Cinnamaldehyde elicits itch behavior via TRPV1 and TRPV4 but not TRPA1

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Abstract

Introduction: Cinnamaldehyde (CA) elicits itch sensation in humans. We investigated if CA elicits scratching behavior in mice and determined the roles for TRPV1, TRPA1, and TRPV4.

Materials and Methods: Scratching behavior elicited by intradermal injection of CA was assessed in wildtype (WT) mice and knockout (KO) mice lacking TRPV1, TRPA1, TRPV4, or deficient in mast cells. We also assessed scratching and wet dog shakes elicited by low-threshold mechanical stimulation of skin treated topically with CA or vehicle. Using calcium imaging we tested if CA activates dorsal root ganglion (DRG) neurons of each genotype.

Results: Intradermal cheek injection of CA elicited dose-dependent hindlimb scratch bouts, with fewer forelimb wipes and facial groom bouts that were not dose-dependent. CA elicited significantly fewer scratch bouts in TRPV1 and TRPV4 KO mice, but not TRPA1KOs, compared with WTs. There were no sex differences across genotypes. The histamine H1 antagonist cetirizine did not affect CA-evoked scratching, which was normal in mast cell deficient mice, indicating lack of histamine involvement. Scores for alloknesis were significantly greater following topical application of CA compared with vehicle. Post-CA alloknesis scores were significantly higher in TRPV4KOs of both sexes and in female TRPV1 and TRPA1KOs, compared with WTs. Low threshold mechanical stimuli also elicited significantly more wet dog shakes in mice treated topically with 20% CA, with significantly fewer in TRPV1, TRPA1, and TRPV4KOs compared with WTs. In calcium imaging studies, CA excited 24% of WT DRG cells, significantly fewer (11.5%) in cells from TRPV4KOs, and none in TRPA1KOs. Responses of cells of all genotypes exhibited significant sensitization to repeated CA stimulation. Sensitization was significantly enhanced by IL-4, which itself excited 16% of WT DRG cells and none from TRPA1KOs.

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Discussion: The results indicate that TRPA1 is dispensable for CA-evoked scratching, which depends partly on TRPV1 and TRPV4.

Keywords

itch; cinnamaldehyde; scratching; TRPA1; TRPV1; TRPV4

It has been estimated that itchy skin conditions such as atopic dermatitis or psoriasis affect upwards of 10% or more of the general population with associated annual health care and economic costs in the billions of dollars[1–5]. There is thus a pressing need to better understand itch mechanisms to develop more effective antipruritic therapies. A variety of chemicals elicit itch when delivered to the skin[6–10]. Two major types of itch have been identified: histamine-dependent itch, that is thought to require the thermosensitive TRP channel TRPV1[11,12], and nonhistaminergic itch that requires TRPA1[13,14]. The TRPA1 agonist cinnamaldehyde (CA)[15,16] was recently reported to elicit a sensation of itch when applied topically to the skin[17,18]. However, topical CA also reduced histamine-evoked itch[19], and is associated with pain. Topical CA elicited an equal or greater incidence of warmth and burning pain sensations compared with itch[19,20], as well as thermal hyperalgesia[21–23] and enhancement of spinal dorsal horn neuronal responses to noxious heat[24]. Moreover, CA excited nociceptive neurons in trigeminal subnucleus caudalis[25,26]. Interestingly, topical delivery of the TRPV1 agonist capsaicin to human skin initially elicited a sensation of itch followed by burning pain[27], and topical application of menthol elicited itch in some subjects[20], suggesting that some TRP channel agonists initially activate pruriceptors followed later by nociceptors.

More recently, the osmosensitive ion channel TRPV4 has also been implicated in both histaminergic and nonhistaminergic types of itch[28–30] and TRPC4 has been implicated in itch elicited by α-methyl-5-HT[31]. The primary aim of the present study was to investigate if CA elicits itch-related and/or pain-related behavior in mice, and if so, if CA-evoked behavioral responses involve TRPV1, TRPA1, and/or TRPV4 activity. We additionally investigated if there is a sex difference in CA-evoked behaviors. Finally, we also recorded calcium responses of dorsal root ganglion (DRG) neurons from wildtype (WT) and knockout (KO) mice lacking TRPV1, TRPA1, or TRPV4 to determine if sensory neuron activation by CA is consistent with the behavioral data.

Materials and methods

All experiments were approved by the UC Davis Institutional Animal Care and Use Committee. WT C57Bl/6J and TRPV1KO mice of both sexes, and male Sash (cKit[W-Sh]) mice, were purchased from Jackson Laboratories. TRPA1KO mice were a generous gift from Dr David Julius, UC San Francisco. TRPV4KO mice were originally obtained from Riken, Japan and kindly transferred to our laboratory by Dr Hongzhen Hu, Washington University, St. Louis.

Behavioral experiments

All mice were habituated to the recording arena for 3 days before testing. We used the cheek model[32,33]. Fur on the cheek was shaved 1 week before testing. CA (1%–5% in 10% Tween-80; 10%–20% in 5% or 10% Tween-80) was injected intradermally (id) in the cheek in a volume of 10 μL and animals were then videotaped for 40–60 minutes in a clear enclosure with angled mirrors to allow multiple views of the face. Videotapes were viewed offline by at least 2 blinded reviewers, who scored the number of hindlimb scratch bouts directed to the injected cheek, number of forelimb wipes across the injected cheek, and number of facial grooming bouts. These behavioral responses are described in detail elsewhere[32,33]. WT mice served as controls and were age-matched to the corresponding TRPV1, TRPA1, and TRPV4 knockouts and Sash mice. Data were analyzed by 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, with sex and genotype as factors. To test for dose-dependency, WT mice were injected id with vehicle (5% Tween-80), 1% CA, 2.5% CA, or 5% CA. Dose-response data in WTs were analyzed separately by sex using 2-way ANOVA with Bonferroni post hoc test.

To test for alloknesis and wet dog shakes, the rostral back was treated with topical application of 20% CA or vehicle (10% Tween-80). At 5-minute intervals after treatment, the rostral back skin was then stimulated with a von Frey filament (0.7 mN bending force) delivered 5 times in succession[34]. We recorded if a hindlimb scratch bout was directed to the stimulus site immediately following each von Frey filament application. An alloknesis score was calculated as the total number of immediate von Frey-evoked scratch bouts directed to the stimulus site (maximum alloknesis score = 5). We noticed that the stimulus often elicited wet dog shakes, either alone or following a hindlimb scratch bout, and these were also counted. These shakes were rapid movements similar to a dog shaking water off of its back. We compared the raw counts of touch-evoked scratch bouts or wet dog shakes between sex and genotypes using a 2-way ANOVA with Bonferroni post-test with $P < 0.05$ taken to be significant.

To test the antihistamine cetirizine, it was dissolved in saline and administered ip in doses of either 1.5 or 15 mg/kg. CA (5%) was injected in the cheek 15 minutes after cetirizine administration.

Calcium imaging

Solutions and chemicals—The standard extracellular solution used in all experiments contained (in mM) NaCl, 140; KCl, 4; CaCl$_2$, 2; MgCl$_2$,1; HEPES, 10; NaOH, 4.54; and glucose, 5 (pH 7.4 at 25°C). Chemicals for application were diluted from the following stock solutions: CA 200 mM in ethanol, allyl isothiocyanate (AITC) 100 mM in DMSO, capsaicin 5 mM in ethanol, histamine dihydrochloride 100 mM in water, GSK1016790A 30 μM in DMSO, and recombinant Murine IL-4 300 μM in water. All compounds were from Sigma except AITC (Fluka) and IL-4 (Peprotech). The concentrations of applied chemicals were: CA 300 μM, AITC 100 μM, capsaicin 200 nM, histamine 50 μM, IL-4 300 nM, GSK1016790A 35 nM, KCl 50 mM.

Mouse DRG culture—Experiments were performed using adult male WTs and TRPV1, TRPA1, and TRPV4KOs (18–34 g) under a protocol approved by the UC Davis Animal Care and Use Committee. Mice were euthanized by inhalation of increasing levels of CO_2. DRGs from all spinal levels were excised from adult male mice, transferred to HBSS containing 50 μg/mL gentamicin, treated with 2 mg/mL collagenase and 3 mg/mL dispase for 1 hour. DRG cells were suspended in MEM with Earle's balanced salt solution (Gibco, Life Technologies, Carlsbad, CA) containing 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Life Technologies), 1 × vitamin (Gibco, Life Technologies) and 10% horse serum (Quad Five, Ryegate, MT); plated on poly-d-lysine-coated (200 μg/mL) glass coverslips. NGF at 100 ng/mL (Alomone Labs, Tel Aviv, Israel) was added and cells were cultured for 16–24 hours.

Intracellular ratiometric calcium imaging—DRG cells were incubated in extracellular solution with Fura-2 AM (Life Technologies) 3 μM, 30 minutes at 37°C, containing also 0.02% Pluronic (Invitrogen) and left to recover for about 10 minutes in extracellular solution before recording. Coverslips were mounted on a custom-made aluminum perfusion block and viewed through an inverted microscope (Nikon TS100, Technical Instruments, Burlingame, CA). Fluorescence was excited by UV light at 340 and 380 nm alternately, and emitted light was collected via a CoolSNAP camera attached to a Lambda LS lamp and a Lambda optical filter changer (Sutter Instrument, Novato, CA). Ratiometric measurements were made using NIS-Elements Imaging Software (Nikon Instruments Inc.) and images were acquired at a rate of 0.5–1 Hz. The background intensity was subtracted before calculating the ratio between the fluorescence emitted when the dye was excited at 340 nm and at 380 nm (F340/F380 nm). Changes in fluorescence ratio of 10% or higher were considered responses. The area under the curve of calcium responses was calculated using a MATLAB custom script and compared across groups by ANOVA with genotype and sex as dependent variables.

Results

Scratching behavior

Intradermal cheek injection of CA elicited a significant dose-dependent increase in scratch bouts, but no dose-dependent increase in the number of forelimb wipes or grooming bouts, in WT mice (Fig. 1). The number of CA-evoked wipes was within the range of that reported previously for capsaicin, mustard oil, and bradykinin[33]. There was a significant effect of CA drug concentration on scratch bouts, but no overall effect of gender. Post hoc tests revealed significant differences between saline versus 5% CA for both female and male mice (< 0.05 and <0.01, respectively). Counts of neither scratch bouts nor wipes increased further at higher doses of CA (20% CA: 65.2 ± 16.6 SEM scratch bouts, 19.8 ± 3.9 wipes/40 min, n = 8 male WTs). Topical application of CA to the cheek did not elicit hindlimb scratching, forelimb wiping or grooming above levels elicited by vehicle (ie, <10 scratch bouts, wipes or groom bouts/40 minutes at 30% and 40% CA; n = 8 male WTs).

We then investigated if CA (5%) elicited scratching behavior in TRPV1, TRPA1, and TRPV4KO mice. Figure 2A shows that 5% CA elicited equivalent scratching in female

and male WT mice, as well as in TRPA1KO and male Sash (mast cell-deficient) mice that was significantly greater than that elicited by vehicle injections. Importantly, scratching was significantly reduced by ~50% in TRPV1 and TRPV4KOs (Fig. 2A) compared with WTs ($P < 0.05$, ANOVA). CA did not elicit wipes or groom bouts (Figs. 2B, C) above levels elicited by vehicle, and there were no significant differences by sex or genotype.

Cetirizine

To assess if CA-evoked scratching involves histamine release, we tested the effect of systemic administration of the histamine antagonist ceterizine at a low (1.5 mg/kg) and high (15 mg/kg) dose in WT mice. Neither dose of ceterizine significantly affected the number of scratch bouts elicited by intradermal injection of 5% CA.

Alloknesis (mechanical itch)

We tested if alloknesis occurs following topical application of 20% CA to the rostral back. In WTs, von Frey stimulation within the CA application area elicited significantly more hindlimb scratch bouts (Fig. 3A) compared with vehicle (10% Tween-80) and no treatment for both sexes (Fig. 3B) ($P < 0.05$, ANOVA). Similarly, alloknesis scores were significantly greater following 20% CA compared with vehicle application in TRPV1, TRPA1, and TRPV4KOs (Figs. 3A, B). There were no significant differences in alloknesis scores between sexes of any genotype. Compared with WTs, alloknesis scores after 20% CA were significantly higher for TRPV4KOs of both sexes, and for female TRPV1 and TRPA1KOs (Fig. 3A).

Wet dog shakes

When testing for alloknesis following topical application of 20% CA, von Frey stimulation frequently elicited wet dog shakes. The wet dog shakes occasionally occurred immediately following a stimulus-evoked hindlimb scratch, but more often in the absence of a scratch bout. For all genotypes, mice of both sexes exhibited significantly more stimulus-evoked wet dog shakes following application of 20% CA (Fig. 4A) compared with vehicle or no treatment (Fig. 4B) ($P < 0.01$, ANOVA).

TRPV1, TRPA1, and TRPV4 KOs of both sexes exhibited significantly fewer von Frey-evoked wet dog shakes compared with WTs (Fig. 4, *$P < 0.05$, ANOVA). The observations that CA application resulted in significantly more von Frey-evoked wet dog shakes compared with vehicle, and that animals lacking TRPV1, TRPA1, or TRPV4 exhibited significantly fewer wet dog shakes compared with WTs, suggest that CA elicits some kind of irritant sensation triggering touch-evoked wet dog shakes in a manner that partly involves these TRP channels.

The time courses of von Frey-evoked alloknesis and wet dog shakes appeared to be fairly stable across the 60-minute observation period.

Calcium imaging—A total of 621 DRG cells from WT mice were tested with CA and other chemicals; the percentages of responsive cells are given in Table 1. CA excited ~24% of WT DRG cells (Table 1). An example of CA-evoked responses of DRG cells from WT

mice is shown in Figure 5. Seven of 8 cells exhibited an equal or increased response to a second CA application; one DRG cell (green line in Fig. 5) showed a reduced response. All CA-sensitive cells also responded to AITC, and 2 additionally responded to subsequent capsaicin. The relatively low number of capsaicin-sensitive WT cells (Table 1) may reflect cross-desensitization by AITC which was always tested before capsaicin.

A significantly higher proportion of DRG cells from TRPV1KO mice responded to CA; none responded to capsaicin (Table 1) confirming the absence of TRPV1. CA is an agonist of TRPA1; as expected, no cells from TRPA1KO mice responded to CA and very few responded to AITC, confirming absence of TRPA1 (Table 1). This is consistent with the behavioral observation that CA-evoked scratching was independent of TRPA1. A significantly lower proportion of cells from TRPV4KO mice responded to CA (Table 1), consistent with the behavioral data showing reduced CA-evoked scratching in TRPV4KO mice. Only a small number of cells (1.5%; 5/340) from TRPV4KOs responded to the TRPV4 agonist, GSK1016790A, supporting the absence of TRPV4.

Some CA-sensitive DRG cells also responded to AITC, histamine, capsaicin and IL-4. As shown in Table 1, there was a significant increase in the proportion of DRG cells from TRPV1KOs that responded to AITC and IL-4. In DRG cells from TRPA1KOs, significantly lower proportions responded to AITC or histamine and none responded to IL-4. Finally, there was a significantly lower incidence of capsaicin responsiveness in DRG cells from TRPV4KOs (Table 1).

CA sensitization and desensitization—We calculated the area under the curve of the responses of 76 DRG cells from WT mice to 2 successive applications of CA (CA1 and CA2; normalized to CA1); 73 cells exhibited a larger response ($> 10\%$) to CA2 (Fig. 6; left blue bar). One cell exhibited no change and 2 a decreased CA2 response ($> 10\%$). Similarly, 23/25 cells from TRPV1KO and 7/8 cells from TRPV4KO mice exhibited increases in CA2 of $> 10\%$, with the remainder unresponsive (n = 2) or reduced (n = 1). Mean responses to CA2 were significantly greater for cells from TRPV1 and TRPV4KOs (Fig. 6; middle and right blue bars; $P < 0.05$, ANOVA).

IL-4: CA is a contact sensitizer that triggers a CD-1-restricted T-cell response[35]. Th2 cells release IL-4, among other cytokines, to induce an allergic reaction[36]. We reasoned that CA might cause immune cells to release IL-4, which then excites itch-signaling sensory neurons[37]. We therefore tested if IL-4 activates DRG cells. IL-4 activated 15.8% of DRG cells from WTs, 40.3% from TRPV1KOs and 13.5% from TRPV4KOs (Table 1). Notably, IL-4 did not activate any cells from TRPA1KOs. Most IL-4-sensitive DRG cells also responded to CA, and many also responded to other chemicals tested. Examples of WT cell responses to IL-4 are shown in Figure 7.

We tested if IL-4 further increased sensitization of DRG cell responses to CA. IL-4 was applied immediately before CA2, and resulted in a significantly greater sensitization of responses to CA2 in DRG cells from WT and TRPV1KOs, but not TRPV4KOs (Fig. 6, red bars).

Following application of CA2, AITC was tested either preceded by IL-4 or not. In CA-sensitive cells from WTs, the response to AITC following IL-4 and CA2 was significantly lower compared with the response to AITC following CA2 in the absence of IL-4 ($P <$ 0.01, ANOVA; n = 42 and 76, respectively). The same was true for CA-insensitive cells from WTs ($P <$ 0.01, ANOVA; n = 20 and 36, respectively). The same was also true for CA-sensitive (but not CA-insensitive) cells from TRPV1KOs ($P <$ 0.01, ANOVA; n = 25 and 31, respectively).

Similarly, responses to capsaicin were significantly lower when preceded by CA and IL-4, compared with responses preceded by CA in the absence of IL-4 ($P <$ 0.05, ANOVA, n = 10 and 17, respectively). However, application of IL-4 did not significantly affect responses to capsaicin in CA-insensitive cells or in cells from TRPV4KO mice.

IL-4 did not significantly affect responses of CA-sensitive WT cells to histamine, compared with histamine-evoked responses when IL-4 was not tested (n = 17 and 31, respectively). The same was true for CA-insensitive cells from WTs (n = 14) as well as CA-sensitive and CA-insensitive cells from TRPV4 and TRPV1KOs (n = 5–15). This suggests that IL-4 did not sensitize responses to histamine.

Discussion

The present results using the mouse cheek model show that intradermal injection of CA elicited dose-related hindlimb scratching, but no dose-dependency for forelimb wiping or facial grooming. There was no sex difference in behavioral responses to CA. In addition, topical application of CA resulted in significant increases in touch-evoked scratch bouts (alloknesis or mechanical itch) as well as wet dog shakes. These findings are consistent with human studies showing that topical application of CA elicits itch sensation and alloknesis[17,18]. CA-evoked scratching was significantly lower in TRPV1 and TRPV4KOs, but surprisingly not in TRPA1KOs. In correlative cellular calcium imaging studies, CA excited DRG cells in a TRPA1-dependent manner, and excited significantly fewer cells from TRPV4KOs partly consistent with the behavioral data.

CA-evoked itch and alloknesis (mechanical itch)

Our results are consistent with a human study showing that topical CA (5%) elicited moderate itch sensation that peaked by ~6–8 minutes and then declined, accompanied by alloknesis (itch to brush or low-threshold von Frey stimuli) and pin-prick hyperknesis[18]. There was no sex difference in CA-evoked itch intensity. Consistent with these findings, we observed a dose-related increase in CA-evoked scratching in mice, as well as a significant increase in von Frey-evoked scratch bouts (alloknesis or mechanical itch) in skin topically treated with CA, with no significant sex difference.

The mechanism underlying CA-evoked itch does not appear to involve mast cell degranulation or histamine release in the skin, since the evoked scratching was not significantly affected by the histamine H1 antagonist ceterizine and was not significantly different between mast cell-deficient mice and WTs.

CA-evoked scratching was significantly lower in TRPV1 and TRPV4KOs compared with WTs, suggesting a partial role for these ion channels in mediating CA-evoked itch. TRPV4 in DRG cells was suggested to form a heteromeric complex with TRPV1 to mediate histamine-evoked and chloroquine-evoked itch behavior[30], which might explain the partial requirement of both TRPV1 and TRPV4 for CA-evoked scratching behavior as presently observed. Importantly, CA-evoked scratching in TRPA1KOs was not significantly different from WTs, indicating that TRPA1 is not required for itch elicited by CA even though CA is a TRPA1 agonist.

In contrast to the reduced scratching observed in TRPV1 and TRPV4KOs, alloknesis scores following topical application of CA were significantly higher in TRPV4KOs of both sexes and in female TRPV1 and TRPA1KOs. Thus, it is conceivable that CA acts via TRPA1 expressed in sensory nerve endings[15] or keratinocytes[38] to enhance alloknesis by some unknown mechanism. The spinal pathway for alloknesis has recently been elucidated. Spinal neurons expressing neuropeptide Y (NPY) inhibit mechanical itch[39] while neurons expressing the NPY-1 receptor, which partially overlaps with Ucn$^+$ neurons[40], promote mechanical itch[41]. NPY and NPY-1/Ucn3 expressing neurons receive low-threshold mechanoreceptor input, with inhibition normally dominating to prevent mechanical itch. Activation of Merkel cell/slowly adapting type I mechanoreceptors expressing the mechanotransduction channel piezo2 inhibits alloknesis in chronic itch models, and loss of Merkel cells/piezo2 increases alloknesis[42]. Conceivably, CA may act peripherally to excite pruriceptive nerve endings, but simultaneously to enhance alloknesis via excitation of TRPA1-expressing sensory nerves or keratinocytes. The mechanism is currently unknown, but speculatively may involve reduced mechanoreceptor input (eg, inhibition of slowly adapting type I fibers) which disinhibits NPY interneurons to thereby excite NPY-1 receptor-expressing neurons and thus open the mechanical itch gate. It remains unexplained why alloknesis is increased in TRPV1 and TRPV4KOs, unless this reflects a developmental compensatory increase in TRPA1 expression in neurons lacking TRPV1 and/or TRPV4.

Wet dog shakes

Wet dog shakes are part of the opioid withdrawal syndrome[43] and are triggered by administration of the cooling agent icilin[44,45] and intra-cerebroventricular injection of thyrotropin releasing hormone[46,47]. We previously reported that intradermal injection of 5-HT in rats elicited wet dog shakes that correlated with scratching behavior[48]. We presently observed that low threshold mechanical stimuli elicited wet dog shakes, the number of which was greater following topical application of 20% CA compared with vehicle (Fig. 4). Von Frey stimuli frequently elicited hindlimb scratch bouts followed by wet dog shakes. Assuming that von Frey-evoked scratches reflect alloknesis, perhaps the evoked wet dog shakes do also. Compared with WTs, the number of von Frey-evoked wet dog shakes following application of CA was significantly lower in TRPV1, TRPV4, and TRPA1KOs of both sexes (Fig. 4A), although still significantly greater than in control animals (Fig. 4B). The application of vehicle alone did not elicit any more wet dog shakes compared with the no treatment (dry fur) group (Fig. 4B). We speculate that the increase in numbers of wet

dog shakes following CA application is thus not due to wetness, but rather to an irritant sensation, possibly itch, mediated by TRPV1, TRPV4, and TRPA1.

Calcium imaging

We were interested in testing if CA excited DRG cells in a manner consistent with the behavioral data. CA excited 24% of WT DRG cells, which compares favorably with 17.8% and 13.5% of CA-sensitive mouse DRG and trigeminal ganglion (TG) cells, respectively (E. Carstens, T. Akiyama, M. Iodi Carstens, unpublished observations) and 10.9% and 18.2%–19% of rat DRG and TG cells[49,50]. CA did not excite any cells from TRPA1KOs consistent with CA being a TRPA1 agonist[15]. CA excited significantly fewer (11.5%) cells from TRPV4KOs, consistent with the reduced scratching in this genotype. However, CA excited nearly 40% of cells from TRPV1KOs, which is inconsistent with the significantly lower CA-evoked scratch bouts in this genotype. Since CA (and also AITC and IL-4) excited significantly more cells from TRPV1KOs, one possible explanation is a developmental compensation in which TRPA1 is upregulated in DRG cells of TRPV1KO mice. Capsaicin also excited significantly more cells from TRPA1KOs than WTs, suggesting that TRPV1 may be upregulated in cells lacking TRPA1. However, capsaicin activated a relatively low percentage of WT DRG cells possibly due to desensitization from prior CA and AITC applications.

There was a significant sensitization of DRG cell responses to a second application of CA in WTs and TRPV1 and TRPV4KOs (Fig. 6). We previously reported that rat TG cells' responses to CA exhibited desensitization to repeated application of high (400 μM) but not lower (200 μM) concentrations[49]. The present study used an intermediate concentration (300 μM) that elicited mainly sensitization in mouse DRG cells. In WTs, AITC excited about the same proportion of CA-sensitive DRG cells as CA itself. However, the 2 applications of CA followed by AITC may have cross-desensitized subsequent responses to capsaicin (Table 1) as noted above. In support, repeated application of AITC at short interstimulus intervals elicited a desensitizing pattern of oral irritation, and AITC cross-desensitized capsaicin-evoked irritation[51].

CA-evoked scratching was normal in TRPA1KOs, and thus must have an underlying mechanism that is independent of TRPA1 expressed in sensory neurons or other skin cells. We reasoned that CA activates Th2 cells[35,36] to release cytokines including IL-4 that in turn excite sensory neurons[37]. Indeed, 16% of WT DRG cells were excited by application of IL-4. However, IL-4 did not excite any cells from TRPA1KOs (Table 1). This rules out IL-4 as the mediator of CA-evoked itch which was independent of TRPA1. The exact mechanism underlying CA-evoked itch thus remains unknown. Nevertheless, we were interested to test if IL-4 further sensitized responses of DRG cells to CA or other mediators. Indeed, application of IL-4 before the second application of CA resulted in a further significant sensitization in cells from WTs and TRPV1KOs (Fig. 2). In contrast, IL-4 generally desensitized responses to subsequent application of AITC and capsaicin in CA-sensitive cells from WTs and TRPV1KOs, possibly due to the large calcium response elicited by the prior applications of CA and IL-4. We presently did not observe any

sensitizing effect of IL-4 on responses of DRG cells to histamine, in contrast to a recent report[37].

Methodological issues

Cultured DRG cells are often used as a proxy for peripheral nerve endings, although there are limitations. Cultured DRG cells are axotomized and may not express the same proportions of cell surface proteins as in the nerve endings. This limitation is partly overcome by newer in vivo calcium imaging methods using, for example, GCaMP expression[52]. Moreover, the relationship between calcium influxes and action potentials in DRG cells cannot be directly inferred[53]. Nevertheless, calcium imaging of cultured DRG cells remains a common method to assess the excitability of sensory neurons to chemical agents.

Another issue regards the difference between intradermal injection versus topical application of chemical agents. Clearly intradermal injection delivers the intended concentration of an agent close to the targeted tissue (nerve endings, keratinocytes) much more rapidly compared with topical application. For example, a study measuring skin penetration of topically applied 8% capsaicin reported concentrations of 0.4% and 0.6% of the applied amount in epidermis and dermis, respectively, after 30 minutes[54]. CA is more complex as it is converted to cinnamyl alcohol and cinnamic acid in the skin[55,56] and penetration depends on the delivery vehicle[57]. For topical CA application we used Tween-80 which was estimated to result in a transdermal flux of <20 $\mu g/cm^2$ during the first hour. In our experiments, topical application of 20% CA was insufficient to elicit any scratching or wiping beyond levels elicited by vehicle application, yet presumably resulted in an intradermal concentration of cinnamic acid sufficient to induce alloknesis. In this regard, it is currently not well understood what local concentrations of mediators exist in the skin in close proximity to pruriceptors under pathophysiological conditions, nor the unknown contributions of immune mediators such as IL-4, 13, 31, 33, etc. More data would be very useful in determining the role of endogenous mediators and neuroimmune interactions in the peripheral sensitization of pruriceptors that might contribute to chronic itch.

A final issue is the dissociation of itch and pain when both sensations might coexist, given the role of CA in both itch as well as pain and hyperalgesia. We can only reiterate that itch-related hindlimb scratching behavior increased significantly in a dose-related manner by CA, whereas pain-related forelimb wiping did not (Fig. 1), supporting the notion that these behavioral indices can distinguish between itch and pain in the cheek model.

In conclusion, CA elicits signs of itch (directed scratching, alloknesis) in mice via a mechanism that is independent of TRPA1 and partly requires TRPV1 and TRPV4. The cytokine IL-4 excited CA-sensitive DRG cells but did not appear to mediate CA-evoked itch since it acts via TRPA1. While the exact mechanism of CA-evoked itch remains to be determined, CA may be added to the list of mediators that elicit itch in humans and itch-related scratching behavior in rodents[32,33].

Sources of funding

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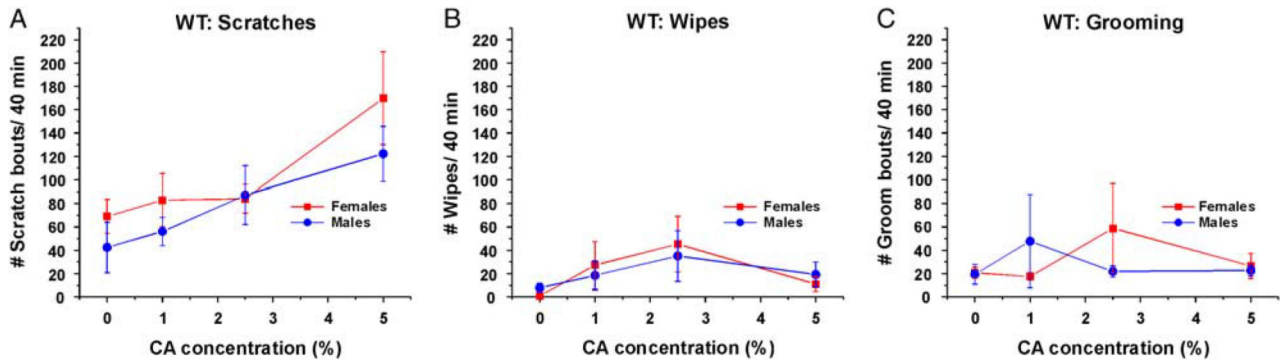
Figure 1.
CA elicits dose-dependent scratching. A, Dose-related increase in scratch bouts elicited by CA injected in the cheek. The increase in scratch bouts was significant ($P < 0.05$, 0.01 for females and males, respectively, analysis of variance followed by post hoc Bonferroni test). There was no significant sex difference. B, CA elicited a low number of forelimb wipes that was not dose-related and there was no sex difference. C, There was no dose-related change in the number of groom bouts and no sex difference. CA indicates cinnamaldehyde; WT, wildtype.
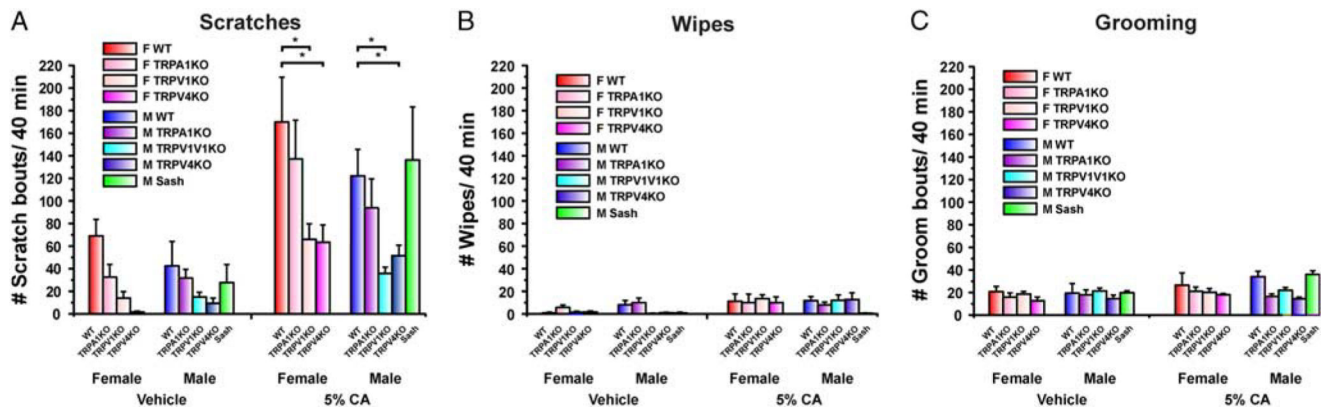
Figure 2.
Scratching elicited by id CA, but not wiping or grooming, was significantly lower in TRPV1 and TRPV4 KO mice of both sexes. A, Scratch bouts elicited by id CA or saline vehicle in different genotypes of female and male mice. CA elicited significantly more scratch bouts in both female and male WTs and TRPA1KOs, compared with vehicle controls. The number of CA-evoked scratch bouts was significantly lower in TRPV1 and TRPV4KOs compared with WTs of both sexes ($P < 0.05$, analysis of variance). B, Wipes. CA elicited low numbers of forelimb wipes that did not differ significantly by sex or genotype. C, Groom bouts elicited by CA did not differ significantly by sex or genotype. CA indicates cinnamaldehyde; WT, wildtype.
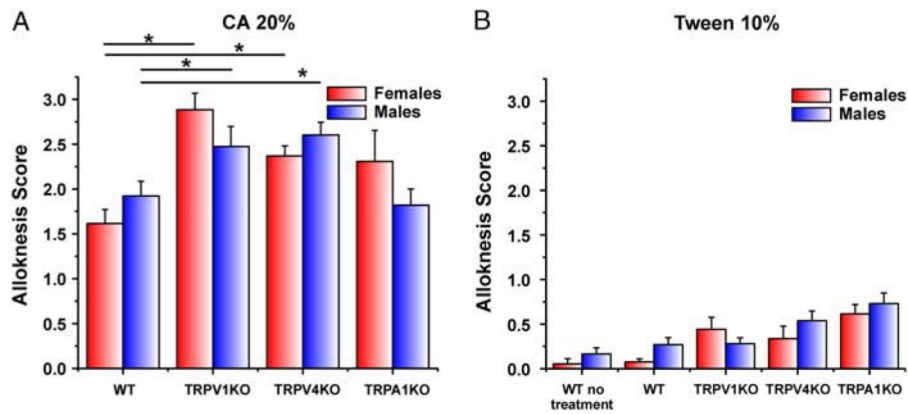
Figure 3.
Topical application of CA enhanced alloknesis. A, Topical application of CA (20% in 10% Tween) resulted in significant increases in alloknesis scores in TRPV4KOs of both sexes and in female TRPV1 and TRPA1KOs. *Significantly different from WT ($P < 0.05$, analysis of variance). B, Vehicle (10% Tween). There was no significant difference in alloknesis scores across genotypes. For all genotypes, alloknesis scores were significantly higher in CA compared with vehicle and no treatment groups ($P < 0.0$, analysis of variance). CA indicates cinnamaldehyde; WT, wildtype.
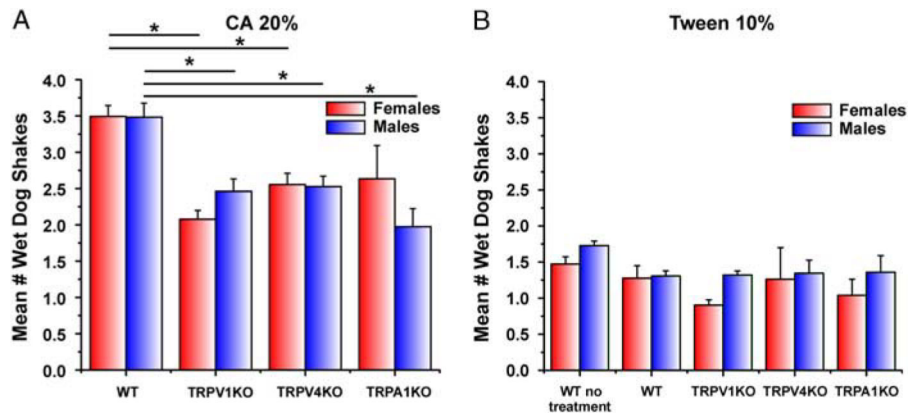
Figure 4.
Wet dog shakes. A, CA (20% in 10% Tween) was applied topically to the rostral back, followed by application of a weak von Frey filament 5 times in succession, every 5 minutes for 1 hour (same as test for alloknesis shown in Fig. 3). Graph plots number of touch-evoked wet dog shakes by sex and genotype groups. *Significantly fewer wet dog shakes in all knockout groups versus WTs ($P < 0.05$, analysis of variance). von Frey stimulus frequently elicited hindlimb scratch followed by wet dog shake. B, Vehicle (10% Tween) and no treatment controls. For all genotypes and both sexes, von Frey stimuli elicited significantly more wet dog shakes following topical application of CA compared with vehicle and no treatment ($P < 0.05$ in all cases, analysis of variance). CA indicates cinnamaldehyde; WT, wildtype.
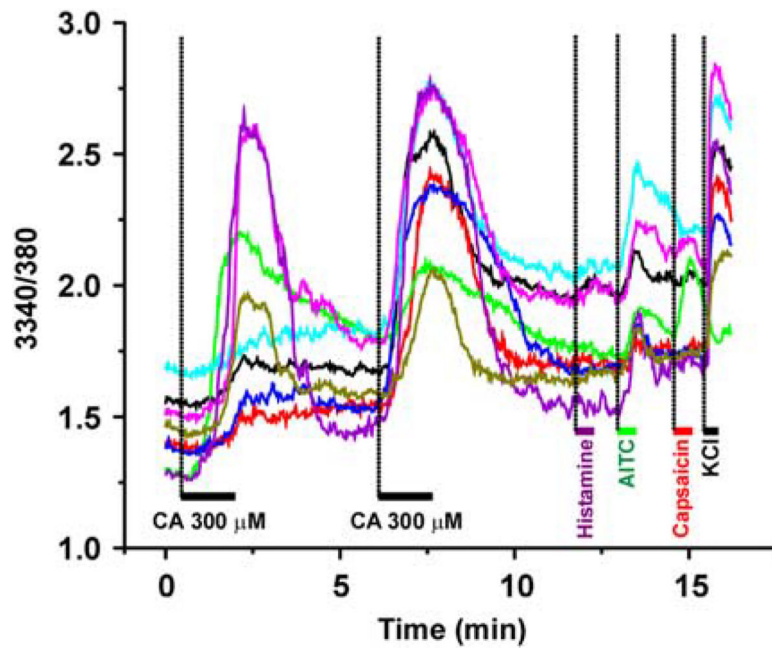
Figure 5.
Responses of dorsal root ganglion cells from WT mice to CA. Graph plots fluorescence ratio versus time. Bars with vertical dashed lines represents times of application of indicated chemicals. Each colored line represents a separate dorsal root ganglion cell. AITC indicates allyl isothiocyanate; CA, cinnamaldehyde.
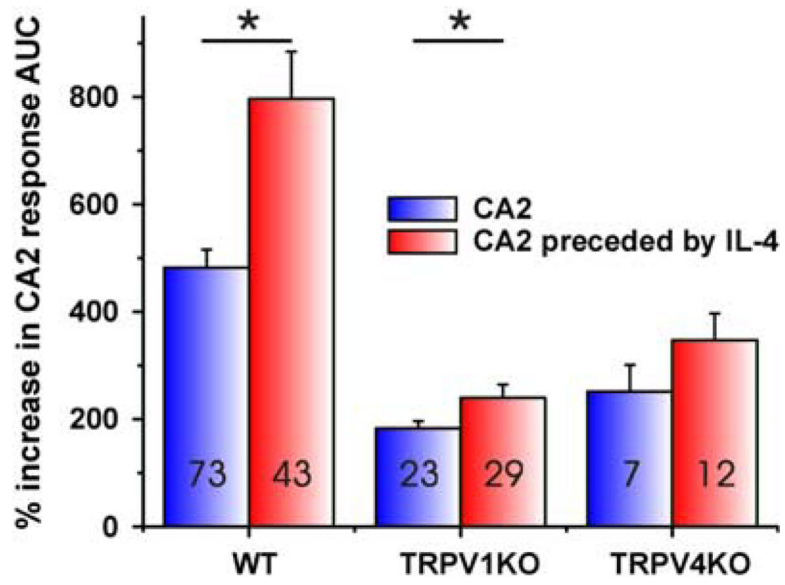
Figure 6.
Sensitization of CA-evoked responses of dorsal root ganglion cells. *Significant difference
($P < 0.05$, analysis of variance). AUC indicates area under the curve; CA, cinnamaldehyde;
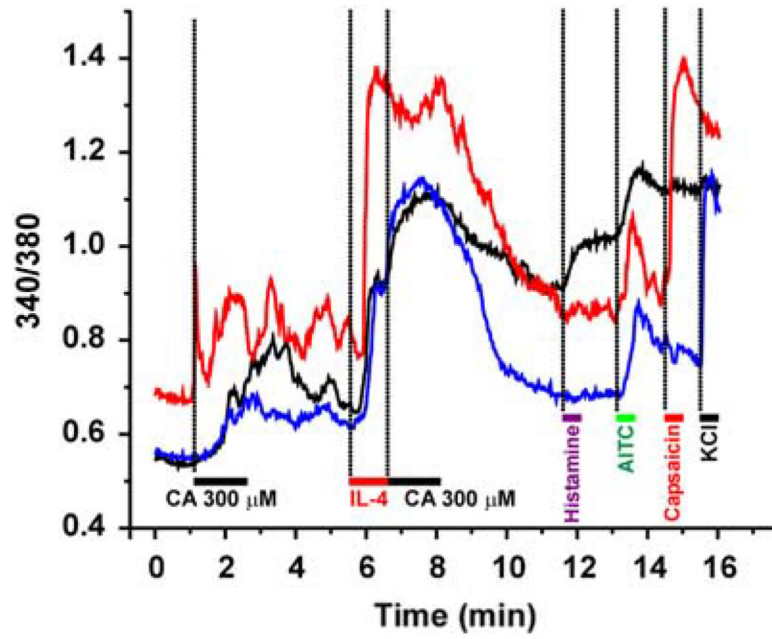WT, wildtype.

Figure 7.
Examples of wildtype dorsal root ganglion cell responses to IL-4 and other chemicals (format as in Fig. 5). AITC indicates allyl isothiocyanate; CA, cinnamaldehyde.

Table 1

Percentages of DRG cells from WT and KO mice excited by various chemicals.

Genotype	CA	AITC	Histamine	Capsaicin	IL-4
WT	23.99 (149/621)	21.9 (136/621)	5.8 (36/621)	7.6 (47/621)	15.8 (35/222)
TRPV1KO	39.9 * (131/328)	37.2 * (122/328)	8.8 (29/328)	0 * (0/328)	40.3 * (65/161)
TRPA1KO	0$ (0/326)	1.5$ (5/326)	1.5$ (5/326)	28.8$ (94/326)	0$ (0/326)
TRPV4KO	11.5# (39/340)	NT	5 (17/340)	3.5# (12/340)	13.5 (23/170)

Parentheses: number of responsive cells/tested.

AITC indicates allyl isothiocyanate; CA, cinnamaldehyde; NT, not tested.

*,$,# Significantly different from WT ($P < 0.05$, χ^2 test).