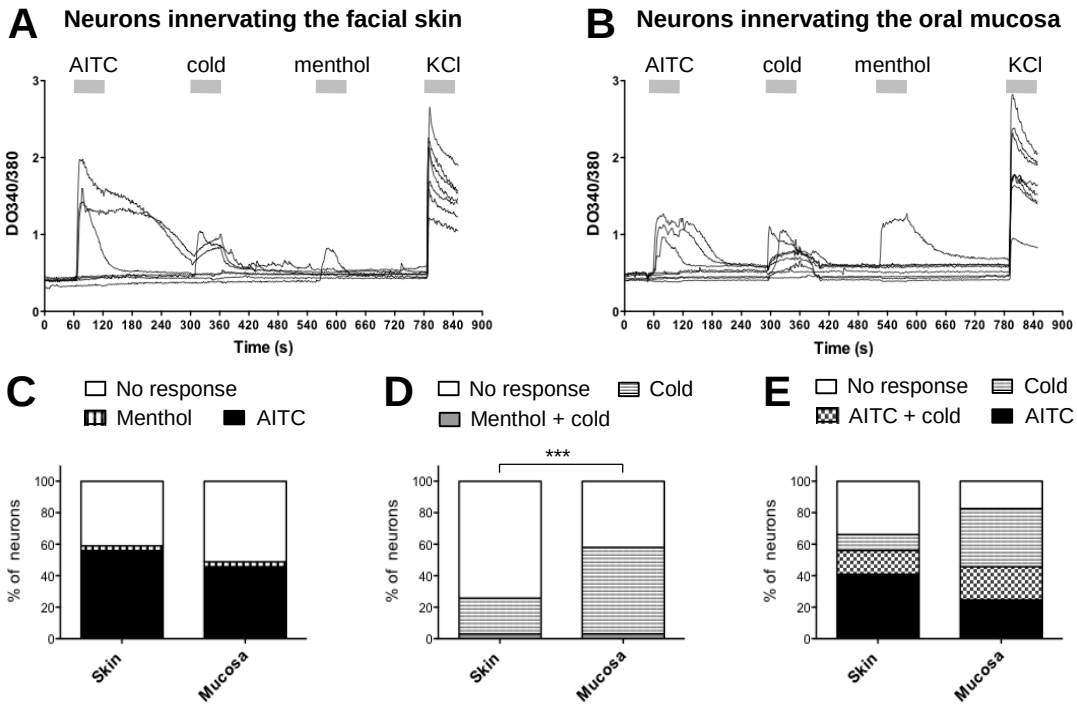
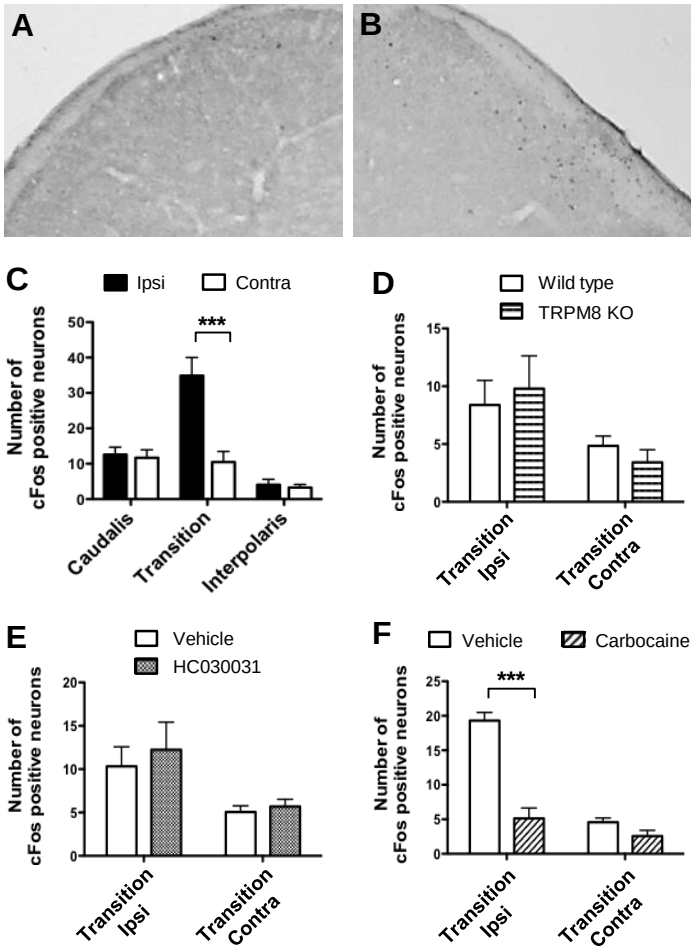

A
TRPM8-
TRPM8+
B
TRPA1-
TRPA1+
C
CGRP-
CGRP+
D
No colocalization
TRPA1+ / CGRP+
% of neurons
100
80
60
40
20
0
Skin
Mucosa
Dental pulp
***
***
E
Fluorogold-labeled neurons
F
TRPA1+ neurons
G
CGRP+ neurons
Large neurons
Medium size neurons
Small neurons

**Figure 4**


A
Neurons innervating the facial skin
AITC
cold
menthol
KCl
DO340/380
Time (s)
B
Neurons innervating the oral mucosa
AITC
cold
menthol
KCl
DO340/380
Time (s)
C
No response
Menthol
AITC
% of neurons
Skin
Mucosa
D
No response
Cold
Menthol + cold
***
% of neurons
Skin
Mucosa
E
No response
Cold
AITC + cold
AITC
% of neurons
Skin
Mucosa

# Figure 5


A
B
C
Ipsi
Contra
Number of cFos positive neurons
***
Caudalis
Transition
Interpolaris
D
Wild type
TRPM8 KO
Number of cFos positive neurons
Transition Ipsi
Transition Contra
E
Vehicle
HC030031
Number of cFos positive neurons
Transition Ipsi
Transition Contra
F
Vehicle
Carbocaine
Number of cFos positive neurons
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Transition Ipsi
Transition Contra