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Trpv1 Mediates Spontaneous Firing and Heat Sensitization of Cutaneous Primary Afferents After Plantar Incision

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Keywords

TrpV1; incision; spontaneous activity; cooling; primary afferent sensitization; skin-nerve preparation

Introduction

TrpV1 is best recognized as the receptor for capsaicin. The channel is expressed primarily by small to medium diameter primary afferent fibers and can be activated by noxious heat (>43°C) and a variety of chemicals present in a pathological milieu [1–4]. In cultured dorsal root ganglia, the channel is activated by 43°C, the same temperature that causes nociception in human skin [5]. It is surprising that the genetic deletion of TrpV1 produced only modest reductions in heat nociception in animals [1,6]. In addition, neurophysiologic experiments demonstrated that primary afferents from TrpV1 knockout (KO) mice had largely normal responses to heat [7].

TrpV1 appears to be much more important for signaling increased behavioral sensitivity to stimuli after tissue injury. This is consistent with many cellular studies demonstrating that chemicals released from injured tissue such as bradykinin, prostaglandins, nerve growth factor (NGF), ATP, serotonin, and hydrogen ions either directly activate or sensitize this channel [8]. The mediators present after an incisional injury include low pH and NGF [9], which can reduce the threshold temperature for activation of TrpV1 [10–12].

Our previous reports demonstrated that incisional injury causes heat sensitization and SA of primary afferent nociceptors [13,14]. The first goal of the present study is to examine the relationship between the marked reduction in heat hyperalgesia after incision in TrpV1 KO mice [15] and the activity of the nociceptors in the normal and injured state in these same KO mice.

The cellular and molecular mechanisms that may underlie SA in nociceptive afferents are largely unknown. It has been hypothesized that lowering of the threshold temperature for activation of afferents may be responsible for SA [16]. This raises the question: are

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thermosensitive ion channels, in part, mediating SA of afferents under certain pathological conditions? The second goal of the present study is to examine the role of TrpV1 in SA of cutaneous afferents after incision and the contribution of TrpV1 to the relationship between SA and heat sensitivity.

In this study, we made a plantar incision in the TrpV1 KO mice and measured pain behaviors. Since the injury site is in the glabrous hind paw skin where pain behaviors are tested, we recorded primary afferents innervating the injured site using an *in vitro* mouse glabrous skin nerve preparation. We compared nociceptors of C57Bl6 and TrpV1 KO for: (1) the percentage and types of fibers that develop SA and heat responses with or without incision, (2) the relationship between skin temperature and SA, and (3) mechanoreceptor responses. Preliminary data were published in abstract form [17].

Methods

Animals

Combined behavioral and electrophysiological experiments were performed on 90 male C57Bl6 and TrpV1 KO mice; 60 animals underwent hind paw incision one day before the electrophysiologic experiments, and 30 were unincised controls. The weight of the mice was 19–25 gm; the age was 6–12 weeks. Two to four animals were housed in plastic cages with bedding in a climate-controlled room under a 14/10 hr light/dark cycle. Mice were purchased from Jackson Laboratory (Bar Harbor, ME). The Animal Care and Use Committee at The University of Iowa, Iowa City, Iowa approved the experiments, and the animals were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals.

Surgery

Animals from each genotype were randomly assigned to two different groups, incision or control. The procedure for plantar incision has been described in our previous report [18]. Anesthesia was induced by placing the animals in a plastic box that contained 4% isoflurane in air. After loss of the righting reflex, anesthesia was maintained by administering 1–2% isoflurane in air through a tightly fitting mask. An 8-mm longitudinal incision was made with a number 11 blade through the skin, fascia, and muscle of the right hind paw, modified from Pogatzki and Raja[19]. The skin was apposed with two single sutures of 6-0 nylon, and the wound site was covered with triple antibiotic ointment. After surgery, anesthesia was discontinued, and the animals were allowed to recover in their cages.

Withdrawal responses to heat

Withdrawal latencies to heat were assessed by applying a focused radiant heat source on the incised area of unrestrained mice placed on a heat-tempered glass floor. The heat stimulus was a light from a 50-W projector lamp, with an aperture diameter of 6 mm, applied from underneath a heat-tempered glass floor (3 mm thick) on the middle of the incision. Paw withdrawal latencies were measured to the nearest 0.1 s. The latency to evoke withdrawal was determined with a cutoff value of 30s. The plantar incision produces robust heat hyperalgesia. We were interested in responses to slow heating of the paw to detect small differences in the exaggerated response after incision. The intensity of the heat was adjusted to produce withdrawal latencies in normal mice of 25 to 30s. Three trials 5–10 min apart were used to obtain the average paw withdrawal latency.

Guarding behaviors

A cumulative pain score was used to assess non-evoked pain behaviors. Unrestrained mice were placed on a stainless steel mesh floor (4×4mm). Using an angled magnifying mirror, the incised and non-incised paws were viewed. Both paws of each animal were closely observed during a 1 min period repeated every 5 min for 1 hr. Depending on the position in which each paw was found during the majority of the 1 min scoring period, a 0, 1, or 2 was given. Full weight bearing of the paw (score = 0) was present if the wound was blanched or distorted by the mesh. If the paw was completely off the mesh, a score of 2 was recorded. If the area of the wound touched the mesh without blanching or distortion, a 1 was given. The sum of the 12 scores (0–24) during the 1 hr session was obtained for each paw. The difference between the scores from the incised paw and non-incised paw was the cumulative pain score for that 1 hr period.

Withdrawal responses to mechanical stimuli

After one to two hours' recovery in clean bedding, the mouse was placed on an elevated stainless steel floor covered with a clear plastic cage top. The animals were allowed to ambulate, explore, and eventually rest lying on the mesh. For testing mechanical responses, von Frey filaments (calibrated thin plastic filaments) were applied adjacent to the wound from least to greatest forces (3.1, 5.9, 13.9, 26.8 mN forces). Each filament was applied for 5 times with at least 30 seconds between applications before the next filament was tested. Data are expressed as the percent of paw withdrawal for each filament.

Experimental protocol for behavioral studies

In order to habituate to the testing environment, the mice were acclimated for two days; no testing was performed. Eight mice were tested at one time; four from each genotype. One person performed all of the behavioral experiments and was blinded to the genotype of animals. Baseline pre-incision testing was performed on the third day. Three days later, the incision was made. Four hours, 1 day, 2 days, 3 days, 5 days, and 7 days after incision, nociceptive tests were performed on each animal, as described above. In our experiments, three nociceptive tests could not be completed within our testing period, 8:00 AM to 1:00 PM. Therefore, no more than two nociceptive tests were performed on each mouse. Testing of guarding pain behavior was always first, followed by testing of heat responses. In other mice, only mechanosensitivity was tested. Testing was performed at approximately the same time each day. Because we recorded nociceptors on postoperative day 1, the 4 hr time point in the behavioral studies is not shown.

Electrophysiological procedures using the mouse glabrous skin nerve preparation

The details of electrophysiology procedure have been published [20] Electrophysiological recordings were performed in four groups of mice: 1. Control, unincised C57/Bl6, 2. Control, unincised TrpV1 KO, 3. C57/Bl6 one day after incision, 4. TrpV1 KO one day after incision. The person performing the recordings (RKB) was blinded to the genotype. Mice were euthanized using CO₂; then the glabrous skin of the mouse hind paw and its intact tibial nerve and plantar branches were dissected free from muscles and tendons. The preparation was then placed in an organ bath and was continuously superfused with a modified Krebs-Hensleit solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂SO₄, 24.4 NaHCO₃, and 20 glucose), which was saturated with a gas mixture of 95% O₂ and 5% CO₂. The temperature of the bath solution was maintained at 32±1°C. After the dissection, the preparation was placed with 'epidermal side down'. The nerves attached to the skin were drawn through one small hole to the second chamber, which was filled with liquid paraffin. The nerves were placed on a fixed mirror, their sheaths removed and nerve filaments repeatedly teased to allow single fiber recording to be made by using double-platinum electrodes (one for recording and

the other for reference). Single nociceptive afferent fibers were recorded extracellularly with a differential amplifier (DAM50, Harvard Apparatus, Holliston, MA). Neural activity was amplified and filtered using standard techniques. Amplified signals were relayed to a digital oscilloscope, an audiomonitor and a computer via a data acquisition system (spike2/CED1401 program).

Feedback controlled mechanical stimulation

Servo-controlled mechanical stimulation (Series 300B dual mode servo system, Aurora Scientific, Canada) was used to measure mechanosensitivity. The flat and cylindrical metal probe (tip size 0.7 mm) attached to the tip of stimulator arm was placed close to the receptive field so that no force was generated. A computer controlled ascending series of force-controlled stimuli was applied to the most sensitive spot of the receptive field at 60-s intervals. Each force stimulus was 5s in duration and started from zero to 5, 10, 20, 40, 80, 120, 160 and 200mN. When an afferent produced a response to a particular force controlled ramp, it received three more ascending series of stimuli. To avoid fatigue, no more than three suprathreshold stimuli were tested. For example, if an afferent responded during the 20mN stimulus, it received the 40, 80 and 120mN stimuli.

Feedback controlled heat stimulation

After mechanical stimulation, a standard feedback controlled heat ramp was delivered by a customized heat stimulator (Bioengineering, University of Iowa, Iowa City, IA). The receptive field of each unit was isolated with a small metal ring, which could seal by its own weight. In some cases silicone grease was added to prevent leakage from the bath into the receptive field within ring. The solution was removed and a thermocouple was gently placed to measure the dermal side temperature. A radiant lamp was placed in the translucent area underneath the organ bath and the light beam was focused onto the epidermal side of the skin. A computer controlled heat ramp was delivered starting from 33 degrees to 49 degrees centigrade over 17 seconds. We determined that the temperature of the epidermal side was about 1 degree centigrade higher than the dermis.

Effects of cooling

The receptive field was isolated from the perfusion chamber as described above. The receptive field temperature was continuously monitored by a thermocouple lightly touching the dermal side of skin. Then, an ice-cold Krebs's solution (temperature approximately 1 to 2°C) was superfused to decrease the receptive field temperature using a rapid exchange system combining superfusion and aspiration. The duration of cooling was two minutes and despite heat conduction from the surrounding perfusate, the temperature was reduced to 12 to 16°C during the superfusion period.

Conduction velocity and fiber categorization

To avoid damaging the receptive field, the conduction velocity was always measured at the end of protocol. The conduction velocity of the axon was determined by monopolar electrical stimulation through an epoxy-coated electrode. The electrical stimulation (1–20 V at 0.2–1 Hz for 0.5–2 ms) was delivered at the most mechanosensitive spot of a receptive field. The intensity of the stimulus started from 0.1V and gradually increased until the shape of spike appeared with constant latency. The distance between receptive field and the recording electrode (conduction distance) was divided by the latency of the action potential. We categorized Aδ-fibers as those slowly adapting to mechanical stimulation with a conduction velocity less than 10 meters per second and greater than 1.2 m/s. The fibers, which had conduction velocity less than or equal to 1.2 m/s and were slowly adapting to mechanical stimulation, were considered C-fibers.

Experimental protocol for electrophysiologic studies

We used a mechanical search stimulus (see above) to identify receptive fields of single nociceptive fibers. Once a single afferent with a mechanosensitive receptive field was identified, mechanical stimuli were applied to the most sensitive spot of the receptive field using a von Frey hair (527 mN) and fire-polished glass rod to determine adaptation properties. Only slowly adapting fibers were studied further. If the fiber had SA, this was recorded for five minutes without any stimulation. After that, the mechanical response properties and the responses to heat were measured as described above. The cold sensitivity was tested in spontaneously active afferents from incised skin followed by conduction velocity.

Data analysis

Action potentials, collected on a computer, were analyzed off-line with a template matching function of spike 2 software. The activity was counted in one-second bins. If the unit discharged at a rate of 0.1 impulses per second or more without any intentional stimuli, it was categorized as spontaneously active. Fibers in which SA was decreased by the initial warming during heat stimulation were considered to be non-nociceptive cold fibers and were excluded from further analysis.

The heat threshold was determined as that temperature which elicited the first action potential in fibers without activity. If SA was present, threshold was determined by the temperature that increased background activity by at least 2 standard deviations greater than the background average measured for 10 sec (in 1 sec bins) before the stimulus. The percentage of fibers responding to heat, total spikes during the 17 sec heat stimulus, peak frequency during heat, and heat responses in 1 sec bins before, during and for 5 sec after the heat stimulus were measured. Background activity was subtracted from any evoked responses; thus, assuming background activity was sustained during the stimulus period.

For mechanical responses, we divided the A and C-fiber nociceptors into low and high threshold groups. The stimulus-response curves were generated from the low threshold fibers that received 5, 10, 20 and 40 mN stimuli and from the high threshold fibers that were tested with 20, 40, 80 and 120 mN stimuli. To quantify the fibers' mechanical response, the total number of spikes during the mechanical stimulus were counted and the peri-stimulus time histograms were averaged. After discharges were rare and not quantified.

During heat stimulation, previously unnoticed, small amplitude units were often excited by heat. These afferents were named as 'unclassified' since attempts to identify mechanical receptive fields of these units were not successful. Consequently, conduction velocity of these units could not be measured. These fibers may be mechano-insensitive, and were noticed primarily because of their vigorous responses to heat during the testing of mechanosensitive fibers. In a separate analysis, the total activity was counted so that the identified mechanosensitive afferent and unidentified heat responsive afferents were included. Care was taken to ensure these multi-unit recordings were not influenced by noise.

Statistical analysis

Behavioral data were analyzed using 2-way analysis of variance (ANOVA) and when significant, post hoc unpaired t-tests for comparison of individual times after incision. Unpaired t-test was used to compare threshold temperature, peak discharge frequency, the rate of SA and subcutaneous hind paw skin temperature. Due to the non-Gaussian data distribution, a non-parametric Mann Whitney U test was used in the analyses of total spikes evoked per heat stimulus. Contingency table comparisons were made for the percent of C-fibers responsive to heat and for the incidence of SA. These were analyzed by Chi-square tests. The stimulus-response curves for heat were compared by 2-way ANOVA. The effect of cooling was

compared using a Wilcoxon matched pair test. Comparisons of mechanical responses between C57BL6 and KO mice were made using 2-way ANOVA. All tests were made with GraphPad Prism software, version 4 (GraphPad, San Diego, CA), and were two-tailed, unless otherwise stated. Values of $p < 0.05$ were considered significant.

Results

Behavior

The guarding pain score increased after plantar incision in both groups (Fig. 1a). On postoperative day 1, the median guarding pain scores in C57BL6 and KO mice were 4.8 and 3.7 respectively. Similarly, incision increased the frequency of withdrawal responses to all four monofilaments; however, there was no difference between C57BL6 and KO mice (Figure 1b–e). There were minimal differences in the heat responsiveness before incision in the KO (25 ± 2 s mean $\pm$ SEM) vs. the C57BL6 (23 ± 1 sec). Incision decreased the withdrawal latency to radiant heat in the C57BL6 mice but the withdrawal latency was not decreased on postoperative day 1 and later in the KO mice (Fig 1f). In general, the pain related behaviors had resolved by postoperative day 7.

Neurophysiology

Extracellular recording was made from a total of 291 cutaneous primary afferents from 90 male mice (Table 1). Only fibers with conduction velocities in the A δ or C-fiber range were studied. Fifty-one afferents were unclassified (See below). Except for the unclassified fibers, all fibers were mechanically sensitive. For comparison of mechanosensitivity, A and C-fibers were further classified into low (<10 mN) and high threshold (≥ 10 mN) groups. Heat sensitivity was more frequently seen in the C-fibers compared to A-fibers (Table 1). Few fibers were excited by cooling to 12–16°C (Table 1). The conduction velocities of the A δ -fibers ranged from 1.5 to 6.2 m/s (3.2 ± 1.4 m/s), and C-fibers were between 0.2 and 1.2 m/s (0.7 ± 0.04 m/s). The lowest and highest number of units we recorded from one preparation was one and five, respectively.

Heat responses of mechanosensitive afferents

Unincised mice—When both strains of normal mice were compared, there was no difference in the heat responses of mechanosensitive C-fibers innervating the glabrous skin (Fig. 2). Sixty percent of mechanosensitive C-fibers (12 of 20) from C57BL6 mice were responsive to heat, and a similar proportion (46%, 14 of 30) from KO mice responded to heat (Fig. 2a). The heat responsive, mechanosensitive afferents were not different in C57BL6 and KO mice in regard to the threshold temperature for activation, total evoked spikes and peak discharge frequency during heat stimulation (Figs. 2b–f). Moreover, when a stimulus response curve was plotted against the temperature increase in the receptive field, the curves for C57BL6 and KO mice were nearly identical (Fig. 2e).

Incised mice—We compared primary afferent activities from incised KO and C57BL6 mice one day after incision. Of the 28 C-fibers tested from incised C57BL6 mice, 18 (64%) responded to heat; a similar proportion of afferents from incised TrpV1 KO mice were heat sensitive (71%, 15 of 21, Fig 3a). In the mechanosensitive C-fibers, the heat thresholds averaged $39 \pm 5^\circ$ C and $42 \pm 3^\circ$ C in the incised C57BL6 and KO mice, respectively ($p < 0.05$, t-test; Fig 3b). The total evoked action potentials and peak discharge frequencies were not different between groups (Figs 3c–d). The stimulus response curve, which was spikes in each 1-second bin versus temperature, showed no significant differences between C57BL6 and KO mice ($F_{1,27} = 1.26$, $p > 0.2$, 2-Way ANOVA; Fig 3e).

In summary, when unincised C57BL6 and KO mice are compared, the lack of TrpV1 receptor did not affect heat responses of the mechanosensitive C-fibers. When incised C57BL6 and KO

mice are compared, the lack of TrpV1 receptor only affected heat threshold of activation of C-fiber afferents (Fig. 3b), despite the striking differences in behavioral responses to heat (Fig 1f).

We evaluated the effect of incision on heat responses of mechanosensitive C-fibers for each mouse genotype by comparing data from Figures 2 and 3. In the unincised (Fig. 2d) and incised (Fig. 3d) C57BL6 afferents, only peak discharge frequencies during the heat response (16.8 ± 2.9 vs. 32.9 ± 5.4 spikes/s, $p < 0.05$) were different as reported [20]. The threshold temperature and total evoked spikes per heat stimulus were not affected by incision. In the unincised and incised KO mice, heat responses of mechanosensitive C-fibers were not different (Fig. 1f).

Heat responses of unclassified afferents

We counted ‘total activity’ of all heat responsive afferents, which included activity from mechanosensitive afferents and unclassified afferents that were not initially identified by mechanical stimulation. Like the single unit analysis, the total multi-unit stimulus response functions showed no difference between unincised C57BL6 and KO mice (Fig. 4b). The stimulus response functions that included unclassified afferents were markedly different in the incised C57BL6 vs. KO mice ($p < 0.0005$; Fig. 4c). A detailed analysis showed that the threshold temperature eliciting activity was greater in the incised KO animals compared to incised C57BL6 mice ($p < 0.0001$; Fig 4d). The average heat thresholds for evoking activity that included unclassified afferents were $35 \pm 2^\circ\text{C}$ and $42 \pm 3^\circ\text{C}$ in the incised C57BL6 and KO mice, respectively. The total action potentials produced in each recording that included unclassified afferents and the peak discharge frequencies during heat stimulation were significantly reduced in the incised KO mice compared to incised C57BL6 mice ($p < 0.0001$; Fig 4e–f). The comparison of the stimulus response curves showed that the total afferent activity from incised C57BL6 increased in response to the rise of temperature in the receptive field up to 48°C (Fig. 4c). The temperature-response curve of heat responsive afferents from incised KO mice was significantly less responsive (Fig. 4c). Since the loss of TrpV1 caused only a small reduction in the heat responses of mechanosensitive afferents, the large differences in the activity from the incised preparations that include unclassified afferents (Fig. 4c) are perhaps due to the loss of TrpV1 receptors in the unclassified afferents. The responses of these fibers may contribute to marked differences in behavior observed in the incised KO and C57BL6 mice.

Relationship between SA and heat sensitization

In the incised C57BL6 mice, 31% of the mechanosensitive C-fibers (10 of 32) had SA. Deletion of TrpV1 did not change the proportion of spontaneously active afferents (22%, 5 of 22) or the rate of SA (Fig. 5a–b). Heat sensitive, unclassified afferents were noted in the 19 of 23 (83%) recordings in the incised C57BL6 mice and in 13 of 17 (76%) recordings in the incised KO mice. In the incised C57BL6 mice, 73% of (14 of 19), unclassified afferents had SA, whereas in the incised KO mice this proportion was 35% (6 of 17) (Fig. 5c; $p < 0.03$, χ^2 -test). The rate of activities was also significantly less in the incised KO mice (3.6 ± 4.6 vs. 1.0 ± 1.1 spikes/s, $p < 0.04$, t-test; Fig. 5d). In addition, spontaneously active, unclassified afferents from C57BL6 mice generally had a lower threshold temperature compared to unclassified afferents without SA (Fig 6a–c). The threshold temperature of spontaneously active unclassified afferents was $34 \pm 1^\circ\text{C}$ whereas the threshold of unclassified afferents without SA was $42 \pm 3^\circ\text{C}$ (Fig 5c; $p < 0.001$, t-test). A notable finding is that the rate of SA was inversely correlated with the threshold temperature ($r, -0.5$; $p < 0.05$; Fig 6d) for heat; thus, unclassified afferents that exhibited a higher rate of SA had lower threshold temperatures for activation by heat.

Cooling the receptive field temperature from $32 \pm 1^\circ\text{C}$ to approximately 12 – 16°C reversibly inhibited SA in 8 of 10 (80%) unclassified afferents and 4 of 4 (100%) mechanosensitive afferents tested from incised C57BL6 mice (Fig. 7). In some cases, cooling itself excited

afferents, which were not identified by mechanical stimulation (data not shown). However, in no instance did cooling excite spontaneously active unclassified afferents. We tested cooling on two incised KO afferents that had SA. In these fibers, the mean rate of SA before cooling was 2.1 spikes/s and during cooling the rate was reduced to 0.6 spikes/s. Thus cooling also could reduce SA in heat sensitive afferents from incised KO mice.

Mechanical responses

Figure 8 summarizes mechanical responses of A- δ and C-fibers from incised and unincised, control KO mice. In this analysis we used C57BL6 data from our previous report[20]. In our earlier study, incision increased mechanical responsiveness of A- δ and C-fibers that responded to weak mechanical stimuli. In agreement with this previous study, incision in TrpV1 KO mice increased the mechanical responses of A- δ and C-fibers that responded to weak mechanical stimuli. There were no differences in mechanical responses between control, unincised C57BL6 and KO mice. Also there were no differences in mechanical responses between incised C57BL6 and KO mice (Fig. 8a–h). In some cases the number of fibers in the KO that were tested for the complete series of mechanical stimuli is small and the variation large.

Discussion

The present study demonstrates that the loss of TrpV1 produced very specific abnormalities: it completely abolished heat hyperalgesia one day after incision and in incised preparations, reduced responses to heat in recordings of all heat sensitive fibers. TrpV1 gene deletion also decreased the proportion of unclassified afferents with SA and the amount of SA. After incision, the rate of SA of unclassified, heat sensitive afferents in vitro, was inversely related to threshold temperature for activation of afferents suggesting that SA may be related to temperature. The heat responses of mechanosensitive C-fiber afferents were affected to a much lesser extent by the loss of TrpV1; only the threshold temperature was less in the afferents from the incised KO mice.

Role of TrpV1 in the normal heat response

Using a radiant heat test with a slower ramp (cut-off 30s), we show that the loss of TrpV1 in the unincised mice produced only a very small deficit in withdrawal latencies to noxious heat. This observation is in agreement with a previous study [21] that showed TrpV1 KO mice may have some deficits in heat evoked pain behaviors but usually the differences are small and dependent upon stimulus intensity. The small difference in the heat responses between KO and C57BL6 is perhaps an example of functional redundancy by other heat sensitive Trp channels [22].

In our study, the heat sensitivity of mechanosensitive C-fiber nociceptors from unincised mice was similar between strains. Neither the prevalence of mechanoheat sensitive nociceptors nor the response properties of heat responsive afferents was affected by loss of TrpV1. Also, the heat responses of unclassified fibers were not different between unincised C57BL6 and KO mice (Fig 4b). This is in contrast with the study by Caterina and his colleagues [21], who found that 13/24 C-fibers and 1/13 A δ -fibers from C57BL6 mice were excited by a noxious heat (peak temperature, 47°C) whereas only 4/24 C-fibers from TrpV1 KO mice were responsive to the same heat stimulus. Among heat responsive afferents, the threshold was unchanged in the KOs, but their mean heat-evoked discharge was significantly reduced[21]. In another study, Woodbury and colleagues[7] showed that lack of TrpV1 actually increased the prevalence of IB4-negative heat sensitive nociceptors (57% vs. 78%) in an ex-vivo preparation. The threshold for heat responses and discharge frequency during the heat stimulus were identical between genotypes. The differences in the results in the above two studies and the current study may be related to the differences in the preparation used. These differences include ex vivo

recordings from DRG vs primary afferents in the skin nerve fiber preparation[7], the hairy [21] vs. glabrous (current study) skin nerve preparation, and mechanical (current study and ref. 21) vs electrical search stimulus [7].

Roles of TrpV1 in heat sensitization after incision

Pogatzki-Zahn et al.[15] demonstrated that TrpV1 mediates incision induced heat hypersensitivity. The current study confirms this using a slower heating rate and strengthens the hypothesis that TrpV1 is a critical mediator for tissue injury related heat hypersensitivity [6,21,23–25].

Our speculation in this study was that the loss of TrpV1 would completely eliminate incision induced heat sensitization of C-fiber afferents. Surprisingly, however, we observed only a greater threshold temperature for activation of afferents from incised KO mice; no differences in the total evoked action potentials and peak discharge frequency were noted. The result gives rise to a paradox because incised KO mice showed severe deficits in the incision induced heat hypersensitivity. Since we recorded afferents from the same anatomical locus used in the behavioral assay, the problem cannot be explained simply as differences in glabrous versus hairy skin afferents or hind paw versus tail afferents, which are histologically and functionally unique [26–28]. One explanation for the difference in heat withdrawal latency after incision in the C57BL6 vs KO is the peak response during heating in the C mechanoheat fibers (Fig. 2d vs 3d and ref. 20). Alternatively, an additional afferent population outside of the mechanosensitive heat responsive units, which are excited by heat and have altered responsiveness after incision, may contribute to this difference in withdrawal latency.

Recently we showed that in the incised mouse glabrous skin nerve preparation, a group of unclassified afferents are responding to heat but are not readily identified by mechanical stimulation [20]. The activity of these afferents was counted by a separate analysis of ‘total activity’ that includes activity of mechanosensitive afferent and these unclassified afferents (See methods). The ‘total activity’ evoked by heat is markedly reduced in the incised TrpV1 KO afferents (Fig 4c) suggesting a contribution of TRPV1 to heat sensitization in afferents which are not mechanically responsive. The result is in agreement with recent observation by Lawson and colleagues [29], who showed that mouse cutaneous C-fibers that responded to both mechanical and heat stimuli do not express TrpV1. The study used a combination of immunocytochemistry and electrophysiology to characterize each afferent. A possible explanation for why this afferent population has been missed in previous studies is the search strategy, which in general has been limited to mechanosensitive afferents. Few studies characterized mechano-insensitive afferents but found that they comprised as many as thirty percent of afferent axons in normal skin [30–32], and were sensitized by mustard oil and capsaicin [33]. In addition, it should be noted that most of the previous studies were conducted on species other than mice. Heterogeneity in the distribution of afferent fiber populations is observed among species. For example, the proportion of polymodal receptors to cutaneous C-fiber afferents is almost 100% in humans, 85–90% in monkeys, 35–40% in cats, about 70% in rabbits and [34]. Perhaps TrpV1 containing mechano-insensitive afferents are more common in mice than other species[29].

In our results, the prevalence of unclassified afferents with SA is significantly reduced in the incised TrpV1 KO mice. This implies a role of TrpV1 in the development of SA in the incised skin. The channel normally opens at approximately 43°C but several factors could influence its responsiveness to heat. For example, tissue acidification could sensitize the temperature-response relation so that it could be activated by body temperature [2,35]. Tissue acidosis is an important characteristic of incisional injury and corresponds well with the occurrence of spontaneous pain behavior and hypersensitivity[9]. However, pH of the incised tissue falls to 6.9 one day after incision, while studies from excised membrane patches indicated that TrpV1

was activated by room temperature when pH was 6.0 or below [2]. Likely other mediators could potentiate TrpV1 responses and work in concert to regulate TrpV1 function. One factor is NGF, which is profoundly increased in incised skin and contributes to guarding pain behavior and heat hyperalgesia after incision [36,37]. NGF potentiates TrpV1 responses to heat and acid [10]. The mechanisms that underlie such potentiation include TrkA signaling pathway that phosphorylates TrpV1 [12]. NGF also inserts new receptors into the membrane surface, which increases current density [12].

The majority of SA in the unclassified afferents was completely eliminated by cooling the receptive field temperature at 12–16°C. The observation could explain a mechanism for reducing postoperative pain using cooling [38–43]. The observation is compatible with previous studies that showed a marked inhibitory effect of cooling on the ongoing discharge of C-fiber nociceptors sensitized by mustard oil [44] or capsaicin [45]. Moreover, cooling reduced capsaicin induced CGRP release from nociceptive nerve fibers in isolated rat skin [46].

Conclusion

In the mouse glabrous skin nerve preparation, KO of TrpV1 did not affect heat sensitivity of mechanosensitive C-fibers. In the incised mice, loss of TrpV1 only decreased the heat threshold of these mechanosensitive C-fibers. The heat responses of other afferents are reduced by the loss of TrpV1 in the incised preparations. These afferents were unclassified because mechanosensitive receptive fields were not identified. SA was related to heat responses and inhibited by cooling in these unclassified afferents from incised mice. KO of TRPV1 did not influence mechanosensitivity of nociceptors or the mechanosensitization of nociceptors by incision.

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Figure 1.

The effects on TrpV1 gene deletion on guarding behavior, heat and mechanical sensitivity in mice before and after plantar incision. Before or after incision, no differences are observed in the guarding behaviors (a) and paw withdrawal frequencies to von Frey hair stimulation (b–e). In a, note that ‘Y’ axis is extended up to the maximal guarding behaviors reported in the rat after incision. f) In C57BL/6 mice, incision produced significant reductions in the withdrawal latencies to a heat stimulus on the incised paw. The decrease was significantly different from the paw withdrawal latencies in the TrpV1 knock out (KO) mice. Asterisks indicate a significant difference on the incised side between C57BL/6 and KO mice (* $P < 0.05$, Repeated measures ANOVA followed by posthoc t test). mN=milli-Newtons.

Figure 2.

Heat responses in mechanosensitive afferents from TrpV1 KO and C57BL6 mice. a) The percentage of heat responsive C-fibers in C57BL6 and KO mice. The threshold temperature for heat response (b), total spikes evoked during heat stimuli (c), and the peak discharge frequency (d) were not different between genotypes. Threshold was the temperature that elicited the first action potential if background activity was absent or at least 2 standard deviations greater than SA (For details, see materials and methods). e) Stimulus-response function for heat response in C57BL6 and KO mice (bin width 1-s, mean number of spikes $\pm$ SE). f: Digitized oscilloscope trace of two typical C-fiber activities during heat stimulus in C57BL6 (left) and KO mice (right). Below, real-time record of skin temperature, and above is the peri-stimulus time histogram.

Figure 3.

Loss of TrpV1 had a small effect on heat responses of mechanosensitive afferents after incision.

a) the percentage of heat responsive C-fibers was not different between incised C57BL/6 and KO mice. b) Threshold temperatures for activation of mechanosensitive C-fiber afferents from incised mice. The horizontal line represents mean $\pm$ SE, $p < 0.05$, unpaired t-test. The total spikes evoked during heat stimulation (c) and peak discharge frequency (d) were not different between incised C57BL/6 and TrpV1 KO mice. e: Stimulus response function for heat in incised C57BL/6 and KO mice (bin width 1-s, mean number of spikes $\pm$ SE). f: Response of two typical C-fiber activities during the heat stimulus in the incised C57BL/6 (left) and TrpV1 KO mice (right).

Figure 4.

The total activity of all afferents during heat stimulation. a) Left: Example recording from incised C57BL/6 mice. In addition to a mechanically identified C-fiber afferent (spikes with large amplitude, filled arrow), small amplitude action potentials from another fiber are noted immediately during the onset of the heat stimulus (open arrow). The small amplitude action potentials are presumably from another class of afferents that are not readily identified by mechanical stimulation. In this study we named these 'unclassified' since they could not be further characterized (for details, see Material and Methods). Analyses of all afferent activity during heat stimulation are shown in b-f. Stimulus response function for heat response of all afferents in unincised (b) and incised (c) C57BL/6 and KO mice (bin width 1-s, mean number of spikes $\pm$ SE, $p < 0.0005$, 2-way ANOVA). d-f: The threshold temperature ($p < 0.0001$, unpaired t-test), total activity ($p < 0.0001$, Mann-Whitney test) and peak discharge frequency ($p < 0.001$, unpaired t-test) of all heat sensitive activity are significantly different between incised C57BL/6 and KO mice.

Figure 5.

Spontaneous activity (SA) of afferents from incised mice. The proportion of mechanosensitive C-fibers with SA was not different in the incised C57BL6 and TrpV1KO mice (a). b) the rate of mechanosensitive C-fibers with SA was similar in the KO and C57BL6. c) The percentage of the unclassified heat responsive afferents that had SA was higher in the incised C57BL6 mice compared to incised KO mice (b, $p < 0.03$, chi-square test). A significantly higher rate of SA was evident in the unclassified, heat responsive afferents from incised C57BL6 mice (d, $p < 0.05$, unpaired t-test). e) Example recording from a mechanosensitive C-fiber afferent with SA.

Figure 6.

Relationships between SA and heat sensitivity of unclassified afferents from incised C57BL6 mice. a: During this recording, a mechanically identified C-fiber afferent had no spontaneous activity and was not heat responsive. An unclassified afferent with SA could not be identified using a mechanical stimulus, had spontaneous activity and responded to heating at 35°C. b: The heat responses of an unclassified afferent without any spontaneous activity. The afferent responded at 41°C. c: Unclassified, heat responsive, spontaneously active afferents had a significantly lower heat threshold when compared with unclassified, heat responsive afferents without SA in the incised C57BL6 mice ($p < 0.001$, unpaired t-test). Pearson non-linear regression analysis was performed on heat threshold temperature of unclassified afferents and rate of SA. The skin temperature is negatively correlated with the rate of SA ($r, -0.5$; $p < 0.05$).

Figure 7.

Effects of cooling on SA. a: Example recording of an unclassified afferent with SA from incised WT mouse. Cooling the receptive field to 12–16°C completely and reversibly eliminated SA from this afferent. b: The effects of cooling on the SA of afferents from incised WT mice. The schematic shows time points chosen for counting before, during and after cooling. Before, 60s before the start of cooling; during, last 60s of cooling; and after, 60s from the point when the dermal temperature returned to the normal bath temperature. The difference between before and during cooling is significant ($p < 0.0002$, Wilcoxon matched pair test). Symbol shows mean response of 14 afferents.

Figure 8.

Mechano-sensitivity of cutaneous primary afferents from unincised and incised C57BL6 (a–d) and KO (e–h) mice. The mechanosensitivity is measured by computer operated feedback controlled force stimuli (tip diameter 0.7 mm). Each mechanical stimulus was 5s in duration and started from zero to 5, 10, 20, 40, 80, 120 mN. The inter-stimuli intervals were one minute. When an afferent produced a response to a particular force controlled ramp, it received three more ascending series of stimuli. To avoid fatigue, in no cases, more than three suprathreshold stimuli were applied; for example, if an afferent produced a response during the 20mN ramp, it received the 40, 80 and 120mN ramp. The total number of spikes was calculated over the

entire 5s of the stimulus. The number of fibers is shown in the parenthesis. Statistical significance is assessed by a two-way ANOVA for KO vs C57BL6.

Table 1
Fiber Classification of Mouse Glabrous Skin Afferents*

Animal type	Fiber type	Number Recorded	Mechano-sensitive	Heat+	Cool+
WT sham	A	38	+	0/16	1/8
	C	35	+	12/20	0/9
KO sham	unclassified	10	-	10/10	0/10
	A	28	+	1/11	0/2
	C	38	+	14/30	2/13
	unclassified	9	-	9/9	0/9
WT 1d incision	A	24	+	2/12	1/6
	C	36	+	18/28	2/19
KO 1d incision	unclassified	19	-	19/19	0/19
	A	20	+	1/20	1/15
	C	21	+	15/21	2/8
	unclassified	13	-	13/13	0/13
	Total	291			

Abbreviation: +, sensitive, -, not sensitive, 1d, one day after plantar incision, WT, wild type C57BL/6 mice, KO, TrpV1 knockout mice

*. Rapidly adapting fibers, cold and warm fibers were not tested. For details see materials and methods.