Immunohistochemical colocalization of TREK-1, TREK-2 and TRAAK with TRP channels in the trigeminal ganglion cells

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(17 pages including table and figures)

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Acknowledgements. This study was supported in part by a Grant-in-Aid from the JSPS, Japan (19380166).

Abstract

TREK belongs to a subfamily of tandem pore domain K^+ channels, and consists of three subunits, TREK-1, TREK-2 and TRAAK. We examined the distribution of TREK-1, TREK-2 and TRAAK immunoreactive neurons in rat trigeminal sensory neurons. In the trigeminal ganglia, 31, 43 and 60 % of neurons were immunoreactive for TREK-1, TREK-2 and TRAAK, respectively. Mean sizes of TREK-1, TREK-2 and TRAAK immunoreactive trigeminal ganglion neurons were 447 ± 185, 445 ± 23 and 492 ± 12 mm^2, respectively. Furthermore, TREK channels were colocalized with polymodal cationic TRP channels, TRPV1, TRPV2 and TRPM8. TREK-1 immunoreactive neurons were colocalized with TRPV1 (57 %), TRPV2 (11 %) and TRPM8 (33 %). TREK-2- immunoreactive neurons were colocalized with TRPV1 (33 %), TRPV2 (9 %) and TRPM8 (19 %). TRAAK immunoreactive neurons were colocalized with TRPV1 (47 %), TRPV2 (10 %) and TRPM8 (22 %). The present results revealed that TREK-1, TREK-2 and TRAAK channels colocalized with thermosensitive TRP channels in some small trigeminal ganglion neurons.

Keywords:

TREK channel, TRP channel, trigeminal ganglion, thermosensation, sensory nerve.

TREK belongs to a subfamily of tandem P domain K^+ channels, and is comprised of three subunits, TREK-1, TREK-2 and TRAAK [7]. In general, these channels are closed at low temperatures (< 30 °C) but are activated by polyunsaturated fatty acid, increasing cellular volume, protons and general anesthetics [5, 7]. It has been demonstrated that the mRNAs and proteins of these channels are widely expressed in both the central and peripheral nervous systems using reverse transcriptase-polymerase chain reaction (RT-PCR; [10]), *in situ* hybridization [13] and immunohistochemistry [4]. In the peripheral sensory ganglion, mRNAs of TREK-1, TREK-2 and TRAAK were detected in the dorsal root ganglion neurons [13], and mRNA for TREK- 1 was also observed in the neurons in the trigeminal, geniculate and petrosal ganglia [9]. Furthermore, TREK-1 immunoreactivity was detected in small and medium-sized neurons in the dorsal root ganglion [8].

On the other hand, it has been reported that several transient receptor potential (TRP) channels play important roles in sensory perception. Several TRP channels with distinct thermal activation thresholds were found in mammals; e.g., 43 °C for TRPV1, > 52 °C for TRPV2, >32 °C for TRPV3 and TRPV4, < 25 °C for TRPM8, <18 °C for TRPA1 [2]. Furthermore, thermosensitive TRP channels are activated by other stimulants such as capsaicin, heat and protons for TRPV1, and menthol for TRPM8 [12]. Alloui et al. [1] reported that 56 % of TRPV1-immunoreactive neurons in the mouse dorsal root ganglion expressed TREK-1 mRNA, and that TREK-1 modulated TRPV1 sensitivity for temperature, protons and mechanical stimuli. They also found that

the saphenous nerve C-fibers in TREK-1 knockout mice showed a lower threshold for heat than controls. Therefore, TREK channels may modulate the activities of several transient receptor potential (TRP) channels.

In the present study, immunohistochemical localization of TREK-1, TREK-2 and TRAAK in the trigeminal ganglion was examined to analyze the characteristics of immunoreactive ganglion cells. We also examined the colocalization patterns of TREK-1, TREK-2 and TRAAK with three TRP channels, TRPV1, TRPV2 and TRPM8 to clarify the modulatory functions of TREK channels for polymodal sensation in trigeminal sensory neurons.

Male Wistar rats (n=10; 8 weeks old) were used. All procedures for animal handling were performed according to the guidelines approved by the local animal ethics committee of Iwate University.

For RT-PCR analysis, rats were euthanized by intraperitoneal injection of an excessive amount of pentobarbital, and the trigeminal ganglia were dissected and frozen with liquid N_2. Total RNA of trigeminal ganglia were corrected using a magnetic beads methods (MELT™ total nucleic acid isolation system, Ambion, Austin, TX). RNA templates were incubated with DNAase I (Takara, Tokyo) for 30 min at 37°C before use. RT-PCR was performed with a Qiagen OneStep RT-PCR kit (Qiagen) with specific primers for TREK-1, TREK-2 and TRAAK. Primers for GAPDH were also used to detect mRNA expression of housekeeping genes. Details of the primers used in the present study are shown in Table 1. Reverse transcription was performed for 30 min at 50 °C and initial PCR activation was incubated for 15 min at 95 °C. After reverse transcription,

PCR amplifications were performed for 40 times as follows: 30 sec at 94 °C for denaturation, 30 sec at 55 °C for annealing, and 1 min at 72 °C for extension. After PCR amplification, samples were applied for 10 min at 72 °C for final extension. PCR endproducts were visualized on 2% agarose gels using ethidium bromide. For negative control experiments, mRNA templates were omitted.

For immunohistochemistry and double immunofluorescence, the animals was anesthetized by pentobarbital (15 mg/kg; intraperitoneal injection) and perfused with Ringer's solution (500 ml) followed with 4% paraformaldehyde containing 0.5% picric acid in 0.1 M phosphate buffer (pH 7.4, 500 ml) from the ascending aorta. The trigeminal ganglia were dissected and further fixed with the same fixative for 5 hours. Then, the tissues were soaked in 30 % sucrose in phosphate-buffered saline (PBS; pH 7.4) and frozen with O.C.T. compound medium (Sakura Finetech, Tokyo). For double immunofluorescence using a TRPM8 antibody, unfixed tissues were immediately frozen with liquid N_2. The frozen tissues were serially sectioned at a thickness of 8 μm by a cryostat, and mounted on glass slides coated with chrome alum-gelatin. The sections were incubated for 20 min with 0.3 % H_2O_2 in methanol, and then for 60 min with non-immune donkey serum (1:50), and rinsed with phosphate-buffered saline (PBS; pH 7.4). Then, the sections were incubated overnight at 4 °C with goat polyclonal antisera against TREK-1 (1:100; sc-11556, Santa Cruz Biotechnology, Santa Cruz, CA), TREK-2 (1:100; sc-11560, Santa Cruz Biotechnology) and TRAAK (1:100; sc-11326, Santa Cruz Biotechnology). After incubation, the sections were washed with PBS again, and treated with biotinylated donkey

antibody against goat IgG (1:500, Jackson Immunoresearch, West Grove, PA) for 30 min at room temperature. After washing with PBS, sections were treated with ABC reagent from a kit (Elite ABC kit, Vector). Finally, the immunoreaction sites were visualized by incubation with a Tris-HCl buffer containing 3,3'-diaminobenzidine, 4HCl (0.2 mg/ml) and 0.003 % H_2O_2. Negative controls were incubated with either PBS or a preabsorbed antibody. For morphometry, digitized images were monitored on a PC monitor, and areas of trigeminal ganglion cells with TREK-1, TREK-2 and TRAAK immunoreactivities were measured using Scion Image software (Scion Corp., Frederick, MD, USA). Histograms of cell profile areas of TREK-1, TREK-2 and TRAAK immunoreactive and negative neurons were made. More than 1,000 neurons from five rats were measured for each antibody; 2,474 neurons for TREK-1, 2,021 for TREK-2 and 1,378 for TRAAK.

Some sections were used for double immunofluorescence for TREK-1, TREK-2 and TRAAK with TRPV1, TRPV2 and TRPM8. Sections were incubated with goat polyclonal TREK-1, TREK-2 or TRAAK together with a rabbit polyclonal antibody against TRPV1 (1:100; PC420, Calbiochem, Darmstadt, Germany), TRPV2 (1:100; ab6183, Abcam, Cambridge, U.K.), or TRPM8 (1:100; KM060, Transgenic, Kumamoto, Japan) for 12 hours at 4˚C. Sections were then incubated with a mixture of TRITC-labeled donkey anti-goat IgG (1:100, Jackson Immunoresearch) and FITC-labeled donkey anti-rabbit IgG (1:100, Jackson Immunoresearch) for 2 hours at 25˚C. The sections were coverslipped with glycerol-PBS, and examined.

PCR products of the predicted size for TREK-1, TREK-2 and TRAAK were amplified from total RNA templates of the trigeminal ganglion (Fig. 1). In a positive control experiment, products for GAPDH were detected. In a negative control experiment, no product was found.

TREK-1, TREK-2 and TRAAK immunoreactivities were found in the small ganglion cells and nerve fibers in the rat trigeminal ganglion (Figs. 2A, C, E). In the trigeminal ganglion, 31, 43 and 60 % of neurons were immunoreactive for TREK-1, TREK-2 and TRAAK, respectively. The immunoreactive neurons for TREK-1, TREK-2 and TRAAK were distributed throughout the ganglion. A few medium-sized neurons were also immunoreactive, but large neurons were not. Histograms of positive neurons for each channel showed a single peak and mean sizes of TREK-1, TREK-2 and TRAAK immunoreactive neurons were 447 ± 185, 445 ± 23 and 492 ± 12 μm^2, respectively (Figs. 2B, D, F). Immunonegative neurons ranged widely in size.

Double immunofluorescence revealed that trigeminal ganglion neurons that were immunoreactive for TREK channels, were also immunoreactive for TRP channels, TRPV1, TRPV2 and TRPM8 (Fig. 3). TREK-1 immunoreactive neurons were colocalized with TRPV1 (57 %), TRPV2 (11 %) and TRPM8 (33 %). TREK-2-immunoreactive neurons were colocalized with TRPV1 (33 %), TRPV2 (9 %) and TRPM8 (19 %). TRAAK-immunoreactive neurons were colocalized with TRPV1 (47 %), TRPV2 (10 %) and TRPM8 (22 %). On the other hand, some neurons that were immunoreactive for TRP channels were also immunoreactive for TREK channels. TRPV1-immunoreactive neurons were

immunoreactive for TREK-1 (54 %), TREK-2 (38 %) and TRAAK (41 %). TRPV2-immunoreactive neurons were immunoreactive for TREK-1 (21 %), TREK-2 (15 %) and TRAAK (10 %). TRPM8-immunoreactive neurons were immunoreactive for TREK-1 (50 %), TREK-2 (17 %) and TRAAK (30 %).

The present study revealed the expression of mRNA and protein for TREK-1, TREK-2 and TRAAK in the rat trigeminal ganglion. The immunoreactivities for TREK-1, TREK-2 and TRAAK were mainly observed in small neurons, and histograms were similar among TREK-1, TREK-2 and TRAAK-immunoreactive neurons. It has been reported that TREK-1-immunoreactive neurons in the rat dorsal root ganglia were small to medium-large in size and showed a dual peak in the mouse [8]. However, in the present study, a single peak was noted in the histogram for TREK-1-immunoreactive neurons in the trigeminal ganglion. Neuron types expressing TREK channels may differ between dorsal root ganglion and trigeminal ganglion. On the other hand, mRNA for TREK-2 and TRAAK were distributed in the rat dorsal root ganglion neurons as shown by in situ hybridization [13], but the detailed distribution patterns of TREK-2 and TRAAK have not been reported. In the present study, the histograms of TREK-2 and TRAAK-positive neurons in the rat trigeminal ganglion were similar to that of TREK-1-positive neurons. The present results indicate TREK channel families, TREK-1, TREK-2 and TRAAK, modulate the sensory function of small neurons in the rat trigeminal ganglion. In the dorsal root ganglion cells of neonatal rats, K^+ channels similar to TRESK, TREK-1, TREK-2 and TRAAK were identified, and

TREK-2 like channels were most active [5]. It is possible that TREK-1, TREK-2 and TRAAK are co-expressed in the same small neurons to form complicated K^+ currents.

Alloui et al. [1] demonstrated that TREK-1 knockout mice are more sensitive to increases in temperature, and suggested that TREK-1 channels modulate thermosensation. In the present study, some TREK-1, TREK-2 and TRAAK-immunoreactive neurons were also immunoreactive for three thermosensitive TRP channels, TRPV1, TRPV2 and TRPM8. Because TREK-2 and TRAAK are closed by low temperatures like TREK-1 [6], TREK channels colocalized with thermosensitive TRP channels appear to regulate the thermosensitivity of the trigeminal sensory neurons. It has been reported that the thermosensitive mechanism of the TRP channel is dependent on the membrane potential [15]. TREK-1, TREK-2 and TRAAK may modulate the threshold temperature of TRPV1, TRPV2 and TRPM8. The variation in colocalization patterns indicates that the three TREK channels do not modulate a specific TRP channel but several channels. In the trigeminal ganglia, it has been reported that TRPA1 is a candidate for cold perception [2]. Because TRPA1 is coexpressed in TRPV1 neurons in trigeminal ganglion neurons [3], it is possible that TREK channels are coexpressed in the TRPA1-expressing cells. Further study is needed to clarify the coexpression patterns of these TREK channels with thermosensitive channels. On the other hand, Nealen et al. [11] reported that the mRNA of TREK-1 was expressed in only 40.0 and 9.4 % of low and high threshold cold-activated neurons. Thut et al. [14] found that inhibition of TREK

channels by Ba^{2+} and/or Gd^{3+} did not affect cold transduction of trigeminal neurons. TREK channels may not be essential for cold perception, but play an important role in modulation of the membrane current in thermosensitive neurons.

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Table 1. Primers for RT-PCR

mRNA (Accession number)	Primer sequences	Position	Product length (bp)
TREK-1 (AF385402)	5'-TGACCTCAGACAGTCGGTAT-3' (sense) 5'-CAAGCCTGCTATACCTCGT-3' (antisense)	2394-2413 2500-2518	125
TREK-2 (AF385401)	5'-GGTAGCAGGTGTCGGCTATT-3' (sense) 5'-GTTCACCAAAGCTTGCGA-3' (antisense)	2239-2258 2701-2718	480
TRAAK (AF302842)	5'-CATCGGTCACATCGAAGC-3' (sense) 5'-TCGCCTGGTACATAATCGC-3' (antisense)	450-467 650-668	219
GAPDH (AF106860)	5'-TGGAGTCTACTGGCGTCTT-3' (sense) 5'-AGTGAGCTTCCCGTTCAG-3' (antisense)	1130-1148 1513-1530	401

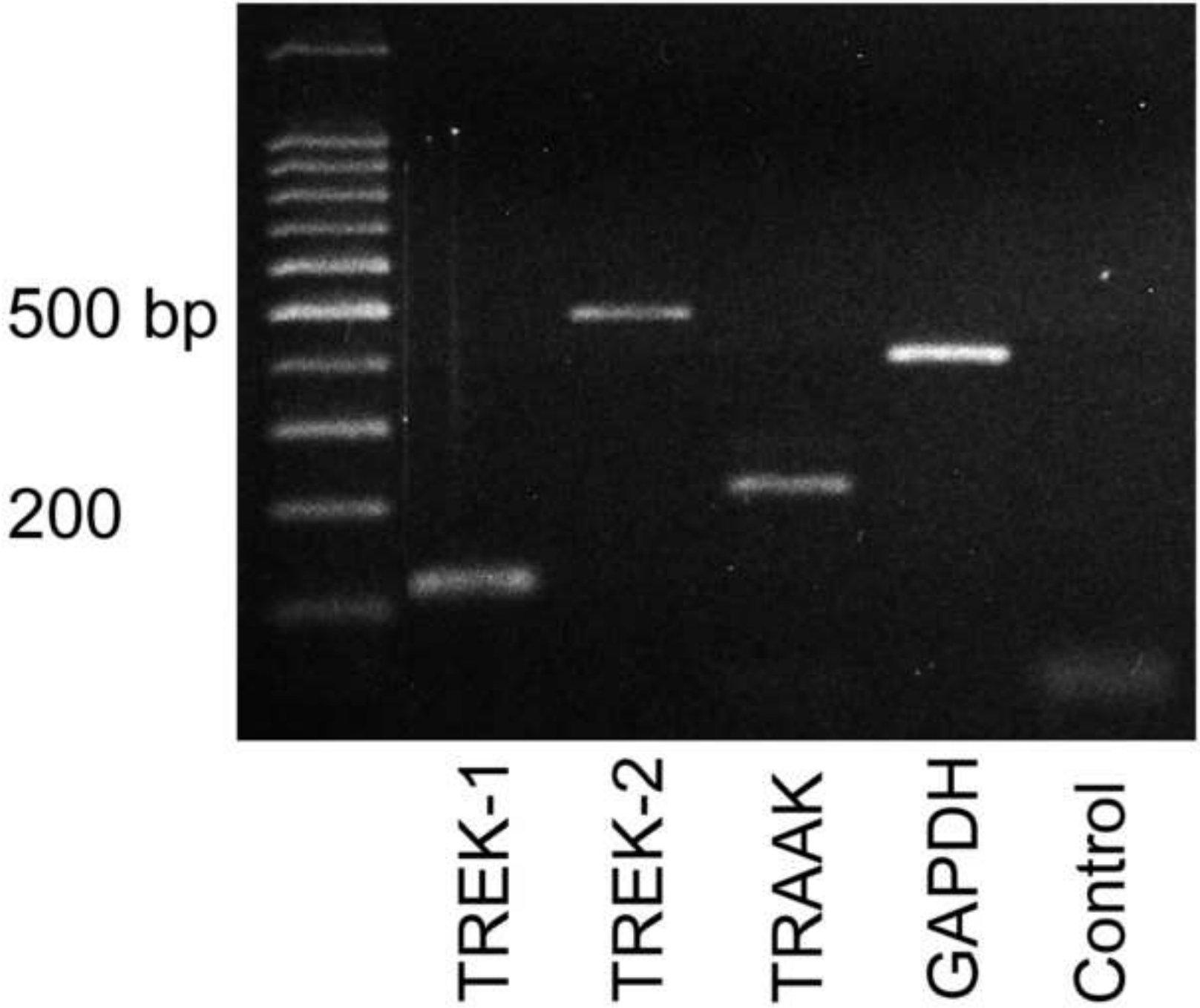
Figure Legends

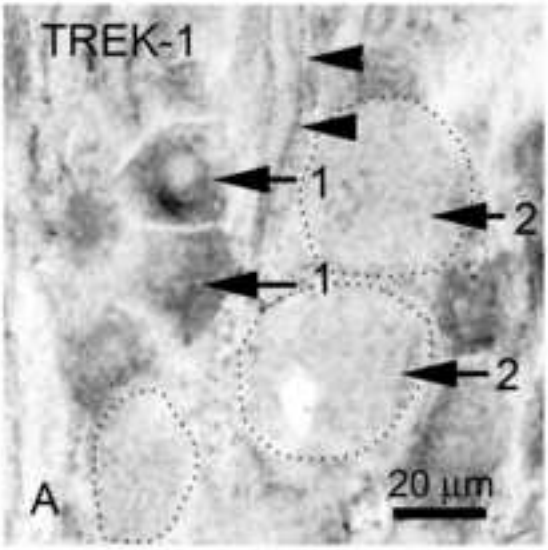
Fig. 1. RT-PCR for TREK-1, TREK-2 and TRAAK in the rat trigeminal ganglion. Appropriate size PCR products are visualized. Lane 1, 100 bp DNA ladder; 2, TREK-1; 3, TREK-2; 4, TRAAK; 5, GAPDH.

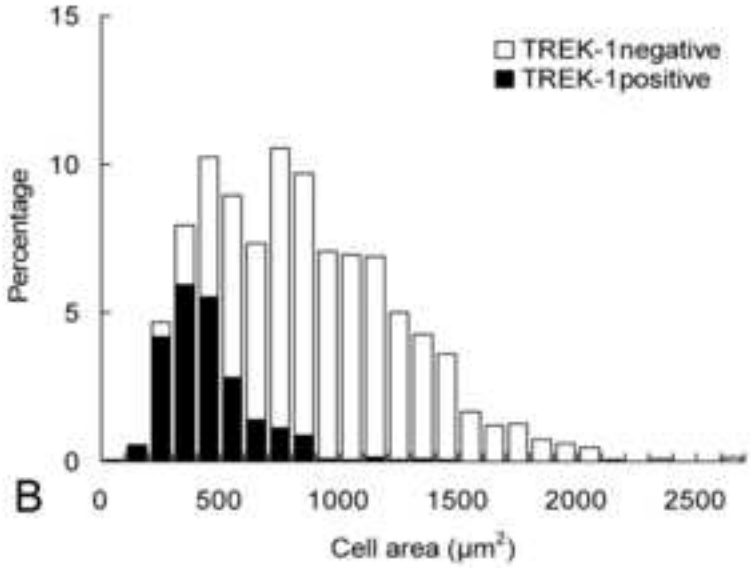
Fig. 2. Immunolocalization of TREK-1 (A), TREK-2 (B) and TRAAK (C) in the rat trigeminal ganglion. Both immunoreactive cells (1) and negative cells (2; outlined with dotted line) are shown. Nerve fibers in the ganglion are also immunoreactive (arrowheads). Histograms of nerve profile areas of TREK-1, TREK-2 and TRAAK-immunoreactive cells are shown in panels D-F, respectively.

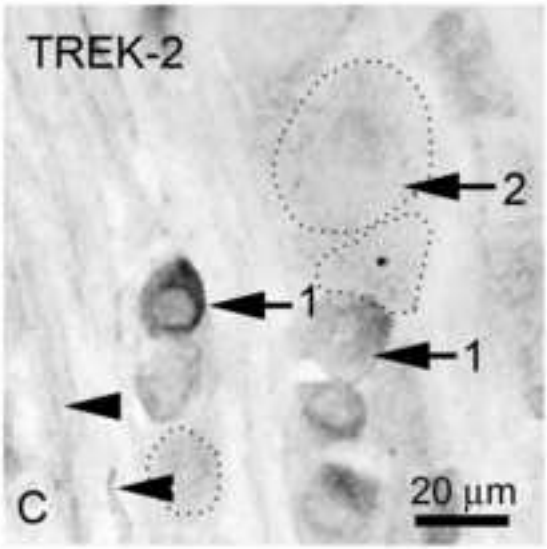
Fig. 3. Merged images of double immunofluorescence for TREK channels, TREK-1, TREK-2 and TRAAK, combined with TRP channels, TRPV1, TRPV2 and TRPM8. Double positive cells (1), cells positive for only TREK channels (2) and cells positive for only TRP channels (3) are shown in all patterns of combination. Graphs indicate percentages of single positive neurons for TREK (red), double positive neurons for TREK and TRP (yellow) and single positive neurons for TRP (green) among neurons with immunoreactivity for TREK channels and/or TRP channels. The percentage of the positive cells of each panel is shown in the bar chart. A-C, TREK-1 with TRPV1 (A), TRPV2 (B) and TRPM8 (C); D-F, TREK-2 with TRPV1 (D), TRPV2 (E) and TRPM8 (F); G-I, TRAAK with TRPV1 (G), TRPV2 (H) and TRPM8 (I).

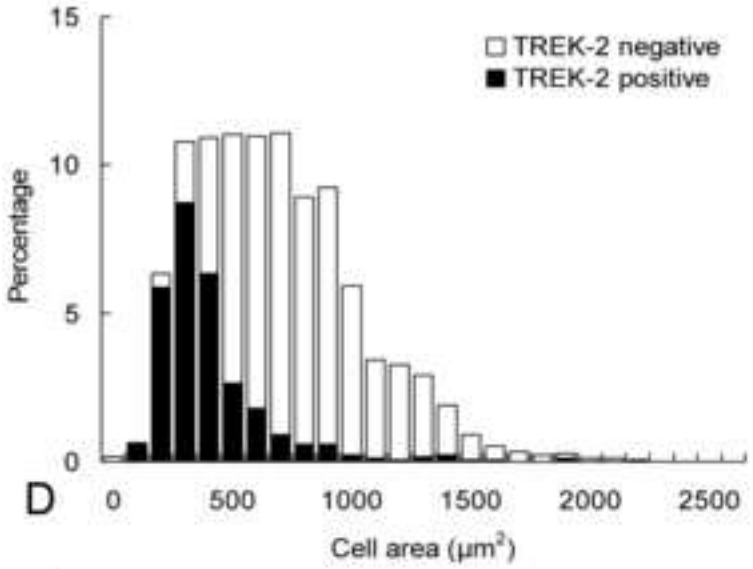
Figure 1

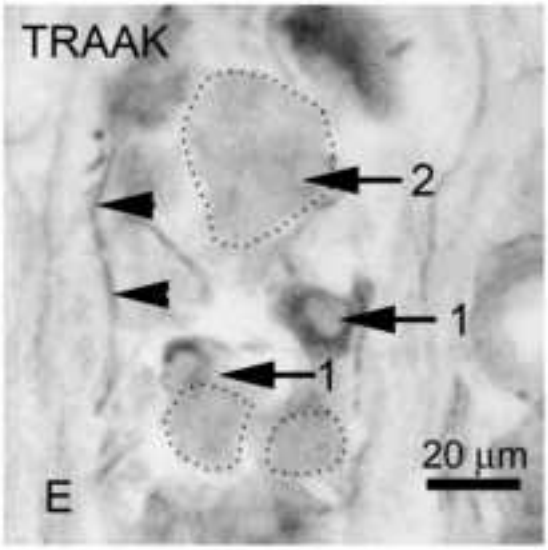












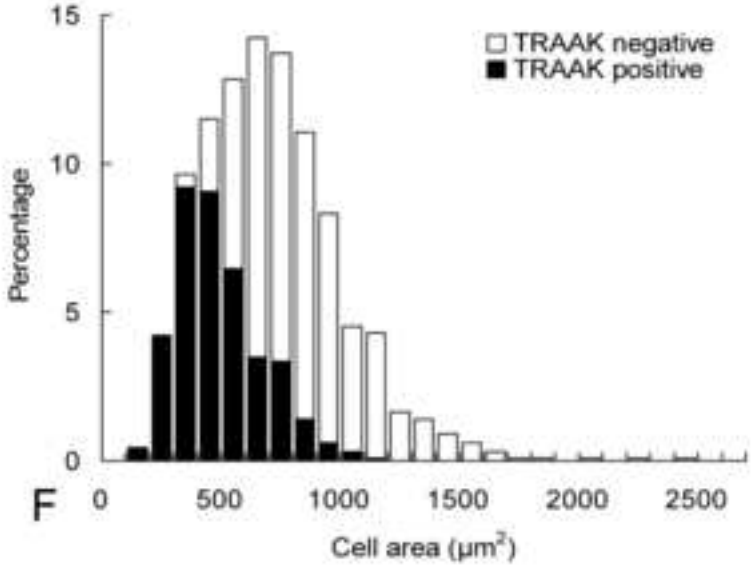
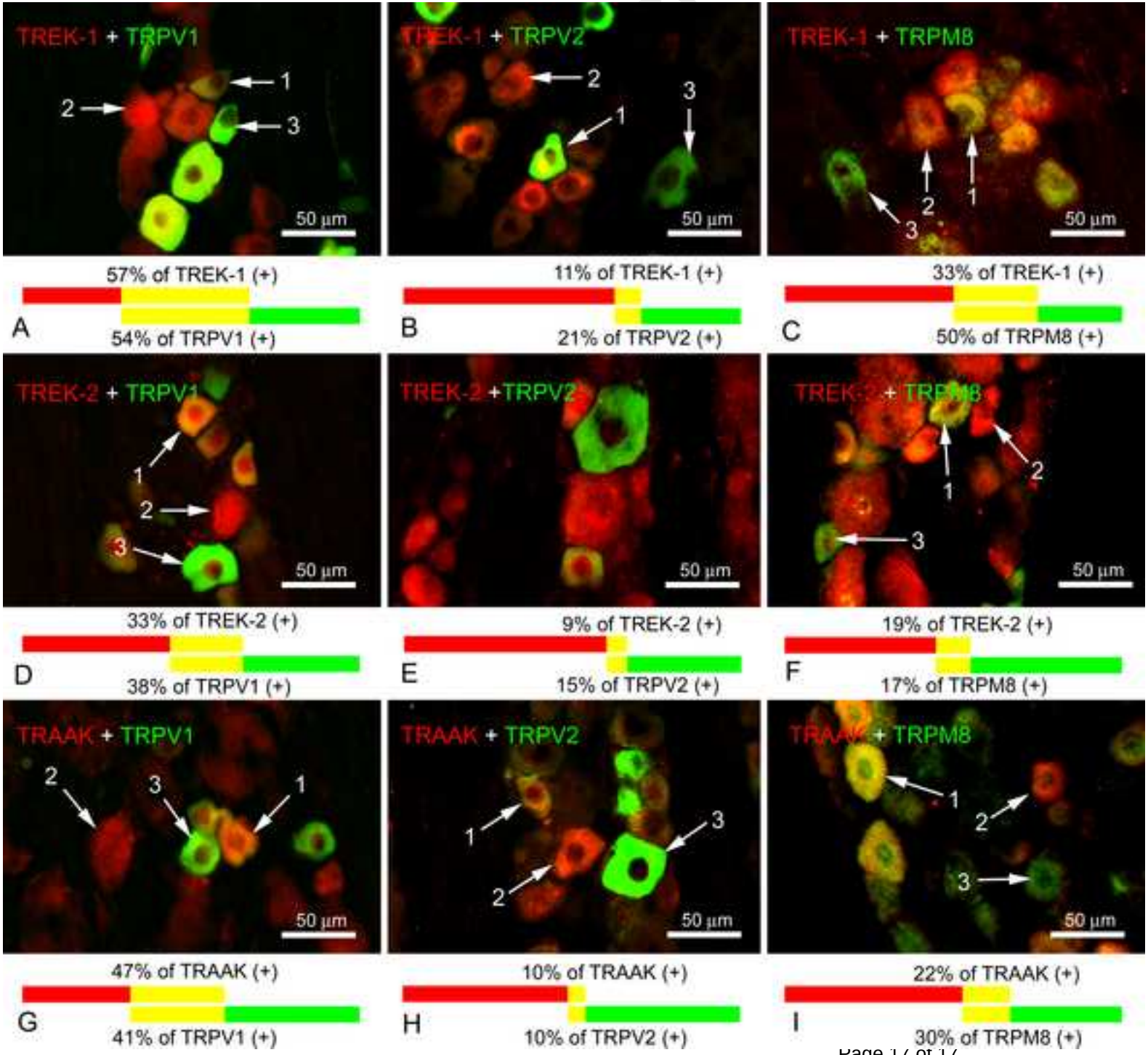


Figure3


TREK-1 + TRPV1
1
2
3
50 µm
57% of TREK-1 (+)
A
54% of TRPV1 (+)
TREK-1 + TRPV2
2
1
3
50 µm
11% of TREK-1 (+)
B
21% of TRPV2 (+)
TREK-1 + TRPM8
2
1
3
50 µm
33% of TREK-1 (+)
C
50% of TRPM8 (+)
TREK-2 + TRPV1
1
2
3
50 µm
33% of TREK-2 (+)
D
38% of TRPV1 (+)
TREK-2 +TRPV2
50 µm
9% of TREK-2 (+)
E
15% of TRPV2 (+)
1
2
3
50 µm
19% of TREK-2 (+)
F
17% of TRPM8 (+)
TRAAK + TRPV1
2
3
1
50 µm
47% of TRAAK (+)
G
41% of TRPV1 (+)
TRAAK + TRPV2
1
3
2
50 µm
10% of TRAAK (+)
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10% of TRPV2 (+)
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2
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50 µm
22% of TRAAK (+)
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30% of TRPM8 (+)