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TRPV1 activation alters the function of Aδ and C fiber sensory neurons that innervate bone

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ABSTRACT

The Transient receptor potential cation channel subfamily V member 1 (TRPV1) is a non-selective cation channel that is activated by capsaicin, low pH and noxious heat. It has been suggested to have a pro-algesic role in a range of conditions that present with bone pain, but the mechanisms by which this occurs are not yet clear. In this study we aimed to determine if TRPV1 is expressed in Aδ and/or C fiber bone afferent neurons, and to explore its role in the activation and/or sensitization of bone afferent neurons to mechanical stimulation. A combination of retrograde tracing and immunohistochemistry was used to determine expression of TRPV1 in the soma of bone afferent neurons that innervate the rat tibial marrow cavity. A novel, *in vivo*, electrophysiological bone-nerve preparation, recently developed in our laboratory, was used to make recordings of the activity and sensitivity of bone afferent neurons in response to application of the TRPV1 agonist capsaicin to the marrow cavity. We found that a substantial proportion of bone afferent neurons express TRPV1. These include both small-diameter myelinated (neurofilament rich) and unmyelinated (neurofilament poor) neurons that are likely to be Aδ and C fiber neurons, respectively. Electrophysiological recordings revealed that application of capsaicin to the marrow cavity increased ongoing activity of C fiber, and to a lesser extent Aδ fiber, bone afferent neurons. Capsaicin also sensitized both Aδ and C fiber bone afferent neurons to mechanical stimulation. This evidence supports a role for TRPV1 in the pathogenesis of pain associated with bone pathology or disease.

1. Introduction

TRPV1 is a nonselective ligand-gated cation channel that detects many physical and chemical stimuli, including noxious heat (> 43 °C), protons (pH < 6), capsaicin and other inflammatory mediators [1]. It is expressed in the nerve terminal endings of peripheral sensory neurons that innervate the bone marrow [2,3] and in dorsal root ganglion (DRG) neurons retrograde labelled from the bone marrow [2,4]. It has been implicated in the pathogenesis of inflammatory and/or proton mediated pain in a number of different bone pathologies, including bone cancer, fracture, osteoarthritis and osteoporosis [2,3,5–13]. In particular, there is an increase in TRPV1 expression in the DRG of animals with cancer-induced bone pain [5–8], fracture pain [9] and ovariectomy induced osteoporosis [10]. Pharmacological blockade of TRPV1 attenuates cancer-induced bone pain [3,5,11] and TRPV1 knockout or ablation prevents pain in animal models of cancer-induced bone pain and osteoarthritis [2,12,13]. Taken together, these findings provide clear evidence that TRPV1 plays a critical role in the generation and/or maintenance of bone pain. However, it is not yet clear how TRPV1 activation affects the function of different populations of sensory neurons that innervate bone (bone afferent neurons).

Pain is transmitted by two main classes of peripheral sensory neurons [14]. Aδ fiber nociceptors are small-diameter myelinated sensory neurons that transmit fast, intense pain, of the sort experienced in response to fracture, acute inflammation or mechanical instability of bone. C fiber nociceptors are small-diameter unmyelinated sensory neurons that encode slow, aching pain of the sort experienced in more chronic conditions such as osteoarthritis or bone cancer. Both Aδ and C fiber nociceptors innervate bone [15–20], contain molecular markers for nociceptors, such as substance P (SP), calcitonin gene-related peptide (CGRP), and tropomyosin receptor kinase A (TrkA) [4,16,19,21–25], and are responsive to noxious chemical and mechanical stimuli [4,17,18,26–28].

We have recently developed a novel *in vivo* electrophysiological bone-nerve preparation that allows us to record, for the first time, the activity of different populations of bone marrow nociceptors [4,29–31]. In these studies, recordings were made from a small nerve, proximal to its entry into bone, in response to noxious mechanical stimulation delivered by increasing intra-osseous pressure with injections of isotonic saline, or in response to known algesic substances, including

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inflammatory mediators. This has provided us with unprecedented insight into how bone marrow nociceptors respond to noxious stimulation of bone, and an assay for use in testing therapeutics to treat bone pain. Here we use this approach to determine how bone marrow nociceptors respond to activation of TRPV1, with a view to identifying mechanisms that we could manipulate for therapeutic benefit in the future.

The specific aims of the present study were to explore TRPV1 expression in small myelinated (Aδ-fiber) and unmyelinated (C-fiber) bone afferent neurons, and to determine how TRPV1 activation by capsaicin affects their function. The findings support a role for TRPV1 in the pathogenesis of pain associated with bone pathology or disease.

2. Material and methods

Male Sprague-Dawley rats weighing between 200 and 250 g were used in this study. Animals were group housed in a 12 h light/dark cycle and were provided with food and water *ad libitum*. All experiments conformed to the Australian National Health and Medical Research Council code of practice for the use of animals in research and were approved by the University of Melbourne Animal Experimentation Ethics Committee.

2.1. Retrograde tracing and immunohistochemistry

Animals were anesthetized with isoflurane in air (4% induction; 2.5% maintenance). A skin incision was made over the medial aspect of the tibia and a small hole was made in the cortical bone on the medial aspect of the tibial diaphysis using a sterile needle. A Hamilton syringe was used to inject the retrograde tracer Fast Blue (2 μl FB; 10% in dH_2O) through the hole and directly into the marrow cavity. The hole was sealed with bone wax to prevent leakage into surrounding tissues and the entire area was washed extensively with 0.1 M phosphate buffered saline (pH 7.4; PBS) and inspected for tracer leakage using a hand-held UV illumination device. There was no evidence of tracer leakage to surrounding tissues. Skin incisions were closed with stainless steel autoclips. Animals were left for a 10-day survival period to allow for transport of the tracer to neuronal cell bodies in the DRG. After 10 days, each animal was given an overdose of ketamine/xylaxine (ketamine 130 mg/kg; xylaxine 10 mg/kg; i.p.), and was perfused *via* the ascending aorta with 500 ml of PBS followed by 500 ml of 4% paraformaldehyde in PBS. Lumbar DRG L3 were dissected and left overnight in PBS sucrose (30%), and were sectioned at 10 μm using a cryostat the next day. Multiple series of sections were collected on gelatinized glass slides (0.1% chrome alum and 0.5% gelatin), air-dried for an hour and processed for immuno-labelling.

Sections were immuno-labelled to determine if retrograde labelled neurons expressed TRPV1 and/or the neurofilament heavy chain marker for myelinated neurons (NF200). Details of the primary and secondary antisera, including references to work that demonstrate specificity of the antibodies, are given in Table 1. All antisera were diluted in PBS containing 0.3% Triton X-100 and 0.1% sodium azide. Sections were washed 3 times in PBS, incubated for 1 h in 10% normal horse serum containing 1% Triton X-100, and then incubated overnight in the primary antisera at room temperature. Following 3 further washes in PBS, they were incubated in secondary antisera for 2 h, and washed again 3 times in PBS. The slides were cover-slipped using DAKO fluorescence mounting medium.

DRG sections were examined and photographed with a 10× objective using a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkocken, Germany) fitted with an AxioCam MRm camera. FITC, Texas Red and UV filter sets were used to discriminate labelling with the AlexaFluor 488, 594 fluorophores and Fast Blue, respectively. Counts, soma size measurements (cross-sectional area of soma) and intensity measurements were made directly from the images using Zen lite software. Only cells with a nucleus visible under the microscope were examined. We determined the proportion of retrograde labelled neurons that expressed each antibody marker for each animal. Data are presented as mean ± SEM. Figures were prepared using CorelDraw software. Individual images were contrast and brightness adjusted. No other manipulations were made to the images.

2.2. Electrophysiological recordings using an in vivo bone-nerve preparation

2.2.1. Recording configuration

We used an *in vivo* bone-nerve electrophysiological preparation, recently developed in our lab [4,29–31], to determine how bone afferent neurons responded to application of the TRPV1 agonist capsaicin to the bone marrow. Rats were anesthetized with urethane (50% w/v, 1.5 g/kg i.p.). Rectal temperature was maintained within the physiological range (36–37 °C) with a servo-controlled heating pad. A fine branch of the tibial nerve that innervates the tibial marrow cavity was carefully teased away from its associated blood vessels and membranes and placed on a platinum hook electrode for extracellular recording. Whole-nerve electrical activity was amplified (1000×) and filtered (high pass 100 Hz, low pass 3 kHz) (DP-311 differential amplifier, Warner Instruments), sampled at 20 kHz (PowerLab, ADInstruments, Australia) and stored to PC using LabChart recording software (ADInstruments). Mechanical stimulation was delivered to the endings of bone afferent neurons by raising intra-osseous pressure in the marrow cavity. This was achieved by injection of heparinised (0.17 IU/ml) physiological saline (0.9% sodium chloride) through a needle that was connected to a feedback-controlled syringe pump (PHD ULTRA pump, Harvard apparatus) with polyethylene tubing. The input pressure to the bone was measured using a bridge amplified (TAM-D amplifier, Harvard Apparatus) signal derived from a pressure transducer (APT300 transducer, Harvard Apparatus), placed to measure the input pressure to the bone. The pump uses this as feedback to adjust flow through the system to control and maintain constant input pressures. We used this feature to apply a ramp-and-hold mechanical stimulus with an initial flow rate of 7 ml/min during the ramp phase, and a constant 300 mm Hg of pressure delivered for 15-s duration during the hold phase. Capsaicin (0.1 or 1 μM), or saline (control), was delivered to the marrow cavity through a second hole using a Hamilton syringe attached with polyethylene tubing. This was infused slowly so there were no

Table 1
Source and concentrations of the primary and secondary antisera used in this study.

Primary antibody antigen	Immunogen	Manufacturer and product code	Dilution used	Specificity/characterization
Rabbit α Transient Receptor Potential Vanilloid 1 (TRPV1)	Intracellular c-terminus of rat TRPV1 (824–838)	Alomone Labs; Rabbit polyclonal; #ACC-030	1:500	[95,96] Manufacturer's information
Mouse α Neurofilament 200 (NF200)	Carboxyterminal tail segment of pig neurofilament H-subunit	Sigma; Missouri, US; Mouse monoclonal; #N0142	1:1000	[97] Manufacturer's information

Secondary antibody	Manufacturer	Dilution used
Donkey anti-Rabbit 594	Molecular probes, Invitrogen; #A21207	1:200
Donkey anti-Mouse 488	Molecular probes, Invitrogen; #A21202	1:200

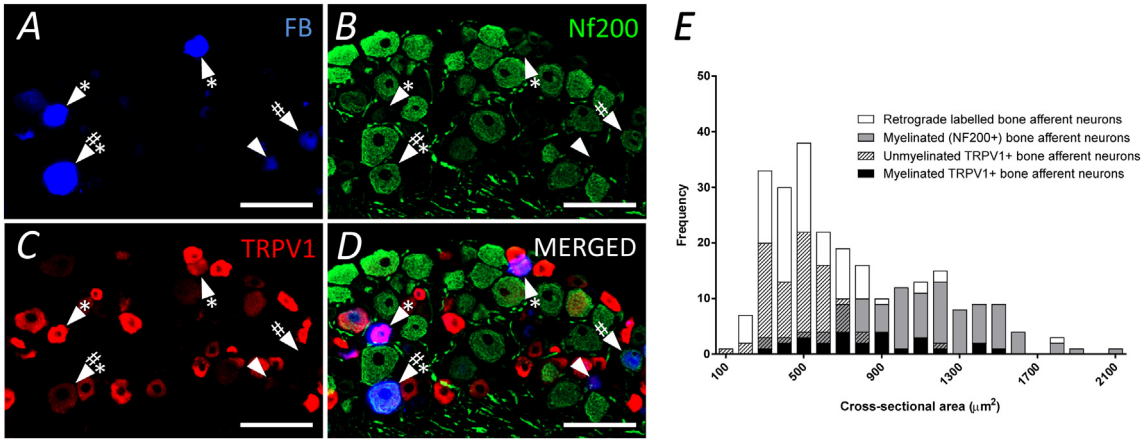

Fig. 1. TRPV1 expression in retrograde labelled bone afferent neurons (n = 3). Panels A-D are representative images of the same field of a single section of the L3 dorsal root ganglion, showing in blue retrograde labelled bone afferent neurons (A), green immunolabelling of NF200+ myelinated afferent neurons (B), red immunolabelling of TRPV1+ bone afferent neurons (C), and a merge of all three channels (D). Retrograde labelled bone afferent neurons are indicated by arrowheads. NF200+ myelinated bone afferent neurons are indicated by hashes (#). TRPV1+ bone afferent neurons are indicated by asterisks (*). Scale bars = 100 μm. E shows the distribution of size/frequency for all retrograde labelled bone afferent neurons analyzed (white), retrograde labelled neurons that are also myelinated (grey), retrograde labelled neurons that are both unmyelinated and express TRPV1 (hashed) and retrograde labelled neurons that are both myelinated and express TRPV1 (black). Most unmyelinated bone afferent neurons express TRPV1 and have a size profile consistent with a C fiber classification. Only a quarter of myelinated bone afferent neurons express TRPV1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

changes in the pressure recorded in the marrow cavity in response to injection.

2.2.2. Classification of spikes derived from Aδ and C fiber bone afferent neurons

We were unable to routinely record conduction velocities in each experiment because we could not electrically stimulate the receptive fields of individual units buried deep inside the marrow cavity. Instead, we classified spikes as originating from C, Aδ or Aβ units on the basis of previously published experiments using the same recording configuration in which we demonstrated a linear relationship between conduction velocity and peak-to-peak action potential amplitude for units activated with mechanical stimulation from within the bone marrow [30,31]. On the basis of this relationship, impulses with amplitudes < 40 μV were defined as originating from C-fibers (conduction velocities < 2.5 m/s) and those with amplitudes between 40 and 145 μV were defined as originating from Aδ fibers (conduction velocities between 2.5 and 12.5 m/s). For a thorough discussion of how this division was selected, see [30].

2.2.3. Recording protocols and analysis

All action potentials (spikes) with positive and/or negative peaks clearly above noise were sampled from the whole-nerve recordings. Subsequent analysis was performed on data derived from spikes with amplitudes consistent with Aδ or C fiber conduction velocities.

For analysis of ongoing activity, a continuous whole-nerve recording was made from 5 min before to 35 min after capsaicin or saline injection. The number of spikes before and after the injection was reported in a frequency histogram generated using 20 s bin widths. For analysis of the onset of capsaicin-induced activity, the number of spikes before and after the injection was determined with a frequency histogram generated using 1 s bin widths and the onset of the effect was defined as the time at which the mean frequency of discharge in two consecutive 1 s bins was at least twice that of the maximum mean frequency of discharge in all pre-injection 1 s bins. This criterion was used to define a change in activity in response to application of capsaicin. 1 s bin widths were used for this latter analysis because the 20 s bins did not provide adequate resolution to capture the rapid onset of capsaicin-induced activity.

For analysis of sensitivity to mechanical stimulation, whole-nerve recordings were made during application of a ramp-and-hold intraosseous pressure stimulus of 15-s duration and 300 mm Hg. Discharge frequency during the pressure stimulus was determined before and at 10 min after application of capsaicin or saline. It was reported over the entire ramp-and-hold pressure stimulus (total response), for the ramp phase of the stimulus (defined as the first 2 s of the ramp-and-hold pressure stimulus), and for the hold phase of the stimulus (defined as the last 5 s of the ramp-and-hold pressure stimulus). Discharge frequency was expressed as fold-change relative to the pre-injection baseline values.

2.3. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Comparison between the effects of capsaicin and saline on the activity of bone afferent neurons was analyzed using a two-way ANOVA with repeated measures over time. Bonferroni's *post hoc* tests were performed only if the ANOVA indicated a significant difference. Comparison between the effects of capsaicin and saline on responses to mechanical stimuli were tested using the non-parametric Kruskal-Wallis test followed by Dunn's *post hoc* tests. $P < 0.05$ was used to define statistical significance. Data are represented as mean ± SEM.

3. Results

3.1. TRPV1 is expressed in both small-diameter myelinated and unmyelinated bone afferent neurons

We used a combination of retrograde tracing and immunohistochemistry to determine the proportion of bone afferent neurons that express TRPV1 and/or NF200 (a marker for myelinated neurons) (Fig. 1; Table 2). A total of 251 retrograde labelled bone afferent neurons were counted in L3 DRG taken from three animals. Almost all bone afferent neurons were small or medium sized (> 99% of those counted were < 1800 μm^2; Fig. 1E). A substantial proportion of bone afferent neurons expressed TRPV1 (47 ± 4.8%; n = 3 animals; Table 2). The majority of TRPV1 expressing bone afferent neurons were unmyelinated (did not express NF200; 78 ± 1.9%; n = 3 animals), but some were myelinated (expressed NF200, 22 ± 1.9%; n = 3 animals)

Table 2
The percentage of retrograde labelled bone afferent neurons that express TRPV1 and/or NF200.

	Percentage (Mean ± SEM)
Percentage of bone afferent neurons that express:	
TRPV1	47 ± 4.8
NF200	46 ± 4.6
Proportion of unmyelinated (NF200−) bone afferent neurons that express TRPV1	68 ± 3.7
Proportion of myelinated (NF200+) bone afferent neurons that express TRPV1	23 ± 2.6
Proportion of TRPV1 expressing bone afferent neurons that are unmyelinated (NF200−)	78 ± 1.9
Proportion of TRPV1 expressing bone afferent neurons that are myelinated (NF200+)	22 ± 1.9

(Table 2). Approximately two thirds of the unmyelinated bone afferent neurons (68 ± 3.7%; n = 3 animals; Table 2) and one quarter of the myelinated bone afferent neurons (23 ± 2.6%; n = 3 animals; Table 2) expressed TRPV1. The unmyelinated TRPV1 expressing bone afferent neurons were almost entirely small, and the myelinated TRPV1 expressing bone afferent neurons were small and medium sized (Fig. 1E).

3.2. Capsaicin applied to the marrow cavity increases ongoing activity in bone afferent neurons

We used the *in vivo* electrophysiological bone-nerve preparation to determine if capsaicin can directly activate bone afferent neurons. Application of capsaicin to the marrow cavity increased ongoing activity in C fibers, and to a lesser extent Aδ fibers (Fig. 2). Fig. 2A shows a typical example of nerve activity in response to application of 1 μM capsaicin. It is clear from this example that capsaicin increased the activity of C fiber (< 40 μV peak-to peak), and to a lesser extent Aδ fiber (> 40 μV peak-to-peak) spikes. Group data show that there was a rapid and transient increase in C fiber spike activity in animals injected with 1 μM capsaicin (Fig. 2B) but not 0.1 μM capsaicin (Fig. 2C) or saline (Fig. 2D). For all recordings made with 1 μM capsaicin, the increased C fiber spike activity began within 1 min of capsaicin application to the marrow cavity (latency to onset = 4.8 ± 1.57 s; mean ± SEM; n = 5 animals), and was maintained for up to 40 s (Fig. 2B). The magnitude of the peak capsaicin-induced activity was up to 5 times that of the baseline. Two-way ANOVA with repeated measures revealed a significant interaction ($F(8,44) = 3.775$ $P = 0.0019$) and time effect ($F(4, 44) = 5.483$ $P = 0.0011$), and Bonferroni's *post hoc* analysis showed it was only apparent at 0–1 min after administration of 1 μM capsaicin (Fig. 2E). In contrast to C fibers, there was only a change in Aδ fiber spike activity in two of the recordings in which 1 μM capsaicin was applied (latency to onset = 7 and 27 s), and one of the recordings in which 0.1 μM capsaicin was applied (latency to onset = 4 s). For the group mean data reported this appears as only a small change in Aδ fiber spike activity in response to 1 μM (Fig. 2F) and 0.1 μM capsaicin (Fig. 2G), but not saline (Fig. 2H). Whilst the magnitude of this change was up to 5 times that of baseline, Two-way ANOVA with repeated measures showed that these changes were not statistically significant (treatment $F(2,11) = 0.7638$ $P = 0.4891$; time $F(4,44) = 1.289$ $P = 0.2889$; interaction $F(8,44) = 0.8727$ $P = 0.5465$) (Fig. 2I).

3.3. Capsaicin applied to the marrow cavity sensitizes bone afferent neurons to noxious mechanical stimulation

To test whether capsaicin could sensitize bone afferent neurons to noxious mechanical stimulation, we assessed the frequency of spikes during responses to a ramp-and-hold pressure stimulus (300 mm Hg), before and following application of saline, 0.1 μM or 1 μM capsaicin (Fig. 3). Fig. 3A and B show examples of whole-nerve recordings, and rasters of both Aδ fiber and C fiber spikes, in response to the pressure stimulus, before (Fig. 3A) and after (Fig. 3B) application of 1 μM capsaicin to the marrow cavity. In this example, there was an increase in both Aδ fiber and C fiber activity in response to the pressure stimulus after application of capsaicin. Group data showed that although not all of the recordings showed evidence of sensitization (increase in whole-nerve discharge frequency), there was a dose dependant increase in whole-nerve discharge frequency after application of capsaicin (Fig. 3C and D). Kruskal Wallis testing with Dunn's *post hoc* analysis of this group data revealed that, when calculated over the entire pressure stimulus (total response), this increase was statistically significant only in animals injected with 1 μM capsaicin compared to those injected with saline (C fiber, Fig. 3C, $H(2) = 8.966$ $P = 0.0113$; and Aδ fiber, Fig. 3D, $H(2) = 7.005$ $P = 0.0301$). We have demonstrated in the past that the activity of discrete sub-populations of bone afferent neurons can be differentially affected by different parts of the pressure stimulus we deliver [31], and this could in part contribute to coding for different types of mechanical stimuli presented to bone. We therefore repeated the analyses using data confined to either the ramp phase of the pressure stimulus (to capture effects of change in pressure on neuronal activity), or the hold phase of the pressure stimulus (to capture effects of sustained pressure on neuronal activity). Kruskal Wallis testing of group data revealed significant differences in activity during both the ramp phase (C fiber, Fig. 3C1, $H(2) = 8.449$ $P = 0.0088$; and Aδ fiber, Fig. 3D1, $H(2) = 7.953$ $P = 0.0187$) and the hold phase (C fiber, Fig. 3C2, $H(2) = 11.38$ $P = 0.0034$; and Aδ fiber, Fig. 3D2, $H(2) = 7.18$ $P = 0.0276$). Dunn's *post hoc* analysis further showed significant (and dose dependant) changes in C fiber activity, at both of the capsaicin concentrations, during only the *hold phase* of the mechanical stimulus when there was sustained pressure within the marrow cavity (Fig. 3C2). It also showed significant (and dose dependant) changes in Aδ fiber activity, at both of the capsaicin concentrations, during only the *ramp phase* of the mechanical stimulus when the pressure was changing (Fig. 3D1). Thus capsaicin dose dependently sensitizes both Aδ and C fiber bone afferent neurons, and there may be differential effects on each that could be related to the type of stimulation delivered.

4. Discussion

The main findings of the present study are that TRPV1 is expressed in a substantial proportion of both small myelinated (Aδ fiber) and unmyelinated (C fiber) bone afferent neurons, and that TRPV1 activation by capsaicin affects the function of both Aδ and C fiber bone afferent neurons. The findings identify mechanisms by which TRPV1 activation could mediate pain associated with bone pathology or disease.

We have previously shown that TRPV1 is expressed in a substantial proportion of bone afferent neurons [4], and others have also reported it is expressed in peripheral nerve terminals that innervate the bone marrow [3]. In this study we have extended these findings by showing that TRPV1 is expressed in two thirds of unmyelinated (C fiber) bone afferent neurons, and in one quarter of small myelinated (Aδ fiber) bone afferent neurons. This expression profile aligns with previous functional data from our group showing that one quarter of the single, isolated Aδ fiber bone marrow nociceptors that we have recorded from have reduced thresholds for activation and increased discharge frequency in response to pressure after the application of capsaicin [31]. Thus the expression profile we report here is consistent with the presence of functional TRPV1 in a subset of Aδ fiber bone afferent neurons. TRPV1 expression patterns that are consistent with this have also previously been reported in DRG and trigeminal ganglion neurons [32–35], and in sensory neurons retrogradely labelled from other tissues [36–44]. Interestingly, the vast majority of TRPV1 expressing visceral afferent neurons that innervate the gut are peptidergic [45,46]. We did not double label bone afferent neurons with antibodies directed against TRPV1 and CGRP in the present study and so cannot comment on

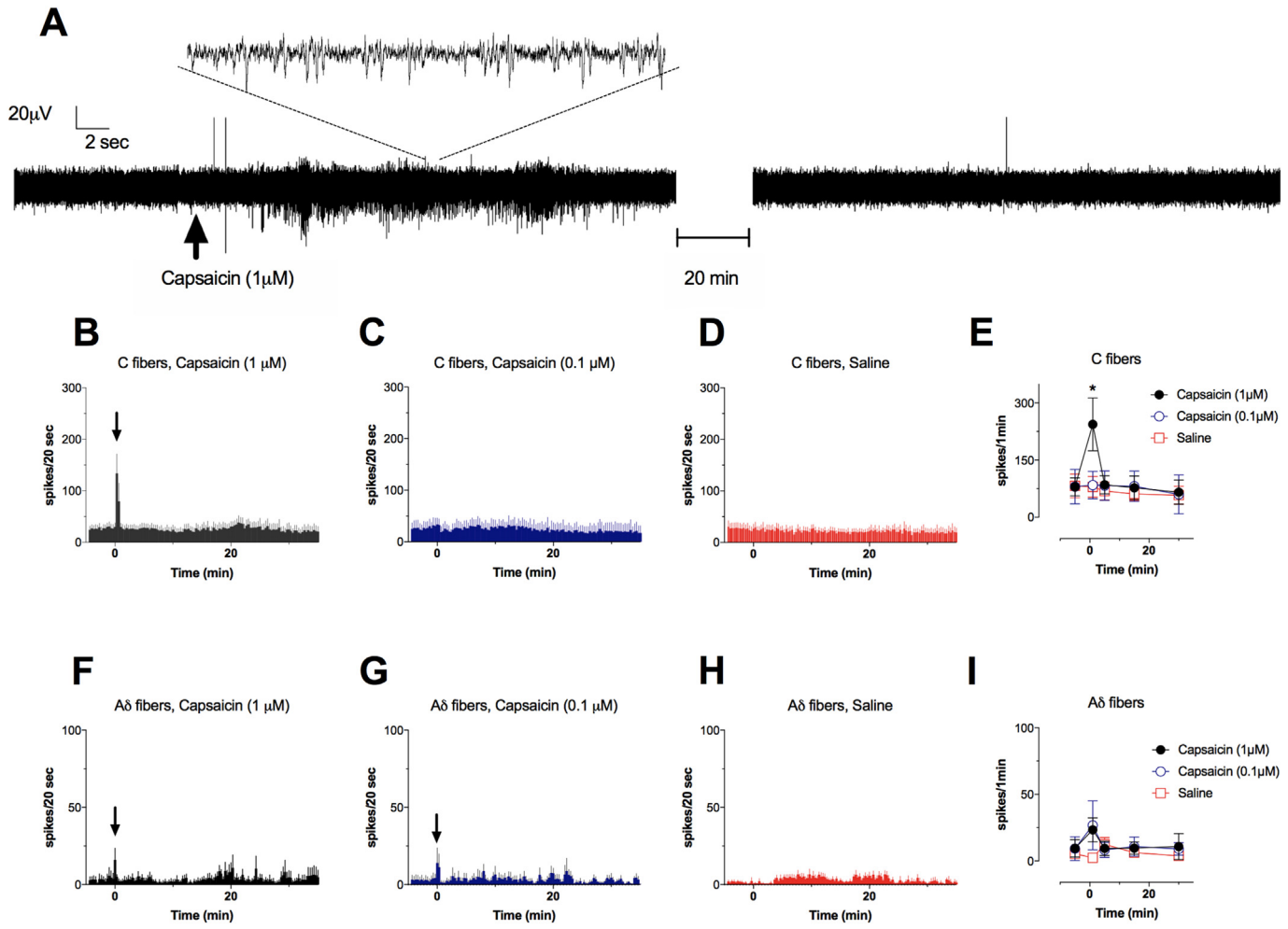

Fig. 2. Capsaicin applied directly to the marrow cavity activates bone afferent neurons. A, An example of a whole-nerve recording after application of 1 μM capsaicin to the marrow cavity. This example shows a rapid and transient increase in activity of small amplitude (C fiber; < 40 μV peak-to-peak) and medium amplitude (Aδ fiber; 40–145 μV peak-to-peak) spikes. B–D, Frequency histograms of the total number of C fiber spikes isolated from whole-nerve recordings before and after application of 1 μM capsaicin (B, n = 5), 0.1 μM capsaicin (C, n = 4) or saline (D, n = 5). Bin width = 20 s. E, Group data showing C fiber spike counts in 1 min bins of time, at discrete time-points over the recording period (5 min before, and immediately, 5, 15 and 30 min after injection of capsaicin or saline). 1 μM capsaicin caused a rapid and transient increase in C-fiber spike activity compared to baseline recordings (asterisk, Bonferroni's *post hoc* test, $P < 0.001$). F–H, Frequency histograms of the total number of Aδ fiber spikes isolated from whole-nerve recordings before and after application of 1 μM capsaicin (F, n = 5), 0.1 μM capsaicin (G, n = 4) or saline (H, n = 5). Bin width = 20 s. I, Group data showing Aδ fiber spikes counts in 1 min bins of time, at discrete time-points over the recording period (5 min before, and immediately, 5, 15 and 30 min after injection of capsaicin or saline). There were no statistically significant differences in Aδ fiber spike counts at any time-point. Data are represented as mean ± SEM.

whether this is also the case for afferent neurons that innervate the bone. TRPV1 expression in peripheral sensory neurons is known to be increased during inflammation [47,48], and this could affect the pattern of expression in bone pathology or disease. Indeed, increased TRPV1 expression has been reported in a number of bone pathologies, including in the synovium of osteoarthritis patients [49], in DRG neurons of rats with osteosarcoma [5], and in a rat model of osteoporosis [10].

Direct activation of TRPV1 by capsaicin causes a rapid depolarization that results in increased action potential discharge in peripheral sensory neurons [50–53]. TRPV1 expression was observed in both Aδ and C fiber bone afferent neurons, and our electrophysiological experiments revealed that application of 1 μM capsaicin to the marrow cavity rapidly and transiently increases discharge frequency in C fiber, and to a lesser extent, Aδ bone afferent neurons. The short latency of the change in activity of bone afferent neurons suggests this effect was likely due to direct activation of TRPV1 by capsaicin. Our findings are consistent with results of other electrophysiological studies of the response of peripheral sensory neurons to capsaicin [54]. For example, capsaicin stimulates the peripheral endings of cutaneous C fiber polymodal nociceptors [55–62], C fiber warmth receptors [55–57,63], and some Aδ fiber polymodal nociceptors [60,64,65].

Sensitivity to mechanical stimulation is a hallmark of a number bone pathologies and it is thought to result from sensitization of peripheral bone afferent neurons [66–68]. Capsaicin has been shown to sensitize both Aδ and C fiber nociceptors to mechanical stimulation in a number of different tissue systems [69–72]. Our findings reveal that both Aδ and C fiber bone afferent neurons can be sensitized to mechanical stimulation by capsaicin, suggesting that TRPV1 activation in both of these populations could contribute to increased sensitivity to mechanical stimuli in patients with bone pathology or disease. How capsaicin alters mechanical sensitivity is not yet known. It is possible that activation of TRPV1 results in phosphorylation of other transduction molecules that sensitize the bone afferent neurons to mechanical stimulation [73]. Another possibility is that TRPV1 activation lowers the voltage threshold for action potential initiation, making these neurons more excitable. It is also possible that they could be sensitized by capsaicin-induced local inflammation [74].

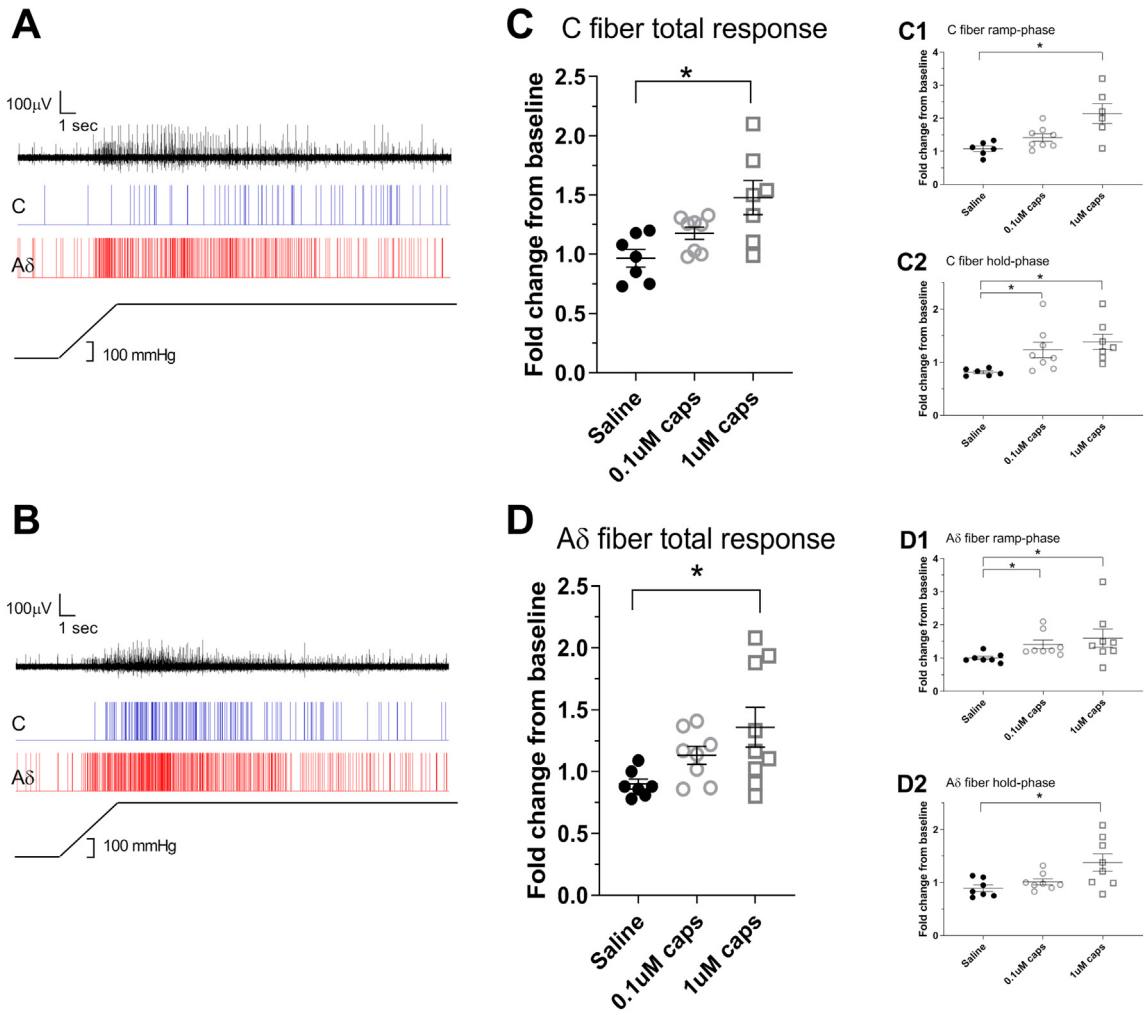

Fig. 3. Capsaicin sensitizes both Aδ and C fiber bone afferent neurons to mechanical stimulation. A and B are examples of a whole-nerve recording and rasters of Aδ and C fiber activity in response to a 300 mm Hg ramp-and-hold pressure stimulus before (A) and 10 min after (B) 1 μM capsaicin application to the marrow cavity. C, Application of 1 μM capsaicin (n = 8) resulted in a significant increase in the discharge frequency of small amplitude (C fiber) spikes across the total response (C, Dunn's test; $P < 0.05$), and during both the ramp (C1, Dunn's test; $P < 0.01$) and hold (C2, Dunn's test; $P < 0.01$) phases of the pressure ramp, relative to saline (n = 7). Application of 0.1 μM capsaicin (n = 8) resulted in an increase in the discharge frequency of small amplitude (C fiber) spikes only during the hold phase (C2, Dunn's test; $P < 0.05$). D, Application of 1 μM capsaicin (n = 8) caused a significant increase in the discharge frequency of medium amplitude (Aδ fiber) spikes across the total response (D, Dunn's test; $P < 0.05$), and during both the ramp (D1, Dunn's test; $P < 0.05$) and hold (D2, Dunn's test; $P < 0.05$) phases of the pressure ramp, relative to saline (n = 7). Application of 0.1 μM capsaicin (n = 8) resulted in an increase in the discharge frequency of medium amplitude (Aδ fiber) spikes only during the ramp phase (D1, Dunn's test; $P < 0.05$). Data are represented mean ± SEM.

Whilst capsaicin is used routinely and to good effect as a TRPV1 agonist in experimental studies, it is not an endogenous ligand for TRPV1, and it is not what interacts with TRPV1 in disease. Inflammation, protons and noxious heat are more relevant stimuli during physiological and/or pathological conditions. Inflammation is a hallmark of many bone pathologies including bone cancers, fractures, intra-osseous engorgement syndrome, osteoarthritis and osteomyelitis [67,75–81]. Agents known to act by reducing inflammatory processes (*e.g.* NSAIDs and specific COX inhibitors) produce partial analgesia in animal models of cancer-induced bone pain [66,82,83] and pro-inflammatory cytokines contribute to mechanically-induced nociceptive responses in fracture models [84]. Osteoclast-mediated bone remodeling is accompanied by the production of extracellular protons (hydrogen ions), which are known to activate and/or sensitize nociceptors in other tissues [85–87]. Increased osteoclast activity is also a hallmark of osteoporosis [11] and can occur in some types of bone cancer [88,89]. Whilst pain is clearly experienced in response to fracture in patients with osteoporosis, there is also pain reported in osteoporosis patients with no evidence of fractures [90], and this could result from activation and/or sensitization of bone afferent neurons by protons released from osteoclast mediated bone resorption [91]. Interestingly, bisphosphonates, which are anti-bone resorption drugs that inhibit osteoclast activity, relieve pain in patients with osteoporosis (even pain in the absence of evidence of fracture) [90,92], and in animal models of bone cancer induced pain [93,94]. Taken together with the results we have presented here, these studies suggest that inflammatory mediators and protons released during bone pathology and/or disease are likely triggers of pain originating in the bone marrow, and that TRPV1 expressing bone afferent neurons, at least in part, contribute to the pain associated with these conditions.

In conclusion, this study has demonstrated that both Aδ and C fiber bone afferent neurons express TRPV1, and that application of a TRPV1 agonist to peripheral nerve terminal endings in the marrow cavity can activate and sensitize Aδ and C fiber bone afferent neurons. The findings support a role for TRPV1 in the pathogenesis of pain associated with bone pathology or disease.

Declarations of interest

None.

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Author contributions

Michael Morgan was involved in Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. Sara Nencini was involved in Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - review & editing. Jenny Thai was involved in Data curation; Formal analysis; Methodology; Writing - review & editing. Jason Ivanusic was involved in Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing - original draft; Writing - review & editing.

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