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Vanilloid receptor TRPV1-mediated phosphorylation of ERK in murine adjuvant arthritis

Y. Chen Ph.D.[a], H. H. Willcockson M.S.*[a] and J. G. Valtschanoff M.D.
Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599, United States

Summary

Objective: The vanilloid receptor transient receptor potential vanilloid 1 (TRPV1), expressed by sensory neurons that innervate joints, is implicated in arthritis but the mechanisms are not fully understood. One possibility is that downstream effects of activation of TRPV1 are mediated by the extracellularly-regulated kinase (ERK). ERK is phosphorylated (p-ERK) in sensory neurons in response to noxious stimuli and its inhibition has been found to be antinociceptive in several pain models. We here wanted to ascertain whether TRPV1 may contribute to the pain hypersensitivity and inflammation of arthritis *via* an ERK-mediated pathway.

Methods: We used a model of adjuvant-induced arthritis (AIA) of the ankle and investigated the changes in expression of p-ERK in sensory afferent neurons in dorsal root ganglia (DRG) and spinal dorsal horn of TRPV1-knockout (KO) mice, compared to wild-type (WT) mice of the same genetic background, using multiple immunofluorescence.

Results: Two to three weeks after inducing AIA in mice, the number of neurons in DRG and spinal cord that expressed p-ERK was significantly higher on the side of AIA than on the contralateral, vehicle-injected side. The fraction of p-ERK-positive neurons in the DRG that also expressed TRPV1 was increased, indicating that activation of ERK occurred preferentially in TRPV1-positive neurons. Moreover, TRPV1-KO mice had reduced activation of ERK in sensory neurons, compared to WT mice. These changes in expression of p-ERK correlated with changes in pain behavior and joint histopathology: TRPV1-KO mice had reduced nociceptive behavior and severity of arthritis, compared to WT mice.

Conclusion: Our results support the idea that activation of ERK in primary afferent neurons is mediated, at least in part, by TRPV1. In the absence of TRPV1, the signs of arthralgia and histopathology in the mouse model of AIA are reduced. We conclude that TRPV1, expressed by neurons in the articular afferent pathway, contributes to the pathogenesis of arthritis *via* an ERK-mediated pathway.

Key words: Complete Freund's adjuvant (CFA), Transient receptor potential vanilloid 1 (TRPV1), Extracellularly regulated kinase (ERK), MAPK, Hyperalgesia, Allodynia, Osteoarthritis, Dorsal root ganglia (DRG), Mouse.

Introduction

Pain is the primary complaint, the main cause for disability, and the main therapeutic target in patients with osteoarthritis (OA). The other two pathogenetic components of arthritis, inflammation and progressive degeneration of joint tissues, appear to be related to the pain's severity, evolution and prognosis, and may share common mechanisms with it.

An important step for our understanding of articular nociception has been the cloning of the transient receptor potential vanilloid 1 (TRPV1). TRPV1, a non-selective cation channel, is activated by heat or H^+ as well as by vanilloids, like capsaicin[1]. TRPV1 is synthesized in sensory neurons in the dorsal root ganglia (DRG) and transported along their peripheral axons to both skin and viscera, and centrally to the spinal dorsal horn[2,3]. TRPV1 in the skin is important for thermal nociception and inflammatory hyperalgesia and allodynia, and has also been implicated in neuropathic pain[4].

Besides the skin, TRPV1-positive sensory afferents may innervate many deep structures of the body[5,6]. The expression of TRPV1 in articular afferents has been suggested by immunostaining of nerve fibers within the rat temporomandibular joint[7,8], rat and human knee joints[9], and mouse knee and ankle joints[10]. TRPV1 has also been demonstrated in synovial fibroblasts from patients with OA and rheumatoid arthritis, suggesting that it may play a role in non-neuronal modulation of nociception in arthritis[11,12]. The involvement of TRPV1 in the mechanisms of joint disease was proposed long before the receptor was first cloned, based on experiments with capsaicin, to which it is the only known receptor[13]. Capsaicin is the active ingredient of numerous preparations sold for relief of arthritic pain; the efficacy of these products is presumably based on capsaicin's ability to temporarily desensitize TRPV1[14]. Capsaicin-sensitive afferents have also been implicated in experimental arthritis[15,16].

More recently, knockout (KO) mice have been used to investigate the role of TRPV1 in acute and chronic nociception using several models of painful inflammatory conditions, including arthritis[17]. The severity of adjuvant-induced arthritis (AIA) of the knee[18,19] and ankle[20] was found to be significantly reduced in TRPV1-KO mice. Also, intraarticular injections of capsaicin demonstrated significant attenuation of the articular vasoconstriction and reduction of blood flow

[a]Equal contribution by these authors.

*Address correspondence and reprint requests to: Helen H. Willcockson, M.S., Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599, United States. Tel: 1-919-962-6882; Fax: 1-919-966-1856; E-mail: hhw@med.unc.edu

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in the knee of KO mice, indicating that these effects are TRPV1-dependent[21].

Although TRPV1 has been implicated in arthritis, the mechanisms are not fully understood. Pain causes expression of immediate early genes in sensory neurons. The biochemical link connecting activity in nociceptors to altered gene expression remains unclear, but one important element may be the ERK pathway. The extracellularly-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family of serine/threonin protein kinases, transduces extracellular stimuli into intracellular post-translational and transcriptional responses. When activated by mitogens, growth factors, and mediators of stress and inflammation, MAPK is translocated to the nucleus and regulates transcription of genes relevant to cellular activation, proliferation, and production of cytokines and other factors, released during inflammation[22,23]. MAP kinases exist in multiple isoforms and are activated by a series of upstream kinases thus forming a biochemical cascade of critical importance for the regulation of the inflammatory process[24,25].

ERK is phosphorylated (p-ERK) in sensory neurons in response to noxious, but not to non-noxious, stimuli applied to the skin or viscera[26,27]. Importantly, inhibition of ERK has been found to be antinociceptive in several pain models[28–30], suggesting that rather than merely providing a convenient experimental probe, this pathway may be an essential link in the biochemical cascade of pain. Recently, it was shown that an intraperitoneal application of an ERK inhibitor reduced capsaicin-induced thermal hyperalgesia while intrathecal application of this inhibitor reduced release of the neuropeptide calcitonin gene-related peptide (CGRP) in skin[31], and attenuated visceral pain behavior[27]. This suggests that ERK plays a role in the development of hyperalgesia, exerting effects both centrally and peripherally. Phosphorylation of ERK is required for the rapid facilitation of heat-induced currents in sensory neurons that are thought to be at the base of the mechanism of thermal hyperalgesia[32], and for the upregulation of TRPV1 expression in sensory neurons by cytokines[33]. A role for ERK has recently been proposed also in arthritis: inhibition of the ERK pathway has resulted in attenuated nociceptive behavior and inflammation in models of arthritis in rats[34,35] and mice[25,36,37].

The goal of the present study was to ascertain whether TRPV1 may contribute to the pain hypersensitivity and inflammation of arthritis *via* an ERK-mediated pathway. For this, we used ankle injections of complete Freund's adjuvant (CFA, known to cause arthralgia and arthritis) and investigated the changes in expression of p-ERK in the sensory afferents of TRPV1-KO, compared to wild-type mice (WT) of the same genetic background. We hypothesized that phosphorylation of ERK in articular afferents is downstream to the activation of TRPV1 receptor. If confirmed, this would provide a mechanism for activation of the ERK signaling cascade with the ensuing changes in expression of genes for relevant receptors and mediators. In turn, this may underlie the sensitization, both peripheral and central, which is a major contributor to the pain and inflammation in arthritis.

Materials and methods

ANIMALS

A total of 31 male mice, including 14 TRPV1-KO and 17 wild-type C57BL/6 mice (Jackson), weighing 25–30 g, were used in this study. Experimental procedures were carried out in compliance with the NRC's Guide for the Care and Use of Laboratory Animals, according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Carolina.

INDUCTION OF ARTHRITIS

Mice were anesthetized with a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine and injected with 10 μl of CFA (20 mg/ml suspension of heat-killed *Mycobacterium tuberculosis* in vehicle, Difco Lab) in each of two sites, front and back of the left ankle, using a 30-gauge needle attached to a 10 μl Hamilton syringe. The right ankle of each mouse was injected with the same volume of vehicle (paraffin oil containing mannide monooleate; incomplete Freund's adjuvant, IFA). Matching numbers of KO and WT mice were sacrificed at 3 days ($n=4$), 7 days ($n=4$), 14 days ($n=8$), and 21 days ($n=12$) after induction of AIA; three WT mice without injections were sacrificed for counts of DRG neurons in naïve animals.

ASSESSMENT OF PAIN BEHAVIOR

Six each of the KO and WT mice were tested for pain behavior at various time points after induction of AIA. Mice were acclimated unrestrained in testing chambers, 2–3 times for 30–60 min prior to testing and nociceptive sensitivity was assessed 2 days and 1 day before induction of AIA (baseline), and then on days 3, 7, 10, 14, and 21 after induction of AIA. On each test day, unilateral von Frey monofilament test[38] was conducted twice with a Hargreaves radiant heat test[39] between the two tests separated by 40 min intervals.

Withdrawal thresholds to tactile stimulation were measured using a standard set of von Frey monofilaments (North Coast Medical Inc.). Each hindpaw was stimulated five times for 1 s with a 1 s inter-stimulus interval using filaments of increasing bending force (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 2, and 4 g), beginning with the lowest. The threshold for mechanical sensitivity was recorded as the bending force of the filament that elicited withdrawal response to three out of five presentations.

The latency to withdrawal from a thermal stimulus was measured using the Hargreaves method with a Model 336 Paw/Tail Stimulator Analgesia Meter (IITC) set at 2% idle light intensity and 40% working light intensity. The stimulus was turned off manually upon the hindpaw withdrawal or automatically if the 20 s cut-off time was reached. Each mouse received two trials of each hindpaw with 10 min between trials and the results were averaged for analysis. Data were expressed as the latency to withdrawal in seconds. An overall mean of post-baseline behavioral data obtained from each test across time was calculated for each of the WT and KO groups.

TISSUE PROCESSING

Mice anesthetized with sodium pentobarbital (80 mg/kg, i.p.) were perfused intracardially with 30 ml freshly prepared solution of 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), followed by 100 ml solution of 4% paraformaldehyde in PB. Tissues, including the L4–L5 spinal cord segments, corresponding DRG bilaterally, and ankle joint preparations, were dissected out, post-fixed in 4% paraformaldehyde for 4 h, and stored in PB. Joint preparations were decalcified in Rapid Cal Immuno (BBC Biochemical) for 3 days. Tissue blocks were further cryoprotected in 30% sucrose in PB for 24–48 h and sectioned on a cryostat. Sections of DRG (10 μm) and joint preparations (20 μm) were thaw-mounted onto slides. Spinal cord was cut at 30 μm and free-floating sections were collected in PB.

IMMUNOHISTOCHEMISTRY

Sections of spinal cord and DRG were permeabilized with 50% ethanol in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) for 30 min, blocked with 10% normal donkey serum (NDS; Jackson) in PBS, and incubated overnight with primary antibodies in PBS: rabbit anti-p-ERK (1:100, Cell Signaling), goat anti-TRPV1 (1:250, Santa Cruz), and mouse anti-NeuN (1:100, Millipore). After several rinses with PBS and incubation with 2% NDS for 30 min, sections were incubated with the appropriate secondary antibodies (fluorescein isothiocyanate- or Cy3-conjugated donkey antibodies; 1:200 in PBS; Jackson) for 2 h, rinsed, mounted, and coverslipped with Vectashield (Vector). Digital micrographs were obtained with a Retiga EX cooled CCD camera (Q-Imaging) attached to a Leitz DMR microscope and saved as TIFF files; contrast and brightness were adjusted with Adobe Photoshop CS2.

All antibodies employed here are well characterized and in routine use in our laboratory. As a matter of control, we processed sections of DRG and spinal cord according to the above protocols, except that primary or secondary antibodies were omitted, or blocking peptides were added. Omission of primary or secondary antibodies or preadsorption with appropriate blocking peptides completely abolished specific staining. We also stained sections of DRG and spinal cord for TRPV1 to verify genotype: staining of material from KO mice was uniformly negative.

ASSESSMENT OF ARTHRITIS

Prior to testing on behavioral test days, the width of each hindpaw at the ankle was measured with calipers, and the skin temperature of the plantar surface of the paw was measured with an infrared thermometer (Fisher Scientific). Swelling of the ankle and local flair were recorded as percent change in hindpaw width and skin temperature at various time points pre- and post-injection. For histopathological evaluation, sections of ankles stained with hematoxylin and safranin-O were examined on a Leitz DMR light microscope; color photomicrographs were acquired with a Retiga EX camera with an RGB filter (Q-imaging). From every joint preparation, 4–6 sections, taken approximately 100 μm apart, were scored for severity of arthritis by an investigator who was blinded to the genotype of the animal and the treatment received[40]. The severity of AIA was scored according to the following scale: 0 – normal, 1 – mild inflammation with minor histological changes, 2 – moderate inflammatory disease, 3 – marked inflammatory changes with erosive arthritis and pannus formation, and 4 – severe destructive arthritis with major bone remodeling. Mean scores for individual joints, derived from scores for different sections of the same joint, were used to calculate composite scores for the various experimental groups.

IMAGE ANALYSIS AND STATISTICS

Digital images were analyzed using Image J 1.38x software (NIH) by an investigator blinded to the source material, including the genetic makeup of the mouse and the side (i.e., the notch identifying the right side in spinal cord sections was blocked out from view and the images were flipped horizontally). For DRG, 12 sections were analyzed per side per mouse (4–8 sections of each of L4 and L5 ganglia, approximately 60 μm apart); neuronal profiles (NP) were identified by their typical cellular morphology. The cut-off brightness level (labeling density threshold) was determined by averaging the integral brightness of three NP per section that were judged to be minimally positive. All NP whose mean labeling density exceeded this threshold were counted as positive. For spinal cord, four transverse sections of the L4–L5 segments, spaced approximately 300 μm apart, were analyzed in every mouse. p-ERK-positive neurons, identified by simultaneous labeling with NeuN, were counted in the dorsal horn on each side, spinal laminae were defined based on relative densities of cells and fibers in images obtained with DIC optics[41].

Data were analyzed using SPSS 11.5x software and graphed using Microsoft Excel. Differences between behavioral measurements and of immunohistochemical scores in different animal groups were studied for significance with one-way analysis of variance (ANOVA), which assessed the overall influence of genotype, injected side, and time after induction of AIA, followed by a *post hoc* general contrast comparison using Tukey's test. Analysis of the histological scores for severity of arthritis was performed using nonparametric Mann–Whitney *U* test. Significance was set at $P < 0.05$.

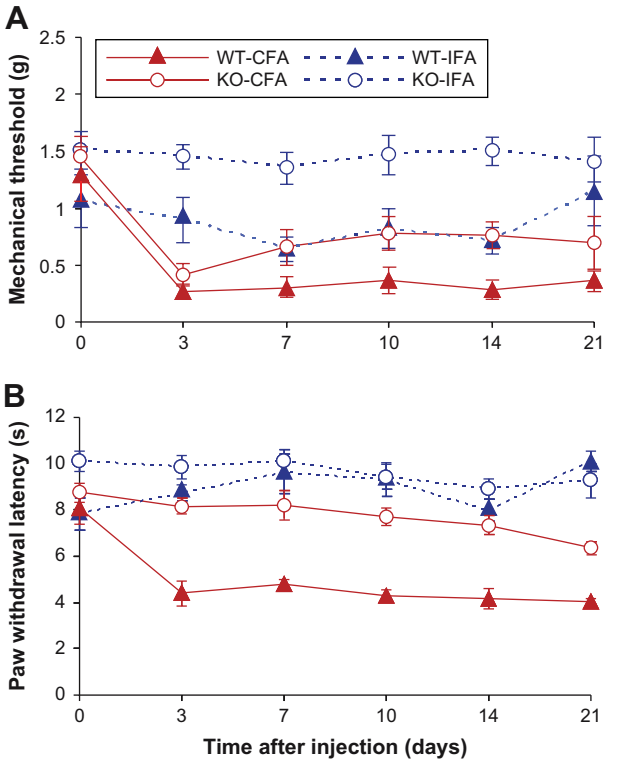

Fig. 1. CFA-induced mechanical allodynia and thermal hyperalgesia were decreased in TRPV1-KO mice. (A) von Frey mechanical testing showed that, compared to baseline sensitivity (time point 0), the CFA-injected side was significantly more sensitive than the IFA-injected side in both phenotypes. Also, the CFA-injected side of KO mice was less sensitive compared to WT mice throughout the testing period and this difference was statistically significant at 14 days. (B) The Hargreaves test showed significantly decreased withdrawal latencies compared to baselines on the CFA-injected side of WT but not in KO mice.

Results

Baseline sensitivity to mechanical or thermal stimuli did not differ significantly between KO and WT mice (Fig. 1). von Frey testing showed that, for all time points, the CFA-injected side was significantly more sensitive to mechanical stimulation than the IFA-injected side for each genotype, and that there was a slight increase in sensitivity on the IFA-injected side of WT but not KO mice (Fig. 1A). Both WT and KO mice were most sensitive on day 3 after CFA but maintained significant allodynia for the duration of the experiment. KO mice were less allodynic than WT on both CFA- and IFA-injected sides throughout the testing period; however, the attenuation of allodynia on the CFA-injected side was significant only on day 14 after injection (Fig. 1A). The Hargreaves test revealed that CFA decreased the withdrawal latencies in both WT and KO mice. In WT mice withdrawal latency decreased to its lowest as early as 3 days post-injection and remained unchanged until day 21, while in KO mice, the latency progressively decreased for the duration of the experiment (Fig. 1B). Importantly, the withdrawal latency on the CFA-injected side in KO mice was not significantly different from baseline or from the IFA-injected side, but was significantly longer than that on the CFA-injected side of WT mice (Fig. 1B).

Outward signs of inflammation were similarly pronounced in WT and KO mice: the paw was reddened and swollen 3 days after injection of CFA and some blistering and/or cracking of the skin appeared in some mice at day 7 and resolved by day 10–14. Redness and swelling of the skin decreased with time but the ankle remained enlarged by ~5 mm (200%) and the skin temperature was elevated by ~3°C throughout the experiment. Upon visual inspection, the IFA-injected ankle appeared unaffected and was used for weight bearing with better overall mobility than the CFA-injected side. Microscopic examination of joint sections revealed that 3–7 days after injection, the joints appeared largely unaffected. However, after 14–21 days, all CFA-injected joints showed clear histological signs of arthritis and, particularly at day 21, partial destruction of cartilage and bone and widespread pannus invasion (Fig. 2A–D). Histopathological scores were significantly higher on the CFA-injected side than on the IFA-injected side in both WT and KO mice at all survival time points. Moreover, at 14 and 21 days after injection, the histological changes in CFA-injected ankles were significantly less severe in KO mice than in WT mice (Fig. 2E).

Fourteen and 21 days after induction of AIA, the fraction of p-ERK-immunoreactive DRG neurons on the CFA-injected

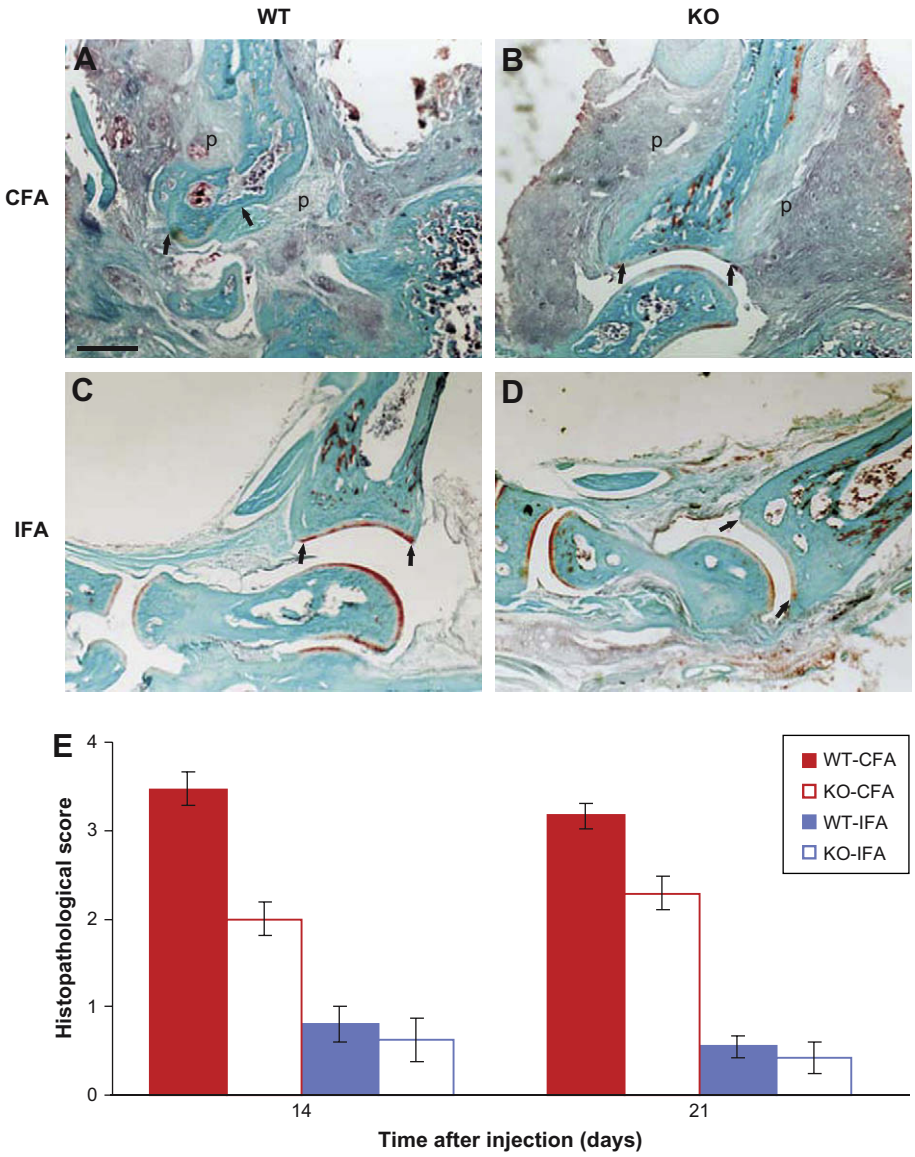

Fig. 2. Histopathological scores were lower in TRPV1-KO than in WT mice with AIA. (A–D) Staining with hematoxylin and safranin-O was used to assess for changes in the articular cartilage (arrowheads) and for pannus invasion (p) of the tibia. More subtle microscopic changes in the joint tissues, including inflammatory infiltration of the joint capsule, hypertrophy and cloning of chondrocytes, and disorganization/fibrillation of the joint cartilage, were also considered in assigning a composite histological score of the overall severity of arthritis. At 21 days, all CFA-injected joints showed obvious signs of advanced OA with partial destruction of cartilage and bone and widespread pannus invasion. (E) At both 14 and 21 days, the overall histological scores (mean ± S.E.M., 14–18 sections per group) on the CFA-injected side were significantly lower in KO mice than in WT mice. Scale bar, 100 μm.

side of KO mice was significantly smaller than that in WT mice, and not significantly different from the fraction on the IFA-injected side (Fig. 3A–E). In parallel with the changes in the DRG, the number of p-ERK-immunoreactive neurons in the superficial dorsal horn of WT mice was also increased 14 and 21 days after CFA injection. In KO mice, this overall increase was significantly smaller, and the number of p-ERK-immunoreactive neurons on the CFA-injected side was significantly smaller than that on the CFA-injected side of WT mice (Fig. 3F–J). At shorter survival time points, the expression of p-ERK in DRG and spinal cord neurons was not significantly different on the CFA-injected and IFA-injected sides of WT and KO mice.

Fourteen and 21 days after induction of arthritis, the fraction of p-ERK-immunoreactive neurons in DRG was 3–4 fold higher on the CFA-injected side than on the IFA-injected side (Fig. 4). The fraction of TRPV1-immunoreactive DRG neurons also increased bilaterally over the values in naïve mice (39.4 ± 0.9% at day 14 and 40.6 ± 0.8% at day 21 vs 28.0 ± 1.7% in naïve mice, mean ± S.E.M.). The neurons that co-expressed p-ERK and TRPV1 constituted a significantly higher fraction of TRPV1-immunoreactive neurons on the CFA-injected side than on the IFA-injected side (Fig. 4H). Moreover, the fraction of p-ERK-immunoreactive neurons that also expressed TRPV1 was significantly higher on the CFA-injected side than on the IFA-injected side (Fig. 4I),

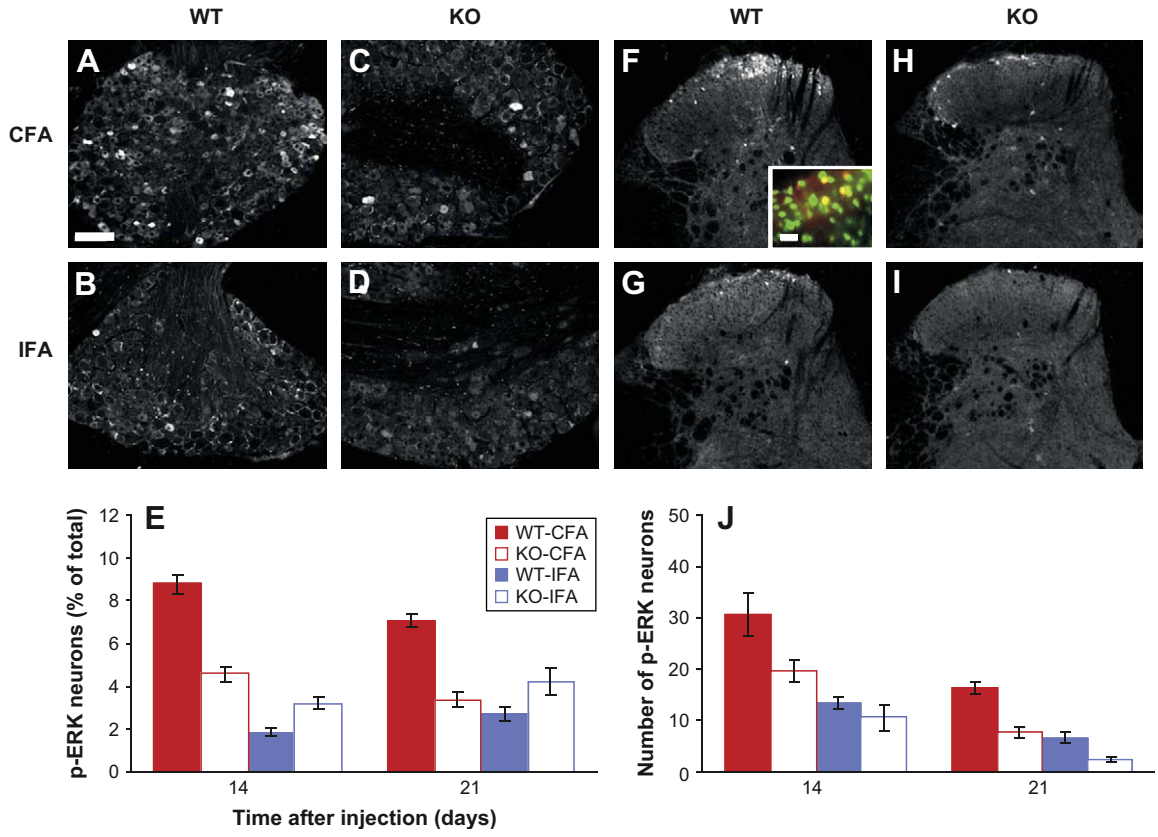

Fig. 3. Expression of p-ERK in DRG and spinal dorsal horn was lower in TRPV1-KO than in WT mice with AIA. (A–E) In DRG, the fraction of labeled neurons on the CFA-injected side in KO mice was significantly smaller than that in WT mice, and not significantly different from the fraction on the IFA-injected side. (F–J) In the spinal cord, the number of p-ERK-immunoreactive neurons on the CFA-injected side in KO mice was significantly smaller than that in WT mice. Photomicrographs are from sections of representative mice with 21 days survival. The insert in (F) illustrates simultaneous immunostaining for p-ERK (red) and the neuronal marker NeuN (green), used to verify that cells in the spinal cord that express p-ERK were neurons. Data in histograms are mean ± S.E.M. from 4 to 8 sections per mouse for DRG and four sections per mouse for the spinal cord in 4–6 mice per time point. Scale bars, 100 μm in (A–I) and 20 μm in the inset in (F).

indicating that CFA had caused p-ERK expression to increase preferentially in TRPV1-positive neurons.

Discussion

The main findings of this study are that 14–21 days after ankle injection of CFA in the mouse, (1) the number of neurons in DRG and spinal dorsal horn at L4–L5 that express the activated form of ERK increased several fold; (2) this increase occurred preferentially in TRPV1-positive DRG neurons, and (3) TRPV1-KO mice had reduced nociceptive behavior, severity of OA, and activation of ERK in sensory neurons, compared to WT mice.

The hypothesis that TRPV1 is involved in inflammatory hyperalgesia was originally supported by evidence that mice lacking this receptor do not develop hyperalgesia to a noxious heat 24 h after cutaneous inflammation[42–44], and that capsazepine, a selective TRPV1 antagonist, reverses the development of mechanical hyperalgesia in experimental inflammation[45]. More recently, the use of transgenic mice to explore the pathways essential to the pathogenesis of human diseases has expanded to include arthritis[46].

In the present study, the mechanical allodynia and thermal hyperalgesia following CFA were reduced in KO, compared to WT mice, in agreement with the results of previous studies of mouse models[18,20]. However, Bolcskei *et al.*[17] found no differences between KO and WT mice in formalin-induced nocifensive behavior, carrageenan-evoked inflammatory mechanical hyperalgesia, and partial sciatic nerve lesion-induced neuropathic mechanical hyperalgesia. In addition, Walker *et al.*[45] reported that pre-treatment with capsazepine reversed the CFA-induced mechanical hyperalgesia in guinea pigs but not in rats or mice. We found that KO mice had normal mechanical sensitivity at baseline[42] and significantly higher mechanoreceptive thresholds on the IFA-injected side than WT mice. This is consistent with the idea that CFA may exert contralateral effects on mechanical, but not on thermal sensitivity, presumably *via* involvement of supraspinal pathways[47], and that this effect may be TRPV1-dependant, as it is missing in KO mice. Keeble *et al.*[19] argued that this suggests an essential involvement of TRPV1 in the control, by the central nervous system, of the phenotypic changes of articular primary sensory neurons during arthritis.

The fraction of p-ERK-positive neurons was higher on the CFA-injected side than on the IFA-injected side in WT but not in KO mice, suggesting that the activation of ERK in nociceptive primary afferents in AIA may be mediated by TRPV1. This is consistent with our previous observation that activation of ERK in primary afferents was significantly

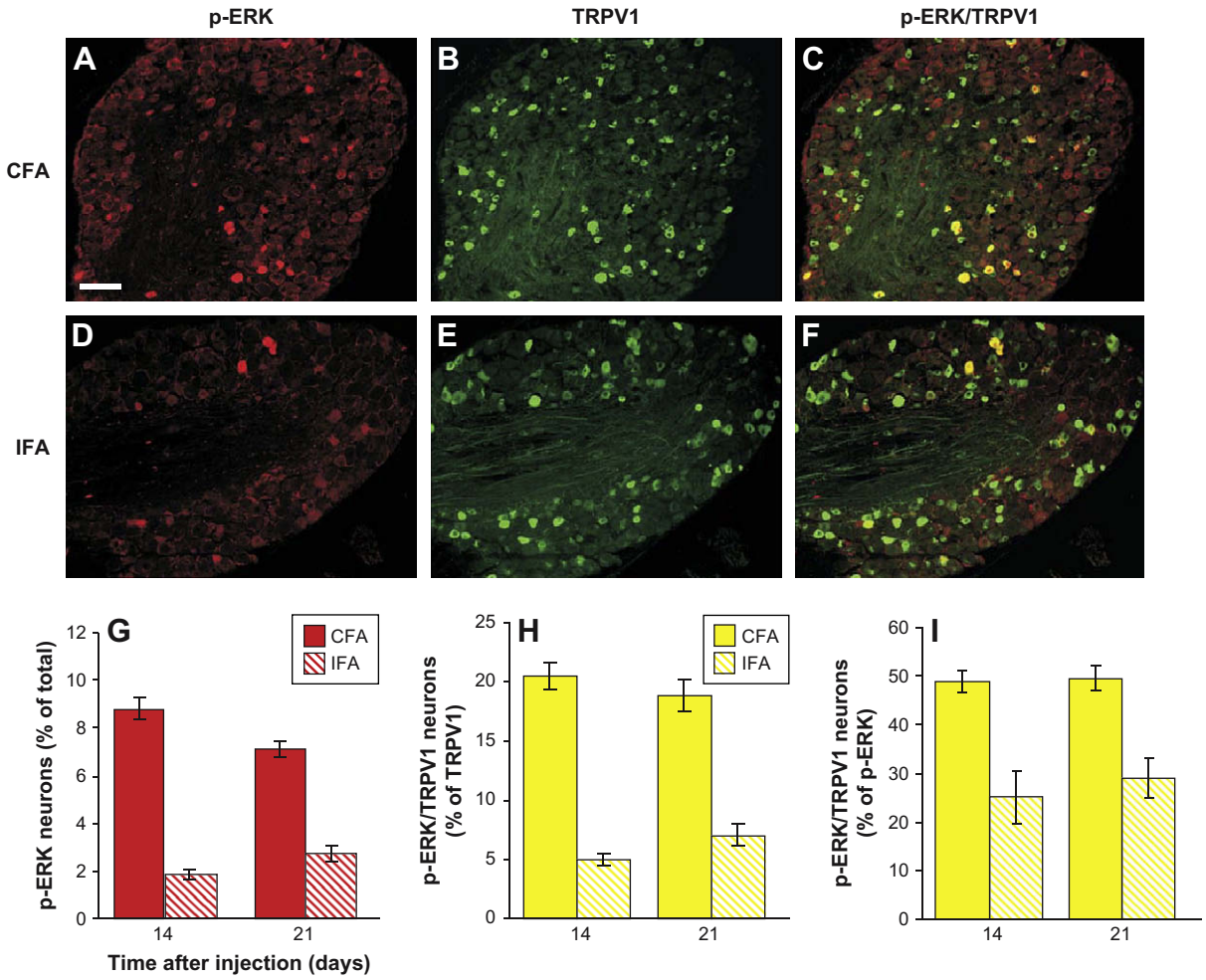

Fig. 4. Expression of p-ERK increased preferentially in TRPV1-positive neurons in DRG after CFA. (A–F) Photomicrographs from ganglia of a representative mouse with 21 days survival, showing that DRG neurons of wild-type mice express p-ERK (red), TRPV1 (green) or p-ERK/TRPV1 (yellow) after injections of CFA or IFA. (G) The fraction of p-ERK-immunoreactive neurons of total neurons was significantly higher on the CFA-injected side than on the IFA-injected side, 14 and 21 days after injections. The double-labeled p-ERK/TRPV1-immunoreactive neurons constituted a significantly higher fraction of TRPV1-immunoreactive neurons on the CFA-injected side than on the IFA-injected side (H) and the fraction of p-ERK-immunoreactive neurons that also expressed TRPV1 was significantly higher on the CFA-injected side (I). Data in histograms are mean ± S.E.M. from 12 sections per side per mouse in 4–6 mice per time point. Scale bar, 100 μm.

higher after intraarticular injections of capsaicin than vehicle[48]. Alternatively, it may be due to the decreased pain and inflammation observed in KO mice and not directly linked to TRPV1. It has been shown that both cutaneous and visceral inflammation leads to nociceptive-specific activation of ERK in spinal neurons[27,49,50]. This may underlie the mechanism of central sensitization at the level of the spinal neuron that receives direct synaptic input from the primary afferent nociceptor. The specific involvement of the ERK pathway in the mechanism of central sensitization is not completely understood but acutely, ERK may modulate excitability of spinal neurons through an interaction with *N*-methyl D-aspartate (NMDA) receptors[51]; chronically, ERK or a downstream product may translocate to the nucleus and regulate transcription of genes involved in nociception such as the neurokinin receptor (NK1)[52,53]. Since TRPV1 can mediate the release of glutamate and substance P (SP)[54,55] from DRG neurons that can induce phosphorylation of ERK in spinal cord neurons[56], we speculate that the increase of p-ERK in AIA may be due, at least in part, to a TRPV1-dependent release of glutamate and SP. Therefore, the reduced activation of ERK in spinal neurons of TRPV1-KO mice may be due to a decreased release of glutamate and SP.

Several recent studies have addressed the role of the ERK pathway in inflammatory joint pain. Using a rat model of AIA, Cruz *et al.*[34] showed that movement of the inflamed joint increased the number of p-ERK-positive neurons in the spinal dorsal horn correlated with the duration of arthritis. In a mouse model of collagen-induced arthritis, upregulation of p-ERK in joints peaked with maximum inflammation, and pharmacological inhibition of the ERK pathway produced a profound antiarthritic effect[25]. Similarly, attenuated activation of ERK in mice with genetic deletion of the scaffold kinase KSR1 correlated with impaired development of arthritis[36]. Taken together, these results support the idea that activation of ERK plays an important role in chronic joint pain and that its inhibition may provide a therapeutic target for arthritis. Providing a functional link between activation of ERK in neurons and their expression of TRPV1, Firner *et al.*[32] showed that ERK phosphorylation is required for rapid facilitation of

capsaicin-induced currents in DRG neurons and concluded that the ERK cascade plays a vital role in the regulation of the TRPV1-positive nociceptors' sensitivity.

In the present study, the edema caused by ankle injections of CFA in KO mice was similar to WT, at variance with previous reports[18–20]. The degree of involvement of extra-articular soft tissues in a particular inflammatory model may be an important determinant of overall swelling of the paw. This may also be why intraplantar injection of vanilloid compounds produced less paw swelling in TRPV1-KO mice[42]. The same reasoning may apply to the discrepant findings regarding the severity of the adjuvant-induced histological changes in the articular and periarticular tissues: the cumulative histopathology scores in KO, compared to WT mice, were found to be significantly lower in the present study and by Szabo *et al.*[20] but not different by Barton *et al.*[18] Thus, while the studies that have utilized KO mice and/or pharmacological manipulations agree that TRPV1 plays an important role in the hyperalgesic and vascular components of OA, the influence of the deletion of the TRPV1 gene on the inflammatory and degenerative components of arthritis remains elusive.

Upregulation of TRPV1 in sensory neurons may represent one mechanism of pain hypersensitivity in arthritis. In this study, the fraction of TRPV1-positive DRG neurons in arthritic mice was significantly higher than that in naïve mice. The value of comparing counts of immunopositive neurons across different groups of animals may be limited by the vagaries of the immunohistochemical protocols. Moreover, we could not identify the articular afferents from the injected ankles, and any discrete changes in this restricted pool may have escaped our analysis. However, our results are consistent with the report that 2 days after injection of CFA in a mouse hindpaw, the percentage of isolectin B4 of *Griffonia simplicifolia* (IB4)-positive DRG neurons that responded to capsaicin or H^+ increased from 24% to 80% and from 54% to 85%, respectively, in parallel with an increased number of TRPV1-immunoreactive neurons[57]. Increased expression of TRPV1 has been reported in DRG neurons[58–61] and in peripheral nerve fibers at the site of inflammation[62,63], although the changes in TRPV1 expression may depend on the particular model of arthritis, survival time, and animal species[64].

Conclusion

In the present study, we have established that activation of the ERK biochemical cascade in nociceptive neurons is mediated, at least in part, by TRPV1. In the absence of TRPV1, the signs of arthralgia and histopathology in the mouse model of AIA are reduced. It will be important to determine the extent of involvement of TRPV1 in ERK activation in nociceptive neurons and to further elucidate the mechanism by which ERK mediates the development of pain and inflammation in arthritis.

Conflict of interest

The authors of the manuscript entitled *vanilloid receptor TRPV1-mediated phosphorylation of ERK in murine adjuvant arthritis* have no conflict of interests with this work.

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