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TRPV4 is necessary for trigeminal irritant pain and functions as a cellular formalin receptor

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ABSTRACT

Detection of external irritants by head nociceptor neurons has deep evolutionary roots. Irritant-induced aversive behavior is a popular pain model in laboratory animals. It is used widely in the formalin model, where formaldehyde is injected into the rodent paw, eliciting quantifiable nocifensive behavior that has a direct, tissue-injury-evoked phase, and a subsequent tonic phase caused by neural maladaptation. The formalin model has elucidated many antipain compounds and pain-modulating signaling pathways. We have adopted this model to trigeminally innervated territories in mice. In addition, we examined the involvement of TRPV4 channels in formalin-evoked trigeminal pain behavior because TRPV4 is abundantly expressed in trigeminal ganglion (TG) sensory neurons, and because we have recently defined TRPV4's role in response to airborne irritants and in a model for temporomandibular joint pain. We found TRPV4 to be important for trigeminal nocifensive behavior evoked by formalin whisker pad injections. This conclusion is supported by studies with *Trpv4*$^{-/-}$ mice and TRPV4-specific antagonists. Our results imply TRPV4 in MEK-ERK activation in TG sensory neurons. Furthermore, cellular studies in primary TG neurons and in heterologous TRPV4-expressing cells suggest that TRPV4 can be activated directly by formalin to gate Ca^{2+}. Using TRPA1-blocker and *Trpa1*$^{-/-}$ mice, we found that both TRP channels co-contribute to the formalin trigeminal pain response. These results imply TRPV4 as an important signaling molecule in irritation-evoked trigeminal pain. TRPV4-antagonistic therapies can therefore be envisioned as novel analgesics, possibly for specific targeting of trigeminal pain disorders, such as migraine, headaches, temporomandibular joint, facial, and dental pain, and irritation of trigeminally innervated surface epithelia.

1. Introduction

The trigeminal sensory system enables vertebrate animals to detect a wide range of environmental stimuli, including noxious cues such as chemical irritants. Detection of environmental irritants by the trigeminal system subserves a sentinel function that has enhanced evolutionary fitness of the respective species. Although some progress has been made, the molecules that sense the chemical irritants and signal the noxious cue in the trigeminal system remain largely elusive.

Formalin is a prototypical irritant that has been formulated in aqueous solution so that it can be injected into tissue. In the study of pain, the formalin model has led to profound insights on analgesic potency of candidate drugs and to elucidation of signaling pathways related to pain [9,14,17,32,34,35]. Formalin is an electrophile and reacts with a variety of amino acids by nucleophilic addition. It also irreversibly cross-links proteins. These chemical properties of formalin have been viewed as contributory to tissue injury. Via these mechanisms, formalin can also directly irritate nerve terminals of nociceptor neurons. Together, these events are viewed as

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causal for pain behavior following formalin injection. Whether there are specific protein targets that are critical for formalin-evoked pain and their eventual identity is largely unknown. However, TRPA1 channels have been implicated as critical regulators of the formalin behavioral response as well as in the response to formalin and other electrophilic irritants in heterologous cellular systems [23,24]. This raises the question whether other molecules, in particular another TRP ion channel, could play a role in response to formalin.

Practically, extratrigeminal somatosensory exposure to formalin in humans virtually does not occur as a pain-eliciting or -facilitating condition [38], yet airborne trigeminal exposures facilitate headaches and local painful irritations of conjunctivae of the eyes, sinuses, and upper airways [25,29]. However, most reported applications of the formalin model is extratrigeminal, with formalin injection to the hind paw. Therefore, one of our main rationales was to adapt the formalin model to trigeminally innervated areas [5,22] in a mouse model. In terms of which mouse line to subject to trigeminal formalin injections, we decided to use *Trpv4*$^{-/-}$ mice so that the role of TRPV4 in trigeminal irritant pain could be assessed, based on our previous findings that TRPV4 was critical for airborne irritation in upper airways [19] and a key contributor to temporomandibular joint (TMJ) pain induced by arthritogenic irritant [8].

Our results demonstrate an essential role for TRPV4 in the trigeminal formalin response in vivo, in TRPV4 activation by formalin in trigeminal ganglion (TG) sensory neurons, and in heterologous cellular systems. Our findings reiterate the concept that TRPV4 forms a relevant target in trigeminal pain via its expression in TG sensory neurons.

2. Materials and methods

2.1. Animals

The pan-null phenotype of *Trpv4*$^{-/-}$ mice relies on excision of the exon encoding transmembrane domains 5 and 6. Mice were outcrossed to C57BL/6J background and genotyped by PCR [21]. *Trpa1*$^{-/-}$ mice (B6;129P-*Trpa1*^{tm1Kykw}/J) were from the laboratories of Drs Robert W. Gereau (Washington University) and Ru-rong Ji (Duke University). Male wild-type (WT) (C57BL/6J), *Trpv4*$^{-/-}$, and *Trpa1*$^{-/-}$ mice, 2 to 2.5 months of age, were used for all experiments.

Epidermal-specific, Tamoxifen (tam)-inducible *Trpv4* knockdown mice were created as previously described [26]. In brief, the *Trpv4* genomic locus was engineered so that loxP sites surrounded exon 13, which encodes TM5–6. This mutation was propagated in mice that were crossed to K14-CRE-ER^{tam} mice, so that ((*Trpv4*$^{lox/lox}$)X(K14-CRE-ER^{tam})) mice could be induced by tam administration via oral gavage for 5 consecutive days at 6 mg/day in 0.3 mL corn oil, at age 2 to 2.5 months of age, plus a one-time booster 2 weeks after the last application. Control animals received the same volume of corn oil. Efficiency of targeting was verified by qRT-PCR and immunohistochemistry for *Trpv4* expression in skin at the gene and protein level, respectively [26].

Male dominant-negative mitogen-activated protein kinase kinase (dnMEK) transgenic mice, 2 to 2.5 months of age, were used. The neuron-specific and pan-neuronal Tα1 α-tubulin promoter was used to drive the transgene [31]. We documented expression of dnMEK in TG sensory neurons [8].

Animals were housed in climate-controlled rooms on a 12/12 h light/dark cycle with water and standardized rodent diet available ad libitum. All animal protocols were approved by the Duke University IACUC in compliance with NIH guidelines.

2.2. Formalin-induced pain behavior and chemical injections

Mice were allowed to acclimate to a Plexiglas chamber for at least 30 min before testing, and they received 10 μL subcutaneous injection of 4% of formalin (diluted from an aqueous solution of commercial 37% formaldehyde with normal saline) through a 30-gauge needle or saline into the right whisker pad or into the right hind paw [22]. After injection, mice were immediately placed back in chamber, and rubbing behavior was recorded by a Panasonic videocamera for a 45 min observation period. The recording time was divided into 9 blocks of 5 min, and a nociceptive score was determined per block by measuring the time that the animals spent rubbing the injected area predominantly with the ipsilateral forepaw and rarely with hind paw for whisker pad injection, and the time spent licking, flicking, and lifting the injected hind paw. The rubbing behavior with forepaw is evoked by pain, which is distinct from itch behavior [33]. Behavioral analysis was conducted by observers blinded to genotype.

To investigate the effects of the specific TRPV4 inhibitor HC067047 or GSK205 on formalin-induced nociceptive behavior, mice received a single intraperitoneal administration of HC067047 (dissolved in 6% DMSO; Sigma) or received a single subcutaneous injection of GSK205 into the whisker pad (10 μL, dissolved in 4% DMSO) 15 min before formalin injection. For testing the effect of 4α-PDD (10 μL, dissolved in 4% DMSO), a specific agonist of TRPV4, on pain behavior, mice received a subcutaneous injection into the whisker pad. In addition, to investigate the effects of the specific MEK inhibitor U0126 on formalin-induced nociceptive behavior, mice received a single subcutaneous injection of U0126 into the whisker pad (10 μL, dissolved in 20% DMSO) 15 min before formalin injection. Control animals received the same volume of normal saline, or 4%, 6%, or 20% of DMSO.

2.3. Immunohistochemistry, morphometry analysis, and neural tracing

Routine procedures were followed [8]. Briefly, mice were perfused transcardially with 0.01 M phosphate-buffered saline followed by ice-cold 4% paraformaldehyde (PFA) at the experimental time point under study. Their TGs and whisker pad skin were dissected and postfixed in 4% PFA overnight, cryoprotected in 20% sucrose (48 h) and sectioned on a cryostat at 12 and 20 μm, respectively. Sections were blocked with 5% normal goat serum (Jackson) and incubated overnight with primary antibodies: rabbit anti-TRPV4 (1:300, Abcam), mouse anti-Keratin 14 (1:200, Santa Cruz), guinea pig anti-PGP 9.5 (1:500, Neuromics), rabbit anti-phospho-ERK (pERK, 1:250, Cell Signaling Technology), and mouse anti-pERK (1:600, Cell Signaling Technology; for double-labeling with TRPV4 in TG). Immunodetection was accomplished with secondary antibodies (AlexaFluor 594–conjugated goat anti-rabbit; AlexaFluor 488–conjugated goat anti-mouse or anti–guinea pig; all 1:500; Invitrogen) for 2 h, and coverslipped with Vectashield (Vector). Digital micrographs were acquired using a BX61 Olympus upright microscope equipped with a high-resolution CCD camera and with constant acquisition/exposure settings using ISEE software (ISEE Imaging Systems).

To track whisker pad skin innervation of TRPV4 and/or pERK expressing TG neurons, mice were injected with 1.5 μL of neural tracer Fast Blue (2% aqueous solution, Polysciences) into the whisker pad 2 days before normal saline or 4% formalin administration. At 5 min after the last injection, mice were perfused, and tissues were immunostained as described above.

For every animal under study, 4 to 6 TG sections were analyzed per mouse; neurons were identified by morphology. The cutoff density threshold was determined by averaging the density of 3 neurons per section that were judged to be minimally positive by using ImageJ software. All neurons for which the mean density

exceeded the threshold >25% were judged as positive. Positive cells were expressed as the percentage of total counted TG neurons.

2.4. Western blot analysis

Routine procedures were followed [8]. Briefly, snap-frozen TGs and whisker pad skin were protein extracted in CHAPS, then electroblotted to nitrocellulose membranes after gel separation of proteins in a 10% polyacrylamide gel. Membranes were blocked with 5% dry milk; pERK and ERK were specifically detected with primary antibodies (rabbit anti-pERK and anti-ERK, both at 1:500; Cell Signaling Technology), secondary antibody (anti-rabbit peroxidase-conjugated, 1:5000; Jackson ImmunoResearch), and chemoluminescence substrate (ECL-Advance, GE Healthcare). Abundance was quantified using ImagePro Plus software. β-Actin, as a control, was detected with a mouse monoclonal anti-β-actin antibody (clone AC-5, 1:500; Abcam).

2.5. Cell culture and Ca^{2+} imaging

N2a cells were cultured on poly-D-lysine and laminin coated coverslips in a 24-well plate containing DMEM (Sigma) supplemented with 10% bovine serum and penicillin/streptomycin. After 24 h, cells were transfected with rTRPV4-GFP or GFP alone (control) using the Lipofectamine 2000 protocol (Invitrogen).

TGs from 1.5- to 2-month-old male WT and *Trpv4*$^{-/-}$ mice were dissected and digested with 1 mg/mL collagenase (Worthington, CSL1) and 5 mg/mL dispase (Invitrogen) for 45 min, then triturated [6]. The resulting cell suspension was filtered through a 70 μm cell strainer (BD Falcon) to remove debris. Neurons were cultured in DH10 medium (1:1 DMEM:Ham F12, Invitrogen) with 10% fetal bovine serum (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco), and 50 ng/mL nerve growth factor (USBiological) on coverslips coated with poly-D-lysine and laminin (Invitrogen), and incubated with 5% CO_2 at 37°C. Ca^{2+} imaging was performed the day after culture.

Primary mouse keratinocytes were cultured as previously described [26]. The epidermis from back skin of newborn WT mice was separated from the dermis by a 1 h dispase (BD Biosciences) treatment. Then the keratinocytes were dissociated from the epidermis using trypsin (Gibco). Keratinocytes were plated on collagen-coated dishes or glass coverslips and grown in keratinocyte serum-free media (Gibco) supplemented with bovine pituitary extract and epidermal growth factor (R&D Systems), 100 pmol cholera toxin (Calbiochem), and 1× antibiotics/antimycotics (Gibco) in an incubator at 5% CO_2 and 37°C.

Ca^{2+} imaging of primary TG neurons and epidermal keratinocytes in response to formalin was conducted after loading with 2 μM fura2-AM (Invitrogen) for 30 min, following a ratiometric Ca^{2+}-imaging protocol with 340/380 nm blue light for dual excitation [26]. Ratios of emissions were acquired at 0.5 Hz. $\Delta R/R_0$ was determined as the fraction of the increase of a given ratio over baseline ratio, divided by the baseline ratio.

To investigate the effects of the specific TRPV4 inhibitor GSK205 [26,28] or TRPA1 inhibitor A-967079 [7,30] on formalin-induced Ca^{2+} influx, cells were incubated with GSK205 or A-967079 15 min before formalin stimulation.

2.6. Electrophysiology

Heterologously transfected N2a cells were recorded as described previously [19]. Patch clamp recordings were performed 24 h after transfection. Briefly, cultured cells on individual coverslips were incubated for 10 min at 37°C in extracellular solution containing (mM) 1 $MgCl_2$, 10 glucose, 10 HEPES, 145 NaCl, and 2 $CaCl_2$ (pH 7.4, 310 mOsM). Cells were then transferred to a recording chamber staged on an inverted Leica microscope that was equipped with fluorescent filters. Each GFP labeled cell was identified for whole cell currents recording using a glass electrode. The glass electrodes were pulled (P-80/PC; Sutter Instruments) from borosilicate glass capillaries (Warner Instruments) and had resistances of 2.5 to 3.0 MΩ. Before recording, the glass electrodes were filled with intracellular solution containing (mM) 140 CsCl, 10 HEPES, 1 EGTA, 0.3 Na-GTP, 2 Na_2-ATP, and 2 $MgCl_2$ (pH 7.4, 295 mOsM). Whole cell currents were recorded using pclamp 9 software (Molecular Devices) and Axopatch 200B amplifier (Axon Instruments). The cell was first clamped at −65 mV in voltage clamp mode, and a 1 s voltage ramp from −110 mV to +120 mV was applied every 2 seconds for 15 to 20 sweeps.

Formalin was only added after a stable baseline was established. For recording the effect of TRPV4 inhibitor on formalin, cells were first incubated for 10 min with 10 μM GSK205 before adding formalin. The recorded data were filtered at 2 kHz and digitized at 10 kHz. The capacitance was monitored during recording and any data with a ±5 pF change were excluded from analysis. Post-recording analysis was conducted using clampfit 9 software.

2.7. Statistical analysis

All data are expressed as mean ± SEM. Two-tailed *t* tests or 1-way ANOVA followed by Tukey post hoc test were used for group comparison. $P < .05$ indicated statistically significant differences.

3. Results

3.1. Trpv4 in TG sensory neurons is essential for formalin-evoked irritant pain

Subcutaneous injections of minute amounts of diluted formalin elicit an acute response that lasts approximately 5 min directly after injection. An intermediary phase is characterized by less pain behavior for the next 10 min [11]. This is followed by a tonic or late response for the next 30 to 45 min, again with increased nocifensive behavior, which is sustained by maladaptive neural responses, demonstrated for sensory relay neurons in the spinal cord dorsal horn [40].

In laboratory mice of a C57BL/6J genetic background, we found a biphasic response after whisker pad injection of 10 μL of 4% formalin (Fig. 1A). The term "biphasic" has to take into account that the interphase did not equal lack of nocifensive behavior after saline injections but was clearly less pronounced than acute or tonic phase. The acute phase was characterized by half the time spent with nocifensive behavior, and the tonic phase a third of the time (Fig. 1B). We selected 4% formalin in a 10 μL injection volume because this application can produce the most robust nocifensive behavior [22].

Having elaborated these basics of the irritant behavioral response to formalin in the trigeminal system, we next subjected *Trpv4*$^{-/-}$ pan-null mice to formalin injections on the basis of the rationales laid out above. We found significantly attenuated nocifensive behavior in these mice for all phases (Fig. 1C, D). This result indicates that *Trpv4* is necessary for both the acute response to tissue injury by formalin in the whisker pad and the protracted tonic response, which is neurally mediated by the trigeminal system. In order to confirm and extend these findings, we preapplied a TRPV4-selective inhibitor, HC067047 [10], by systemic application. We obtained similar results as for genetically encoded deletion in *Trpv4*$^{-/-}$ mice. In addition, attenuation of the tonic phase of the response was dose dependent (Fig. 1E, F). Acute block of TRPV4 function that leads to a similar result as genetically encoded gene

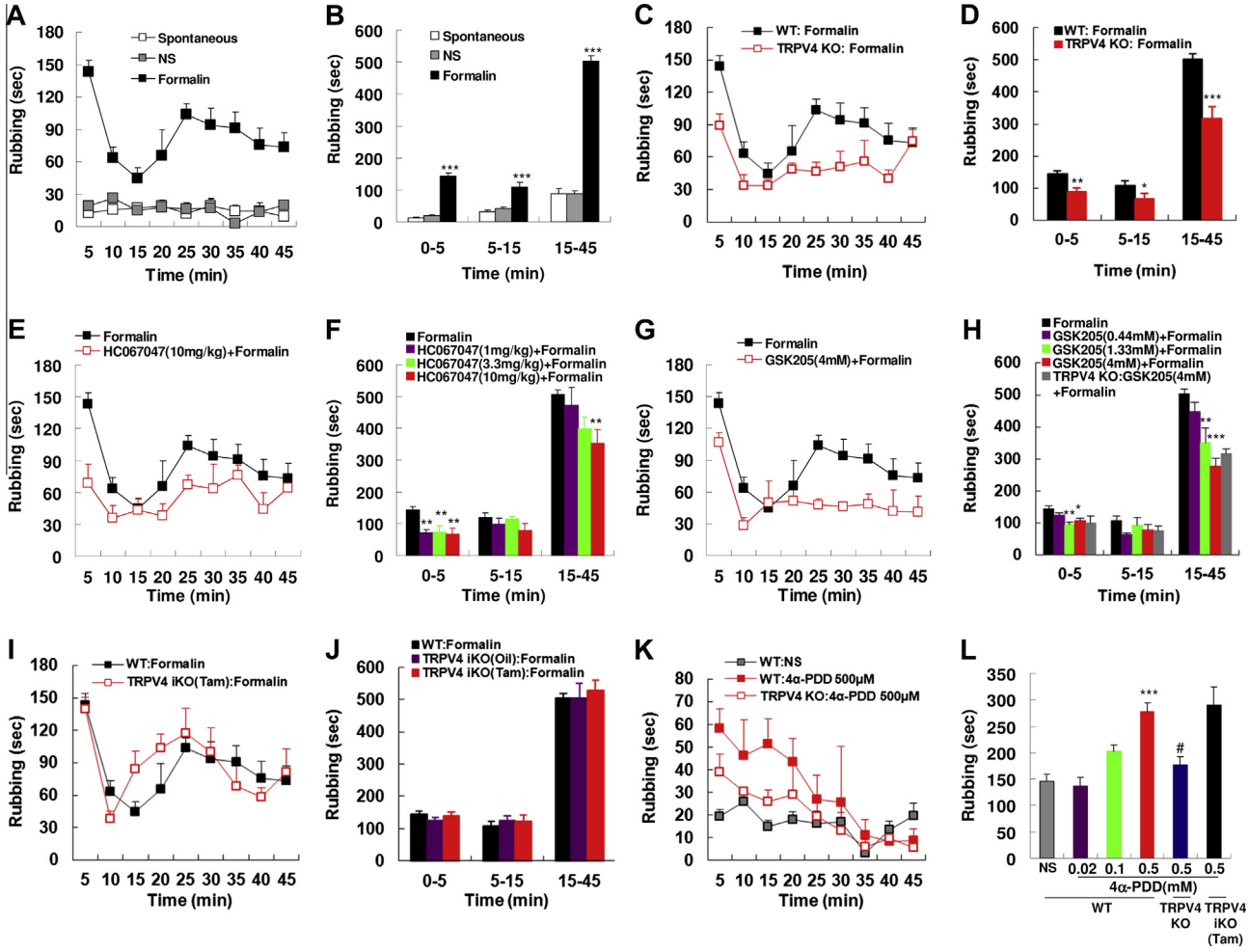

Fig. 1. *Trpv4* in TG sensory neurons is essential for formalin-evoked irritant pain. (A) Time course of the face-rubbing activity observed in naive mice and in mice after subcutaneous injection of normal saline (NS) or formalin (4%) into the whisker pad. Time that mice spent rubbing is plotted for each 5 min block over 45 min. (B) Quantification of the formalin response binned into different phases: acute phase (0–5 min), interphase (5–15 min), and tonic phase (15–45 min) (***$P < .001$ vs NS). Significant attenuation of irritant pain induced by formalin was observed in mice lacking *Trpv4* (C, D; *$P < .05$, **$P < .01$ and ***$P < .001$ vs WT: formalin), systemically pretreated (intraperitoneally) or subcutaneously pretreated with the TRPV4 inhibitor HC067047 (E, F; **$P < .01$ vs formalin) or GSK205 (G, H; *$P < .05$, **$P < .01$ and ***$P < .001$ vs formalin; the absence of off-target effects of GSK205 is demonstrated in *Trpv4*$^{-/-}$ mice), but not in mice with inducible *Trpv4* deletions in keratinocytes (I, J). In addition, mice pretreated with the selective inhibitors showed dose-dependent reduction of rubbing activity for the tonic phase (F, H). Animals injected with the TRPV4 selective agonist 4α-PDD displayed significant rubbing behavior of single phase in a dose-dependent manner that was absent in *Trpv4*$^{-/-}$ mice, but was unaffected by ablating *Trpv4* in keratinocytes (K, L; ***$P < .001$ vs NS and $^{\#}P < .05$ vs WT: 0.5mM 4α-PDD); $n = 5–9$/group; 1-way ANOVA with Tukey post hoc test was used for statistic analyses.

deletion of *Trpv4* indicates that TRPV4 channels contribute to the trigeminal formalin response as observed here, importantly without apparent contribution by genes that are up-regulated in a compensatory manner when deleting *Trpv4*.

On the basis of the dose-dependent attenuation of the formalin response by systemic application of TRPV4-inhibitor, HC067047, we also wanted to learn whether whisker pad injection of a TRPV4 inhibitor had a similar effect or perhaps more pronounced impact on one specific response phase. We used the rapid-onset, rapid-clearance TRPV4-selective blocker GSK205 [19,28,41], which we have used previously in a sunburn pain model where the compound proved effective upon topical application and devoid of off-target effects [26]. Surprisingly, the protracted tonic phase was attenuated more robustly, also in a clearly dose-dependent manner, by GSK205 than its effects on the acute and interphase (Fig. 1G, H). One reason for this effect could be the protracted diffusion to reach the compound's targets for the acute phase. However, differences in attenuation of the acute phase between different *Trpv4* loss-of-function experiments were moderate.

Importantly, all 3 experiments share the feature that the protracted neural phase is regulated by TRPV4. The effect we noticed the most was the response to whisker pad injection of GSK205. GSK205 off-target effects could not be seen in our trigeminal formalin model (Fig. 1H). This result, together with established insights on the tonic phase of the formalin model, and with known functional expression of TRPV4 channels in TG sensory neurons [8,20,21], suggests that the critical site of action for TRPV4 in the trigeminal formalin response could be in TG sensory neurons. However, given the robust TRPV4 expression in epidermal keratinocytes [26] (Fig. 5A, B), we also considered a co-contribution by epidermal TRPV4 to the formalin response, essentially for all 3 phases. We did not find evidence in favor of this hypothesis because we recorded identical abundance of nocifensive behavior in a *Trpv4* keratinocyte-specific, inducible knockout that we have developed previously [26]. Induced and sham-induced mice showed the same robust nocifensive behavior for all 3 phases (Fig. 1I, J). Thus, TRPV4 channel expression by TG sensory neurons, including their peripheral projections to the whisker pad, appear to be key expression sites for the observed nocifensive response in all 3 phases. TRPV4 expression in keratinocytes plays no role, and rapid migration of other TRPV4-expressing nonneural cells to the site of injection that subsequently regulate the tonic phase of the

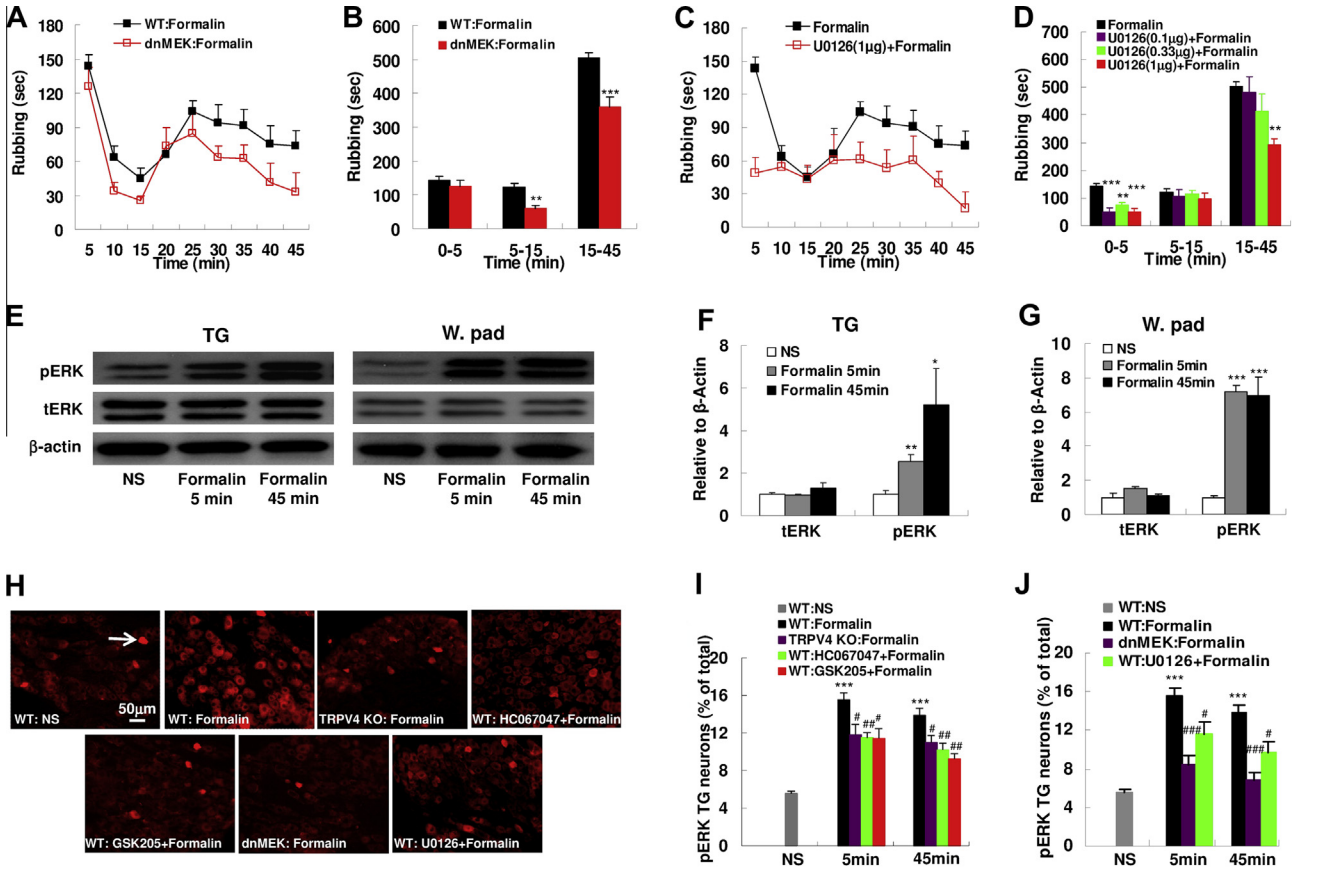

Fig. 2. Extracellular signal–related kinase (ERK) signaling downstream of TRPV4 is critical for formalin-evoked irritant pain. Reduced tonic phase of irritant pain was observed in the dominant negative mitogen-activated protein kinase kinase (dnMEK) mice (A, B; $n = 7–9$/group, $^{**}P < .01$ and $^{***}P < .001$ vs WT: formalin). Different from the dnMEK mutant mice, animals subcutaneously pretreated with the selective MEK inhibitor U0126 not only showed reduction of the tonic phase of pain, but also of the acute phase (C, D; $n = 5–9$/group, $^{**}P < .01$ and $^{***}P < .001$ vs formalin). (E–J) Increase of phospho-ERK (pERK) expressing TG neurons in response to formalin depends on Trpv4. Micrographs show pERK and total ERK (tERK) expression in the TG and whisker pad skin homogenates as revealed by Western blot analysis (E). Right bar graphs depict quantitation. Note early and robust increase of pERK expression and unchanged tERK expression in the TG and whisker pad (W.pad) skin after formalin (F, G; $n = 5$/group, $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs NS). (H, I) Increase of pERK in TG is restricted predominantly to neurons and significantly inhibited in mice lacking *Trpv4* or pretreated with the TRPV4 selective inhibitors ($n = 4–5$/group, $^{***}P < .001$ vs NS, $^{\#}P < .05$ and $^{\#\#}P < .01$ vs WT: formalin). (H, J) Increase of pERK is inhibited in dnMEK mutant mice and in mice pretreated with the MEK selective inhibitor U0126, as expected ($n = 4–5$/group, $^{\#}P < .05$, and $^{\#\#\#}P < .001$ vs WT: formalin). One-way ANOVA with Tukey post hoc test was used for (B) and (D), and 2-tailed *t* test was used for (F), (G), (I), and (J).

trigeminal formalin behavioral response appears unlikely and is not supported by current understanding of the formalin model. Therefore, TG sensory neurons are the most likely sites of TRPV4 function in response to whisker pad injection of formalin. This conclusion is highly likely for the neurally mediated tonic phase. For the acute phase, a major contributory role of TRPV4 appears likely.

In order to conduct a gain-of-function study on the role of trigeminally expressed TRPV4, we injected TRPV4-selective activators, 4α-PDD and GSK101 [4,37], into the whisker pad. This led to a monophasic behavior, reminiscent of the formalin acute phase, but more protracted and lacking subsequent phases (Fig. 1K, L). GSK101 proved slightly more potent than 4α-PDD (data not shown). The elicited behavior was dependent on TRPV4, as shown by the greatly attenuated response of *Trpv4*$^{-/-}$ mice (Fig. 1K, L). However, there was also a minor effect in the null background, pointing toward a marginal off-target effect by 4α-PDD as a trigeminal irritant. When using again the keratinocyte-specific and inducible *Trpv4* knockdown mouse [26], there was identical nocifensive behavior in these animals when inducing *Trpv4* knockdown compared with WT (Fig. 1K, L). These findings suggest that TRPV4 is needed for the nocifensive response to whisker pad injections of a known TRPV4-selective activator, 4α-PDD, yet suggest that TRPV4 expression in keratinocytes of the whisker pad skin plays no role in this behavior.

Taken together, loss-of-function experiments on the role of TRPV4 in the trigeminal formalin response, as well as gain-of-function experiments on the role of TRPV4 in nocifensive behavior in response to whisker pad injection of selective TRPV4 activators, both suggest that TRPV4 is an irritant receptor expressed by and functional in TG sensory neurons. Robust expression of TRPV4 by whisker pad skin keratinocytes does not play a role in the nocifensive behavioral response to whisker pad injections of formalin or selective chemical TRPV4 activators. Furthermore, we excluded an exclusive trigeminal role of TRPV4 in the trigeminal formalin response by eliciting a footpad formalin response and demonstrating similar *Trpv4* dependence using *Trpv4*$^{-/-}$ mice (Supplementary Fig. 1).

3.2. ERK signaling downstream of TRPV4 in TG sensory neurons is critical for formalin-evoked irritant pain

On the basis of our previous observations of MEK-ERK MAP-kinase signaling likely downstream of TRPV4 expressed in TG sensory neuron [8], as well as related findings in sensory neurons and airway epithelial cells [1,19], we tested the trigeminal formalin response of mice with dominant-negative MEK expression directed to neurons, including TG sensory neurons [8]. We observed significantly reduced nocifensive behavior in response to whisker pad

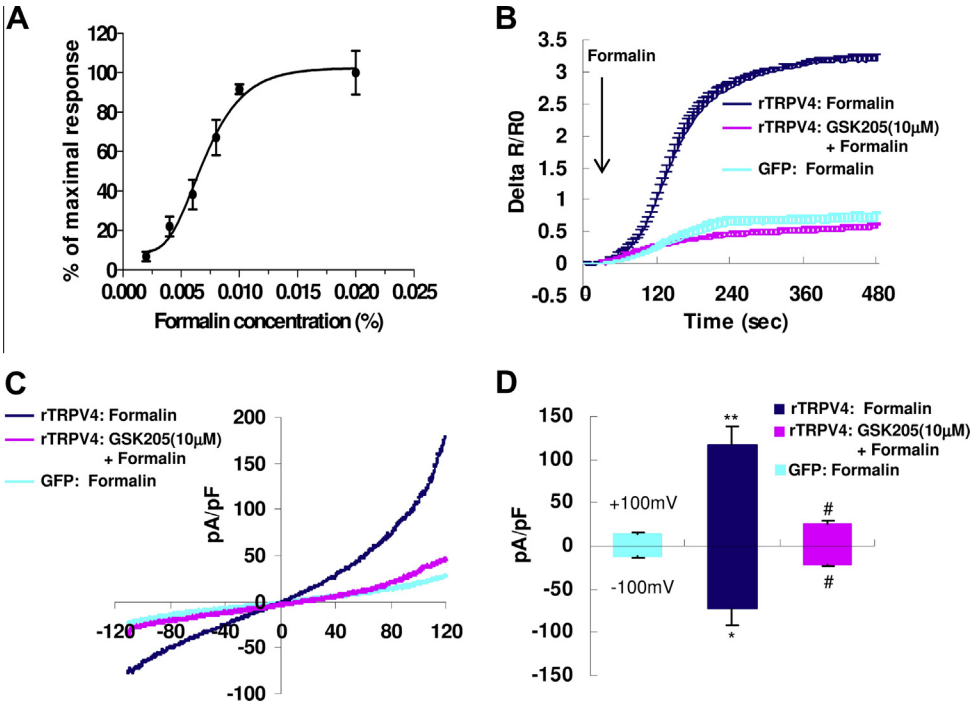

Fig. 3. Formalin activates heterologously expressed TRPV4. (A) Concentration-dependent curve of Ca^{2+} influx induced by formalin in N2a cells transfected with rTRPV4 (64–312 cells/concentration). (B) Ca^{2+} responses caused by formalin (0.01%) were reversed by pretreatment of the TRPV4 selective inhibitor GSK205 to a level equivalent to control cells transfected with green fluorescent protein (GFP, 89–133 cells/group). (C) Inhibition of formalin (0.01%)-evoked currents by GSK205 in N2a cells transfected with rTRPV4. (D) Mean current density measured at +100 and −100 mV in response to formalin (0.01%) and coapplication of GSK205 (6–12 cells/group, $^{**}P < .01$ vs GFP: formalin and $^{\#}P < .05$ vs rTRPV4: formalin, 2-tailed *t* test).

Table 1
Percentage of cultured TG sensory neurons responding to formalin.

Mouse	Treatment	Responsive TG neurons
WT	Formalin	27.66% (143/517)
WT	GSK205 (10 μM) + formalin	9.69% (35/361)
TRPV4 KO	Formalin	32.12% (62/193)
WT	4α-PDD (5 μM)	24.69% (41/166)
WT	A-967079 (10 μM) + formalin	15.79% (66/418)
TRPV4 KO	A-967079 (10 μM) + formalin	3.24% (14/432)

TG = trigeminal ganglion; WT = wild type; KO = knockout.

injections of formalin in the interphase and tonic phase, not in the acute phase (Fig. 2A, B). This result suggests that MEK-ERK is likely functioning downstream of TRPV4 in TG sensory neurons in formalin-evoked nocifensive behavior, and that this mechanism is relevant for interphase and tonic phase of the formalin response. Dominant-negative MEK is also effective in central neurons that transmit pain [16], but this location as a cause of its effect in the trigeminal formalin response is less likely because of the following result. We complemented assessment of the dnMEK mouse with whisker pad injections of specific MEK inhibitor, U0126 [15]. As a result, we observed significant attenuation of nocifensive behavior. This effect, for the tonic, neurally mediated phase, was dependent on the dose of U0126 applied (Fig. 2C, D). The acute phase was sensitive to even the lowest dose of U0126. The acute phase therefore shows an interesting profile: it is *Trpv4* dependent (Fig. 1) but at the same time not MEK-ERK dependent in TG neurons (Fig. 2A, B), although robustly MEK-ERK dependent locally in the whisker pad (Fig. 2C, D). These observations during the acute phase can be reconciled with TRPV4 functioning in nonneural cells upstream of MEK-ERK, such as endothelial cells and macrophages, both of them known to express TRPV4 [2,12,13,27,36]. These cells participate in sensitizing peripheral nerve endings to formalin. Together, our results suggest that MEK-ERK signaling, downstream of TRPV4-mediated Ca^{2+} signaling, functions as a significant mechanism in the organismal trigeminal formalin response. In keeping with this conclusion, we detected ERK phosphorylation in response to whisker pad injection of formalin within 5 min after injection, both in whisker pad and TG extracts (Fig. 2E–G). The increased phosphorylation level was sustained in both tissues at the 45 min time point (Fig. 2E–G). Immunolabeling studies in the TG demonstrated phosphorylated ERK to be strictly neuronal (Fig. 2H).

We next asked whether this regulation did indeed depend on *Trpv4*. We uncovered affirmative results by use of $Trpv4^{-/-}$ mice, systemic application of HC067047, and whisker pad injections of GSK205. This was observed at both the 5 and 45 min time points, indicative of the rapid as well as sustained dynamics of the TRPV4-dependent regulation (Fig. 2H, I). Confirming the specificity of the approach, ERK phosphorylation in TG neurons was reduced in dnMEK transgenic mice and in mice with whisker pad preinjections of U0126 in response to whisker pad injections of formalin (Fig. 2H, J). These results validate the above conclusion of TRPV4 functioning upstream of MEK-ERK. Therefore, this signaling mechanism in TG sensory neurons likely underlies the tonic neural phase of the trigeminal formalin behavioral response.

3.3. TRPV4 is activated by formalin in cultured heterologous cells and TG sensory neurons

To complement and extend our in vivo studies, we conducted experiments in dissociated TG sensory neurons and in heterologous cells with directed expression of TRPV4. For the latter, we observed a concentration-response relationship for formalin application using Ca^{2+} imaging as a readout in N2a cells (Fig. 3A; EC_{50} = 0.00638% formalin). A similar response was detected in TRPV4-expressing HEK293T cells (data not shown). Control-transfected N2a cells displayed a minimal response to formalin (0.01%).

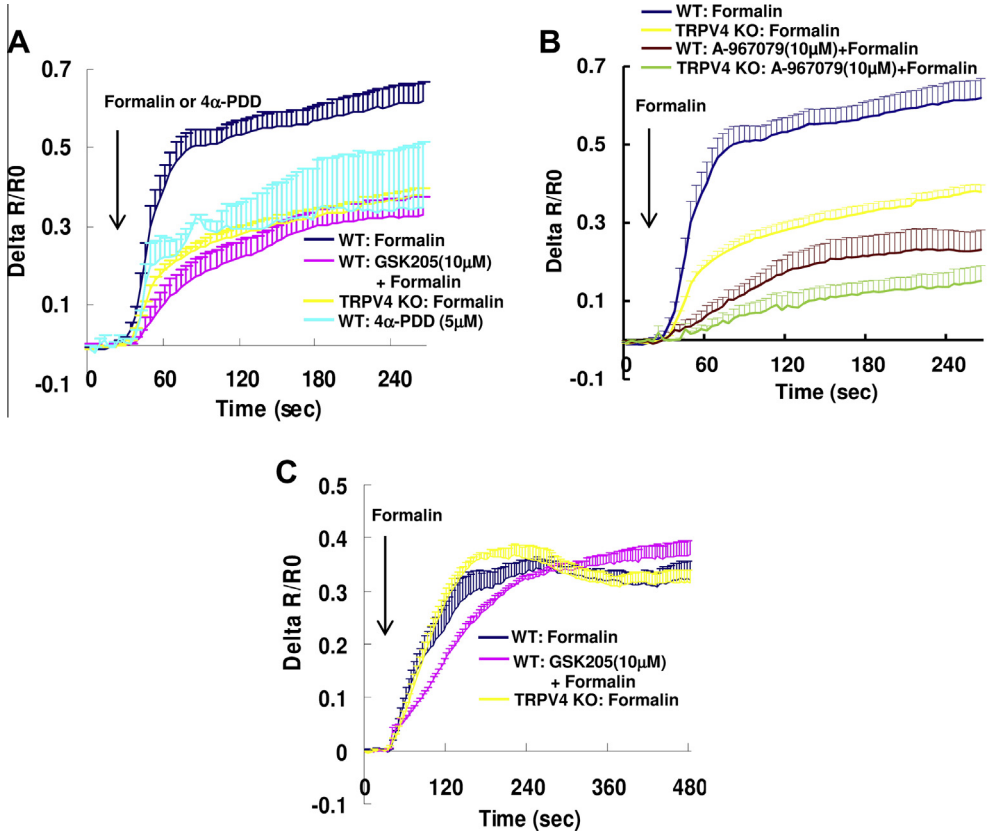

Fig. 4. Ca^{2+} influx caused by formalin in TG neurons is TRPV4 dependent. (A) Formalin (0.01%)-evoked Ca^{2+} influx was attenuated in the presence of TRPV4-selective inhibitor GSK205 in cultured TG neurons, as well as in TG neurons from *Trpv4*$^{-/-}$ mice. In addition, Ca^{2+} influx was evoked by the TRPV4 selective agonist 4α-PDD (166–517 cells/group). (B) A-967079, a specific TRPA1 inhibitor, led to an attenuated Ca^{2+} signal in WT TG neurons and to further reduction in neurons from *Trpv4*$^{-/-}$. (C) Formalin (0.01%)-evoked Ca^{2+} signaling was not affected by GSK205 in cultured keratinocytes and remained completely unaffected in keratinocytes derived from *Trpv4*$^{-/-}$ mice (170–457 cells/group).

The Ca^{2+} response to formalin (0.01%) could be completely reverted to background levels by use of 10 μM GSK205 (Fig. 3B). We recapitulated this experiment using patch clamp electrophysiology, where we noticed the typical outward rectification in TRPV4-expressing N2a cells that were stimulated with formalin. Channel activation was reverted to background levels in the presence of 10 μM GSK205 (Fig. 3C, D). These results indicate that heterologous expression of TRPV4 channels is sufficient to confer upon a minimally responsive cell robust responsiveness to dilute formalin. This response is mediated by TRPV4. TRPV4 is therefore an ionotropic formalin receptor, or it supplies the critical channel element to a signaling chain that is present in N2a and HEK293 cells.

We next investigated TG sensory neurons' response to formalin, using Ca^{2+} imaging. We observed an appreciable Ca^{2+} response to formalin (0.01%) in 27.66% of the TG neurons (Table 1). This response rate was not different from that elicited by stimulation with 4α-PDD (5 μM). Indicative of a critical involvement of TRPV4, the response rate decreased to 9.7% when applying GSK205 (10 μM). In addition, for responsive cells, intracellular Ca^{2+} evoked by formalin was reduced 50% when directly antagonizing TRPV4 with GSK205 (10 μM, preexposure for 15 min). Genetically encoded absence of *Trpv4* led to a slightly increased response rate as in WT (32.12% vs 27.66%, Table 1). Neurons from *Trpv4*$^{-/-}$ mice showed a formalin-evoked Ca^{2+} response that was decreased by 50% in terms of intracellular concentration (Fig. 4A). When examining the formalin response of dissociated keratinocytes, we found it to be independent of TRPV4, evidenced by its presence in keratinocytes derived from *Trpv4*$^{-/-}$ mice, and when applying GSK205, all together not different from that of WT mice (Fig. 4C). Of note, these cells do respond to specific chemical activation of TRPV4 by 4α-PDD and GSK101 [26].

Thus, TG sensory neurons do respond to formalin directly. This response is sustained by TRPV4 ion channels that these neurons express. Antagonism of TRPV4 channels reduced the number of formalin-responsive neurons and reduced the Ca^{2+} signal by ½ in the remainder responsive neurons. Genetically encoded absence of *Trpv4* attenuated the Ca^{2+} signal by ½, not reducing the number of responsive cells, indicating gene-regulatory compensation in the *Trpv4*$^{-/-}$ pan-null mouse. These findings suggest a significant contribution of TRPV4 ion channels to the formalin response of TG sensory neurons, but also the presence of another formalin-responsive pathway. In the first place, the established formalin receptor, TRPA1 comes to mind as a candidate mechanism to underlie the non-TRPV4-related formalin response [23,24]. We began to explore this question by using the potent and specific TRPA1-inhibitory compound A-967079 [7,39] in our experiments. It led to an attenuated Ca^{2+} signal in WT TG neurons and a further reduction in *Trpv4*$^{-/-}$ TG neurons in response to formalin (Fig. 4B). The percentage of responsive cells was reduced from 27.7% to 15.8% when acutely blocking TRPA1 and to a mere 3.2% in *Trpv4*$^{-/-}$ TG neurons plus TRPA1 inhibitor (Table 1). Thus, TRPV4 and TRPA1 appear to co-contribute to responsiveness of TG sensory neurons to formalin.

Together, cellular experiments reveal that directed expression of TRPV4 is sufficient to render an otherwise minimally responsive cell formalin responsive, and that the primary sensory neurons of highest interest in our investigation—TG sensory neurons—can be activated directly by formalin in a TRPV4-dependent manner.

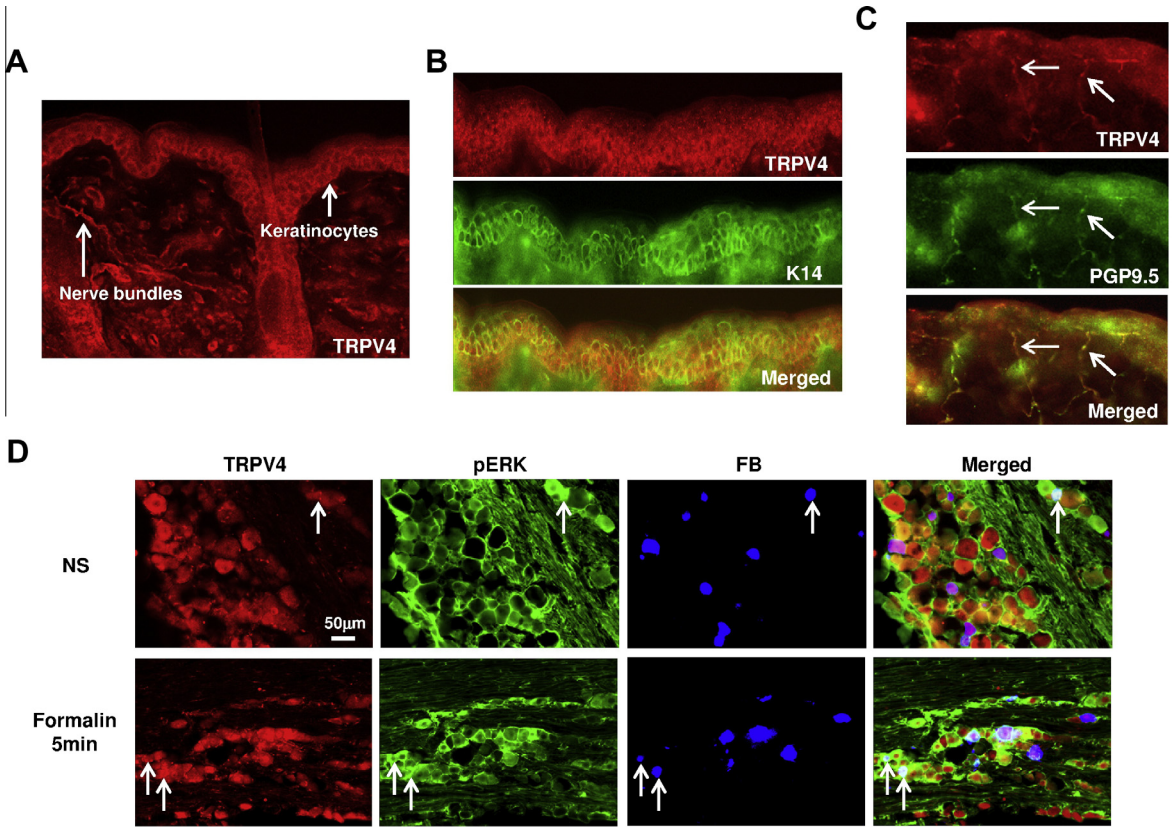

Fig. 5. TRPV4 and pERK coexpressing TG neurons innervate the whisker pad skin. (A) Immunostaining revealed TRPV4 expression in epidermal keratinocytes and whisker pad skin nerve fibers, as demonstrated by colabeling with the specific keratinocyte marker K14 in WT mice (B) and peripheral nerve fiber marker PGP9.5 in (*Trpv4*$^{lox/lox}$ X K14-Cre-ER^{tam}) keratinocyte-specific and inducible *Trpv4* knockout mice (C) respectively. (D) TRPV4 coexpresses with pERK in TG neurons that innervate the whisker pad, as identified with Fast Blue (FB) tracing that is originating from the whisker pad.

Table 2
Increase of TRPV4 and pERK coexpressing TG neurons innervating the whisker pad skin in response to formalin.[a]

Treatment	TRPV4/total	pERK/total	FB/total	TRPV4 + pERK/TRPV4	TRPV4 + pERK + FB/FB
Normal saline	32.15 ± 1.02%	5.44 ± 0.57%	6.53 ± 1.21%	5.67 ± 1.08%	11.25 ± 0.83%
Formalin 5 min	33.72 ± 0.56%	15.54 ± 0.73%***	6.88 ± 0.73%	10.35 ± 0.46%**	18.04 ± 0.61%***

TG = trigeminal ganglion.
[a] Quantification illustrates the increase for TRPV4-pERK coexpressing TG neurons of all TRPV4 expressing neurons, also the increase for whisker pad-innervating TRPV4-pERK coexpressing neurons of all whisker pad-innervating neurons in response to formalin (**$P < .01$ and ***$P < .001$ vs normal saline, 2-tailed t test). Note the unchanged percentage of TRPV4-expressing neurons and fast blue (FB)-labeled neurons; $n = 4$ mice/group.

3.4. TRPV4 and pERK coexpressing TG neurons innervate the whisker pad skin

On the basis of these insights, we returned to the TG of control and formalin-injected mice. First, our immunostaining analysis revealed TRPV4 to be expressed in both epidermal keratinocytes and whisker pad skin nerve fibers (Fig. 5A–C). Using trigeminal ganglia sampled 5 min after whisker pad injections, we combined coimmunolabeling of TRPV4 and phosphorylated ERK with neural tracing from the whisker pad skin (Fig. 5D; Table 2). Our results are telling in that they demonstrate a greater number of phosphorylated ERK-immunoreactive neurons in the population of TRPV4-expressing sensory neurons, an increase of ~100% (Table 2, 5.67% vs 10.35%). Importantly, in TRPV4-expressing neurons that colabel with whisker pad injected tracer, we found an increase of phosphorylated ERK by ~75% (11.25% vs 18.04%). This is an interesting finding when compared to all TG sensory neurons, where phosphorylated ERK expressing neurons tripled from 5.44% to 15.54% (Table 2). This means that whisker pad injections of formalin can possibly activate TG sensory neurons that do not innervate the whisker pad, which is even more astounding given the time interval between whisker pad injection of formalin and sampling of the TG, namely 5 min. As a measure of the validity of our approach, there was no significant change in abundance of neurons labeled with neural tracer (6.53% vs 6.88%) and of neurons showing appreciable TRPV4 expression, evidenced by the fact that TRPV4 could be detected in a straightforward manner by simple 2-step immunolabeling (32.15% vs 33.72%). Together, these findings indicate that TRPV4-expressing TG sensory neurons that innervate the whisker pad skin become activated to phosphorylate ERK within 5 min after formalin injection. This activation is likely to underlie the TRPV4-dependent neurally mediated tonic phase of the trigeminal formalin response that we have characterized here.

3.5. Co-contribution of TRPA1 and TRPV4 to the trigeminal formalin response

On the basis of our findings of a co-contribution of TRPA1 and TRPV4 to the formalin response of TG sensory neurons, we compared nocifensive behavior in pan-null mice for both genes, then

applied a TRPV4 inhibitor to *Trpa1*$^{-/-}$ mice. Our results demonstrate attenuation of nocifensive behavior in *Trpv4*$^{-/-}$ and *Trpa1*$^{-/-}$ mice to similar degree of the formalin response (Fig. 6A, B). When pretreated with whisker pad injection of TRPV4-inhibitor, GSK205 (Fig. 1G, H), only the tonic phase was significantly attenuated in *Trpa1*$^{-/-}$ mice (Fig. 6A, B). This suggests that both channels co-contribute to the in vivo effects of formalin, sustaining in particular the tonic phase of the response. We also investigated pERK expression in TG sensory neurons as the intracellular downstream signaling function. We found a significant percentage reduction in both null genotypes, indicating that Ca^{2+} influx via either channel can activate MEK-ERK MAP-kinase signaling in TG sensory neurons (Fig. 6C, D). Our in vivo findings therefore dovetail with the concept of a co-contribution of TRPA1 and TRPV4 to the trigeminal formalin response in vivo.

4. Discussion

Here we demonstrate an important role for TRPV4 ion channels in the trigeminal formalin pain response. TRPV4 is contributory to all phases of the trigeminal formalin pain response. Its functional expression in TG sensory neurons is key for the protracted, neurally mediated tonic phase of pain behavior. TRPV4 channels function upstream of MEK-ERK phosphorylation in whisker pad–innervating TG sensory neurons to evoke the delayed phase of the pain behavior. In keeping with a key role in transduction of the irritant stimulus, TRPV4 expression in heterologous cellular systems confers robust responsiveness to formalin. TG sensory neurons respond to formalin directly with Ca^{2+} influx in a TRPV4-dependent manner. These results imply TRPV4 as a novel irritant receptor in the trigeminal system in addition to the known TRPA1 [3,24]. Targeting TRPV4 for trigeminal pain disorders might be a rational and possibly fruitful new strategy.

With respect to mechanisms of action of formalin on TG sensory neurons, future studies can address the following interesting questions: What is the mechanism of the demonstrated co-contribution of TRPV4 and TRPA1 to the trigeminal formalin response (Ca^{2+} signaling mechanism, channel mechanism, membrane mechanism)? For TRPV4 channel activation mechanisms by formalin, can direct activation be deconstructed, or is formalin acting on a yet-to-be-identified receptor, other membrane protein, or membrane–lipid complex, which subsequently activates TRPV4?

In regard to trigeminal pain disorders, TRPV4 is likely a molecule of interest that regulates the transformation of TG sensory neurons into pain generators. In this process, the Ca^{2+}-permeable channel functions upstream of MEK-ERK MAP-kinase signaling. This is in keeping with another study that we have conducted involving longer-term inflammation of the TMJ using complete Freund adjuvant [8]. In this model, which lasts almost 2 weeks, we observed longer-term changes of pain-enhancing gene regulation in the TG, dependent on TRPV4. MEK-ERK phosphorylation was also found dependent on TRPV4. In the current study, MEK-ERK phosphorylation depends again on *Trpv4,* but the time dynamics of the formalin model is so rapid—45 min—that gene dysregulation cannot be implied as causative. However, we are struck by the common feature of both studies, namely that TRPV4 is critical for pain behavior, that its expression in TG sensory neurons appears key, and that MEK-ERK signals downstream of TRPV4

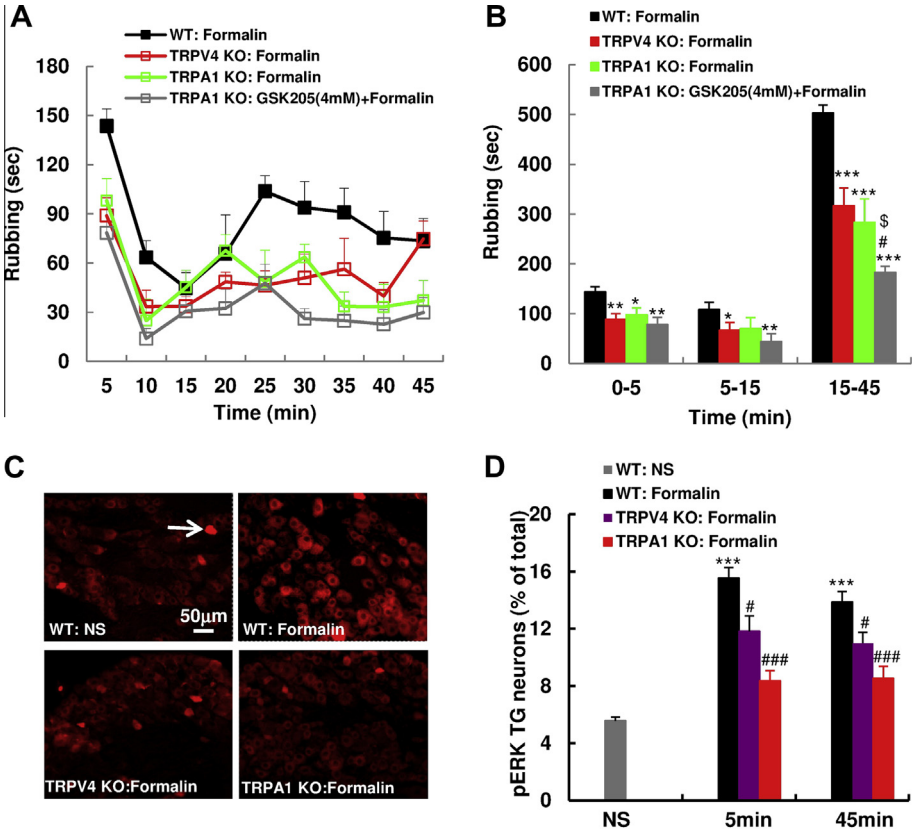

Fig. 6. Co-contribution of TRPA1 and TRPV4 to the trigeminal formalin response. (A, B) Significant attenuation of irritant pain induced by formalin was observed in mice lacking *Trpa1,* and this attenuation was further reduced by subcutaneous pretreatment with the TRPV4 inhibitor GSK205 for the tonic phase ($^{**}P < .01$ and $^{***}P < .001$ vs WT: formalin, $^{\$}P < .05$ vs TRPA1 KO: formalin, and $^{\#}P < .01$ vs TRPV4 KO: formalin; $n = 5–9$/group). (C, D) Increase of pERK in TG neurons caused by formalin injection is significantly reduced in mice lacking *Trpa1* ($n = 4–5$/group, $^{***}P < .001$ vs WT: NS, $^{\#}P < .05$ and $^{\#\#\#}P < .01$ vs WT: formalin). One-way ANOVA with Tukey post hoc test was used for (A) and (B), and 2-tailed *t* test was used for (C) and (D).

as a critical effector mechanisms in the animals' pain behavior. This means that TRPV4 is now a more validated, bona fide target for relief of pain [18] in the trigeminal system. This includes headaches (eg, migraines and tension-type headaches), TMJ pain, sinus pain, trigeminal neuropathic pain, trigeminal neuralgia, tooth pain, and orofacial sensory disorders with a pain-equivalent component (eg, burning mouth syndrome). The relative contribution of TRPV4 to these pain disorders needs to be established in future studies, which can now be conducted in a more rational manner, based on our results presented here and in our previous study [8]. We note that mechanical hypersensitivity, such as hyperalgesia, allodynia, and inflammation-facilitated pain, are a common feature of these disorders. This clinical argument reinvigorates TRPV4 an appealing target molecule.

Beyond classic trigeminal pain disorders, TRPV4 might be functional in trigeminal irritation disorders evoked by airborne exposures of trigeminally innervated external barriers (eg, cornea and conjunctiva of the eye; mucous membranes of the nasal cavity, nasopharynx, sinuses, oral cavity, and pharynx) to formalin and related electrophilic irritants [19].

Specific inhibition of TRPV4 can therefore be a novel therapeutic principle for trigeminal pain as well as irritation disorders. Especially for the latter, topical application of TRPV4 inhibitors might be a suitable and effective remedy.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2014.09.033.

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