TRPV1-lineage neurons are required for thermal sensation

Santosh K Mishra, Sarah M Tisel, Peihan Orestes, Sonia K Bhargoo and Mark A Hoon*

Molecular Genetics Unit, Laboratory of Sensory Biology, NIDCR, NIH, Bethesda, MD, USA

The ion-channel TRPV1 is believed to be a major sensor of noxious heat, but surprisingly animals lacking TRPV1 still display marked responses to elevated temperature. In this study, we explored the role of TRPV1-expressing neurons in somatosensation by generating mice wherein this lineage of cells was selectively labelled or ablated. Our data show that TRPV1 is an embryonic marker of many nociceptors including all TRPV1- and TRPM8-neurons as well as many Mrg-expressing neurons. Mutant mice lacking these cells are completely insensitive to hot or cold but in marked contrast retain normal touch and mechanical pain sensation. These animals also exhibit defective body temperature control and lose both itch and pain reactions to potent chemical mediators. Together with previous cell ablation studies, our results define and delimit the roles of TRPV1- and TRPM8-neurons in thermosensation, thermo-regulation and nociception, thus significantly extending the concept of labelled lines in somatosensory coding.

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Introduction

Our senses provide an internal representation of aspects of the external world that are relevant to our survival, health and happiness. Among these, thermosensation provides animals with critical information about their environment and for us triggers perceptual responses that range from contentment to pain. Recent evidence suggests that a family of transient receptor potential (TRP)-related ion channels act in combination to detect environmental temperature (Jordt *et al*, 2003; Patapoutian *et al*, 2003). For example, the receptor for capsaicin (the ‘hot’ compound from chili peppers), TRPV1, is expressed in somatosensory neurons and thought to be the major mammalian sensor of noxious heat (Caterina *et al*, 1997). Similarly, TRPM8 is activated by menthol and cool temperatures (McKemy *et al*, 2002; Peier *et al*, 2002) while TRPV2 and TRPA1 have been suggested as sensors for extreme heat and cold, respectively (Caterina *et al*, 1999;

Story *et al*, 2003; Jordt *et al*, 2004). In addition, TRPV3 (Moqrich *et al*, 2005) and TRPV4 (Lee and Caterina, 2005) have been reported to mediate warm responses although not by directly activating sensory neurons. Surprisingly, however, knockout mice lacking one or more of these TRP channels exhibit only modest deficits in temperature sensation (Caterina *et al*, 2000; Lee *et al*, 2005; Moqrich *et al*, 2005; Bautista *et al*, 2006, 2007; Kwan *et al*, 2006; Colburn *et al*, 2007; Dhaka *et al*, 2007) implying redundancy in the detection of heat and cold. In contrast, knockout of TRPV1 had greater effects on inflammatory pain sensation (Caterina *et al*, 2000) perhaps suggesting that its primary role may be as an integrator of several noxious signals rather than purely a thermosensor (Tominaga *et al*, 1998).

The problem of distinguishing and responding to different sensory modalities is often solved by selectively tuned receptors that are hardwired to trigger appropriate behaviour for example, the sweet- (attractive) versus bitter- (aversive)-labelled lines (Mueller *et al*, 2005). If a similar logic applies to somatosensation then removal of one class of neurons might eliminate specific sensory modalities without effecting responses to other types of stimuli. In contrast, if instead, stimuli are sensed by broadly tuned receptor neurons (e.g., multimodal nociceptors (Cain *et al*, 2001)), cellular ablation would never have such selective an effect.

Recently, resiniferatoxin-mediated killing (Mishra and Hoon, 2010) or capsaicin-induced deafferentation (Cavanaugh *et al*, 2009) of TRPV1 neurons were shown to attenuate responses to heat but not to mechanically induced pain or cold. Moreover, ablation of a different subset of neurons expressing the mas-related G-protein-coupled receptor MrgD resulted in mechanosensory deficits (Cavanaugh *et al*, 2009) indicating that distinct cellular substrates function as sensors for different classes of noxious stimuli. These studies are important in defining the properties of specific subsets of sensory cells but silencing TRPV1 neurons was potentially incomplete and was relatively poorly defined (Cavanaugh *et al*, 2009; Mishra and Hoon, 2010). In addition, effects on mechanosensation were detected only when MrgD neurons were killed in adult animals but not if these cells were eliminated during development (Cavanaugh *et al*, 2009). Interestingly, diphtheria toxin-mediated killing of somatosensory neurons expressing the ion-channel Nav1.8 throughout development eliminated the majority of TRPV1 expression but had no effect on responses to heat (Abrahamsen *et al*, 2008) and instead altered mechanosensory responses and cold detection. Thus, there are indications that distinct classes of somatosensory neurons may selectively respond to particular sensory input but interpretation of results is limited by the ability to target the appropriate cells and redundancy or plasticity of the system. In this study, we used a molecular genetic approach to generate mice lacking all neurons in the TRPV1 lineage. Notably, these animals display no responses to thermal stimuli but retain normal proprioception and mechanosensation, thus delimiting

*Corresponding author. Molecular Genetics Unit, Laboratory of Sensory Biology, NIDCR, NIH, Building 49, Room 1A16, 49 Convent Drive, Bethesda, MD 20892, USA. Tel.: +1 301 402 6613; Fax: +1 301 480 3590; E-mail: mark.hoon@nih.gov

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Figure 1 TRPV1-ai9 mice reveal the extent and limit of the TRPV1 lineage. Mice expressing tdTomato under the control of TRPV1-Cre-mediated recombination were perfused and tissue was removed for fluorescent imaging. Sections through the DRG (A) and trigeminal ganglion (B) reveal tdTomato expression in a subset of neurons that project to superficial lamina in the dorsal horn (C) and trigeminal tract (D), respectively. Whole-mount imaging of the cornea (E) and skin (F) illustrate the peripheral projections of these fibres. No fluorescent cell bodies were observed in the hypothalamus (HYP) (G) although widely scattered neurons in the cortex (H) were labelled. At the front of the tongue (I) fluorescent processes surround fungiform taste buds, highlighted by TRPM5 staining (green); however, taste receptor cells are not tdTomato positive. Scale bars: A, B, D, I, H 50 μ m; C and E 100 μ m; F and G 1 mm.

plasticity in the development of the thermosensory-labelled line. The mutant mice also exhibit markedly reduced ability to regulate their body temperature in response to a variety of challenges, demonstrating the importance of peripheral thermosensation in this homeostatic process.

Results

We engineered BAC transgenic mice in which Cre recombinase was expressed under the control of TRPV1 (TRPV1-Cre, see Materials and methods and Supplementary Figure S1 for details). To examine the selectivity of Cre expression, TRPV1-Cre animals were crossed with various reporter lines including ai9 ROSA-stop-tdTomato mice (Madisen *et al*, 2010) to generate TRPV1-ai9 mice. Figure 1 demonstrates that, as expected, the dorsal root ganglia (DRG) and the trigeminal ganglia of TRPV1-ai9 mice contain tdTomato marked neurons. In addition, we saw labelling of cells in blood vessels (data not shown) but importantly only very few scattered fluorescent cells were observed outside these tissues for example, in the cortex (Figure 1H). Notably, other areas of the brain including the hypothalamus and hippocampus were devoid of labelling, as were the fungiform taste buds (Figure 1G, I and data not shown). Direct *In situ* hybridization (ISH) or immunohistochemistry also revealed no evidence for the

expression of TRPV1 in the brain or taste tissue. Because of the intense fluorescence of tdTomato in these TRPV1-ai9 mice, peripheral and central projections of the labelled neurons were beautifully revealed (see Figure 1 for detail). We further characterized the specificity of Cre expression in the ganglia using double label ISH and probes that selectively recognize the native and transgene transcripts (see Materials and methods). There was complete correspondence of Cre and TRPV1 expression in adult tissue (Figure 2A) and Cre-mediated excision and TRPV1 in embryonic tissue (Supplementary Figure S2a).

TRPV1-DTA mice selectively lose thermal sensation

We crossed the TRPV1-Cre mice with a ROSA-stop-DTA line (Ivanova *et al*, 2005) to generate animals (TRPV1-DTA) in which a genetically specified population of sensory neurons was ablated. We note that the resulting TRPV1-DTA mice appear healthy and show no obvious phenotypic abnormalities highlighting the restricted nature of TRPV1 expression. For example, TRPV1-DTA animals do not show any signs of self-mutilation unlike mice in which the sciatic nerve is lesioned (Wall *et al*, 1979), there is no obvious change in wound healing (after skin burns or fight wounds) nor any deficit in taste responses (data not shown). In addition, analysis of markers of interneurons in the dorsal horn

Figure 2 In adult mice, TRPV1-Cre is restricted to the TRPV1 neurons that are lost in TRPV1-DTA animals. Sections through the DRG of adult TRPV1-Cre mice were examined using *in situ* hybridization (A). Double labelling with probes for endogenous TRPV1 (green) and Cre (red) reveal complete co-expression (see merged image, right). (B) Staining for TRPV1 (left panels) and TRPA1 (right panels) demonstrate that >95% of positive neurons are lost in TRPV1-DTA mice (lower panels); in contrast (C) only a subset of TRPV2 cells are eliminated in these mutant animals.

indicated no noticeable differences between mutant and control animals (Supplementary Figure S3).

Figure 2B demonstrates that the TRPV1-DTA mice have lost all TRPV1- and TRPA1-expressing neurons in agreement with previous studies that demonstrate that TRPA1 is co-expressed in a subset of TRPV1 neurons (Story *et al*, 2003; Mishra and Hoon, 2010). Consistent with this, responses to capsaicin and mustard oil were completely abolished in standard eye wipe and paw injection paradigms (Supplementary Figure S4). Moreover, several well-characterized behavioural paradigms revealed that TRPV1-DTA mice were completely insensitive to noxious heat. For example, the mutant mice never reacted within the cutoff time when placed on a 55°C hot plate (Figure 3A) even after injection of carageenan to cause paw inflammation. TRPV1-DTA mice also failed to withdraw their tails (within the cutoff time) from radiant heat sources that burned the skin in a modified Hargreaves assay (Figure 3B) and most importantly showed no preference when given the choice between a 30°C platform and another at an elevated temperature (45 or 50°C) that normal mice strongly disliked (Figure 3C). Given the remarkable lack of protective thermosensory responses of the mutant animals, we also examined whether these mice had lost TRPV2- and other candidate thermosensory TRP-ion channels-expressing neurons using ISH (Figure 2C and Supplementary Figure S5). Although mutant animals showed a reduced number of TRPV2-containing sensory neurons,

~20% of positive cells remained, consistent with double labelling for TRPV1 and TRPV2 (data not shown). Finally, as predicted by their behavioural insensitivity to heat, there was also no heat-induced activation of *c-fos* in the dorsal horn of TRPV1-DTA mice (Figure 3D).

In marked contrast to their loss of heat sensation, TRPV1-DTA animals exhibited completely normal mechanosensory responses even after sensitization by inflammation or nerve ligation (Figure 4). We show that the behavioural responses of TRPV1-DTA and littermate controls are indistinguishable in assays for touch (Figure 4A and C) and pinch (Figure 4B). Importantly, recordings from the sciatic nerve to stimulation of the foot by brush, vibration and von Frey microfilaments were the same in TRPV1-DTA and control animals (Figure 4D and E). Moreover, the mutant mice displayed no loss of proprioceptive function for example, in rotarod assays (Figure 4F).

Cre-recombinase mediates DNA excision or rearrangement that is stable in all daughter cells. This can provide valuable information about development but means that recombination can occur in cells that no longer express Cre in the adult. In fact, TRPV1-ai9 animals appear to have many more tdTomato fluorescent neurons than expected based on TRPV1 (or Cre) ISH (compare Figures 1 and 2). Therefore, we carried out double label ISH for TRPV1 and tdTomato in sections through the ganglia of adult TRPV1-ai9 mice (Figure 5A). Approximately twice as many cells expressed the tdTomato as expressed TRPV1 or Cre, reflecting recombination

Figure 3 TRPV1-DTA mice lose all responses to heat. Compared with wild-type controls (grey bars), TRPV1-DTA mutant mice (black bars) exhibit no heat pain sensitivity in a hot-plate assay both before and after carageenan-induced inflammation (**A**) or in a tail-flick test (**B**) ($*P < 0.0001$, Student's *t*-test); mutant mice did not react before the cutoff was reached in both tests (dotted line). Similarly, (**C**) a two-plate choice assays demonstrates that mutant animals fail to distinguish noxious (45 or 50°C) and normal (30°C) temperatures unlike normal animals ($*P < 0.0001$, Student's *t*-test). Immersion of the paw in 55°C water (**D**, **E**) induces fos mRNA expression in the ipsilateral (arrowed) dorsal horn of wild-type but not in TRPV1-DTA mutant mice (**D**); fos-positive cell bodies in laminae I and II are quantified in (**E**) revealing a significant difference between genotypes ($*P < 0.0001$, Student's *t*-test). Data represent means $\pm$ s.e.m.; $n \geq 6$ animals.

during development. Interestingly, double labelling showed that all TRPM8 cold sensing neurons expressed tdTomato (Figure 5B). Correspondingly, at early stages of embryonic development, most TRPM8-expressing neurons co-express TRPV1 (Supplementary Figure S2d) and this overlap disappears around birth (Supplementary Figure S2e and f). Therefore, we hypothesized that TRPM8 neurons would be ablated in TRPV1-DTA mice. Indeed, ISH (Figure 5C) shows that TRPV1-DTA mutants completely lack TRPM8-expressing neurons. These mice show no 'wet-dog shakes' to injection of icilin a compound that gives the percept of cooling and robustly induces this characteristic behaviour in control animals (Figure 5D). Notably, the mutant TRPV1-DTA mice also show absolutely no observable reaction to exposure to a cold plate at -5°C (Figure 5E) or preference for a 30°C environment over one at temperatures as low as 5°C in two choice preference assays (Figure 5F).

TRPM8-expressing cells contribute a relatively small proportion of the extra tdTomato-labelled neurons in the TRPV1-ai9 mice (Figure 5). Therefore, we examined other markers of somatosensory neurons that are not co-expressed with TRPV1 (Figure 6). TrkB and TrkC expression defines a class of completely non-overlapping cells but many of the Mrg neurons co-express tdTomato (Figure 6). Importantly, all tdTomato cells hybridize to a mixed probe including TRPV1, TRPM8 and several different Mrgs (Figure 6C) establishing that TRPV1-Cre-mediated recombination is limited to neurons expressing these markers. There is also a population of Mrg-expressing neurons that does not contain tdTomato in TRPV1-ai9 animals. Consistent with these findings, we

observed that TRPV1-DTA animals retained 10–20% of the normal complement of Mrg-expressing neurons (Figure 6D).

Peripheral thermoreceptors and their role in setting body temperature

It has been shown that IP injection of capsaicin causes profound hypothermia in normal mice but not in TRPV1-KO animals (Caterina *et al*, 2000). We reasoned that ablation of hot and cold sensing neurons might dramatically alter thermoregulation and thus implanted internal temperature probes to measure core body temperature of TRPV1-DTA animals. The mutant animals exhibited a resting body temperature that was indistinguishable from that of littermate controls (Figure 7C) indicating that this temperature is maintained independent of peripheral somatosensory input. As expected, TRPV1-DTA animals lack responses to IP-injected capsaicin. As TRPA1 is expressed in a subset of the TRPV1-expressing neurons (Story *et al*, 2003; Mishra and Hoon, 2010), we also tested the effect of IP injection of mustard oil and found that it induced profound hypothermia in normal but not in the mutant mice suggesting that TRPA1-expressing TRPV1 neurons contribute to thermal homeostasis. Notably, we also found that when environmental temperature was changed (e.g., elevated to 35°C or reduced, 4°C) TRPV1-DTA mutant animals were far less able to maintain their body temperature than wild-type controls (Figure 7D and E).

Given the homeostatic deficit shown by TRPV1-DTA animals in response to thermal stimuli, we investigated whether responses to non-thermal stimuli that induce changes in body temperature were affected by TRPV1-

Figure 4 TRPV1-DTA mice retain normal touch, mechanical pain and proprioceptive responses. Measurements of touch and of mechanical pain following carageenan-induced inflammation (A) or sciatic nerve ligation (C) were made using von Frey microfilaments; mechanical pain was also assessed using a Randall–Selitto apparatus (B) both in normal and inflamed tissue. In addition, nerve recordings of the sciatic were measured in control and mutant mice (D) (responses were normalized to stimulation with a 6 g von Frey microfilament (nr)). Examples of summated responses to repeated 60 Hz vibrations are shown in (E). No significant difference was observed between wild-type and TRPV1-DTA mutant animals in any assay. Similarly, the two groups showed equivalent proprioception and motor function in a rotarod assay (F). Data represent means $\pm$ s.e.m.; $n \geq 5$ animals.

mediated cell ablation. Hypothermia is a hallmark of anaphylaxis caused by mast cell-induced vasodilation and loss of heat through the skin (Gilfillan *et al*, 2009). Normal mice become hypothermic upon injection of an antigen but relatively quickly re-establish normal resting temperature (Figure 7F). In contrast, TRPV1-DTA mutant mice had much deeper hypothermic response to antigen with delayed recovery. Fever is another pathophysiological thermal response that instead elevates body temperature. The cytokine IL1 β mimics this pathway and induces a mild fever in normal mice (Komaki *et al*, 1992). Again we found that TRPV1-DTA animals exhibited more pronounced temperature change in response to IL1 β than controls indicating that peripheral thermosensation has a role in reducing fever (Figure 7G).

TRPV1-DTA animals lose responses to painful and itch-inducing chemical stimuli

Many chemicals can induce pain when injected into tissue, among these is a class of noxious compounds that are believed to be released at sites of injury and include ATP, prostaglandins, bradykinin, histamine and serotonin (termed the 'inflammatory soup' (Kessler *et al*, 1992)). Receptors for

the components of the inflammatory soup have been identified, but it has been unclear which classes of neurons are necessary for nociception. Therefore, we examined whether TRPV1-DTA mice responded to ATP or the whole 'inflammatory soup' and show (Figure 8A and B) that normal responses are completely lost in mutant animals. Thus, although purinergic receptors are expressed by neurons that are not ablated in the TRPV1-DTA mice (see Figure 8C), it appears that these cells are not involved in the noxious response to ATP or other components of the 'inflammatory soup' in the mutant. The simplest explanation is that these mice lack all nociceptors to this class of compounds. However, TRPV1 neurons express a variety of neuropeptides including CGRP and substance P, which have potential roles in neurogenic inflammation and sensitization of responses to the 'inflammatory soup'; their loss may dampen responses in other cells. Indeed, ISH (Figure 8C) demonstrated complete loss of substance P and almost total loss of CGRP in sensory ganglia from TRPV1-DTA mice predicting a significant loss in neurogenic inflammation. Measurements of swelling confirm that significantly less inflammation occurs in TRPV1-DTA animals challenged with carageenan (Figure 8D).

Figure 5 TRPV1-Cre mediates recombination in TRPM8 neurons and TRPV1-DTA mice lose cold sensation. Double-label ISH of sections through the DRG of TRPV1-ai9 mice (**A**, **B**) establish that recombination and tdTomato expression (red) occurs in more neurons than those expressing TRPV1 (**A**, green); all cells that express TRPM8 (**B**, green) also express tdTomato (cells co-expressing TRPM8 and tdTomato are arrowed). TRPV1-DTA mice lose all neurons expressing the TRPM8-cold receptor (**C**), and exhibit severely attenuated responses ('wet-dog shakes') to icilin (50 mg/kg) (**D**). In a cold-plate assay (**E**), mutant mice (black bars) failed to respond to -5°C even after carrageenan-induced inflammation. Moreover, unlike wild-type controls (grey bars) TRPV1-DTA mice (black bars) display no preference for warm (30°C) over a cooler (20°C) or cold (5°C) environments in long-term choice assays (**F**). Data represent means $\pm$ s.e.m.; $n \geq 6$ animals; significant differences between genotypes (Student's *t*-test) are indicated by **P* < 0.0001 and ***P* < 0.01.

In addition to chemical, mechanical and thermal pain, somatosensory neurons transmit responses to pruritogenic agents that lead to the itch response (Shim and Oh, 2008). We found that TRPV1-DTA mutant mice had a complete loss of behavioural responses to subcutaneous injection of three major pruritogenic compounds: histamine, serotonin and a protease-activated receptor agonist (Figure 8E). Thus, TRPV1-DTA animals show profound deficits in response to many but not all nociceptive stimuli.

Discussion

In this study, we have used a molecular genetic approach to examine a defined subset of somatosensory neurons that express (or are derived from cells that express) a heat-

sensitive ion channel by driving Cre-recombinase under the control of *TRPV1*. Our data strongly imply that TRPV1 expression is almost completely restricted to sensory neurons and a subset of cells lining blood vessels and/or their precursors. Strikingly, we see no evidence for TRPV1 expression in fungiform taste receptor cells (Figure 1I) contrary to the proposal that this channel mediates amiloride-insensitive salt taste responses (Lyll *et al*, 2004). Moreover, despite suggestions that hypothalamic TRPV1 might have a role in temperature homeostasis (Mezey *et al*, 2000), this region of the brain was devoid of labelled cells (Figure 1G). In adult animals, all somatosensory neurons that express TRPV1 show Cre-mediated recombination. In addition, recombination selectively occurs in all TRPM8 cold-sensitive cells as well as a large subset of the Mrg-expressing neurons. These

Figure 6 TRPV1-Cre recombination occurs in a restricted population of sensory neurons. The extent of TRPV1-Cre-mediated recombination in somatosensory neurons was assessed using ISH. Sections through the DRG of TRPV1-ai9 mice (A–C) were probed for Cre-mediated recombination using a tdTomato antisense probe (red). TdTomato is not expressed in TrkB- and C-neurons (green; A) but does mark most MrgD-expressing neurons (green and red, B); a few MrgD cells not co-labeled by tdTomato are arrowed. A mixed antisense probe to TRPV1, TRPM8 and Mrgs (green, C) labels all cells in which recombination (and tdTomato expression, red) occurs. As expected from (B), there is a subset of cells that hybridize to the pooled probe that do not express tdTomato (arrowed in C). Single labelling for individual classes of Mrg (D) demonstrates that 80–90% of MrgA, MrgB and MrgD-expressing neurons are eliminated in TRPV1-DTA mice.

data confirm previous reports suggesting that TRPM8- and many Mrg-neurons are derived from embryonic cells that express TRPV1 (Hjerling-Leffler *et al*, 2007; Luo *et al*, 2007; Takashima *et al*, 2010). We used Cre-mediated expression of DTA to ablate this entire class of neurons and generated mice that were completely insensitive to hot or cold. In marked contrast, knockout of TRPV1 (Caterina *et al*, 1999) had almost no effect on detection of heat, and TRPM8 knockout

mice (Bautista *et al*, 2007; Colburn *et al*, 2007; Dhaka *et al*, 2007) displayed only loss of cool but not noxious cold sensation. The TRPV1-DTA mutants also lacked responses to several noxious chemicals including components of the ‘inflammatory soup’ and pruritogenic agents. In addition, we show that although TRPV1-DTA mice normally maintain body temperature, they exhibit deficits in thermoregulation both in response to thermal and non-thermal challenges. Finally, our data reveal that DTA-mediated ablation of this large class of cells, including almost all the peptidergic neurons has no effect on mechanosensation.

TRPV1 is expressed in the development of many somatosensory neurons

Previous studies have shown that peptidergic TRPV1-, non-peptidergic Mrg- and the cold-sensitive TRPM8-expressing nociceptors are derived from common precursors whose development is under the control of the growth factor receptor TrkA (Chen *et al*, 2006; Luo *et al*, 2007; Shibasaki *et al*, 2010). It has been postulated that these precursor cells express TRPV1, as recordings from cultured embryonic cells show overlap in responses to TRPV1 and TRPM8 agonists (Hjerling-Leffler *et al*, 2007), and labelling studies indicate that many TRPV1-expressing neuron co-express TRPM8 (Takashima *et al*, 2010). Our data establish that TRPV1 is expressed early in the development of all TRPM8 neurons and a large subset (but not all) of Mrg neurons. However, because TRPV1-knockout mice do not exhibit major differences in the number or distribution of these classes of cell (Caterina *et al*, 2000), TRPV1 itself is not essential for their specification. In contrast to the expression of TrkA in presumptive nociceptors, TrkB and TrkC are believed to define mechanosensitive and proprioceptive neurons (Huang *et al*, 1999). We see no evidence for TRPV1-Cre expression in these neurons and correspondingly TRPV1-DTA animals show no discernible deficits in either mechanosensation or proprioception.

Recently, ablation of MrgD neurons in adult mice, but not throughout development, was reported to affect mechanosensation (Cavanaugh *et al*, 2009). Taken together with our data, these results indicate significant plasticity in the development of the mechanosensory system. Importantly, the restricted phenotype reported for mice lacking MrgD neurons suggests that this class of neurons is likely to have a very minor role in the behavioural deficits that we observe in TRPV1-DTA animals.

Although TRPV1-DTA mice have lost a large subset of nociceptors, including all TRPV1 cells, they retain a small population of neurons that express CGRP as well as neurons that express purinergic receptors (Figure 8). Interestingly, nociceptive responses to ATP injection are completely lost in these animals as are responses to a broader range of algescic mediators known as the ‘inflammatory soup’. We suggest that a subset of the TRPV1-expressing neurons are likely responsible for painful reaction to all these compounds. Moreover, as subcutaneous injection of some of these agents (as well as additional compounds) causes itch and this is also lost in the TRPV1-DTA mutants, it seems likely that a subset of TRPV1 cells produce the puritogenic response. These data extend the recent proposals that TRPV1 and TRPV1-expressing neurons have a role in itch and that there are distinct labelled lines for itch and pain responses (Imamachi *et al*, 2009; Liu *et al*, 2009).

Figure 7 TRPV1-DTA mice show defective thermal homeostasis. Implanted thermal sensors were used to study the core temperature of TRPV1-DTA (□) and wild-type control animals (○). Control animals exhibit pronounced hypothermia following injection of 20 µg of capsaicin (A) or 0.5 µg of mustard oil (B), but as expected, TRPV1-DTA mutant mice are completely unresponsive to these chemicals. Although mutant animals maintain normal body temperature at 25°C (C), they show defective control of temperature homeostasis following exposure to increased (35°C, D) and decreased (4°C, E) environmental temperatures. Non-thermally induced changes in body temperature are also more pronounced in TRPV1-DTAs than in control animals; for example, temperature decline following anaphylaxis (F) and IL1 β -induced fever (G) are increased in the mutant mice. Data represent means $\pm$ s.d.; $n = 5$ –6 animals, significant differences between genotypes (Student's *t*-test) are indicated by ** $P < 0.01$ and * $P < 0.05$.

The receptors and cells for thermosensation

Several different TRP channels are believed to provide animals with a molecular thermometer (Jordt *et al*, 2003; Patapoutian *et al*, 2003) with different TRPs activated over distinct temperature ranges. Of these, the best characterized are TRPV1, inactive below 42°C, (Caterina *et al*, 1997) and TRPM8, inactive above 25°C (McKemy *et al*, 2002; Peier *et al*, 2002). In addition TRPV2, TRPV3, TRPV4 and TRPA1 have been proposed as additional and differentially tuned sensors (Jordt *et al*, 2003; Patapoutian *et al*, 2003; Karashima *et al*, 2009; Knowlton *et al*, 2010). Interestingly, elimination of TRPV1 (Caterina *et al*, 2000), TRPV3 (Moqrich *et al*, 2005), TRPV4 (Lee *et al*, 2005) and TRPA1 (Bautista *et al*, 2006; Kwan *et al*, 2006) have only very modest effects on thermosensation, while TRPM8 knockout animals (Bautista *et al*,

2007; Colburn *et al*, 2007; Dhaka *et al*, 2007) exhibit a reduction in their responses to cool $<20^{\circ}\text{C}$ but not cold $<5^{\circ}\text{C}$. By contrast, animals lacking the cells expressing TRPV1, TRPA1 and TRPM8 have no response to either hot or cold. As pharmacological silencing of the TRPV1 sensory input (Cavanaugh *et al*, 2009), which includes the TRPA1 cells (Story *et al*, 2003), affects hot but not cold responses we conclude that TRPM8 cells carry much of the critical cold sensory information; that is, there are separate hot- and cold-labelled lines.

The mild phenotypes of TRPV1, TRPA1 and TRPM8 knockout animals (Caterina *et al*, 2000; Bautista *et al*, 2006, 2007; Kwan *et al*, 2006; Colburn *et al*, 2007; Dhaka *et al*, 2007) suggest that there might be additional thermosensory receptors including TRPV2, TRPV3 and TRPV4 (Caterina *et al*,

Figure 8 TRPV1-DTA mice lack algescic and pruritogenic chemical responses. Control mice (grey bars) exhibit pronounced and stereotypic pain responses to intraplantar injection of ATP (**A**) or a mix of agents known as the ‘inflammatory soup’ (**B**). In contrast, TRPV1-DTA mice (black bars) do not react to these chemicals (**A**, **B**). *In situ* hybridization of sections through the DRG (**C**) demonstrates that TRPV1-DTA mice have lost the majority (but not all) of the neurons expressing the purinergic receptor P2X3 and the neuropeptide CGRP as well as all cells expressing substance P (SubP). Mutant mice exhibit a decrease in neurogenic inflammation as measured by paw swelling in response to carageenan injection (**D**). In addition, TRPV1-DTA animals are much less sensitive to sub-dermal injection of a variety of compounds that induce itch than normal control mice (**E**). Data are mean $\pm$ s.e.m.; $n = 6$ animals; significant differences between genotypes (Student’s *t*-test) were $P < 0.0001$ for responses to ATP, inflammatory soup and pruritogenic compound and $P < 0.01$ for neurogenic inflammation.

1999; Lee *et al*, 2005; Moqrich *et al*, 2005). Our data demonstrate that sensory input through any such proteins must be missing in TRPV1-DTA animals and because many neurons still express TRPV2 in TRPV1-DTA mice (Figure 2), we suggest that TRPV2 probably has a non-thermosensory role.

Why do the knockouts of individual TRP channels have such a minor effect on behaviour, when our data from TRPV1-DTA animals and recent reports using pharmacological agents (Cavanaugh *et al*, 2009; Mishra and Hoon, 2010) suggest that the cells expressing these channels are crucial for detecting hot and cold? Given the large number of TRP-related and other potential thermosensors (Jordt *et al*, 2003; Patapoutian *et al*, 2003), it is likely that some redundancy accounts for this difference. However, the complete loss of

both hot and cold responses in TRPV1-DTA mutants shows that only a limited subset of somatosensory neurons conveys thermal information.

Peripheral thermosensation and the regulation of body temperature

Capsaicin rapidly induces hypothermia in normal, but not TRPV1-knockout mice showing that activation of TRPV1 affects temperature homeostasis. Thus, it has been suggested that TRPV1-expressing neurons provide the central control circuits in the hypothalamus with information about the temperature of the periphery (Caterina *et al*, 2000). It should be noted that although this hypothesis is attractive and likely substantially correct, it has not yet been formally

possible to rule out peripheral effects in the response to capsaicin; for example, systemic activation of TRPV1 neurons may result in release of neuropeptides that affect body temperature through vasodilation. Regardless of the exact mechanism, recent data showing that TRPV1 antagonists produce hyperthermic responses (Gavva *et al*, 2008) demonstrate that some TRPV1 fibres and TRPV1 channels must be active at normal body temperatures. The TRPV1-DTA mice that we describe in this study significantly extend our knowledge of the role of peripheral thermosensation in control of body temperature. Notably, TRPV1-DTA mutants generally maintain a standard body temperature revealing that additional (presumably central) thermosensors must have the major homeostatic function. However, these mice exhibit exaggerated fluctuations when placed under various types of thermal and non-thermal stress suggesting peripheral sensory neurons may have a role in feedback control (see Figure 7). In contrast, TRPV1-knockout mice were found to have nearly wild-type temperature homeostasis in response to similar challenges (Iida *et al*, 2005). Thus, just as in temperature sensing, our data studying body temperature reveal that TRPV1 expression marks the neurons that are crucial for detecting temperature throughout the periphery and that TRPV1 is likely to only be one of several thermosensors that activate the neurons.

Concluding remarks

In this study, we have engineered mice that help establish the selectivity of TRPV1 expression primarily in a subset of somatosensory neurons. TRPV1 is an early developmental marker of all the hot- and cold-sensing neurons meaning that TRPV1-DTA mice are completely insensitive to their environmental temperature. Nonetheless, these mutant animals exhibit perfectly normal mechanical responses and proprioception, highlighting that sensory neurons are specified to respond to distinct modalities even where their activation ultimately results in the common sensation of pain.

Materials and methods

Animal models

Mice were 20–30 g (2- to 4-month old) TRPV1-cre, Rosa-DTA (Ivanova *et al*, 2005) or Rosa-tdTomato (Madisen *et al*, 2010) and were intercrossed to generate experimental animals as described in the text; the TRPV1-Cre was hemizygous and knock-in backgrounds heterozygous. The TRPV1-IRES-Cre construct was generated by recombination as described previously (Lee *et al*, 2001) using the RP23-181P10 BAC, importantly a region in the last exon was deleted from the transgene that included the last 20 codons and all the predicted 3' UTR (this fragment was used to probe for native transcript by ISH). Inflammation was induced in the plantar surface of a hind paw or the tail by injection of 20 µl of a 2% carageenan solution (Sigma) in PBS. We monitored inflammation in animals using a plethysmometer device (IITC Life Science, USA). In a model of neuropathic pain, a partial nerve ligation was made by tying a tight ligature around the sciatic nerve (Seltzer *et al*, 1990). Core body temperature was measured using intraperitoneal implanted telemetric temperature probes (Data Sciences Inc.). Incisions were made through the skin and probe inserted under the abdominal wall. Experiments were initiated more than 1 week after surgery. Procedures followed the NIH Guidelines for the care and use of laboratory animals, and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee.

Behavioural assays

Chemical sensitivity. Eye-wipe assays were performed to investigate the afferent functions of the ophthalmic branch of the trigeminal nerve. Capsaicin and mustard oil-induced eye wipes were counted for 1 min after delivery of 50 µl of solution (100 µM capsaicin or 10 mM mustard oil in PBS). We chose this concentration of mustard oil because it has been shown to have no effect on TRPA1-KO mice (Bautista *et al*, 2006; Kwan *et al*, 2006; Mishra and Hoon, 2010); that is, it is completely TRPA1 specific and does not activate other receptors including TRPV1. Peripheral responses to capsaicin (1 µg) and mustard oil (10%) injection (10 µl) in the hind paw were recorded as described in (Caterina *et al*, 2000). Wet-dog shakes were induced by i.p. injection of 50 mg/kg icilin (Sigma) and number of whole-body shakes counted over 30 min as described (Dhaka *et al*, 2007).

Thermal responses. We used a semi-automated tail-flick test (IITC Life Science) to measure thermal responses. Animals were habituated for 10 min in individual chambers before experiments. A radiant heat source was focused on the tail, and the time from the initiation of the radiant heat until withdrawal was measured. A maximum cutoff of 20 s was used to prevent tissue damage. Hot/cold-plate test was used to assess acute temperature sensitivity, mice were placed on single hot plate at 55°C or –5°C and latency to display withdrawal of hind limbs was measured. Cutoffs were set at 30 s (55°C) and 1 min (–5°C) to prevent tissue damage. Two choice temperature preference/aversion assays were employed to determine thermal thresholds; mice were placed in an apparatus that had plates at 30°C and a variable test temperature (T2CT, Bioseb, France). Assays were initiated by placement at the test temperature; each animal was tested twice wherein the order of initial placement was reversed. Mouse position was tracked over 5 min; to ensure data were collected from trials in which animals were exposed to both test temperature and 30°C, only assays in which mice sampled both temperatures in the first minute were counted.

Mechanical responses. Threshold-force-induced paw withdrawal was measured using an automated von Frey apparatus (Ugo Basile Varese, Italy); in addition, a modified Randall–Selitto device (IITC Life Sciences) was used to automatically measure responses when pressure was applied to the tail.

Sciatic nerve recordings. Mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). A deep level of anaesthesia was maintained throughout the experiment using additional sodium pentobarbital, and body temperature was maintained between 35 and 37°C. Briefly, the common peroneal branch of the sciatic nerve was accessed through an incision in the lateral side of the left thigh. To expose the nerve, the biceps femoris was cut. The nerve was then dissected free from surrounding tissue, cut near the branch point from the rest of the sciatic nerve and placed on a (2–4 MEG) tungsten microelectrode (Frederick Haer & Co). A reference electrode was placed in nearby muscle tissue. The incision cavity was then filled with halocarbon oil (Sigma) to slow fibre desiccation. Digitization of nerve responses was performed using Clampex 10.2 of the pClamp software package (Molecular Devices, Sunnyvale, CA), running on an IBM-compatible computer. All responses were amplified using a Grass P511AC amplifier (Grass Technologies, West Warwick, RI) and digitized with Digidata 1322A (Molecular Devices). Data were analyzed using Matlab 7 (Mathworks, Lyme, NH). Three types of stimuli were used to describe mechanical touch sensation on the dorsal side of the foot: brush, 60 Hz vibration and von Frey filaments (4 and 6 g). Animals were tested with individual stimuli three times and these tests were repeated three times, response maxima (versus baseline) were calculated and normalized against the average response from a 6 g von Frey stimuli. Assessment of proprioceptive and motor performance was performed on an accelerating rotarod (4–40 r.p.m. in 5 min; Med Assoc. Inc.). Hypothermia was induced by i.p. injection of 20 µg of capsaicin or 0.5 µg of mustard oil (in 20 µl of saline). The dose of mustard oil in this experiment is ~100 lower than that shown to be selective for TRPA1 (see above eye-wipe assays). Initially, following IP injection the mice are quite different, capsaicin treatment leads to a short period of intense agitation and nociferous behaviour directed to the site of injection, mustard oil injected mice in contrast show much less reaction (possibly because

such a low dose was used). During the period when mice are hypothermic both treatments result in reduced movement and a curling-up behaviour that are indistinguishable. This is consistent with mustard oil activating a different receptor and cell type than capsaicin.

Anaphylaxis. Mice were sensitized by i.v. injection of 20 µg mouse mAb IgE anti-dinitrophenol (DNP) and 20 h later, anaphylaxis was induced by i.v. injection of 1 mg DNP-coupled human serum albumin (Sigma). Temperature was monitored for up to 2 h after which mice were euthanized.

Fever. Recombinant IL1β (R & D Systems) was administered by i.v. injection (100 ng).

Scratching behaviour. Hair was removed from the nape of the neck, and pruritogens administered by 10 µl subcutaneous injection (500 nMol histamine trifluoromethyl toluidide (HTMT; Tocris), 100 µg SLIGRL-NH₂ (Tocris), 30 µg Methyl-serotonin (Me-5HT; Sigma)) and PBS was used as a control. Bouts of scratching were counted over a 30-min observation period.

Algesic substances. Numbers of flinches, guarding and licks were monitored over 10 min after intraplantar injection of 10 µl of ATP (500 nmol), or inflammatory soup (serotonin, histamine, PGE₂ and bradykinin; 1 µmol each). PBS was used as a control and vehicle.

In situ hybridization

ISH was performed at high stringency (washed 30 min, 0.2 × SSC, 70°C) as described previously (Hoon *et al*, 1999). ISH of molecular markers was performed on tissue from >10 TRPV1-DTA and control mice. Serial sections from >10 sections per mouse were hybridized and numbers of cells counted in order to quantize ablation of neurons.

Fos expression. Mice were anaesthetized with avertin (2.5% 20 ml/kg) before immersion of hind paw in water at 55°C, three times for 30 s each with a 1 min interstimulus interval; 30 min after thermal challenge, mice were euthanized and L4/5 spinal cords harvested. ISH was performed with fos riboprobe on tissue taken from three control and TRPV1-DTA animals; counts of fos-positive stained cells were made on 10 sections per animal.

For double-label ISH, we used fluorescein- and digoxigenin-labelled probes that were detected with antibodies coupled to horseradish peroxidase and alkaline phosphatase, respectively, together with tyramide-FITC and fast-red (Adler *et al*, 2000). Images were collected using a Microphot FX microscope (Nikon) and confocal microscopy (1 µm optical sections) was with a Leica TCS SP2 (Leica Microsystems) and images were processed with Adobe Photoshop.

Gene array analysis

Total RNA was isolated from DRG using an RNeasy minikit (Qiagen), cDNA was produced, and amplified cRNA prepared as described by the manufacturer (one-step probe synthesis kit; Agilent). Gene arrays (GE 4 X 44K V2; Agilent) were hybridized, washed and scanned as recommended by the manufacturer. Data were analyzed using Gene Spring (version 11) software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author Contributions: SKM carried out and analyzed the majority of the experiments, SMT and PO contributed data for Figures 8 and 4 respectively, SKB data from nerve ligation studies and MAH designed the study, generated and analyzed data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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