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TRPM8 Is Required for Cold Sensation in Mice

Ajay Dhaka,[1] Amber N. Murray,[1] Jayanti Mathur,[2] Taryn J. Earley,[1] Matt J. Petrus,[2] and Ardem Patapoutian[1,2,*]
[1] Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, USA
[2] Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA
*Correspondence: apatapou@gnf.org
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SUMMARY

ThermoTRPs, a subset of the Transient Receptor Potential (TRP) family of cation channels, have been implicated in sensing temperature. TRPM8 and TRPA1 are both activated by cooling; however, it is unclear whether either ion channel is required for thermosensation in vivo. We show that mice lacking TRPM8 have severe behavioral deficits in response to cold stimuli. In thermotaxis assays of temperature gradient and two-temperature choice assays, TRPM8-deficient mice exhibit strikingly reduced avoidance of cold temperatures. TRPM8-deficient mice also lack behavioral response to cold-inducing icilin application and display an attenuated response to acetone, an unpleasant cold stimulus. However, TRPM8-deficient mice have normal nociceptive-like responses to subzero centigrade temperatures, suggesting the presence of at least one additional noxious cold receptor. Finally, we show that TRPM8 mediates the analgesic effect of moderate cooling after administration of formalin, a painful stimulus. Therefore, depending on context, TRPM8 contributes to sensing unpleasant cold stimuli or mediating the effects of cold analgesia.

INTRODUCTION

Peripheral neurons of the dorsal root ganglia (DRG) and trigeminal ganglia sense thermal and mechanical stimuli within the skin and relay this information to the spinal cord (Hensel, 1981). These neurons are functionally heterogeneous. For example, 7%–15% of cultured DRG neurons respond to moderate cold temperatures (e.g., McKemy et al., 2002; Reid and Flonta, 2001b; Story et al., 2003).

The molecular receptors for sensing thermal and mechanical stimuli are still largely unknown. Many candidates have been suggested to play a role in cold sensation in DRG neurons. For instance, cold-induced closure of a K channel such as TREK-1 could cause depolarization and activation of cold-sensitive neurons (Maingret et al., 2000; Reid and Flonta, 2001a; Viana et al., 2002). Cold-induced inhibition of a Na^+/K^+ ATPase and activation of members of the degenerin (DEG) family of Na channels have also been suggested to play a role in cold transduction (Askwith et al., 2001; Pierau et al., 1974). However, gene ablation studies of TREK-1 and DEG channels in mice have not yet pointed to required roles in cold sensation (Alloui et al., 2006; Price et al., 2000, 2001).

Recent evidence suggests that a subset of Transient Receptor Potential (TRP) cation channels plays an important role in thermosensation. Four TRP Vanilloid (TRPV) and three TRP Melastatin (TRPM) family members are activated by heat, and mouse knockout studies have demonstrated roles for three TRPV ion channels in innocuous and noxious heat sensation (reviewed in Dhaka et al., 2006). Two TRP channels are activated by cold. TRPM8 is activated by innocuous cooling (<30°C) and is a receptor for menthol and icilin (mint-derived and synthetic cooling compounds, respectively) (McKemy et al., 2002; Peier et al., 2002). TRPA1 (Ankyrin family) is activated by noxious cold (<17°C), icilin, and a variety of pungent compounds (Bandell et al., 2004; Jordt et al., 2004; Macpherson et al., 2005; Story et al., 2003). Antisense knock down of TRPA1 in rats has shown a requirement for this ion channel in inflammation-induced and nerve injury-induced cold allodynia (a nociceptive response to an innocuous stimulus) (Obata et al., 2005). TRPA1-deficient mice show reduced sensitivity to cold nociception (Kwan et al., 2006). However, TRPA1 activation by cold and its requirement for cold sensitivity in mice has been disputed (Bautista et al., 2006; Jordt et al., 2004).

Unlike TRPA1, which is expressed in a subset of putative nociceptive neurons, TRPM8 is expressed in small-diameter neurons that do not coexpress known markers of nociceptive fibers (Story et al., 2003). In vitro studies with cultured DRG neurons have suggested that under certain conditions TRPM8 can be coexpressed with the noxious heat nociceptor TRPV1. These findings have led some to speculate that in addition to transmitting sensations associated with innocuous cooling, TRPM8 may also have a role in transmitting the painful signals associated with noxious cold temperature (reviewed in Dhaka et al., 2006). In addition, it is well accepted that cold application is an effective method of pain management (Sauls, 1999). Menthol and, more recently, TRPM8 have been suggested to play an analgesic role (Galeotti et al., 2002; Proudfoot et al., 2006). Here, we explore the role of TRPM8 in cold thermosensation in vivo.

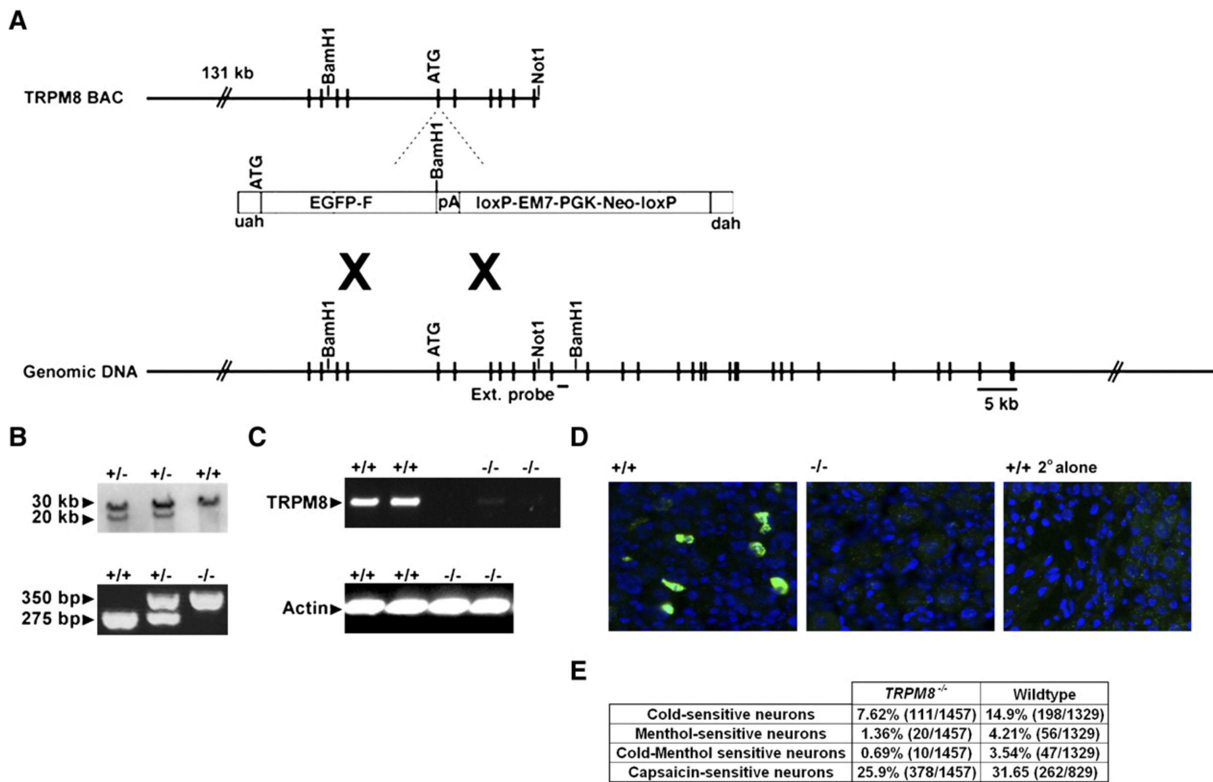

Figure 1. Generation of TRPM8-Deficient Mice

(A) Targeting strategy for the disruption of the *TRPM8* gene. In homologous recombinants 27 residues following the start codon in exon 5 were excised. Exons are shown as black bars on BAC and genomic maps. uah, upstream arm of homology; dah, downstream arm of homology.

(B) Confirmation of homologous recombination. (Top) Southern blot analysis. Genomic ES cell DNA samples were digested with BamHI and hybridized with a 3′ flanking probe. (Bottom) PCR analysis. Genomic tail DNA samples were amplified using a three-primer system, with a common forward primer upstream of the deletion site, a primer restricted to the deleted region, and a primer specific to EGFP-F. *TRPM8* genotypes are indicated above each lane.

(C) RT-PCR analysis using total RNA derived from DRG of wild-type and mutant mice. After 28 cycles a faint band was detected in one of two mutant samples using a primer set spanning exon 6 to exon 9. β-actin was used as a positive control.

(D) In situ hybridization analysis of DRG. *TRPM8* message (green) was readily detected in wild-type DRG (left). No signal above background was detected in *TRPM8*$^{-/-}$ *DRG* (middle). Wild-type DRG, no probe control (right).

(E) DRG responses to cold, menthol (250 μM), and capsaicin (1 μM). Responses are listed as a percentage of total DRG neurons. Response counts are indicated in parentheses. A response is defined as an increase in signal of 30% or more above baseline.

RESULTS AND DISCUSSION

Reduced Number of Cold- and Menthol-Responding Neurons in TRPM8-Deficient Mice

To examine the role of TRPM8 in vivo, we used a targeting construct to delete amino acid residues 2–29 and knocked in a farnesylated enhanced green fluorescent protein (EGFP-F) followed by an SV40polyA tail in frame with the start condon of *TRPM8* (Figures 1A and 1B). The SV40polyA tail should prevent transcription of the full *TRPM8* transcript and thus create a TRPM8-deficient mouse. TRPM8-deficient mice were viable and were generated in the expected Mendelian ratio. DRGs from wild-type and mutant animals were morphologically identical (data not shown). RT-PCR of a region spanning exons 6–9 was used to evaluate the expression of *TRPM8* in the mutant mice. A very faint band was sometimes detected in mutant mice, indicating that a truncated splice variant of TRPM8 may be expressed at low levels (Figure 1C). Sequencing verified that this band represents *TRPM8* cDNA (data not shown). However, we also evaluated *TRPM8* expression using in situ hybridization and were unable to detect any signal above background in *TRPM8* mutant mice, whereas robust wild-type message levels were observed (Figure 1D).

We also tested if we could distinguish the absence of TRPM8-like DRG neuron responses by Ca imaging. We compared cold, menthol, and capsaicin (control) responses of cultured DRG neurons from wild-type and TRPM8-deficient mice. Fourteen point nine percent of cultured DRG neurons from wild-type and seven point six percent from TRPM8-deficient mice responded to a cold stimulus (10°C) (Figure 1E). These results confirm previous findings that multiple cold populations are present in DRG

neurons (Babes et al., 2004; Story et al., 2003). Four point two percent of wild-type DRGs responded to two hundred fifty micromolars of menthol, and the majority of these were within the cold population (Figure 1E). Three-fold fewer menthol-responsive neurons were present in TRPM8-deficient DRGs, and only half of these responded to cold. This suggests that TRPM8 is not the only receptor for menthol in DRGs. Indeed, previous work has shown that only a subset of menthol-responsive DRG neurons expresses detectable TRPM8 and that menthol can induce Ca release in a TRPM8- independent manner (Mahieu et al., 2006; Nealen et al., 2003). Alternatively, a truncated TRPM8 with residual activity in DRG neurons of TRPM8-deficient mice may explain the remaining menthol responses. However, the lack of detectable TRPM8 RNA by in situ hybridizations (Figure 1D) and the absence of behavioral response to icilin (see below and Figures 4C and 4D) in TRPM8-deficient mice argue against a hypomorphic TRPM8 allele. Interestingly, Kwan et al. (2006) report a similar situation for TRPA1 and its agonist mustard oil (MO): a partial elimination of MO responses via Ca imaging and a complete lack of in vivo nociceptive responses in TRPA1-deficient mice.

TRPM8 Is Required for Cool Thermosensation

We assayed the ability of TRPM8-deficient mice to recognize cold temperatures. We first used an apparatus in which mice are videotaped as they move freely in individual compartments (100 cm by 7.6 cm each) along a surface temperature gradient of 15°C to 53.5°C (Figure 2) (Lee et al., 2005; Moqrich et al., 2005). This gradient was virtually divided into 20 adjacent zones with increasing surface temperature ranges, and the amount of time spent in each zone was calculated. We focused our analysis on three consecutive 30 min intervals. During the first 30 min, wild-type, but not TRPM8-deficient, mice showed some preference to warm temperatures, spending approximately twice as much time in the zones near 35°C than in other zones (Figure 2A). During the next hour, clearer temperature preference patterns emerged (Figures 2B and 2C). Wild-type mice showed preference for a relatively narrow range of warm temperatures (30°C–38°C). In contrast, TRPM8-deficient mice showed a wider range of preferred temperatures, spending significantly more time in cooler zones (23°C–30°C) and less time in warm zones (30°C–38°C). This is quantitatively represented by the lower weighted average of occupied temperature for TRPM8-deficient mice (Figure 2D) (Lee et al., 2005). Severe cold (16°C–20°C) and hot (41°C–53.5°C) temperatures are largely avoided by both wild-type and TRPM8-deficient mice. The most straightforward interpretation of these studies is that TRPM8-deficient mice have a specific impairment in sensing cold temperatures, and that

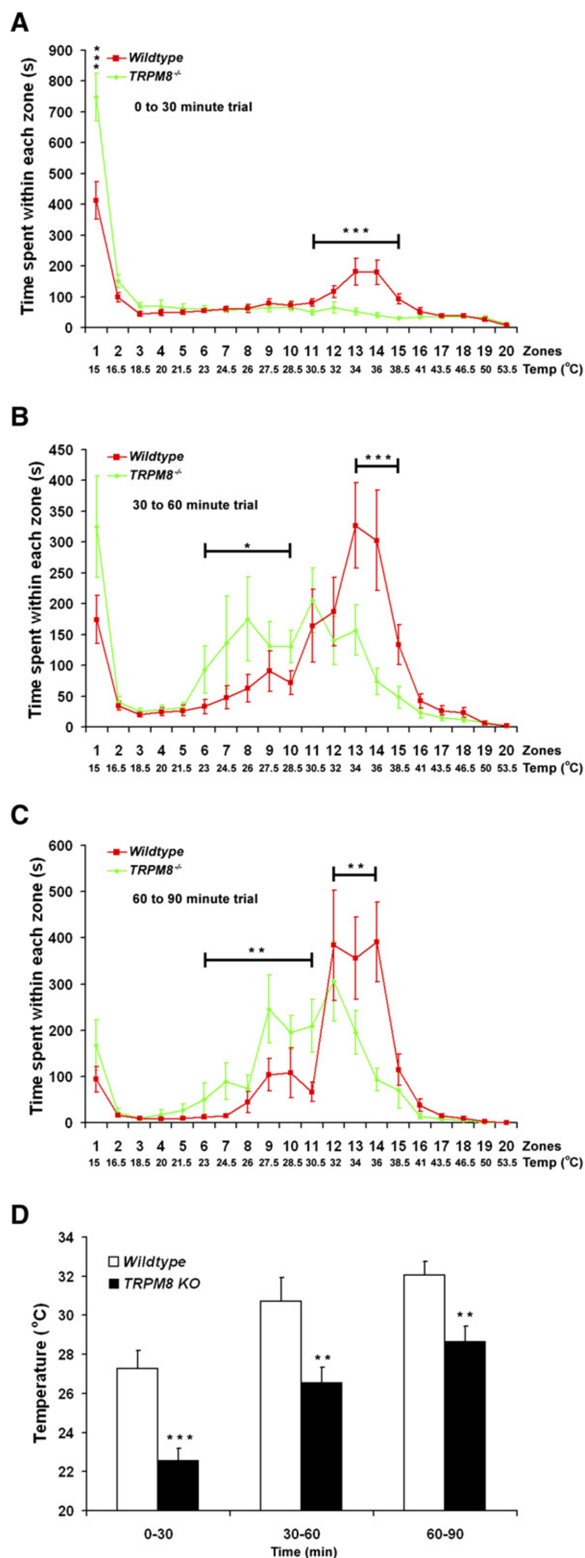

Figure 2. TRPM8-Deficient Mice Show Reduced Avoidance of Cool Temperatures on a Temperature Gradient Assay

(A–C) Time spent at different temperature zones for wild-type and TRPM8-deficient mice (n = 19) during a 90 min assay shown as three consecutive 30 min periods. (D) Weighted average of occupied temperature for wild-type and TRPM8-deficient mice during each 30 min period. *p < 0.5, **p < 0.01, ***p < 0.001 for individual or combined zones. All error bars represent SEM.

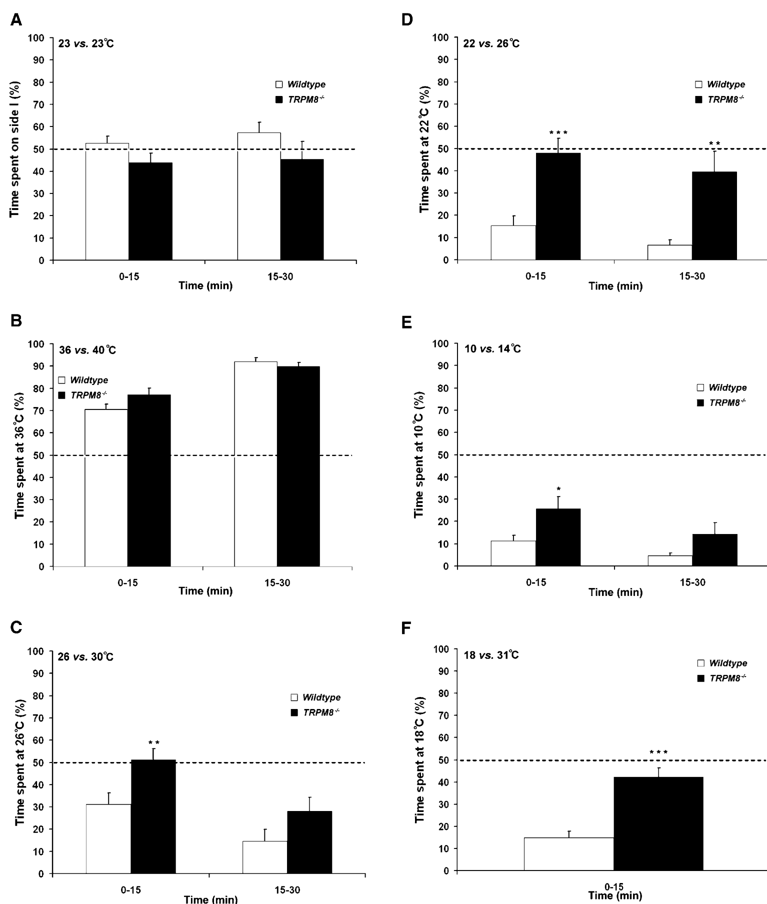

Figure 3. TRPM8-Deficient Mice Show Severe Deficits in Two-Temperature Choice Assays in Response to Innocuous Cool Temperatures

(A–F) Time spent on side I of a two-temperature choice assay (n = 14 wild-type, n = 16 knockout; males). *p < 0.5, **p < 0.01, ***p < 0.001. All error bars represent SEM.

this is manifested as a reduced avoidance of these temperatures relative to wild-type mice, indicating that in this context TRPM8 provides an unpleasant signal that leads mice to seek warmer temperatures. The coldest zone (15°C) is a preferred zone for both genotypes, most likely due to the strong preference in mice for corners, as seen when no temperature gradient is formed over the compartment (data not shown). While the highest temperature of 53.5°C is noxious enough to deter mice from this corner, 15°C may not be (we did not use temperatures lower than 15°C, as these temperatures caused condensation and interfered with recordings).

The gradient assay provides a general indication of the thermosensory capability of mice. In order to more accurately determine temperature preference or detection of particular temperature ranges, we performed two-temperature choice assays (Figure 3) (Lee et al., 2005; Moqrich et al., 2005). Mice are placed on a platform consisting of two identical juxtaposed surfaces (25 cm × 10 cm each) that can each be set to a unique temperature. We monitor time spent in each compartment over 30 min, and analyze the data in two consecutive 15 min bins. When both sides are set at room temperature (23°C), wild-type and TRPM8-deficient mice showed no significant preference for either side, as expected (Figure 3A, time spent on side I is at or close to 50%). We then challenged the mice with a variety of 4°C differential choice tests. Wild-type and TRPM8-deficient mice showed identical preference for 36°C over 40°C, suggesting that TRPM8 is not directly involved in sensing warm temperatures (Figure 3B). Wild-type mice prefer 30°C over 26°C, 26°C over 22°C, and 14°C over 10°C (less than 50% time spent on the cooler side I). TRPM8-deficient mice showed reduced avoidance of the cooler side on all three of these choice assays (Figures 3C–3E). Indeed, the knockouts do not show any significant preference in the 22°C versus 26°C choice assay (statistically not different from a hypothetical 50% choice, one-sample two-tailed t test, $p > 0.05$), arguing that TRPM8 is the only receptor required to discriminate between these two innocuous cold temperatures (Figure 3D). Strikingly, in a 15 min 18°C versus 31°C choice test, TRPM8-deficient mice did not show any significant zone preference ($p > 0.05$), whereas wild-type mice strongly preferred the 31°C zone (Figure 3F). This indicates that TRPM8 plays a substantial role in temperature discrimination over a large 13°C temperature differential encompassing the entire innocuous cool and perhaps some of the warm temperature ranges.

Warm and noxious cold receptors could play an overlapping role with TRPM8 in the 30°C versus 26°C and

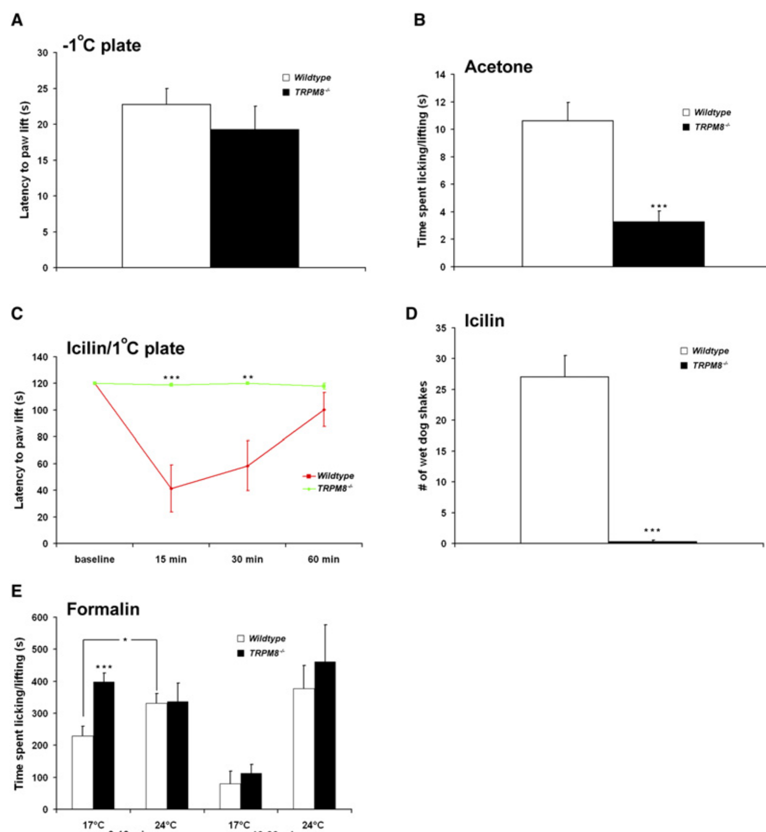

Figure 4. TRPM8-Deficient Mice's Responses to Noxious Cold, Formalin, and Cooling Compounds

(A) Latency of paw withdrawal of wild-type and TRPM8-deficient mice on a −1°C cold plate. Hindpaw lifts are measured. No significant difference in response is observed between the two genotypes (n = 9 knockout, n = 9 wild-type).

(B) Acetone is applied to the hindpaw of animals and total time spent licking and lifting the paw during a 1 min period is counted. (n = 13 knockout, n = 9 wild-type; males.) ***$p < 0.001$.

(C) Twenty-four micrograms in ten microliters is injected into the right hindpaw of wild-type and TRPM8-deficient mice and the latency of paw withdrawal was measured before and fifteen, thirty, and sixty min post injection (n = 10 knockouts, n = 8 wild-type; females.) **$p < 0.01$, ***$p < 0.001$.

(D) Fifty milligrams per kilogram of icilin is injected i.p. in wild-type and TRPM8-deficient mice, and the number of wet-dog-like shakes within twenty minuntes is counted. (n = 6; males.) ***$p < 0.001$.

(E) Time spent licking and lifting hindpaws injected with 0.75% formalin. Wild-type and TRPM8-deficient mice are placed either on a 17°C plate or on a room temperature (24°C) plate (n = 7 knockout, n = 9 wild-type, for room temperature; n = 8 knockout, n = 9 wild-type for 17°C; females). *$p < 0.5$, ***$p < 0.001$. All error bars represent SEM.

the 14°C versus 10°C choice tests, respectively (Figures 3C and 3E). Indeed, both the gradient and choice assays suggest the presence of another sensor for colder temperatures. One other candidate for cold thermosensation is TRPA1 (Story et al., 2003). Further analysis of TRPA1 knockout and TRPM8/TRPA1 double knockout mice in these choice assays could clarify the role of TRPA1 in thermosensation (Bautista et al., 2006; Kwan et al., 2006). Similarly, the residual choice of 30°C over 26°C in TRPM8-deficient mice in the 15 to 30 min portion of the assay might be due to the involvement of heat-activated receptors in sensing the warm 30°C plate. The analysis of mice lacking TRPM8 and TRPV3/4 could address the overlapping role of heat- and cold-activated ion channels in innocuous thermosensation (Lee et al., 2005; Moqrich et al., 2005). Together, the thermotaxis data suggest that innocuous cold thermosensation is severely disrupted in TRPM8-deficient mice, but that other temperature sensation is intact.

A Role for TRPM8 in Cold Nociception?

The thermotaxis assays suggest a crucial role for TRPM8 in avoiding cold temperatures. Avoidance could be due to innocuous or noxious stimuli. Hot plates are routinely used as an assay for heat nociception, as plate temperatures above 45°C cause mice to lick or flick their hindlimbs or to jump. Similarly, cold plates have also been used to assay cold nociception (Bautista et al., 2005; Kwan et al., 2006). To test if TRPM8 is involved in sensing noxious cold, we placed wild-type and TRPM8-deficient mice on a −1°C cold plate. Both genotypes showed identical nociceptive behaviors with similar latencies (Figure 4A). Because these cold temperatures could potentially cause tissue damage to the skin, the cold plate behavioral responses may not be solely due to temperature sensation. However, no paw edema was observed after testing on a −1°C cold plate (data not shown). Temperatures above 0°C did not elicit any nociceptive response in wild-type or TRPM8-deficient mice (data not shown).

We then tested the role of TRPM8 in response to cooling chemicals. Acetone is thought to cause rapid evaporative cooling when applied on the skin, though one cannot rule out non-temperature-mediated chemical effects of acetone. Mice respond to acetone application to the surface of the paw by shakes, lifts, and licks (Bautista et al., 2006; Kwan et al., 2006). TRPM8-deficient mice show a significantly reduced response to acetone application (Figure 4B). Injection of icilin, a synthetic compound that activates TRPM8 and, to a much lesser extent, TRPA1, into the hindpaw of wild-type mice causes the rapid induction of hindpaw withdrawal when the mice are placed on a 1°C cold plate (McKemy et al., 2002; Story et al., 2003; Wei and Seid, 1983). This behavior is completely ablated in TRPM8-deficient mice, suggesting that TRPM8

activation can elicit a nociceptive-like response (Figure 4C). Taken together, the data indicate that TRPM8 is involved in sensing unpleasant or noxious cold. The remaining response observed in TRPM8-deficient mice to acetone as well as the lack of behavioral differences to the −1°C cold plate suggest that other cold receptors are also involved in noxious cold sensation (Kwan et al., 2006).

Intraperitoneal (i.p.) injections of icilin in rodents cause vigorous shaking movements reminiscent of cold-induced wet-dog shakes. Indeed, it has been suggested that these wet-dog shakes are due to the cooling effect of icilin (Tse and Wei, 1986). Strikingly, the robust behavior of icilin-induced wet-dog shakes was essentially eliminated in TRPM8-deficient mice (Figure 4D). This data confirms that it is intense activation of TRPM8 by icilin that causes shivering-like body shakes. Menthol also activates TRPM8 and is widely used in over-the-counter products for its cooling effect. We explored behavioral assays in response to menthol. However, up to 15 mM of intradermal injection or 50 mg/kg of i.p. injection of menthol did not elicit any consistent responses in wild-type mice. Because higher concentrations of i.p. injections of menthol induced ataxia and were avoided, we were likely unable to reach the significant levels of specific TRPM8 activation observed with icilin.

TRPM8 Can Account for the Analgesic Effect of Cold in the Formalin Test

Next we tested if cold can be analgesic in response to a formalin injection in the paw, and if this analgesia was mediated by TRPM8. After a 2% injection of formalin, wild-type mice on a room temperature (24°C) plate showed the characteristic two-phase nociceptive response: first 10 min is thought to account for an acute response; 10–30 min, an inflammatory response (Figure 4E). Wild-type mice on a 17°C cold plate displayed a reduced nociceptive response during both the acute and inflammatory phases, asserting that cold is analgesic to the formalin injection. Formalin responses in TRPM8-deficient mice at room temperature were similar to that of wild-type mice (Figure 4E). On the 17°C cold plate, TRPM8-deficient mice did not show the cold-induced analgesia observed in wild-type mice during the first 10 min (Figure 4E). Therefore, in the context of a painful stimulus such as formalin, TRPM8 activity can reduce the intensity of pain. Both wild-type and TRPM8-deficient mice showed similar reduced responses during the inflammatory phase ($p < 0.01$) (Figure 4E).

In sum, our results point to an essential role for TRPM8 in cold sensation. TRPM8-deficient mice have severe deficits in avoiding cold temperatures and in paw withdrawal responses to acetone and icilin, suggesting that TRPM8 activation sends an unpleasant signal to the brain. Whether these responses can be classified as nociceptive is difficult to assert. However, in the context of a more noxious stimulus such as formalin, TRPM8 activity is analgesic, perhaps as a distracting signal.

EXPERIMENTAL PROCEDURES

TRPM8 Gene Disruption

To target the *TRPM8* locus, we employed a modified version of VELOCIGENE, a BAC-based targeting approach used to generate homologous recombination in embryonic stem (ES, CJ7/129S1) cells (Valenzuela et al., 2003). In this approach a modified BAC targeting construct (pl452) is used to generate homologous recombinants in BACs containing the gene of interest. The recombined BAC is then linearized with an appropriate restriction enzyme and then used to target ES cells. Using this approach we deleted amino acid residues 2–29 of TRPM8. Briefly, *EGFP-F*-SV40polyA (Clontech) was fused in frame to a 156 bp arm upstream of the *TRPM8* start codon (upstream arm of homology; uah), and PCR amplified from BAC clone RP23-180P9 containing the *TRPM8* gene from the C57Bl/6 strain using the following primers: 5′-AAAAACGCGTTATAGATGCAGGGTACAATG-3′ and 5′-CTCGCCCTTGCTCACCATCTTGCCTGCGAG-3′. uah-EGFP-F-SV40polyA was then cloned into the BAC targeting vector (pl452) downstream of an EM7-PGK-Neo selection cassette. A 132 bp fragment of *TRPM8* located 85 bp downstream of the uah was amplified using these primers: 5′-AAAAGACGTCGGAGCACAGACGTGTCCT AC-3′ and 5′-AAAAGTCGACTCGCAAAACAAATACTACCC-3′. It was then cloned immediately downstream of the selection cassette. This BAC targeting construct was then used to generate homologous recombination in RP23-180P9. The recombined BAC was then excised using NotI, creating a 131 kb arm of homology 5′ to the start codon and a 9.4 kb arm of homology 3′ to the selection cassette. The targeting construct was then subcloned into the pBACe6 vector. This final construct was then linearized with NotI and electroporated into ES cells. G418-resistant clones were screened for homologous recombination by southern blot, using 3′ flanking and neomycin probes. Targeted ES clones were confirmed for normal karyotype and injected into C57Bl/6 blastocysts. Chimeric males were mated to C57Bl/6 females. F1 heterozygous offspring were intercrossed to generate F2 littermates used in all studies. Germline transmission of the mutated allele was verified by PCR analysis using the following primers: M8F, 5′-GGGATGTCATAGTGCTGAAAGGCAGA-3′, M8delR, 5′-CCGGGT GCTGCCCATAGTACCATTTC-3′, EGFPFR, 5′GGTGCAGATGAACTT CAGGGTCAGCT-3′. Preliminary investigations showed no GFP fluorescence in DRGs of mice carrying a single allele of the TRPM8 transgene.

All experiments described below were performed blind with respect to genotype and were conducted with the approval of the The Scripps Research Insitute Animal Research Committee.

Expression Analysis

TRPM8 in situ hybridization analysis was performed as described (Peier et al., 2002). For RT-PCR, total RNA was isolated from DRG of wild-type and TRPM8-deficient mice. Point five micrograms was used to generate first-strand cDNA (Superscript II, Invitrogen). Four micrograms of cDNA from each sample was used in each PCR amplification with the following primers: M8 160F, 5′-GTGTCTTCTTTACCAGA GACTCCAAGGCCA-3′, M8 640R, 5′-TGC CAA TGG CCA CGA TGT TCT CTT CTG AGT-3′, ActinF, 5′-GTTTGAGACCTTCAACACCCC-3′, ActinR 5′-GTGGCCATCTCCTGCTCGAAGTC-3′.

Calcium Imaging

Dissociation and culturing of mouse DRG neurons was performed as described with the following modifications (Story et al., 2003). Dissected DRGs were dissociated by incubation for 1 hr at 37°C in a solution of culture medium (Ham's F12/DMEM with 10% Horse Serum, 1% penicillin-streptomycin) containing 0.125% collagenase (Worthington Biochemicals) followed by a 30 min incubation in 10 ml of culture media plus 1.25 units papain. Ca imaging was performed essentially as described (Story et al., 2003). Growth media was supplemented with 100 ng/ml nerve growth factor. Experiments were performed 24 hr

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after plating and the threshold for activation was defined at 30% above baseline.

Behavior

All behavior analysis was conducted on littermate mice 6–16 weeks old. Unless otherwise noted, males and females were combined if they behaved similarly. For other experiments males or females were used based on availability. Animals were acclimated for at least 20 min to their testing environment prior to all experiments. Students' t test was used for all statistical calculations.

Thermal Gradient Test

Seven mice were individually tracked for 90 min in seven different arenas separated by opaque plexiglass walls at the same time. Each arena measures 100 cm long, 7.6 cm wide, and 10 cm high. A well-controlled and stable temperature gradient of 15°C–53.5°C is maintained using two Peltier heating/cooling devices positioned at each end of the aluminum floor (constructed by The Genomic Institute of the Novartis Research Foundation). Each arena is virtually divided into 20 zones of equal size (5 cm) with a distinct and stable temperature using EthoVision tracking system (Noldus Information Technology). One day prior to testing on the thermal gradient, the mice were acclimated on the gradient apparatus wherein the whole floor was at room temperature for 90 min.

Two-Temperature Choice Assay

To construct the two-temperature choice assay testing apparatus, we joined two cold/hot plate analgesia meters (Columbus Instruments) with a 1 inch metal plate separated at the midway point with a thin plastic spacer to thermally isolate each plate. An opaque plexiglass rectangular box divided lengthwise was used to create two lanes measuring 9.5 cm in width across the two plates with walls at a height of 20 cm. The choice test apparatus was illuminated with low, diffuse white light. Mice were placed in each lane simultaneously and tracked for 30 min using the EthoVision tracking system. For the 18°C versus 31°C choice test, mice were tracked for 15 min.

Acetone

Mice were acclimated for 30 min in a Von Frey Apparatus chamber with a mesh floor (Ugo Basile). Point fifteen milliliters of acetone was sprayed onto the hindpaw using a one-milliliter syringe, and the duration of withdrawal, flicking, biting, and licking behavior was measured for one minutes (Kwan et al., 2006). Acetone was applied independently to each hindpaw and the duration of behavior was calculated from the average of the two responses.

Cold Plate

For the −1°C cold plate assay, ceramic plates were cooled in a −20°C freezer. Plates were placed on a on a bed of ice and allowed to warm to −1°C as measured by two independent temperature probes. At this temperature the plates were able to hold this temperature for approximately 2 min. A clear plexiglass cylinder with a diameter of 7 cm and height of 12 cm was placed on the plate and the mice were placed on the plate as well. The onset of brisk hindpaw lifts and/or flicking/licking of the hindpaw was assessed. Prior to both assays mice acclimated in an equivalent chamber at room temperature for 20 min.

Icilin Injections

Mice were acclimated for 20 min. For testing at +1°C, mice were placed on a cold plate (TECA) and the onset of brisk hindpaw lifts and/or flicking/licking of the hindpaw was recorded. Two independent temperature probes were used to confirm the surface temperature of the plate. Two point four milligrams per milliliter icilin was dissolved in eighty percent DMSO/twenty percent PBS, ten microliters was injected into the right hindpaw, and the onset of response on the cold plate was measured at fifteen, thirty, and sixty minutes after injection.

i.p. icilin injections were performed essentially as described (Werkheiser et al., 2006). Briefly, 12.5 mg/ml icilin was suspended in 1% Tween-80/distilled water and then sonicated. Icilin was administered at a concentration of 50 mg/kg. Wet-dog shakes were counted for 20 min.

Formalin Injections

Formalin injections were performed essentially as described (Karim et al., 2006). Mice were acclimated for 20 min in a transparent plexiglass box at room temperature. Ten microliters of two percent formalin solution was injected subcutaneously into the right hindpaw. The total time spent licking, flicking, or lifting the injected paw on a 17°C cold/hot plate analgesia meter (Columbus Instruments) or at room temperature (approx 24°C) was recorded for 30 min.

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