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Valentine Bouvier[1], Yann Roudaut[1], Nancy Osorio[1], Jean-Marc Aimonetti[2], Edith Ribot-Ciscar[2], Virginie Penalba[1], Thierry Merrot[3], Nicolas Lebonvallet[4], Christelle Le Gall-Ianotto[4], Laurent Misery[4], Patrick Delmas[1] and Marcel Crest[1]

In the skin, Merkel cells connect with keratinocytes and Aβ nerve fibers to form a touch receptor that functions as a slow adapting mechanoreceptor (slow adapting type 1). In human and mouse Merkel cells, we observed an increased concentration of intracellular Ca^{2+} ions in response to cold temperature and transient receptor potential melastatine 8 (TRPM8) ion channel agonists. A reduction in the response to cooling and TRPM8 agonists occurred after the addition of TRPM8 antagonists, as well as in TRPM8 knockout mice. Cold temperature and TRPM8 agonists also induced a current that was inhibited by a TRPM8 antagonist. Our results indicate that Merkel cells sense cooling through TRPM8 channels. We hypothesized that cooling modulates the slow adapting type 1 receptor response. Cooling mouse skin to 22°C reduced the slow adapting type 1 receptor discharge frequency. Interestingly, we observed no such reduction in TRPM8 knockout mice. Similarly, in human skin, a temperature of 22°C applied to the slow adapting type 1 receptive field reduced the spiking discharge. Altogether, our results indicate that Merkel cells are polymodal sensory cells that respond to mild cold stimuli through the activation of TRPM8 channels. Thermal activation of Merkel cells, and possibly other TRPM8-expressing non-neuronal cells, such as keratinocytes, potentially adapts the discharge of slow adapting type 1 receptors during cooling.

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INTRODUCTION

Merkel complexes are slowly adapting mechanoreceptors (slow adapting type 1 [SA1]) with narrow, well-defined receptive fields and low mechanical threshold (Vallbo et al., 1995). They participate in tactile discrimination and recognition of textured surfaces (Maricich et al., 2009, 2012). Merkel complexes are composed of 5−50 Merkel cells (MCs) associated with keratinocytes and sensory Aβ nerve fiber endings. During touch, the response of SA1 receptors involves a dynamic spike discharge at the onset of pressure application, followed by a static discharge during the plateau phase of the stimulus. MCs mainly signal static mechanical stimuli, such as maintained pressure, whereas sensory Aβ nerve fiber endings transduce the dynamic phase of stimuli (Ikeda et al., 2014; Maksimovic et al., 2014; Ranade et al., 2014; Woo et al., 2014). However, factors that modify Merkel receptor activity remain poorly studied, particularly the role of temperature.

Thermal transduction is attributed to thermo-transient receptor potential channels located at the free endings of sensory Aδ and C fibers (Li et al., 2011). The contribution of accessory cells, such as keratinocytes, has been postulated due to their response to both warm (Mandadi et al., 2009) and fresh (Tsutsumi et al., 2010) stimuli, as well as harboring functional transient receptor vanilloid type 3 and transient receptor potential melastatine 8 (TRPM8) ions channels (Denda et al., 2010; Moqrich et al., 2005; Peier et al., 2002). However, to our knowledge, the contribution of MCs and keratinocytes to thermal regulation of touch has not been investigated. Previous studies using cat, monkey, and rat models revealed that SA1 receptors are sensitive to cooling (Duclaux and Kenshalo, 1972; Hensel and Zotterman, 1951; Hunt and McIntyre, 1960; Iggo and Muir, 1969; Tapper, 1965). These experiments focused on the effects of cooling on the static phase of SA1 discharge evoked by a constant pressure applied to the skin. It was shown that skin cooling has a biphasic effect on SA1 static discharge; an initial brief increase of firing in response to a drop in temperature, followed by a tonic inhibition once the temperature had stabilized. This observation led Iggo (1969) to dub Merkel complexes as "spurious thermoreceptors." The role of MCs in the thermal regulation of SA1 response, including the molecular mechanisms involved, remains unclear.

We therefore investigated the response to cooling in both mouse and human MCs, demonstrating the activation of TRPM8 channels in MCs. Constitutive deletion of TRPM8

[1]*Aix Marseille Université, Centre National de la Recherche Scientifique, Centre de Recherche en Neurobiologie-Neurophysiologie de Marseille, UMR 7286, Marseille, France;* [2]*Aix-Marseille Université, Centre National de la Recherche Scientifique, Laboratoire de Neurosciences Intégratives et Adaptatives, UMR 7260, Marseille, France;* [3]*Aix Marseille Université, Assistance Publique-Hôpitaux de Marseille, Hôpital Nord, Service de Chirurgie Infantile, Marseille, France; and* [4]*Université de Brest, Laboratoire de Neurosciences, Brest, France*

Correspondence: Marcel Crest, Aix Marseille Université, Centre National de la Recherche Scientifique, Centre de Recherche en Neurobiologie-Neurophysiologie de Marseille, UMR 7286, Faculté de Médecine Campus Nord, 51 Bd Pierre Dramard, CS80011, 13344, Marseille, France. E-mail: marcel.crest@univ-amu.fr

Abbreviations: $[Ca^{2+}]i$, intracellular calcium ion concentration; KO M8, transient receptor potential melastatine 8 knockout; MC, Merkel cell; SA1, slow adapting type 1; TRPM8, transient receptor potential melastatine 8; WT, wild-type

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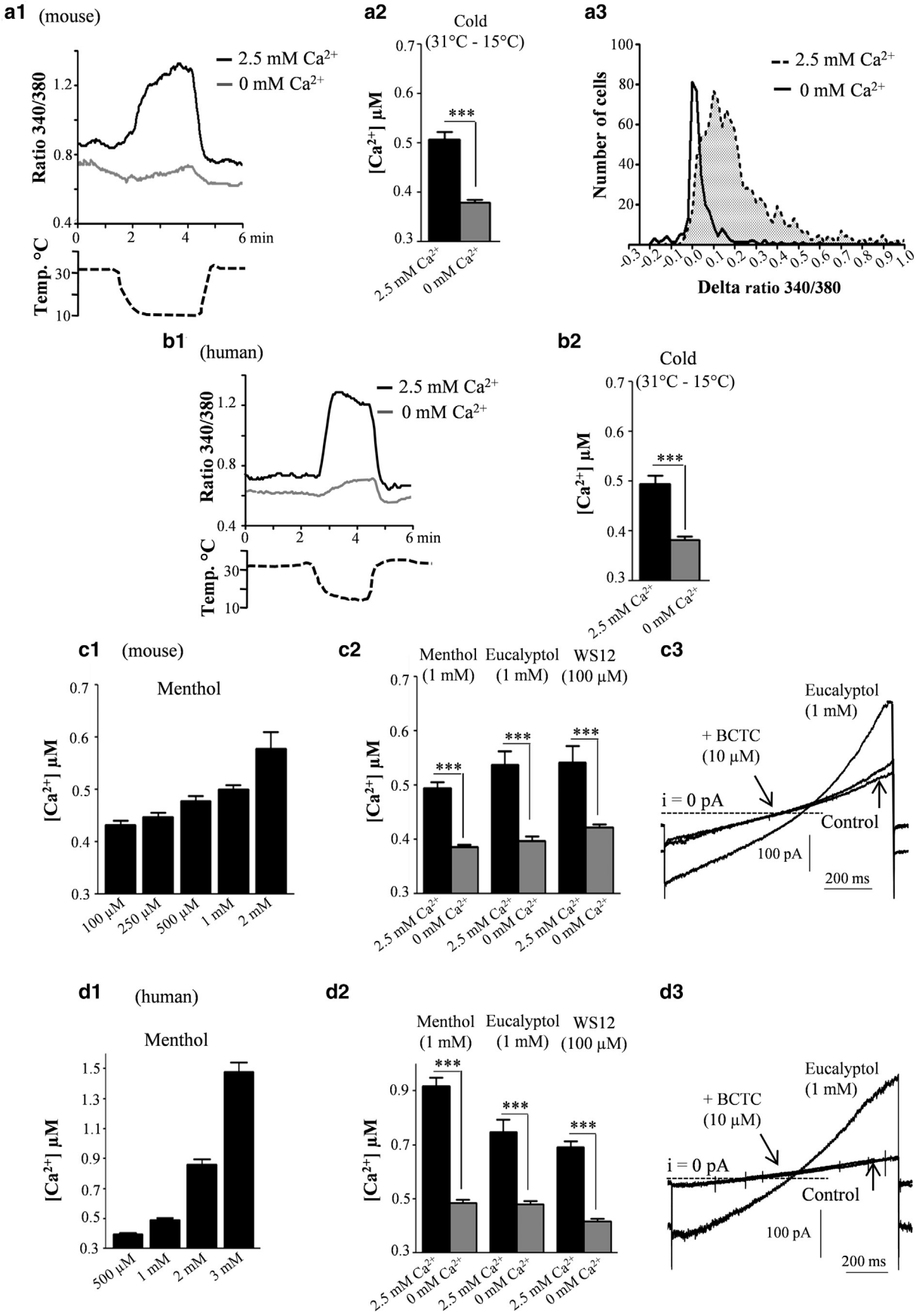

Figure 1. Merkel cells (MCs) sense cooling. (**a**) Mouse MCs sense cooling: (**a1**) Representative intracellular calcium ions concentration ($[Ca^{2+}]i$) increase due to a reduction in temperature from 31°C to 15°C in mouse cultured MCs in the presence (2.5 mM Ca^{2+}) or absence of extracellular Ca^{2+} ions (0 mM Ca^{2+}). (**a2**) Histogram

channels in mice appeared to reduce the effect of cold temperature on SA1 discharge. This suggests that TRPM8, possibly expressed in MCs and/or keratinocytes, regulates touch sensitivity of Merkel receptor complexes during cooling.

RESULTS

Identification of MCs

CK20 labeling of epidermal cell cultures from Atoh1$^{GFP/GFP}$ mice identified MCs, which represented 10% of cells (Supplementary Figure S1a online). Human MCs were selected by CD56 expression (Boulais et al., 2009). Among CD56 selected cells (CD56$^+$ cells), >90% were labeled with CK20, corresponding to MCs (Supplementary Figure S1b). Conversely, CD56$^-$ cells represented other epidermal cells, mainly keratinocytes.

TRPM8 mRNA is expressed in human MCs

We compared TRPM8 mRNA expression levels between human MCs (CD56$^+$ cells) and keratinocytes (CD56$^-$ cells) using real-time quantitative PCR. The migration of PCR products showed the amplicons of TRPM8 and GAPDH at the expected sizes of 119 bp and 140 bp, respectively. Relative mRNA expression appeared similar in CD56$^+$ cells (7.6 ± 0.61 units) and in CD56$^-$ cells (8.4 ± 0.95 units) (Supplementary Figure S1c).

MCs in culture responded to cooling

By decreasing the bath solution temperature, we examined the response to cooling of cultured MCs using calcium imaging. Decreasing the temperature from 31° to 15°C increased the concentration of intracellular calcium ions ([Ca^{2+}]i) in both mouse and human MCs (to 0.51 ± 0.09 μM, n = 1,031, and 0.49 ± 0.11 μM, n = 163, from a basal concentration of 0.30 μM, respectively) (Figure 1a, 1b). The absence of extracellular Ca^{2+} ions consistently reduced cooling responses in mouse (0.39 ± 0.05 μM) and human (0.38 ± 0.05 μM) MCs. In mice, fluorescence increases >0.1 unit appear to correlate with the entry of extracellular Ca^{2+} ions through ion channels expressed at the cell surface, as shown by the distribution of cooling responses in Figure 1a3.

TRPM8, not TRPA1, contribute to the cooling response in MCs

Two TRP channels involved in cold detection have been described previously: TRPM8 and TRPA1. Therefore, we investigated the presence of TRPM8 in cultured MCs using specific agonists. Application of menthol (from 1 mM), eucalyptol (1 mM), and (1R,2S,5R)-N-[(4-methoxyphenyl)-5-methyl-2-(1-methylethyl) cyclohexane-carboxamide] (WS12, 100 μM) all increased [Ca^{2+}]i in both mouse (Figure 1c) and human MCs (Figure 1d). The absence of extracellular Ca^{2+} ions greatly reduced the agonist-induced [Ca^{2+}]i increases (Figure 1c2, 1d2). The TRPM8 antagonist 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC, 10 μM) reduced cooling responses by 56% ± 7.2% and 72% ± 2.3% in mouse and human MCs, respectively (Figure 2a1, 2a2). Another TRPM8 antagonist, hydrochloride N-(2-aminoethyl)-N-[[3-methoxy-4-(phenylmethoxy)-phenyl]methyl]-2-thiophenecarboxamide hydrochloride (M8B, 1 μM), also reduced the cooling response in mouse MCs by 53% ± 5.1% (Figure 2a1). In patch-clamp experiments, eucalyptol (1 mM) generated a current that was inhibited by 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl) phenyl]-1-piperazinecarboxamide (BCTC, 10 μM) in both mouse and human MCs (Figure 1c3, 1d3). 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC) also abolished the current observed at 22°C in mouse (Figure 2b1) and human MCs (Figure 2b2), yet we observed no effect on currents recorded at 30°C.

Next, we investigated the cooling response in TRPM8 knockout mice (KO M8). As expected, we observed a smaller [Ca^{2+}]i increase in response to menthol and eucalyptol in cultured MCs from KO M8 mice compared to wild-type (WT) mice (Figure 3a2). Interestingly, KO M8 cells also showed a 67% reduction in response to cooling compared to WT cells (n = 972) (Figure 3a1). Figure 3a3 shows the distribution of response amplitudes, demonstrating a shift to lower values for KO M8 cultured MCs compared to WT. Moreover, the cooling-induced increase of [Ca^{2+}]i in KO M8 with or without extracellular Ca^{2+} ions does not differ significantly (Figure 3b1).

Therefore, we next investigated the possible contribution of TRPA1 in the cooling response of cultured MCs. The specific TRPA1 agonist, cinnamaldehyde (1 mM), induced only small responses in cultured MCs from both WT and TRPA1 knockout mice (Figure 3c), as well as human MCs (not illustrated), suggesting the absence of TRPA1 channels in cultured MCs. In addition, cultured MCs from TRPA1 knockout mice showed a similar cooling response to WT

showing the mean cooling-induced [Ca^{2+}]i variations in mouse cultured MCs in the presence (2.5 mM Ca^{2+}, n = 1,031 cells) or absence of extracellular Ca^{2+} ions (0 mM Ca^{2+}, n = 308 cells). (**a3**) Distribution of Δ[Ca^{2+}]i variations in the presence or absence of extracellular Ca^{2+} ions. (**b**) Human MCs sense cooling: (**b1**) Representative [Ca^{2+}]i increase due to a reduction in temperature from 31°C to 15°C in human cultured MCs in the presence (2.5 mM Ca^{2+}) or absence of extracellular Ca^{2+} ions (0 mM Ca^{2+}). (**b2**) Histogram showing the mean cooling-induced [Ca^{2+}]i variations in human cultured MCs in the presence (2.5 mM Ca^{2+}, n = 163 cells) or absence of extracellular Ca^{2+} ions (0 mM Ca^{2+}, n = 91 cells). (**c**) Mouse MCs are activated by transient receptor potential melastatine 8 (TRPM8) agonists: (**c1**) Mean [Ca^{2+}]i variations in response to increasing concentrations of menthol (from 100 μM to 2 mM, n = 45, 30, 48, 556, and 39 cells, respectively); (**c2**) mean [Ca^{2+}]i variations in response to 1 mM menthol in the presence (2.5 mM Ca^{2+}, n = 556 cells) or absence (0 mM Ca^{2+}, n = 139 cells) of extracellular Ca^{2+} ions. Same experiments with eucalyptol (n = 334 and 57 cells, respectively) and (1R,2S,5R)-N-[(4-methoxyphenyl)-5-methyl-2-(1-methylethyl) cyclohexane-carboxamide] (WS12) (n = 80 cells and 46 cells, respectively); (**c3**) Currents induced in the presence of 1 mM of eucalyptol (reversal potential: 7.6 mV) and by eucalyptol + 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC) (10 μM). Currents were induced by a voltage ramp from −60 mV to + 60 mV (0.12 mV/ms) from a holding potential of −50 mV. (**d**) Human MCs are activated by TRPM8 agonists: (**d1**) Mean [Ca^{2+}]i variations in response to increasing concentrations of menthol (from 500 μM to 3 mM, n = 41, 71, 55, 41 cells, respectively); (**d2**) Mean [Ca^{2+}]i variations in response to menthol in the presence (2.5 mM Ca^{2+}, n = 55 cells) or absence (0 mM Ca^{2+}, n = 39 cells) of extracellular Ca^{2+} ions. Same experiments with eucalyptol (n = 70 and 33 cells, respectively) and WS-12 (n = 45 and 43 cells, respectively). (**d3**) Currents induced by 1 mM of eucalyptol (reversal potential: 1.7 mV) and by eucalyptol + BCTC (10 μM). Currents were induced by a voltage ramp from −60 mV to +60 mV (0.12 mV/ms) from a holding potential of −50 mV.

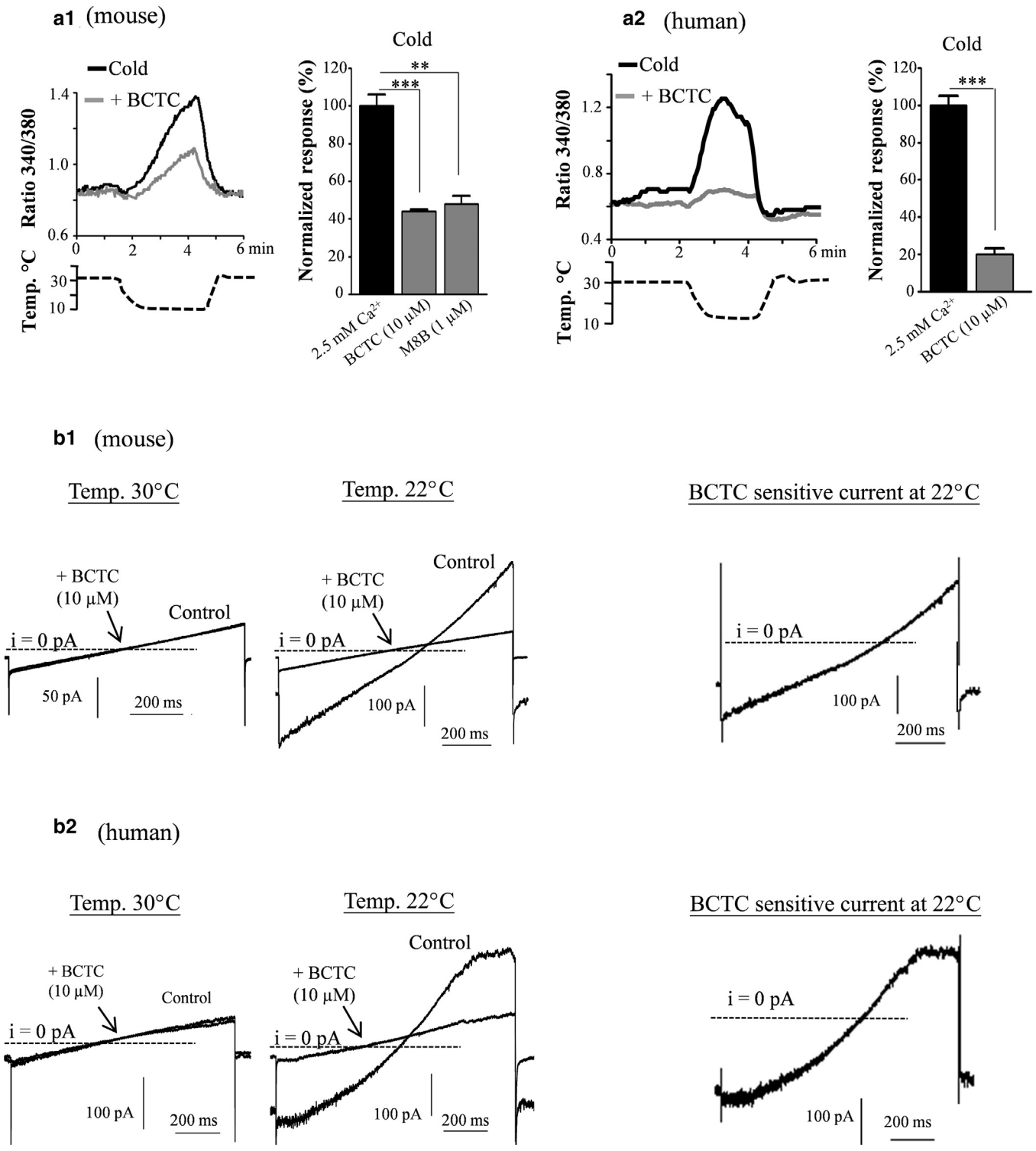

Figure 2. Cooling responses in mouse and human cultured Merkel cells (MCs) are blocked by transient receptor potential melastatine 8 (TRPM8) antagonists. (**a**) Cooling-induced $[Ca^{2+}]i$ increase is blocked by TRPM8 antagonists: (**a1**) Mouse MCs, Left: representative reduction by 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC) (10 µM) of the cooling-induced $[Ca^{2+}]i$ increase (from 31°C to 15°C). Right: histogram of the mean inhibition by BCTC (10 µM, n = 313 and 206 cells) and hydrochloride N-(2-aminoethyl)-N-[[3-methoxy-4-(phenylmethoxy)-phenyl]methyl]-2-thiophenecarboxamide hydrochloride (M8B, 1 µM, n = 313 and 92 cells) of the $[Ca^{2+}]i$ variations; (**a2**) Human MCs. Left: representative reduction by BCTC (10 µM) of the cooling-induced $[Ca^{2+}]i$ increase (from 31°C to 15°C). Right: histogram of the mean inhibition by BCTC (10 µM, n = 43 and 43 cells) of the $[Ca^{2+}]i$ variations. (**b**) Cooling-induced current is blocked by TRPM8 antagonist: (**b1**) Mouse MCs. Left: Current at 30°C (reversal potential: −7.3 mV) in control conditions and in the presence of BCTC (10 µM). Middle: The current at 22°C (reversal potential 1.3 mV) is partly blocked by BCTC (10 µM). Right: Part of the current induced at 22°C and blocked by BCTC (reversal potential 3.3 mV). (**b2**) Same experiments performed in human Merkel cells. Reversal potential of the current induced at 22°C and blocked by BCTC: 3.7 mV.

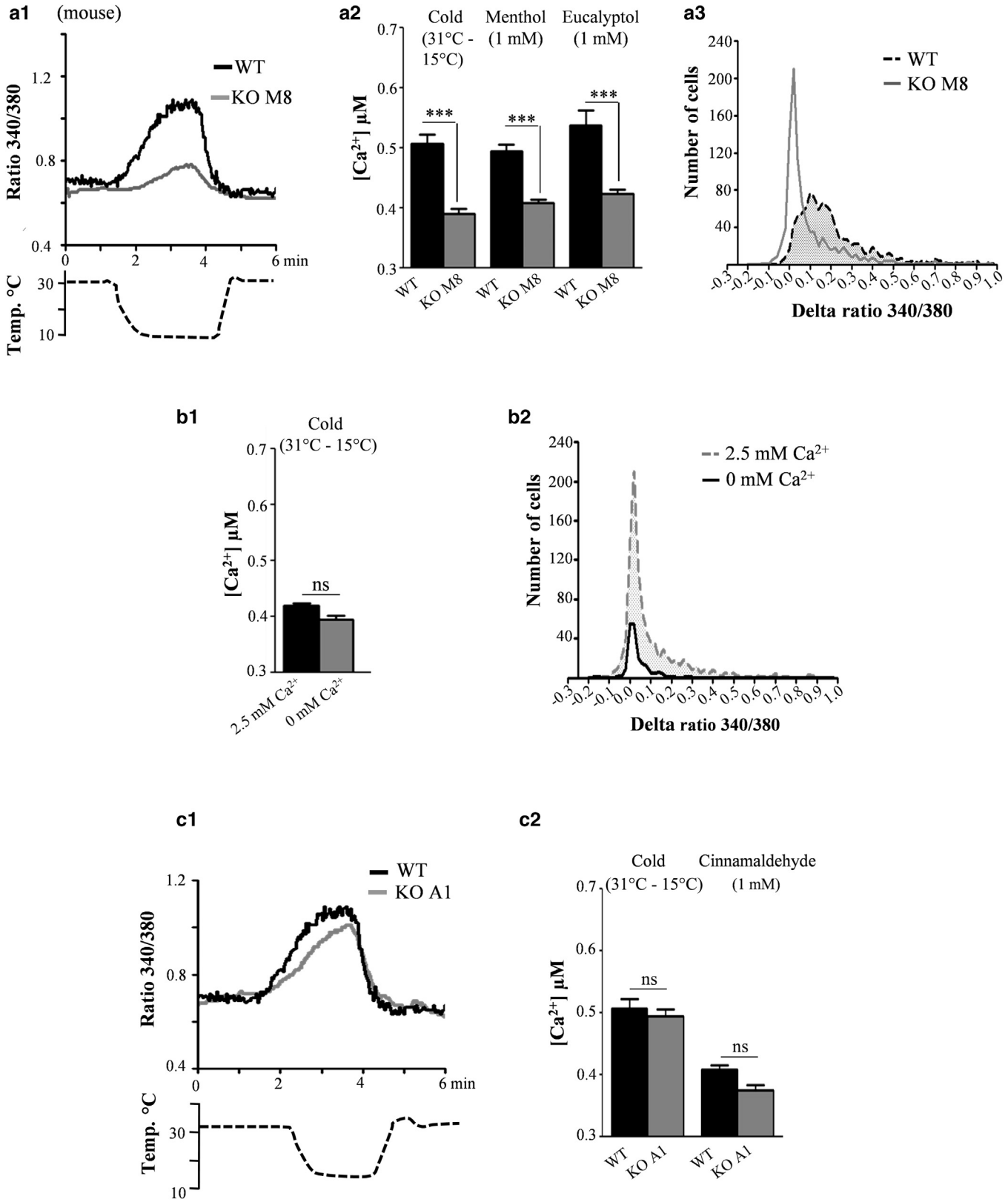

Figure 3. Transient receptor potential melastatine 8 (TRPM8) channels contribute to cooling responses in mouse Merkel cells (MCs). (**a**) Wild-type (WT) and TRPM8 knockout (KO M8) mouse MCs: (**a1**) Representative reduction in KO M8 mouse MCs of the $[Ca^{2+}]i$ increase due to a reduction in temperature from 31°C to 15°C. (**a2**) Histogram of the mean response to cooling in WT mice (n = 1031 cells) and KO M8 mice (n = 972 cells), to menthol (1 mM, n = 556 and 939 cells) and to eucalyptol (1 mM, n = 334 and 727 cells). (**a3**) Distribution of responses to cooling in both WT and KO M8 mice. (**b**) KO M8 mouse MCs: (**b1**) Ca^{2+} variations in KO M8 mice in the presence (2.5 mM Ca^{2+}, n = 972 cells) or absence (0 mM Ca^{2+}, n = 202 cells) of Ca^{2+} ions in the extracellular saline. (**b2**) Distribution of responses to cooling in KO M8 mice. (**c**) TRPA1 channels do not contribute to the cooling response in mouse MCs: (**c1**) Representative response in TRPA1 knockout (KO A1) mouse of the $[Ca^{2+}]i$ increase due to a reduction in temperature from 31°C to 15°C. (**c2**) Ca^{2+} variations in response to cooling in WT mice (n = 1031 cells) and KO A1 mice (n = 396 cells) and after application of cinnamaldehyde in WT mice (n = 50 cells) and KO A1 (n = 42 cells).

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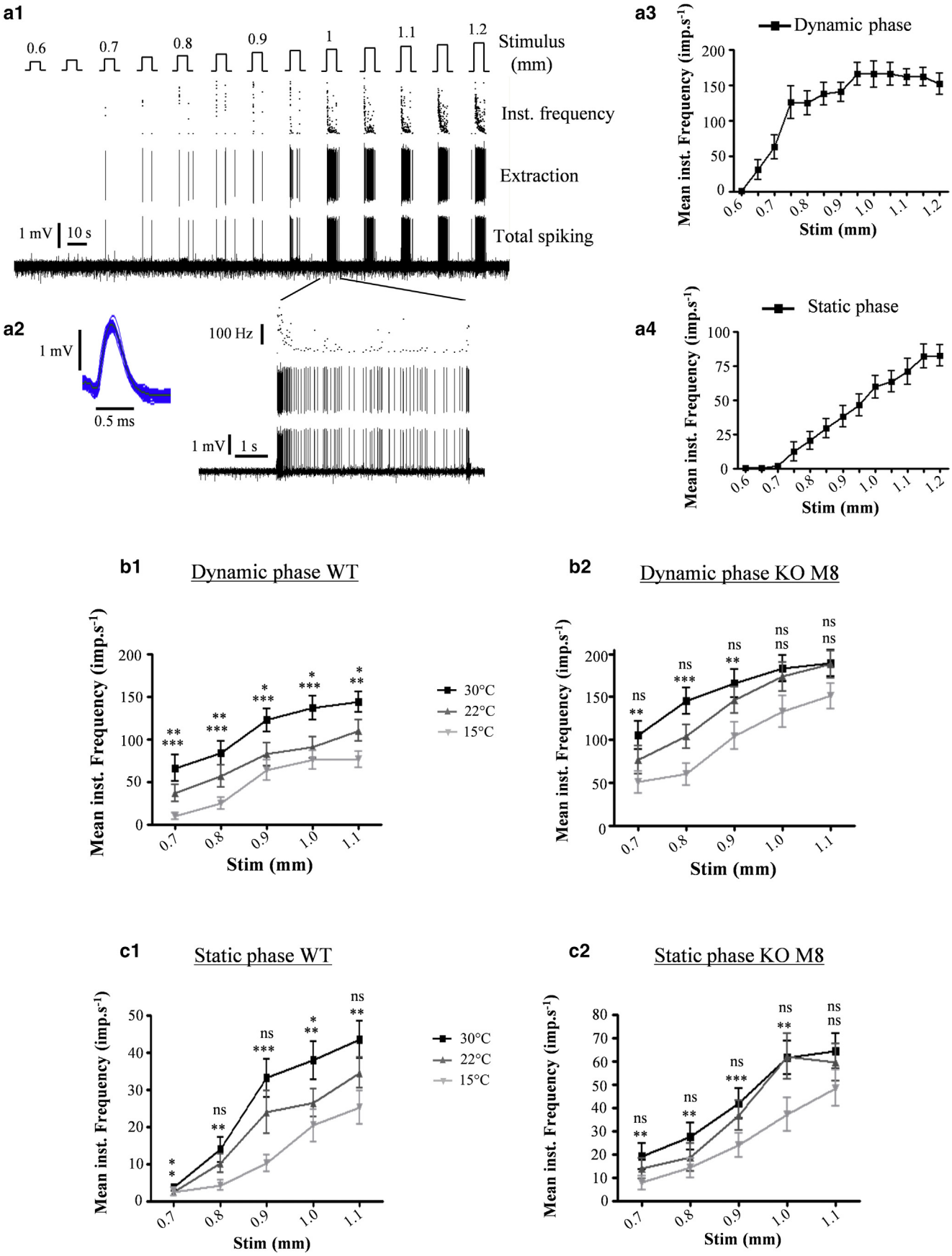

Figure 4. Comparison of the cooling effects on slow adapting type 1 (SA1) discharge between wild-type (WT) and transient receptor potential melastatine 8 knockout (KO M8) mice. (**a**) Characterization of the mechanical response of SA1 receptors in skin nerve preparation: (**a1**) Representative spiking induced by successive mechanical

MCs (TRPA1 knockout: 0.49 ± 0.07 μM; WT: 0.51 ± 0.09 μM, Figure 3c).

Overall, our results demonstrate the contribution of TRPM8, not TRPA1, to the cooling response in MCs.

Cooling reduced the SA1 discharge in WT mice rather than KO M8 mice

To evaluate how cooling affects the mechanosensitive role of SA1 receptors, we performed isolated skin-nerve recordings. The skin-nerve technique measures the activity of SA1 mechanoreceptors in the saphenous nerve upon mechanical stimulation of the corresponding receptive field in the skin, while maintaining the temperature of the skin at 30°C, 22°C, or 15°C. To characterize the SA1 discharge in WT mice, we used 13 successive stimuli, each lasting 5 seconds, with increasing depth amplitude from 0.6 mm to 1.2 mm (steps of 50 μm). We defined the dynamic phase as the nerve fiber response during the first 200 milliseconds and the static phase as the nerve fiber response during the following 4.8 seconds. Figure 4a1 and 4a2 show a typical nerve fiber discharge considered as SA1 discharge. Figure 4a3 and 4a4 show the mean instantaneous frequency of dynamic and static phases for 24 SA1 receptors recorded at 30°C. Based on these results, we selected five probe displacements from 0.7 mm to 1.1 mm (steps of 0.1 mm) and determined the mean instantaneous frequencies of dynamic and static phases at 30°C, 22°C, and 15°C.

In WT mice, cooling at 22°C and 15°C significantly reduced the mean instantaneous frequency of the dynamic phase (n = 31; Figure 4b1). Cooling the receptive fields to 22°C also decreased the mean instantaneous frequency of the static phase, although this was only significant for 0.7 mm and 1 mm stimuli (Figure 4c1). At 15°C the mean instantaneous frequencies of the static phase was reduced for all stimuli (Figure 4c1).

We repeated these experiments in KO M8 mice and observed no significant differences in the mean instantaneous frequencies between 22°C and 30°C in either dynamic or static phase for all mechanical stimuli (n = 33; Figure 4b2, 4c2). However, at 15°C, the mean instantaneous frequency was significantly reduced during mechanical stimuli (Figure 4b2, 4c2).

We compared mean instantaneous frequencies at 30°C, 22°C, and 15°C between WT and KO M8 mice. KO M8 mice exhibited higher frequencies in the dynamic and static phases compared to WT at all 3 temperatures, although this effect was only statistically significant at 22°C (Figure 5a).

To discriminate between the effects of TRPM8 deletion versus cold temperature on nerve conduction, we combined mechanical and electrical stimuli of the SA1 receptor. Figure 5b1 shows the spikes induced successively by mechanical and electrical stimulation at the same point inside the receptive field of an SA1 receptor in a WT mouse. We confirmed the recording of an Aβ nerve fiber as the conduction velocity was >15 milliseconds^{-1}. Using electrical stimuli, we determined the relative spike thresholds and ability to sustain a high-frequency discharge when the temperature inside the receptive field decreased from 30°C, to 22°C and 15°C (n = 7, Figure 5c). Outside the receptive field, the temperature remained at 30°C (see Supplementary Figure S2 online).

A temperature reduction to 15°C increased the spike threshold in WT as well as KO M8 mice (Figure 5b2, 5b3). Applying successive stimuli at 10, 20, 30, and 40 Hz over 1 second slightly increased the number of failures at 15°C in WT and KO M8 mice (Figure 5c). Interestingly, a temperature of 22°C had no effect in either WT or KO M8 mice, indicating that cooling the skin to 22°C has no effect on nerve conduction of an Aβ nerve fiber. Moreover, we failed to observe Aβ nerve fiber spiking induced by cold temperature in the absence of mechanical stimulus, demonstrating a lack of thermosensitivity of the nerve terminal itself.

We therefore conclude that the reduction in the SA1 response to mechanical stimuli at 22°C is possibly the consequence of activating TRPM8 channels present in the receptive field.

Moderate cooling of human skin to 22°C reduced the SA1 discharge

Finally, we performed microneurographic experiments in three healthy volunteers to determine the effects of moderate skin cooling on SA1 receptor discharge. Figure 6a shows a recording of an SA1 discharge during a plateau pressure of 5 seconds. The mechanical stimuli contained a dynamic phase of 1 second and static phase of 4 seconds. At 30°C, we recorded mean instantaneous frequencies in dynamic and static phases of 29.3 Hz and 14.8 Hz, respectively. In the dynamic phase, cooling to 22°C and 15°C reduced the frequency by 24% and 56%, respectively. In the static phase, 22°C reduced the frequency by 23% and 15°C reduced it by 53%. Because a temperature of 22°C particularly affected the dynamic phase in WT mice, we used sinusoidal stimuli because the rising phase of sinusoid corresponds to a dynamic stimulus and spiking is concentrated during the dynamic phase. At 30°C, the SA1 receptor mostly responded with spike triplets for each sinusoid, however, the response dropped to doublets and single spikes per cycle at 22°C and 15°C, respectively (Figure 6b). The mean instantaneous frequency at 30°C (4.1 Hz) reduced by 38% at 22°C and by 77% at 15°C. We

stimuli of the skin inside a SA1 receptor field. Displacement of the stimulus probe from 0.6 to 1.2 mm, increment of 0.05 mm, duration 5 seconds. (**a2**) Left: superimposition of spikes during a 5-second stimulus. Right: Detail of the spiking discharge during the stimulus of 1 mm. Note the typical response of SA1 receptor, including an initial dynamic phase with fast spiking and a static phase with irregular spiking. The duration of dynamic and static phases were fixed to 0.2 s and 4.8 seconds, respectively. (**a3**) Mean instantaneous frequency of the dynamic phase collected from 24 SA1 receptors. (**a4**) Mean instantaneous frequency of the static phase collected from 24 SA1 receptors. (**b**) Cooling effects on the dynamic phase of the SA1 discharge in WT and KO M8 mice: (**b1**) Mean instantaneous frequency of the dynamic phases obtained in WT mice during 5 successive mechanical stimuli of increasing amplitude (from 0.7 to 1.1 mm, increments of 0.1 mm) applied at 30°C, 22°C and 15°C (n = 32 SA1 receptors). Statistical: upper symbol: 30°C versus 22°C, lower symbol: 30°C versus 15°C. (**b2**) Same protocol in KO M8 mice (n = 33 SA1 receptors). (**c**) Cooling effects on the static phase of the SA1 discharge in WT and KO M8 mice: (**c1**) Mean instantaneous frequency of the static phase obtained in WT mice at 30°C, 22°C and 15°C (n = 32 SA1 receptors). (**c2**) Same protocol in KO M8 mice (n = 33 SA1 receptors).

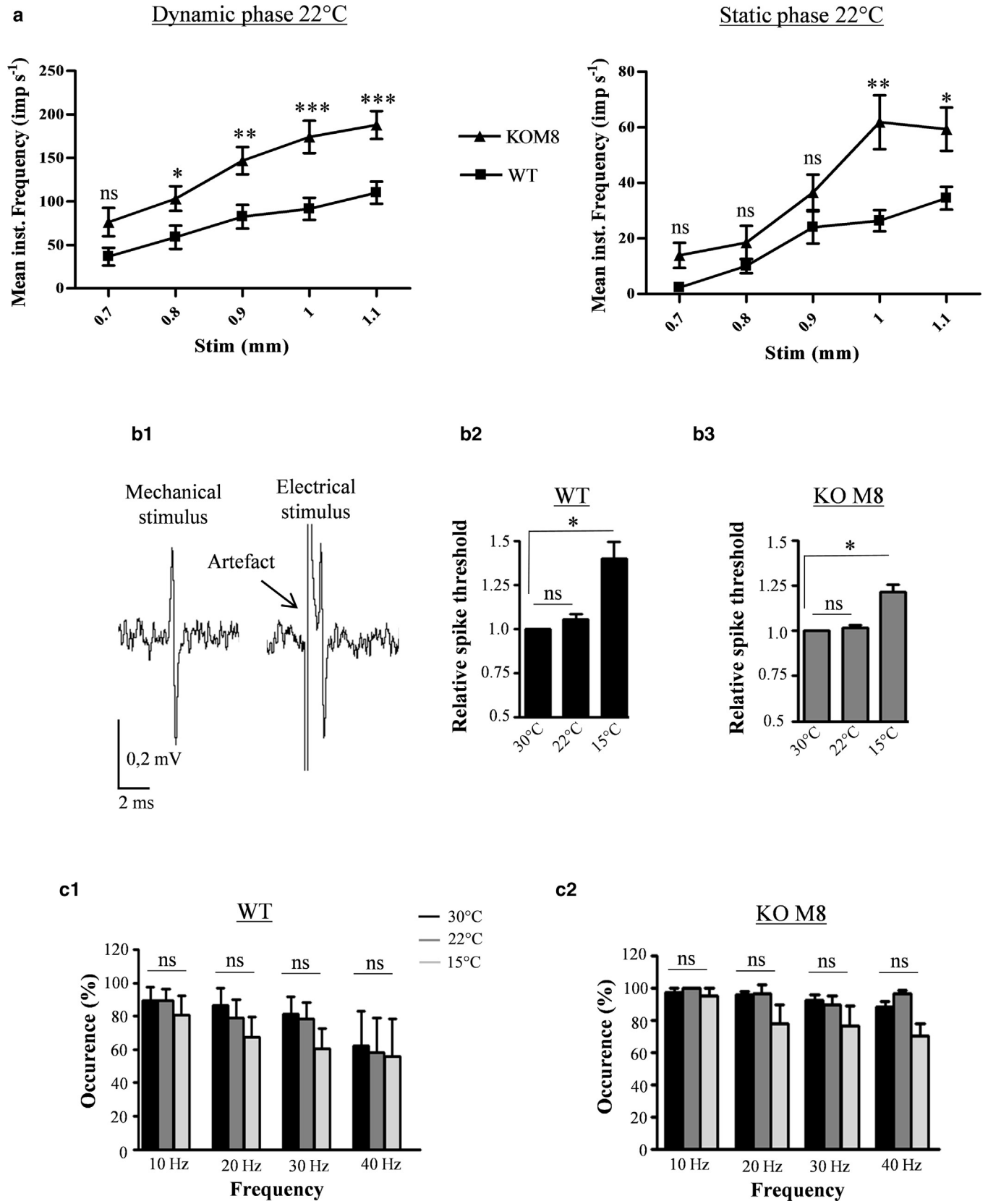

Figure 5. Effects of skin cooling on slow adapting type 1 (SA1) discharge and SA1 nerve fiber conduction: (**a**) Comparison of the SA1 discharge at 22°C between wild-type (WT) and transient receptor potential melastatine 8 knockout (KO M8) mice: Mean instantaneous frequency of the dynamic and the static phases of SA1 receptor discharge at 22°C in WT and KO M8 mice. (**b**) Effects of cooling on nerve conduction in mice: (**b1**) Mechanically (left) and electrically (right) evoked spikes recorded from the same SA1 receptor field. (**b2**) Relative spike threshold induced by electrical stimuli at 30°C, 22°C, and 15°C in WT mice (n = 7) and (b3) KO M8 mice (n = 8). (**c**) Change of the SA1 discharge during repetitive electrical stimulation: (**c1**) WT mice: percentage of spike occurrence observed during spike trains of 1 second at 10, 20, 30, and 40 Hz. Experiments were repeated at 30°C, 22°C, and 15°C (n = 7). (**c2**) Same experiment in KO M8 mice (n = 8). ns, not significant.

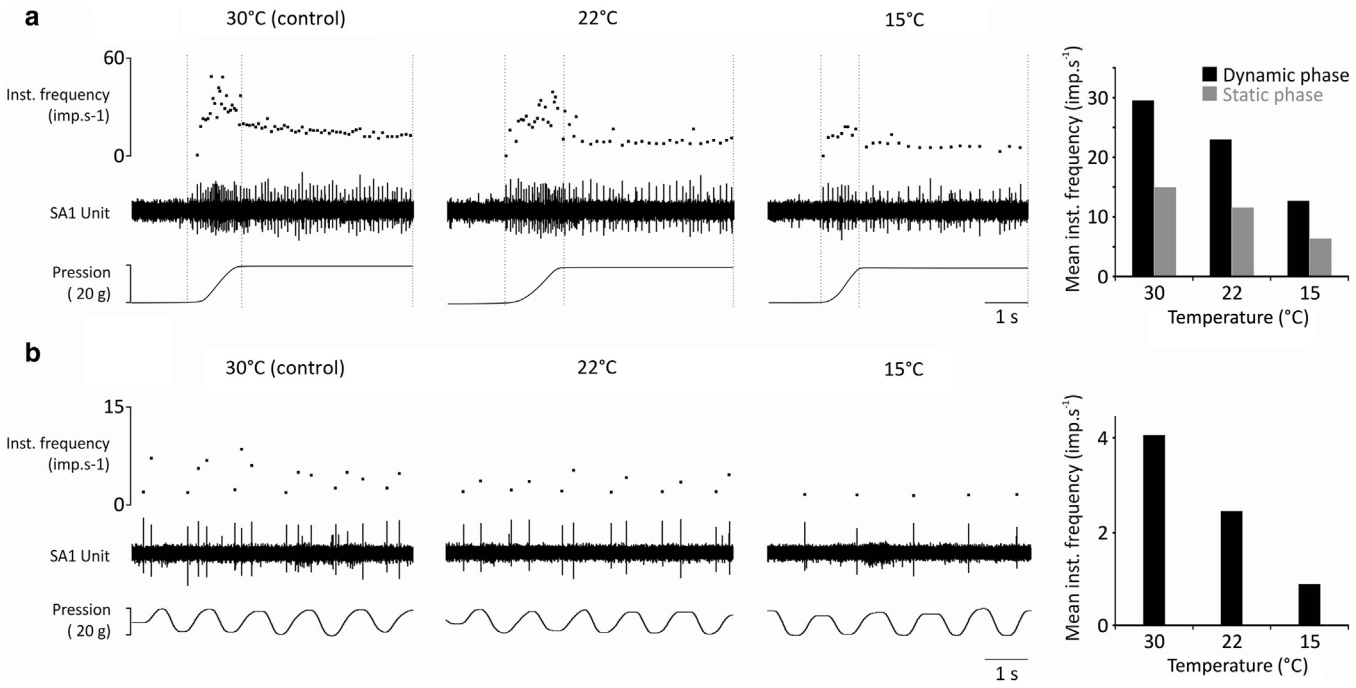

Figure 6. Cooling reduces the slow adapting type 1 (SA1) discharge in human skin. (**a**) Example of spiking induced by mechanical pressure of 20 g applied at 30°C, 22°C, and 15°C inside the receptor field of an SA1 unit located in the skin of the left leg. Note the reduction of the mean instantaneous frequency observed at 22°C. (**b**) Representative spiking induced by successive sinusoidal pressures of 20 g (each cycle lasting 1 second) applied at 30°C, 22°C, and 15°C inside the receptor field of a different SA1 unit located in the skin of the left leg. Note that triplet spiking at 30°C reduced to doublet spiking at 22°C and single spikes at 15°C.

therefore conclude that cooling human skin to 22°C reduces the SA1 discharge.

DISCUSSION

TRPM8 channels have been described in the endings of Aδ and C fibers in sensory skin nerves (Bautista et al., 2007; Dhaka et al., 2008; McKemy et al., 2002; Takashima et al., 2007), as well as in human keratinocytes (Denda et al., 2010). However, the role of TRPM8 in non-neuronal cells of the skin remains unclear, and to our knowledge has never been described in touch sensory complexes. We have shown that mouse and human MCs respond to a decrease in temperature by increasing the concentration of intracellular Ca^{2+} and generating a current that reverses around 0 mV. We demonstrated a contribution of TRPM8 to this cooling response in MCs from KO M8 mice by a 67% reduction in response compared to WT MCs. TRPM8 mRNA was previously identified in mouse epidermal cells by Bidaux et al. (2015) and, in addition, we detected similar expression levels of TRPM8 mRNA in human MCs as in human keratinocytes. We also detected reduced responses in KO M8 MCs to all TRPM8 agonists although not cinnamaldehyde, a TRPA1 agonist. The TRPM8 antagonist, 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC, 10 μM) (Almeida et al., 2012), inhibited the Ca^{2+} increase and the current induced by cooling and eucalyptol. 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide (BCTC) has been described previously as a blocker of both transient receptor vanilloid type 1 (Valenzano et al., 2003) and TRPM8 (Weil et al., 2005). Therefore, we ensured that mouse and human MCs express no transient receptor potential vanilloid type 1, as they were not activated by capsaicin (not illustrated).

Considering MCs express TRPM8 channels, we sought to determine the consequences of cooling on SA1 mechanotransduction. Cooling can affect spike threshold, discharge frequency, and conduction velocity. However, we isolated the SA1 receptive field from the rest of the skin within a small ring, minimizing the impact of cooling on nerve fibers. Using electrical stimulation of the SA1 receptor at 22°C, we observed no significant modification in spike threshold, spiking at 40 Hz or conduction of the Aβ fiber. However, in WT mice, a temperature of 22°C significantly reduced the mean instantaneous frequency of the dynamic phase. We also noted a reduced frequency in the static phase, although it required larger displacements. We exclude the possibility of thermosensitivity in the Aβ nerve terminal because a temperature of 22°C generated no spontaneous spiking or a decrease in spike threshold. Altogether, these data suggest that modulation of SA1 response by cooling arises from auxiliary cells, and not Aβ nerve terminals.

Modulation of SA1 receptor response by cooling has been described previously (Duclaux and Kenshalo, 1972; Hensel and Zotterman, 1951; Hunt and McIntyre, 1960; Iggo and Muir, 1969; Tapper, 1965). However, these studies differed from ours because they explored the effect of cooling on static discharge resulting from a constant pressure applied to the skin. Interestingly, they similarly observed a reduced spike frequency of static discharge under steady cold temperature. These authors also reported a transient increase in firing during the cooling ramp. The mechanism of this cooling-induced transient increase in SA1 static discharge

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remains to be determined. They also reported no spiking activity in the absence of mechanical stimuli similar to our observation that no spiking of Aβ nerve fibers occurred during cooling.

Interestingly, as mentioned, our protocol allowed us to study both static and dynamic discharges of the SA1 receptor. We observed an attenuation of the dynamic discharge during cooling; an effect that was absent in KO M8 mice. The higher mean instantaneous frequency of the dynamic phase in KO M8 mice at 22°C compared to WT mice possibly suggests an inhibitory effect of TRPM8 channel activity on the mechanical SA1 response. The mechanism of this regulation and the specific role of MCs remain unclear. Similar to MCs, keratinocytes express TRPM8 channels and are in close proximity to the Aβ nerve terminal. We suggest that the activation of TRPM8 channels in keratinocytes and/or MCs, releases mediators, which tonically dampen the excitability of Aβ nerve terminals, therefore, reducing both dynamic and static discharges of SA1 receptors. The use of global KO M8 mice precluded the analysis of MC and keratinocyte relative contribution of TRMP8 channels. However, further experiments using mouse models with either MCs or keratinocytes specifically deleted for TRPM8 channels are required to fully define the role of MC TRPM8 channels in regulating SA1 discharge.

What is the function of such an inhibitory mechanism mediated by TRPM8 channels? A recent analysis of the somatosensory circuit for cooling perception in mice indicates that the primary somatocortical area S1 is critical for thermal and tactile perception (Milenkovic et al., 2014). Patch-clamp recordings of S1 and L2/3 cortical neurons showed that a large proportion of neurons respond to both mechanical and cooling stimuli. Therefore, cortical integration of tactile and cooling sensory messages may be coordinated by the same neuron. It is tempting to speculate that inhibition of SA1 discharge during mild cooling reduces the frequency of tactile input, favoring simultaneous integration of cooling input.

In conclusion, we have shown that MCs are thermosensitive by expressing functional TRPM8 channels. Cooling to 22°C reduced the discharge frequency of the SA1 receptor and the deletion of TRPM8 in the skin abolished this effect. Further research is needed to determine the mechanisms of such a regulation and explore other potential functions of TRPM8 channels in MCs.

MATERIALS AND METHODS

Animals

All transgenic animals were backcrossed to a C57Bl/6J inbred strain. Atoh1$^{GFP/GFP}$ mice are Atoh1$^{tm4.1Hzo}$ mice (JAX, stock # 13593; Jackson Laboratory, Bar Harbor, ME) containing a green fluorescent protein fused to the Atoh1 gene (Woo et al., 2014). KO M8 mice were Trpm8^{tm1Jul}/J mice (JAX, stock # 008198; Jackson Laboratory) deleted for the TRPM8 gene. TRPA1 knockout mice were Trpa1^{tm1Kykw}/J mice (JAX, stock # 006401; Jackson Laboratory) deleted for the TRPA1 gene. Adult mice were anesthetized and killed with isoflurane. Animals were used in accordance with the European community guidelines outlining the care and use of animals (2010/63/UE).

Tissue samples and culture of MCs

Human foreskins were provided from healthy subjects of the pediatric surgery service, North Hospital, Marseille Public University Hospital System, Marseille, France. Patient consent for experiments was not required because French laws consider human tissue left over from surgery as discarded material. Mouse skin samples were extracted from their paws. Epidermis was separated from dermis using dispase solution (25 U/ml, Invitrogen, Carlsbad, CA) over 4 hours for human skin and 1 hour for mouse. Epidermis cells were dissociated for 5 minutes using trypsin solution (0.05%, Invitrogen) (Azorin et al., 2011). After centrifugation the pellets were diluted in 500 µl CNT-02 medium (CellNTech, Bern, Switzerland). Human MCs were selected as CD56^{+} cells (Boulais et al., 2009) according to the Miltenyi Biotec (Bergisch Gladbach, Germany) procedure.

Calcium imaging

Cultured MCs were loaded with Fura2-AM (2 µM, Sigma Aldrich, St Louis, MO) for 40 minutes. Experiments were performed in Krebs solution containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 10 glucose (300 mOsm, pH: 7.35). For Ca^{2+}-free solution, $CaCl_2$ was replaced with 0.4 mM EGTA. The temperature of media varied between 10°C and 40°C at the rate of 1°C/3 seconds (temperature controller CL100; Warner Instruments, Hamden, CT, and heater-cooler pencil; Harvard Apparatus, Cambridge, MA). Results were expressed as the 340/380 fluorescence ratio variation between the peak and base line (Delta ratio 340/380) and secondary converted to calibrated Ca^{2+} ions concentration. For details, see Supplementary Material (online).

Patch-clamp

Currents were recorded with the voltage-clamp technique in whole-cell mode. Currents were elicited every 5 seconds by a voltage depolarizing ramp from −60 to +60 mV (0.12 mV/ms) from a holding potential of −50 mV. Currents were filtered at 1 kHz and recordings sampled at 20 kHz. Patch pipettes had a resistance of 3−5 MΩ and were filled with intracellular medium containing (in mM): 130 CsCl, 10 HEPES, 8 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 1 MgATP, and 0.4 Na_2GTP. Extracellular medium was similar to that employed for calcium imaging experiments.

Skin-nerve preparation in mice

The saphenous nerve and dorsal part of the hind limb were dissected and placed corium side up in the main chamber perfused with synthetic interstitial fluid containing (in mM): 120 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 2 $CaCl_2$, 1.7 NaH_2PO_4, 5 $NaHCO_3$, 9.5 Na-gluconate, 5.5 glucose, 7.5 sucrose, 10 HEPES, pH 7.4, at 30°C and continuously oxygenated. The neural activity was amplified $\times 10^4$ (AC Differential Amplifier DAM 80; WPI, Sarasota, FL), 10 Hz-10 kHz filtered, and sampled at 25 kHz with an analog-to-digital converter (1401; Cambridge Electronic Design Limited, Cambridge, UK). Spike discrimination was performed off-line using Spike2 software (version 7, CED). Results were expressed as the mean instantaneous frequency during the dynamic and static phases of the mechanical response. For details, see the Supplementary Material.

Microneurography in human skin

The study was approved by the local ethics committee (CPP Sud-Méditerranée I). Experiments were conducted on three healthy volunteers (mean age 23 years old). Cutaneous afferent activity was recorded with an insulated tungsten microelectrode (Frederick Haer & Co, Bowdoin, ME). The electrode was manually inserted into the common peroneal nerve. The neural activity was amplified,

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300–3000 Hz filtered, and sampled at 10 kHz. The data were processed off-line using Spike 2 software (CED). For details, see the Supplementary Material.

Statistical analysis

For calcium imaging comparison of WT and KO M8 mice in skin nerve experiments, statistical analyses were performed using Mann-Whitney test with two-tailed P values and 95% confidence interval. For comparison of the SA1 discharge at 30°C, 22°C, and 15°C, statistical analyses were performed using one-way analysis of variance with nonparametric Friedman test and Dunn's post-test to compare all pairs of columns with 95% confidence intervals. Significant differences are represented by symbols: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.11.004.

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