TRPM8 mRNA is expressed in a subset of cold-responsive trigeminal neurons from rat

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Running head: TRPM8 expression in a subset of cold-responsive neurons

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Abstract

Recent electrophysiological studies of cultured dorsal root and trigeminal ganglion neurons have suggested that multiple ionic mechanisms underlie the peripheral detection of cold temperatures. Several candidate “cold receptors”, all of them ion channel proteins, have been implicated in this process. One of the most promising candidates is TRPM8, a non-selective cationic channel expressed in a subpopulation of sensory neurons that is activated both by decreases in temperature and the cooling compound menthol. However, evidence for the expression of TRPM8 in functionally defined cold-sensitive neurons has been lacking. Here, we combine fluorometric calcium imaging of cultured rat trigeminal neurons with single-cell RT-PCR to demonstrate that there are distinct subpopulations of cold responsive neurons, and that TRPM8 likely contributes to cold transduction in one of them. TRPM8 is preferentially expressed within a subset of rapidly responsive, low-threshold (~30°C), cold-sensitive neurons. A distinct class of slowly responsive cold-sensitive neurons that is activated at lower temperatures (~20°C) generally lacks detectable TRPM8 mRNA. Together with previous findings, our data support the notion that cold responsive neurons are functionally heterogeneous.
Introduction

The ability of the peripheral nervous system to perceive changes in environmental temperature is a critical first step in behavioral and physiological thermoregulation. While there has been significant recent progress in defining the means by which peripheral neurons detect heat stimuli (Souslova et al. 2000; Caterina and Julius 2001; Peier et al. 2002b; Smith et al., 2002; Xu et al., 2002), the molecular mechanisms underlying the detection of cool and painfully cold stimuli remain less well understood. Cooling-evoked changes in membrane potential, ionic conductance, or intracellular calcium can be recapitulated in sensory neurons cultured from dorsal root (DRG) and trigeminal ganglia (TG) (Suto and Gotoh 1999; Reid and Flonta 2001a, b, 2002; Viana et al. 2002; McKemy et al. 2002; Okazawa et al. 2002; Thut et al. In Press). Data from several recent electrophysiological studies have revealed two general mechanisms that appear to underlie cold transduction in these neurons. One mechanism involves the cooling-induced activation of a non-selective cationic current that is present in approximately 10% of cultured sensory neurons (Reid and Flonta 2001a, 2002; Okazawa et al. 2002) and can be potentiated by the cooling agent menthol (Reid and Flonta 2001a, 2002). A second mechanism entails the cooling-induced inhibition of certain potassium currents, which results in net membrane depolarization (Reid and Flonta 2001b; Viana et al. 2002). In addition, a distinct potassium current has been reported to mask cooling-induced depolarization in a subset of apparently cold-insensitive sensory neurons (Viana et al. 2002).
Several candidate cold transducing molecules have been identified based upon their behavior in heterologous expression systems and upon their endogenous expression in sensory neurons. One member of the two-pore potassium channel family, TREK-1, appears to be inhibited by cooling when expressed in Xenopus oocytes (Maingret et al. 2000). In contrast, the epithelial sodium channel, ENaC, mediates a ligand-independent, amiloride-sensitive current that can be stimulated by cooling (Askwith et al. 2001). A third candidate cold receptor, TRPM8, is a non-selective cation channel expressed in a subset of small diameter sensory neurons (McKemy et al. 2002; Peier et al. 2002a). In heterologous expression systems, this channel can be activated by either cold temperature or menthol (McKemy et al. 2002; Peier et al. 2002a), making it a particularly strong candidate for a cold transducer.

To better understand the different patterns of cold responses observed in vitro, we used the single cell reverse transcription – polymerase chain reaction (RT-PCR) method to assay the expression of candidate cold transducing molecules in functionally characterized cultured trigeminal neurons.
Methods

Cell Culture and Calcium Imaging

Adult male Sprague Dawley rats (150-300g, Harlan Sprague-Dawley, Indianapolis IN) were housed in a vivarium under a 12hr light/dark cycle and were fed standard rat diet and water ad libitum prior to use in experiments. Rats were anesthetized with a cocktail of ketamine, xylazine, and acepromazine, then decapitated. All procedures performed on rats were approved by institutional animal care and use committees.

Trigeminal ganglia were removed, dissociated in 0.125% collagenase (Roche, Indianapolis IN), trituated in 0.25% trypsin (Worthington, Lakewood NJ), and separated on a Percoll (Sigma, St. Louis MO) gradient (Eckert et al. 1997). Cells were plated onto poly-L-ornithine coated coverslips and incubated in MEM with vitamins, penicillin/streptomycin, 10% fetal bovine serum, and 20 ng/mL NGF (Invitrogen, Carlsbad CA) for 1 day (3% CO₂, 37°C). Cells were loaded with 2.5 µM fura-2-acetomethoxy ester (TEF Labs, Austin TX) for 20 minutes at room temperature (~22°C), followed by a 20 minute incubation in bath solution (see below) to enable de-esterification of the fura. For 10 minutes of this second incubation period, cells were stained with 10 µg/ml fluorescein-tagged IB4 (Sigma) (Stucky and Lewin 1999). Coverslips were placed in a recording chamber and continuously perfused (2 ml/min) with bath solution containing (in mM): 130 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 10 HEPES, 10 glucose (pH 7.4 with Tris-Base,
adjusted to 325 mOsm with sucrose). Coverslips were held at 34°C, then cooled at a rate of 2.5°C/s to 14°C, or stimulated with 100 µM menthol for 60 seconds at 34°C. Prolonged cold stimuli (5 min at 14°C) were applied to a subset of cells, as indicated. Temperature was controlled by a Cell Micro Control system (Norfolk VA) consisting of a Peltier unit and a resistive heater with feedback control, and was monitored by a thermistor placed within 150 µm of the microscopic field. Fluorescent measurements were made on a Nikon inverted microscope (Image Systems, Gaithersburg MD) using a CCD camera (Roper Scientific, Trenton NJ), filter wheel (Sutter, Novato CA), and Metafluor software (Universal Imaging, West Chester PA). The ratio of fluorescence emission (510 nm) at 340 nm/380 nm excitation was measured at 1s intervals. Kinetic fitting of responses for half time ($t_{1/2}$) calculations was performed using Prism software (Graphpad, San Diego CA).

**Single Cell RT-PCR**

Functionally characterized neurons were collected with large bore (~30 µm) glass pipettes and expelled into microcentrifuge tubes containing RT mix (Dulac 1998). RT-PCR was performed as described elsewhere (Dulac 1998) except that an anchored primer (5’-ttttttttttttttttttttnt-3’; v = a, c, or g; n = a, c, g, or t) and 50U Superscript II (Invitrogen) were used for reverse transcription. For each experiment, negative controls were performed by omitting reverse transcriptase or
using a cell-free bath aspirate as template. Nine percent of the first strand cDNA from a given cell was used as template in a PCR reaction containing 1X Titanium Taq PCR buffer (Clontech), 0.4 μM outer primers, 0.2 mM dNTPs, and 0.5 μL Titanium Taq (Clontech); primer sequences are listed in Table I. Reactions were incubated at 94°C for 4 min, then cycled 35 times at 94°C/30 sec, 68°C/1 min, before a final extension step of 68°C for 4 min. Four percent of each initial PCR product served as template in a subsequent PCR reaction using a nested primer pair. For each PCR experiment, whole TG-derived cDNA was used as a positive control template. Negative controls included no input template. Twenty percent of each PCR product was electrophoresed on 2% agarose-ethidium bromide gels and photographed. Only neurons producing detectable amplification of a housekeeping gene (cyclophilin) were analyzed further. For each gene assayed, specificity was confirmed by sequencing the nested PCR product amplified from whole TG. Unless otherwise indicated, all quantitative comparisons are presented as mean ± sem and statistical comparisons were performed using Chi squared analysis.
Results

Consistent with previous findings (McKemy et al. 2002; Thut et al. In Press), a subset of rat trigeminal neurons (213/1282 neurons, 20.49 ± 1.9%, n = 10 experiments) exhibited a substantial (i.e., ≥20%) increase in fura fluorescence ratio in response to a decrease in ambient temperature from 34°C to 14°C. Closer inspection revealed the existence of two major patterns of cold responsiveness (Figure 1A, B). The first group (low threshold cool, LTcool, 98 neurons, 7.8 ± 1.1%, n=10 experiments) was characterized by a low threshold for activation (mean threshold = 29.0 ± 1.0 °C (n = 14)) and a relatively rapid increase in intracellular calcium concentration (t1/2 = 5.6 ± 2.7s, n = 14 neurons), whereas the second group (high threshold cool, HTcool, 115 neurons, 12.7 ± 2.2%, n=10 experiments) exhibited a much higher threshold for activation (mean threshold = 20.3 ± 0.8°C, n = 32) and a slower rise time (t1/2 = 33.0 ± 6.5s, n = 32 neurons, p<0.0005 vs. LTcool, Figure 1C). The remaining 1069 neurons (79.5 ± 1.9%, n=10 experiments) exhibited a ≤ 5% change in fura ratio and were therefore designated as cold unresponsive (UN). LTcool neurons were significantly smaller (soma diameter 23.92 ± 0.58µm) than HTcool neurons (26.37 ± 0.90µm, p<0.05 vs. LTcool) or UN neurons (26.57 ± 0.54µm, p<0.005 vs. LTcool, Student’s t-test). A small difference in peak response amplitudes was measured between LTcool (55.2 ± 9.3% increase in fura ratio, n = 14) and HTcool neurons (35.5 ± 2.5% increase, n = 32, p<0.05 vs. LTcool). However this latter difference is somewhat arbitrary in nature, given that relative intracellular calcium levels in HTcool neurons (but not LTcool neurons) were still
increasing at the time the cold stimulus was removed (Figure 1B, C). Prolonged (i.e., 5 min) stimulation of HTcool neurons with cold typically resulted in responses that reached a plateau within 3-4 minutes (Figure 1D).

In order to gain further insight into the nature of these functionally distinct classes of cold-responsive neurons, we examined their ability to bind the isolectin, B4 (IB4). Small diameter neurons can be generally divided into two classes based upon their ability to bind this isolectin. IB4 positive neurons tend to be nonpeptidergic, and project to inner lamina II of the spinal cord dorsal horn. In the adult, the phenotype of these neurons is maintained by glial cell line derived neurotrophic factor (GDNF). IB4 negative neurons, on the other hand, are peptidergic, project to more superficial spinal laminae, and their phenotype is dependent upon NGF. Certain gene knockout and in vitro electrophysiological experiments have provided evidence that these two classes of neurons are functionally distinct (Snider and McMahon 1999; Stucky and Lewin 1999). Moreover, it has been reported that in the mouse, TRPM8 mRNA is preferentially expressed within IB4 negative neurons (Peier et al, 2002a). In our culture system, a large proportion of LTcool neurons failed to bind IB4 (44/50, Figure 2A), whereas HTcool neurons largely did bind this lectin (37/56, Figure 2A, LTcool vs. HTcool, \( p<10^{-6} \)). Together, these findings suggest that LTcool and HTcool neurons differ not only in cold responsiveness, but also in developmental subtype, in agreement with our previous results (Thut et al. In Press).
In order to further compare individual LT$_{cool}$ and HT$_{cool}$ neurons at a molecular level, we performed single cell RT-PCR. To validate this technique, we first assayed the expression of mRNAs encoding molecules known to be differentially expressed between IB4+ and IB4- sensory neurons. mRNA encoding the ATP-gated ion channel, P2X3, which is expressed in 67-87% of IB4+ neurons (Bradbury et al. 1998; Zwick et al. 2002), was detected in 16/23 IB4+ neurons, versus only 1/7 IB4- neurons ($p<0.001$). Conversely, message encoding the neurotrophin receptor, trkA, which is expressed predominantly among IB4- sensory neurons in the adult rat (Averill et al. 1995), was detected in 24/58 IB4- neurons, versus only 8/48 IB4+ neurons ($p<0.01$).

We next evaluated the expression pattern of TRPM8 in the cultured neurons. TRPM8 mRNA could be detected in 23/98 cold responsive neurons (Figure 2B). A more detailed analysis revealed that the expression of TRPM8 was significantly more prevalent among LT$_{cool}$ neurons (18/45) than among HT$_{cool}$ neurons (5/53, $p<0.0005$ vs. LT$_{cool}$) or UN neurons (11/87, $p<0.0005$ vs. LT$_{cool}$) (Table II). Although LT$_{cool}$ neurons are largely IB4 negative, this feature alone could not account for the greater prevalence of TRPM8 expression observed among these cells since eight of the twelve UN neurons in which we could detect TRPM8 were IB4 positive. The apparently low prevalence of TRPM8 expression among HT$_{cool}$ and UN neurons also could not be explained solely on the basis of assay sensitivity, since a virtually identical pattern was
observed among 44 additional neurons in an independent experiment where input template for the PCR reaction was increased by ten-fold (Table II).

Because menthol can activate TRPM8 in heterologous expression systems, we next explored the relationship between responsiveness to this agent and expression of TRPM8 mRNA. Of 288 neurons examined, 40 (13.9%) responded to menthol stimulation (100µM, 60s) with a robust, reversible increase in relative intracellular calcium levels (data not shown). Single-cell PCR analysis revealed that 9/33 menthol-responsive cells expressed detectable levels of TRPM8 mRNA, whereas only 1/37 menthol unresponsive cells were TRPM8 positive (p< 0.005).

Among LT_{cool} neurons, 17/24 (70.8%) were menthol responsive, compared with 20/40 (50%) of HT_{cool} neurons and 2/223 (0.9%) of cold-unresponsive neurons. When all three parameters were assayed together, TRPM8 was found in 5/15 menthol responsive LT_{cool} cells and in 3/16 menthol responsive HT_{cool} cells. However, nearly all TRPM8 positive cells tested (9/10) were menthol responsive, consistent with the reported pharmacological properties of this channel protein (McKemy et al., 2002; Peier et al., 2002a).

The correlation between TRPM8 expression and low threshold responses to cold stimuli, in combination with evidence suggesting that TRP channels can heteromultimerize (Xu et al. 1997; Strubing et al. 2001; Tobin et al. 2002), led us to question whether any other TRPM family member was associated with one or both groups of cold responsive neurons. Of the seven other
subtypes, mRNAs encoding TRPM1, TRPM3, TRPM6, and TRPM7 were detectable at the single cell level (Table II). TRPM2, TRPM4, or TRPM5 mRNAs were not detected in any single cell tested, although they were detected in samples prepared from whole TGs (data not shown). RT-PCR revealed no association between TRPM1 or TRPM6 and any of the three cold response patterns. TRPM3 was detected in a larger proportion of LT_{cool} neurons (5/28) than in all other neurons (5/88, \( p < 0.05 \)). Given the relatively low number of TRPM3 expressing cells observed, however, we cannot exclude the possibility that this subtype’s apparently disproportionate representation represents a sampling artifact. TRPM7 was relatively over-represented among IB4 positive neurons (17/42 vs. 8/40 IB4 negative neurons, \( p < 0.05 \)), but its expression was not correlated with any cold response pattern (Table II).

Because the potassium channel TREK-1 has also been implicated as a possible transducer of cold stimuli, we examined its expression pattern among functionally characterized sensory neurons. TREK-1 mRNA was detected in 10/47 neurons examined. By RT-PCR, we could not detect any significant difference in expression of TREK-1 between LT_{cool} neurons (4/12) and HT_{cool} (2/13, \( p > 0.1 \) vs. LT_{cool}) or UN neurons (4/22, \( p > 0.1 \) vs. LT_{cool}). This channel also did not appear to be expressed differentially between IB4+ and IB4- cells. Moreover, we observed no clear relationship between the expression of TRPM8 and TREK-1 at the single cell level. These data cannot rule out the possibility that TREK-1 contributes to cold-transduction; however, the small percentage of HT_{cool} neurons expressing TREK-1 makes it unlikely that this channel is the major
cold transducer among HTcool neurons. mRNAs encoding another candidate cold transducer (the α, β, and γ isoforms of the ENaC channel) could not be assayed with sufficient sensitivity to evaluate their expression among individual cold sensitive neurons.
Discussion

Our findings, together with previously reported data (McKemy et al. 2002; Peier et al. 2002a; Thut et al. In Press), support a model in which TRPM8 serves as a transducer of cold stimuli in at least a subset of LTcool neurons (Table III). Evidence in support of such a model is as follows. First, both LTcool neurons and nonneuronal cells transfected heterologously with TRPM8 respond to cooling below 29°C with a rapid increase in intracellular calcium. Second, LTcool neurons, like TRPM8-expressing neurons, are predominantly IB4 negative. Third, we have demonstrated that TRPM8 mRNA is detectable in a significantly larger proportion of LTcool neurons than in HTcool or UN neurons.

There are several possible explanations for the incomplete concordance between TRPM8 expression and low threshold cold responsiveness in our experiments. 1) Alternative mechanisms for cold transduction may exist in LTcool neurons that do not require the expression of TRPM8. 2) TRPM8 splice variants may not be detected by the primers chosen for our RT-PCR analysis. The existence of alternatively spliced TRPM8 variants has not been established, although in one published study, a TRPM8 probe recognized two distinct bands in a Northern blot of sensory ganglion RNA (McKemy et al. 2002). It is unclear whether the additional band represents a splice variant, or merely an incompletely spliced message. 3) Neurons in culture may exhibit a disparity between TRPM8 mRNA and protein expression. If so, very low levels of TRPM8 mRNA could have prevented us from detecting the gene’s expression in some cells. 4)
In cold unresponsive TRPM8 positive neurons, K⁺ channels or posttranslational modifications of the TRPM8 protein might mask cold responses, as previously suggested (Reid and Flonta 2001b; Viana et al. 2002). Immunostaining studies with TRPM8-specific antibodies may help distinguish between these possibilities. 5) The amplification of TRPM8 from some cells classified as cold unresponsive may indicate that these cells expressed TRPM8 protein at a level below our calcium imaging detection threshold, had become desensitized to cold during the fura loading period, or were otherwise functionally compromised at the time of the assay.

In our experiments, cold-responsive neurons (both LT\text{cool} and HT\text{cool}) were more likely than the general population to respond to menthol. However, only about one-third of menthol responsive cells expressed detectable TRPM8 mRNA. This difference might be explained in one of two ways. First, it may reflect the relative sensitivity of the single-cell PCR method, in comparison to menthol stimulation, as a predictor of TRPM8 protein expression. Alternatively, it might indicate that menthol is capable of activating cells not only via TRPM8, but also via one or more TRPM8-independent mechanisms. Strong support for this latter hypothesis comes from the fact that whereas low doses of menthol produce a sensation of cooling, high doses of menthol exhibit irritant or anesthetic properties (Eccles 1994). The molecule(s) that mediate such non-cooling responses might be enriched among cold-responsive neurons, many of which appear to fall within the size range of nociceptors.
HT_{cool} neurons were largely TRPM8 and TREK-1 negative. These data suggest that HT_{cool} neurons detect cold via a TRPM8-independent mechanism and/or that these cells express TRPM8 at a level substantially lower than that observed in LT_{cool} neurons. In either case, this finding supports the idea that HT_{cool} neurons constitute a subpopulation of cold responsive neurons that are molecularly distinct from LT_{cool} neurons. As presented here and elsewhere (Thut et al. In Press), HT_{cool} neurons also differ from LT_{cool} neurons in the threshold and kinetics of their responses to cooling, cell body size, and IB4 binding. The notion that there are distinct populations of sensory neurons responsive to a decrease in temperature is consistent with electrophysiological and psychophysical studies suggesting that at least two unique populations of cold-sensitive neurons exist in vivo: one population that is responsive to innocuous cooling and a second responsive to noxious cold (Bessou and Perl 1969; Iggo 1969; Darian-Smith et al. 1973, Georgopoulos 1976). LT_{cool} neurons and HT_{cool} neurons may therefore represent cool fibers and cold nociceptors, respectively. Moreover, differences in IB4 binding observed in these two neuronal classes suggests that high- and low-threshold cold-evoked responses may be differentially regulated by neurotrophins or during pathological pain states.
Acknowledgements

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References


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Figure Legends

**Figure 1.** Two distinct patterns of cold responsiveness are evident among cultured trigeminal neurons.  A. Top three rows illustrate relative intracellular calcium levels in fura-2 loaded cells at baseline (pre), and at two times (5s, 60s) after application of the cold stimulus shown in panel B. Examples of low threshold (LT<sub>cool</sub>), high threshold (HT<sub>cool</sub>), and cold unresponsive (UN) neurons are shown. Colorimetric scale bar indicates fura-2 fluorescence emission ratios at 340/380 nm excitation. Bottom row shows selective binding of fluorescein-conjugated isolectin B4 (IB4). White scale bar, 50 µM.  B. Representative changes in intracellular calcium levels during a cold stimulus. Top, percent change in relative [Ca<sup>2+</sup>]<sub>i</sub> is plotted as a function of time in representative LT<sub>cool</sub> (filled circles), HT<sub>cool</sub> (open circles), or unresponsive (open squares) neurons. Bath temperature is at bottom.  C. Kinetic comparison between the rising phases of 14 LT<sub>cool</sub> responses (black traces) and 32 HT<sub>cool</sub> responses (red traces) normalized to the maximal response during the cold stimulus. Traces are aligned at beginning of each neuron's response to facilitate comparison.  D. Representative response of HT<sub>cool</sub> neuron to a prolonged cooling stimulus. Top, percent change in relative [Ca<sup>2+</sup>]<sub>i</sub> is plotted as a function of time. Bath temperature is at bottom.

**Figure 2.** IB4 binding and TRPM8 gene expression differ among functionally defined classes of cold-responsive cultured trigeminal ganglion neurons.  A. Percent of cells in each cold response
category that bind IB4. (LTcool, n=50; HTcool, n=56; UN, n=88; LTcool vs. all others, p<10^-6) B.

TRPM8 RT-PCR products amplified from representative LTcool and HTcool neurons (four of each). Corresponding cyclophilin (cyclo) RT-PCR products are shown below each sample. Nine percent of the cDNA derived from a single cell was used for each PCR reaction. (+, positive control PCR reaction generated from whole TG; –, no input template; bp, base pairs)

**Table I.** DNA primers used for PCR amplification. Sequence is indicated with 5’ at left. a, adenosine; c, cytosine; g, guanosine; t, thymidine; r, a or g; m, a or c; s, g or c; y, c or t; k, g or t; w, a or t. Outer primers were used for the first round of PCR, nested primers for the second round. Sequences used for primer design are indicated at right. Where rat sequences were not available, PCR primers were designed from mouse and/or human sequences. bp, base pairs.

**Table II.** Summary of gene expression analysis in individual cold responsive (LTcool or HTcool) and cold unresponsive (UN) neurons. For each functional class, the fraction of cells expressing mRNA encoding a particular candidate cold transducer is indicated at left, and the corresponding percentage at right. For TRPM8(10X), 90% of the cDNA derived from each cell was used for PCR; in all other cases, 9% of each cell’s cDNA was used.
Table III. Comparison between properties of TRPM8 and those of LT_{cool} and HT_{cool} neurons.

Threshold values are means ± sem. *From nonneuronal cells heterologously transfected with TRPM8 (Peier et al. 2002a; McKemy et al. 2002). †From mouse DRG in situ hybridization (Peier et al. 2002a). ‡Percent of cells expressing TRPM8 mRNA. n/a, not applicable.
Table I. DNA primers used for PCR amplification

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Table II. Gene expression analysis in individual cold responsive (LT_{cool}, HT_{cool}) and cold unresponsive (UN) neurons

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<td>27.3</td>
<td>15/41</td>
<td>36.6</td>
</tr>
<tr>
<td>TRPM8</td>
<td>18/45</td>
<td>40.0</td>
<td>5/53</td>
<td>9.4</td>
<td>11/87</td>
<td>12.6</td>
</tr>
<tr>
<td>TRPM8(10X)</td>
<td>5/11</td>
<td>45.5</td>
<td>1/11</td>
<td>9.1</td>
<td>4/22</td>
<td>18.2</td>
</tr>
<tr>
<td>TREK-1</td>
<td>4/12</td>
<td>33.3</td>
<td>2/13</td>
<td>15.4</td>
<td>4/22</td>
<td>18.2</td>
</tr>
</tbody>
</table>
Table III. Relationship between TRPM8 and cold-response properties of cultured sensory neurons

<table>
<thead>
<tr>
<th></th>
<th>TRPM8</th>
<th>$LT_{\text{cool}}$</th>
<th>$HT_{\text{cool}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>threshold</td>
<td>25-29°C*</td>
<td>29.0±1.0°C</td>
<td>20.3±0.8°C</td>
</tr>
<tr>
<td>cold response rate</td>
<td>rapid*</td>
<td>rapid</td>
<td>slow</td>
</tr>
<tr>
<td>desensitization</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IB4</td>
<td>– †</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>menthol responsive</td>
<td>+ ‡</td>
<td>70.8%</td>
<td>50%</td>
</tr>
<tr>
<td>TRPM8 expression</td>
<td>n/a</td>
<td>40%</td>
<td>9.4%</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

% IB4 positive

LTcool  HTcool  UN

B

bp

LTcool  HTcool  +  -

TRPM8

cyclo

300
250
300
250