MECHANICALLY SENSITIVE Aδ NOCICEPTORS THAT INNERVATE BONE MARROW RESPOND TO CHANGES IN INTRA-OSSEOUS PRESSURE

Sara Nencini[a] and Jason Ivanusic[a]*

[a] Department of Anatomy and Neuroscience, University of Melbourne, Victoria 3010, Australia

Key words: Bone pain, Mechano-nociceptor, Skeletal pain

Running Title: Mechanical sensitivity of bone nociceptors

*** Correspondence:**

Dr. Jason Ivanusic

Department of Anatomy and Neuroscience,

University of Melbourne,

Melbourne, VIC 3010, Australia

Tel: +61 3 8344 7254

Fax +61 3 9347 5219

Email: j.ivanusic@unimelb.edu.au

KEY POINTS SUMMARY

- Sensory neurons that innervate the bone marrow provide the central nervous system with information about pain associated with bone disease and pathology, but little is known of their function
- Here we use a novel *in vivo* bone-nerve electrophysiological preparation to study how they respond to noxious mechanical stimulation delivered by increasing intra-osseous pressure
- We provide evidence that sensory neurons that innervate the bone marrow respond to high threshold noxious mechanical stimulation, have response properties consistent with a role in nociception, provide information about different features of an intra-osseous pressure stimulus, and express the Piezo2 mechano-transducer molecule
- Our findings show how some bone marrow nociceptors signal pain in bony diseases and pathologies that involve a mechanical disturbance or increased intra-osseous pressure, and that the Piezo2 mechano-transducer may be involved

ABSTRACT

Whilst the sensory neurons and nerve terminals that innervate bone marrow have a morphology and molecular phenotype consistent with a role in nociception, little is known about their physiology or the mechanisms that generate and maintain bone pain. In the present study, we provide evidence that Aδ nociceptors that innervate the bone marrow respond to high threshold noxious mechanical stimulation, exhibit fatigue in response to prior stimulation, and in some cases can be sensitized by capsaicin. They can be classified on the basis of their response properties as either phasic-tonic units that appear to code for different intensities of intra-osseous pressure, or phasic units that code for the rate of change in intra-osseous pressure. Three different subclasses of mechanically sensitive Aδ units were observed: phasic units that were sensitized by capsaicin, phasic units that were not sensitized by capsaicin and phasic-tonic units (that were not sensitized by capsaicin). These could also, in part, be distinguished by differences in their thresholds for activation, mean discharge frequency, latency to peak activation and peak-to-peak action potential amplitude. The majority of small diameter myelinated sensory neurons projecting to the bone marrow expressed Piezo2. Our findings indicate that Aδ mechano-nociceptors are likely to play an

important role in generating and maintaining pain in response to bony pathologies that involve a mechanical disturbance or increased intra-osseous pressure, and imply that Piezo2 signalling may be involved in mechano-transduction in these receptors.

ABBREVIATIONS

ISI, inter-stimulus interval; AP, action potential; IOP, intra-osseous pressure; DRG, dorsal root ganglia; MIA, mechanically-insensitive afferent

INTRODUCTION

Pain associated with bony pathology causes a major burden (both in terms of quality of life and cost) on individuals and health care systems worldwide. A feature common to most bony pathology is the presence of a mechanical disturbance to the bone marrow, and this is a likely trigger for the pain. Bone cancers, fractures, intra-osseous engorgement syndrome, osteoarthritis and osteomyelitis produce inflammation and/or an increase in intra-osseous pressure which results in compression that mechanically activates sensory nerve terminals within the bone marrow (Lemperg & Arnoldi, 1978; Arnoldi *et al.*, 1980; Haegerstam, 2001; Kidd *et al.*, 2004; Urch, 2004; Starr *et al.*, 2008; Mantyh, 2014). Pain is also experienced on needle aspiration of bone marrow, and is distinct from the pain associated with needle insertion through the periosteum (Niv *et al.*, 2003). Furthermore, destruction of bone by osteolytic processes, excessive stress or trauma can lead to mechanical injury or distortion of bone that can mechanically activate sensory nerve terminals in bone marrow (Haegerstam, 2001; Bove *et al.*, 2009; Mantyh, 2014). Whilst the sensory neurons and nerve terminals that innervate bone marrow have a morphology and molecular phenotype consistent with a role in nociception (Mach *et al.*, 2002; Ivanusic, 2009; Jimenez Andrade & Mantyh, 2010; Mantyh, 2014), little is known about their physiology or the mechanisms that generate and maintain bone pain (Nencini & Ivanusic, 2016).

Bone marrow is located deep within the body and is encased by an outer shell of hard compact bone, making experimental access difficult. This has hampered attempts to study the physiology of nociceptors in the bone marrow. Only two published studies have investigated the physiology of peripheral sensory neurons supplying the bone marrow cavity (Furusawa, 1970; Seike, 1976). In these studies, whole-nerve recordings were made from a branch of the tibial nerve whilst noxious stimuli were applied to the marrow cavity of the canine tibia.

Increases in whole-nerve activity were evoked by mechanical and chemical stimulation, but no attempts were made to explore the activity of single units, and so it is not known how individual marrow cavity nociceptors respond to noxious mechanical stimuli, if they can be sensitized by known algesic substances, or if there are multiple subclasses that each respond to different features of a mechanical stimulus.

Piezo2 is a newly discovered mechanically gated ion-channel that has recently received significant attention because of its remarkable structure and because of its defined role in innocuous mechanical sensibility (Coste *et al.*, 2010; Coste *et al.*, 2012; Kim *et al.*, 2012). However, it is not yet clear if Piezo2 mediates responses of small and medium diameter sensory neurons to noxious mechanical stimulation, or even if it is expressed in sensory neurons physiologically defined as mechano-nociceptors.

The aim of the present study was to determine how peripheral sensory neurons that innervate bone marrow respond to noxious mechanical stimulation, and to test whether they express Piezo2. Electrophysiological recordings were made, using a novel *in vivo* bone-nerve preparation, to examine the effects of increased intra-osseous pressure on sensory neurons that innervate the marrow cavity of the rat tibia. Retrograde tracing and immunohistochemistry were used to identify expression of Piezo2 in the soma of sensory neurons that innervate the marrow cavity of this same bone.

MATERIALS AND METHODS

A total of 42 male Sprague Dawley rats weighing between 200-300 g were used in this study. They were sourced from the Biomedical Sciences Animal Facility at the University of Melbourne. Animals were housed in pairs or groups of four, in a 12/12 hour 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*. Experiments also comply with the ethical principles outlined in the editorial by Grundy (2015).

Preparation and recording configuration

Experiments were performed in rats anesthetized with urethane (50% w/v, 1.5 g/kg i.p). A fine branch of the tibial nerve that innervates the marrow cavity of the rat tibia was identified. The nerve was exposed on the postero-medial aspect of the tibia by reflecting the

overlying skin and the medial head of the gastrocnemius muscle. It was carefully teased away from its associated blood vessels over a distance of approximately 1 cm and placed over a platinum hook electrode. A second, indifferent electrode was implanted into nearby muscle. The nerve was protected from dessication by using the skin and muscle flaps to create a paraffin-filled pool that was maintained at room temperature. Rectal temperature was maintained at 36-37 °C using a heating pad. The sciatic and femoral nerves were transected high in the limb to prevent reflex activation of muscle or sympathetic efferent fibres in the nerve that we recorded from. 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 or 40 kHz (Powerlab, ADInstruments, Australia) and stored to PC using the recording software LabChart (ADInstruments) (Fig 1A). The nerve impulses recorded were biphasic (positive-negative) and only impulses that were at least 40 μV from peak-to-peak amplitude (approximately twice the background noise level) were analysed. Nerve impulses arising from single, mechanically sensitive units were discriminated from the whole nerve recordings by their similar amplitude and duration using Spike Histogram software (LabChart 8, ADInstrument) (Fig 1B). In most of the whole-nerve recordings, 2-3 single mechanically sensitive units could be discriminated. Animals were killed by decapitation, whilst still anesthetized by urethane, the end of each recording experiment.

Mechanical stimulation

Intra-osseous pressure was applied by injecting heparinized physiological saline (0.9% sodium chloride) into the marrow cavity through a needle implanted in a small hole in the proximal tibia (size: 0.8 mm). The hole for needle implantation was made by puncturing the cortical bone with a fine gauge syringe needle. The implanted needle was connected to a feedback controlled syringe pump (PHD ULTRA pump, Harvard apparatus) via polyethylene tubing. Changes in the intra-osseous pressure were 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 (Fig 1A). 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 ramp-and-hold stimuli, of 20-sec duration, to maintain a constant (hold) pressure (typically in 100 mmHg steps to 400 mmHg) during the static phase, and to alter the initial flow rate (6, 8 or 10 ml/min) to control the rate of pressure increase during the dynamic phase at the start of the stimulus (ramp)(Fig 2). The

pressure data were stored to PC in parallel with the nerve recordings. Because the marrow cavity of long bone is open to the systemic circulation, absolute intra-osseous pressure is likely affected by flow rate. Thus the pressures we report here are only an approximation of tibial intra-osseous pressures. For the purposes of comparing response properties of single units, the response to the entire 20 sec ramp-and-hold stimulus was reported as the total response. In addition, discharge frequency was determined for the dynamic phase of the stimulus (defined as the first 2 sec of ramp-and-hold stimulus), and for the static phase of the stimulus (defined as the 5 to 10 sec interval after which the increase in pressure of the ramp-and-hold stimulus had plateaued) (Fig 2). Preliminary experiments revealed that 300 mmHg provides supra-threshold stimulation for all single units. The normal physiological intra-osseous pressure in the absence of a pressure stimulus, recorded when the needle was first implanted into the tibia, was between 8-15 mmHg (mean: 11.04 mmHg ± 3.07 SD, n=15).

Conduction velocity

Single unit conduction velocities were determined in a series of experiments using two recording electrodes, mounted on the same arm of a micromanipulator, with a distance of 7 to 10 mm between electrodes. Pressure stimuli of short duration were delivered at supra-threshold (≥300 mmHg) pressures to activate mechanically sensitive neurons. We discriminated single units during mechanical stimulation on the basis of time-locked impulses on each of the two electrodes. This is consistent with both impulses originating in the same axon. For these units conduction velocities were estimated by dividing conduction time by the distance between electrodes. To explore the relationship between conduction velocity and impulse amplitude, conduction velocity was plotted against the peak-to-peak amplitude of the nerve impulses (averaged from 5 consecutive impulses originating from each single unit).

Adaptation kinetics

To investigate adaptation kinetics, instantaneous single-unit discharge frequency in response to a 300 mmHg ramp-and-hold stimulus was plotted as a function of time and the decay phase was fitted with non-linear regression to a mono-exponential function (Graphpad Prism, Graphpad Software). The time constant *tau* (τ), is the reciprocal of the decay constant calculated for the regression, and was used as the measure of the rate of adaptation.

Stimulus-response relationship

To determine if repetitive presentations of intra-osseous pressure can lead to changes in the response properties of single mechanically sensitive units, we delivered pairs of 300 mmHg ramp-and-hold pressure stimuli at inter-stimulus intervals (ISIs) of 30 sec, 2, 5 and 10 min and determined the discharge frequency during the dynamic phase of each pressure ramp. In these experiments, the initial flow rate during the ramp-and-hold stimulus was maintained at 8 ml/min. To assess the conditioning effect of the ISIs, the discharge frequency at the second stimulus was measured as a percentage of that at the first (baseline) stimulus. For experiment, the different ISIs were presented in random order.

To determine if single mechanically sensitive units are capable of coding the absolute intensity of applied intra-osseous pressure, we applied a series of four ramp-and-hold pressure stimuli with the hold phase set at 100, 200, 300 or 400 mmHg, and with the initial flow rate during the ramp phase maintained at 8 ml/min (Fig 2A). In these experiments, the different stimulus intensities were presented in ascending order. Changing the order of presentation did not alter the response of single units; however, the ascending order was preferred because the highest intensity pressure tested (400 mmHg) produced a long-lasting after discharge in some units and we wanted to avoid any confounding effects of this discharge on responses to subsequent stimulus presentations. To determine if these units are capable of coding the rate of increase in intra-osseous pressure in the dynamic phase of the ramp-and-hold stimulus, we applied a series of ramp-and-hold pressure stimuli with the hold phase maintained at 300 mmHg and the initial flow rate during the ramp phase changed to 6, 8 and 10 ml/min (Fig 2B).

Sensitization by capsaicin

To determine if some of the mechanically sensitive units could be sensitized, we recorded their threshold for activation and discharge frequency in response to a ramp-and-hold stimulus (300 mmHg, 8 ml/min) before and after application of capsaicin (0.1 μM, 10 μl) or saline (10 μl). Capsaicin or saline was delivered to the marrow cavity through a second hole in the bone (26 gauge needle, hole size: 0.5 mm) using a Hamilton syringe and polyethylene tubing (Fig 1A). We monitored and confirmed that there was no change in intra-osseous pressure during the injection of capsaicin. The threshold for activation and discharge frequency of each unit following application of capsaicin were expressed as a percentage of the baseline values (pre-injection).

In preliminary experiments we made recordings from the nerve before and then also immediately after making holes in the tibia to get access for saline and capsaicin injections. There was no difference in whole-nerve spontaneous activity under these two conditions. In addition, there was little or no ongoing activity in the whole-nerve recordings or the single mechanically activated units we report in this study, and thresholds for mechanical activation of single units in the absence of capsaicin were typically very high. These findings suggest sensitization did not occur as a result setting up for our recordings, and in particular making holes to access the marrow cavity.

Data analysis

Threshold for activation and discharge frequency for each of the single units were determined. Each unit was classified as phasic if its response to increased intra-osseous pressure was predominantly in the dynamic phase of the pressure ramp or phasic-tonic if it also responded to sustained pressure beyond the dynamic phase of the pressure ramp. The latency to peak activation was the time calculated between the start of the stimulus and the peak instantaneous discharge frequency for each unit. At the end of each experiment the periosteum, nearby muscles and the knee joint were stimulated with innocuous and noxious mechanical stimuli using blunt and sharp probes respectively to determine if each of the units activated by changes in intra-osseous pressure also responded to stimuli applied outside of the marrow cavity. Those that had receptive fields outside of the marrow cavity were excluded from our data set.

Statistical analyses were performed using Prism (Graphpad Prism, Graphpad Software). Differences in the rate of adaptation (τ) between phasic and phasic-tonic units were assessed using Student's unpaired t-test. The effects of different ISIs on single-unit responses were tested using a one sample t-test with Bonferroni's adjustment for multiple comparisons. For this test, we used a theoretical mean of 100 to represent the percentage predicted when there is no change in discharge frequency at the second stimulus relative to the first. Comparison of differences in the effect of the different ISIs on phasic-tonic vs phasic units were made using a two way ANOVA with Bonferroni's post hoc analysis. Stimulus-response functions for intensity and rate coding were evaluated using repeated measures ANOVA with Bonferroni's post-hoc analysis. Differences in the response of phasic-tonic vs phasic units to each intensity or rate stimulus were assessed with a Student's t-test with Bonferroni's adjustment for multiple comparisons. Capsaicin-induced differences

in the response properties of phasic-tonic vs phasic units were tested using either Student's unpaired t-test (for two groups) or an ANOVA with Bonferroni's post-hoc analysis (for three groups). For tests involving multiple groups or time-points, pairwise comparisons were only made and reported if analysis of variance showed significance at $p<0.05$.

Retrograde tracing and immunohistochemistry

Rats were anesthetized with isoflurane (4% induction; 2.5% maintenance). After a skin incision, a small hole was made at the medial aspect of the tibia and a Hamilton syringe was used to inject the retrograde tracer Fast Blue (2 µl FB; 10% in dH_2O) directly into the marrow cavity. The hole was sealed with bone wax, and the area washed extensively with 0.1M phosphate buffered saline (pH 7.4; PBS) and inspected for tracer leakage using a hand-held UV light. Animals that showed evidence of tracer leakage to surrounding tissues were excluded. Skin incisions were closed with stainless steel autoclips. After a 7-day survival period to allow for transport of the tracer to neuronal cell bodies in the dorsal root ganglia, each animal was given an overdose of sodium pentobarbitone (Lethobarb; 80 mg/kg; i.p.), and was perfused via the ascending aorta with 500 ml of heparinized PBS followed by 500 ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). L3 dorsal root ganglia were dissected and left overnight in a solution containing the above fixative and 20% sucrose. They were washed in PBS, frozen in liquid nitrogen cooled isopentane, and sectioned at 14 µm using a cryostat the next day. Sections were collected on gelatinized glass slides (0.1% chrome alum and 0.5% gelatin) and processed for immuno-labelling to determine if retrograde labelled neurons expressed Piezo2 and/or NF200. Details of the primary and secondary antisera are given in Table 1. All antisera were diluted in 0.1M PBS containing 0.3% Triton X-100 and 0.1% sodium azide. Sections were washed 3 times in 0.1M PBS and incubated overnight in the primary antisera at room temperature. Following 3 further washes in 0.1M PBS, they were incubated in secondary antibody for 2 h, and washed again 3 times in 0.1M PBS. The slides were cover-slipped using DAKO fluorescence mounting medium and were examined and photographed with a 10x objective using a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkocken, Germany) fitted with an AxioCam MRm camera. Counts and size measurements (cross-sectional area of soma) were made directly from the images using Zen lite software. We determined the proportion of retrograde labelled neurons that expressed Piezo2 and/or NF200. To prevent double counting and to avoid cell size bias,

only cells with a visible nucleus were counted. Figures were prepared using CorelDraw software. Individual images were contrast and brightness adjusted. No other manipulations were made to the images.

It is possible that the retrograde tracer injected in this study could spread into the systemic circulation via the vasculature of the bone marrow. To control for the possibility that this could result in retrograde labeling of DRG neurons that innervate tissues other than the bone marrow, we also processed contralateral DRG to check for the presence of retrograde labeling. No retrograde labeling was observed in the contralateral DRG. This confirms that even if such spread occurred, it did not result in labeling of neurons innervating tissues other than the bone marrow we injected.

RESULTS

Relationship between conduction velocity and amplitude of action potentials

In preliminary experiments, we determined conduction velocities for 30 units and plotted them against their peak-to-peak action potential (AP) amplitude. There was a linear relationship between AP amplitude and conduction velocity for units that we were able to unambiguously isolate with our spike discrimination protocol (Fig 2). Units with conduction velocities <2 m/s had small amplitude APs (<40 μV, n=5; Fig 3, represented with grey circles). The conduction velocities for these small amplitude units are consistent with a C fibre classification. Units with conduction velocities in the range 2 to 12.5 m/sec had medium size AP amplitudes (range: 47-145 μV peak-to-peak, n=16; Fig 3). The conduction velocities for these medium amplitude units are consistent with an Aδ fibre classification. On probing of tissue surrounding the tibia, we found some units with low-threshold receptive fields outside the marrow cavity and large amplitude APs (>120 μV peak-to-peak; Fig 3, represented with white circles). These units had fast conduction velocities (≥14.3 m/sec, n = 9) and were only activated by very high intensity pressure applied to the marrow cavity (mean threshold ± SD = 241 ± 54 mmHg). It is possible that these units represent low threshold mechano-receptors, in surrounding periosteum or muscle, that were mechanically activated by leakage of saline from the marrow cavity, through Volkmann's canals and into surrounding tissue. They were relatively rare and were excluded from further analysis. Small units with peak-to-peak amplitude <40 μV were also excluded from further analysis because

variation in their amplitude and duration made them difficult to isolate unambiguously. Thus the units presented in the rest of this paper are exclusively Aδ fibres.

Threshold for activation

The threshold for activation of multi-unit activity in whole-nerve recordings was between 49 and 107 mmHg (mean ± SD = 87.2 ± 20.5; n=32). This is approximately 3-10 times that of the normal intra-osseous pressure we recorded for the rat tibia (8-15 mmHg; n=15). However, it was clear that the threshold pressure for activation of *single* mechanically sensitive Aδ units that we isolated using spike discrimination was often much higher (threshold for activation 49-230 mmHg; mean ± SD = 133 ±41.1; n=91). Thus thresholds for activation of single units could be between 3 and 20 times that of the baseline IOP we recorded in the rat tibia.

Adaptation profile to sustained intra-osseous pressure stimulation

All units adapted slowly to the 300 mmHg ramp-and-hold pressure stimulus during the 20 seconds over which it was applied. Peak instantaneous frequency usually occurred during the dynamic phase of the pressure stimulus (Fig 4A). Single unit responses fell into one of two categories. Phasic units (n=34/69) had a burst of activity in the dynamic phase of the stimulus (< 2 Hz) and then adapted during the early part of the static phase (Fig 4A, dark grey). Phasic-tonic units (n= 35/69) had a larger initial burst of activity in the dynamic phase (≥ 5 Hz), and continued to fire APs with low frequency during the static phase (< 3 Hz), often lasting for the duration of the stimulus (Fig 4A, light grey). The time constant of the adaptation curves was estimated by fitting the data for each unit to a mono-exponential function (Fig 4B). Phasic units had significantly shorter time constants (τ) than phasic-tonic units (Fig 4C; t-test, $p<0.0001$).

Effect of repetitive stimulation

Repetitive stimulation can lead to significant changes in the response properties of nociceptors. To test if this occurs in mechanically sensitive bone nociceptors, pairs of 300 mmHg ramp-and–hold pressure stimuli (with the initial rate of infusion set at 8 ml/min) were delivered at 30 sec, 2, 5 and 10 min ISIs, and the size of the response to the second stimulus was compared with that to the first stimulus (Fig 5). To assess the conditioning effect of the ISIs, the change in discharge frequency at the second stimulus was measured as a percentage

of that at the first (baseline) stimulus. For this assessment we chose to restrict our analysis to the dynamic phase of the response because there is little activity of phasic units during the static phase of the stimulus. Fatigue (a decrement in response that results from a prior stimulus) was observed in both phasic-tonic and phasic units at the shortest ISIs and declined at longer ISIs. Phasic-tonic units had significantly reduced discharge frequencies, relative to baseline, at ISIs of 30 sec and 2 min (one-sample t-test; at 30sec $p=0.0009$, at 2 min $p=0.0191$; Fig 5A) and phasic units had significantly reduced discharge frequencies at ISIs of 30 sec, 2 and 5 min (one-sample t-test; at 30 sec $p<0.0001$, at 2 min $p=0.0003$, at 5 min $p=0.0065$; Fig 5B). Comparison between the phasic-tonic units and phasic units shows that phasic units were affected more by fatigue than phasic-tonic units (Two-way ANOVA; interaction $p<0.0001$; Fig 5C). Since a 10-min ISI provided sufficient time for complete recovery of the response of both phasic-tonic and phasic units, we used at least 10 min between pressure stimuli in subsequent experiments.

Intensity coding

To determine if bone nociceptors are capable of encoding information about different intra-osseous pressures, we constructed stimulus-response functions for single units (n=17; 8 phasic-tonic, 9 phasic) in response to application of 100, 200, 300 and 400 mmHg ramp-and-hold pressure stimuli applied with the initial rate of infusion set at 8 ml/min (Fig 6). The discharge frequency of phasic-tonic units increased monotonically as the sustained pressure during the hold phase of the stimulus increased to 400 mmHg. The discharge frequency at 200, 300 and 400mmHg was significantly greater than at 100 mmHg, and the discharge frequency at 400 mmHg was significantly greater than at 200 mmHg (repeated measures ANOVA $p=0.0001$; Bonferroni's post hoc analysis $p<0.05$). In contrast, the discharge frequency of phasic units increased to 300 mmHg (repeated measures ANOVA $p=0.005$; Bonferroni's post hoc analysis $p<0.05$), but not beyond this level (Fig 6A). Most of the changes in activity produced by the changes in pressure occurred during the dynamic (Fig 6B), rather than the static phase (Fig 6C) of the stimulus. The responses of phasic-tonic units were always greater than those of phasic units at each of the stimulus intensities tested (Student's t-test with Bonferroni's adjustment for multiple comparisons)(Fig. 6), and although all phasic-tonic units responded to the 100 mmHg ramp-and-hold pressure stimulus, none of the phasic units did. These findings suggest that phasic-tonic units are more responsive and better able to code for different intra-osseous pressures than phasic units.

Rate coding

To determine if bone nociceptors are capable of coding for the rate of pressure change, we constructed stimulus-response functions for single units (n=17; 8 phasic-tonic, 9 phasic) using 3 different initial rates of infusion (6, 8 and 10 ml/min) (Fig 7) to vary the ramp phase of the pressure stimulus with holding pressure set at 300 mmHg. We chose a 300 mmHg ramp-and-hold pressure stimulus because it reliably activates both phasic and phasic-tonic units. The discharge frequency of phasic-tonic units was greater than that of phasic units at each infusion rate tested (Student's t-tests with Bonferroni's adjustment for multiple comparisons)(Fig. 7). However, only for phasic units was there a clear relationship between discharge frequency and rate of infusion (repeated measures ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$; Fig 7A). As expected for the phasic units the effects of infusion rate on nerve activity were evident during the dynamic phase (repeated measures ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.01$; Fig 7B), but not in the static phase (Fig 7C), of the stimulus. This suggests that phasic (but not phasic-tonic) units are able to code for the rate of change in intra-osseous pressure, at least over the range of rates tested in this study.

Sensitization

To determine if bone nociceptors could be sensitized, we assessed their threshold for activation and discharge frequency during responses to a ramp-and-hold stimulus (300mmHg, 8 ml/min) before and after application of capsaicin (0.1 µM, 10 µl, n=23) or saline (10 µl, n=15). Figure 8 shows the threshold for activation and discharge frequency for single units following application of saline or capsaicin as a percentage of the pre-treatment values. Saline injection (10 µl) had no effect on activation threshold (Fig 8A and B) or discharge frequency (Fig 8C and D) in any of the single units tested (n=15, 9 phasic-tonic and 6 phasic units). In only 6 out of the 23 units tested with capsaicin was there a change in their response to pressure stimuli and these were all phasic units (Fig 8B and D). These units had a reduction in their threshold for activation (ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$; Fig 8B) and an increase in their discharge frequency (ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$; Fig 8D) in response to the ramp-and-hold pressure stimulus. In the remaining 7 phasic units tested, capsaicin did not sensitize responses to the

pressure stimulus. Similarly, capsaicin did not change the response of any of the phasic-tonic units to pressure stimuli (Fig 8A and C).

Three distinct subclasses of Aδ mechano-nociceptor innervate the marrow cavity of the rat tibia

The results already presented suggest there may be at least three different subclasses of mechanically sensitive units that innervate bone: phasic-tonic units (not sensitized by capsaicin), phasic units sensitized by capsaicin and phasic units not sensitized by capsaicin. Figure 9 shows the thresholds for activation, mean discharge frequency, latency to peak activation and peak-to-peak action potential amplitude for each of these groups determined before the application of capsaicin.

The three subclasses of mechanically sensitive units had significantly different thresholds for activation (ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$; Fig 9A). Phasic-tonic units had the lowest threshold for activation, whereas the capsaicin-sensitized phasic units had the highest thresholds for activation. As previously observed phasic units had lower discharge frequencies during the rising phase of the pressure stimulus than the phasic-tonic units (ANOVA, $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$), but this measure did not differ between the phasic units that were sensitized by capsaicin and those that were not (Fig 9B). The latency to peak activation was significantly different for each of the three subclasses (ANOVA $p<0.0001$, Bonferroni's post hoc analysis $p\leq0.001$; Fig 9C). Phasic-tonic units had the shortest latency to peak activation and capsaicin-sensitized phasic units had the longest latency. Capsaicin-sensitized phasic units had smaller peak-to-peak amplitudes than phasic units that were not sensitized by capsaicin and the phasic-tonic units (ANOVA, $p=0.0113$, Bonferroni's post hoc analysis $p\leq0.05$; Fig 9D). As our findings indicated that AP amplitude is positively correlated with conduction velocity, the capsaicin sensitized phasic units might conduct more slowly than the other units investigated in this study. Collectively, these data reinforce the notion that there are at least three distinct sub-classes of Aδ mechano-receptors that innervate bone.

Piezo2 is expressed in small to medium sized myelinated sensory neurons that innervate the rat tibia

A total of 290 retrograde labelled bone afferent neurons were counted in the ipsilateral L3 DRG taken from three animals. They were almost entirely small or medium

sized neurons (>95% of those counted were less than 1800 μm^2) and approximately half were myelinated (NF200+; 51 ± 6%; n=3)(Fig 10). Piezo2 was expressed in the majority of myelinated bone afferent neurons (70 ± 4%; n=3)(Fig 10). Piezo2 expression in unmyelinated bone afferent neurons was rare (3 ± 0.9%; n=3)(Fig 10).

DISCUSSION

In the present study, we provide evidence that Aδ nociceptors in bone marrow respond to high threshold noxious mechanical stimulation, are capable of signalling either the intensity or rate of change in intra-osseous pressure, exhibit fatigue in response to prior stimulation, and in some cases can be sensitized by capsaicin. Phasic-tonic units appear to code for different intra-osseous pressures, whereas phasic units code for the rate of change in intra-osseous pressure. At least three different subclasses of mechanically sensitive Aδ units have been observed: capsaicin sensitized phasic units, phasic units not sensitized by capsaicin and phasic-tonic units not sensitized by capsaicin. These could in part be distinguished also by differences in their thresholds for activation, mean discharge frequency, latency to peak activation and peak-to-peak action potential amplitude. Piezo2 is expressed in the majority of myelinated neurons projecting to bone marrow and is thus likely to contribute to mechano-transduction in the Aδ mechano-nociceptors we have recorded from in our bone-nerve preparation. We have not presented data for C fiber recordings in the present study. C fiber nociceptors are also likely to contribute to bone pain and will be the focus of future studies.

Action potential amplitude predicts conduction velocity for mechanically sensitive marrow cavity nociceptors

Action potentials from single, mechanically sensitive marrow cavity nociceptors were discriminated in whole-nerve recordings according to their relative amplitude and duration. We were unable to record conduction velocities routinely in each experiment because we could not electrically stimulate the receptive fields of individual units buried deep inside the marrow cavity, and we could not always isolate the nerve over long enough distances to place two recording electrodes under it. Instead, we classified units as C, Aδ or Aβ on the basis of preliminary experiments in which we determined the existence of a linear relationship between conduction velocity and peak-to-peak action potential amplitude for 30 single units. Units with small amplitude action potentials (<40 μV) conducted in the C fibre range (<2

m/sec). Units with medium action potential amplitudes (range: 47-145 μV) conducted in the Aδ range (2 to 12.5 m/sec). Units with large action potential amplitudes (>120 μV) conducted in the Aβ range, and on probing of surrounding tissue, displayed low-threshold receptive fields outside the marrow cavity. It is possible that the latter might represent low-threshold mechano-receptors, in surrounding periosteum or muscle, that were mechanically-activated by leakage of saline from the marrow cavity, and so they were excluded from this study. We also excluded small amplitude units because it was not always possible to isolate them unambiguously.

The relationship between action potential amplitude and conduction velocity is not new and has been reported in other extracellular recording studies (Hakansson, 1956; Milner *et al.*, 1981). During active conduction, large axons produce greater extracellular current flux at the site of recording than small axons, and so for large axons the extracellularly recorded action potentials have larger amplitudes than those of small axons (Hakansson, 1956). Peak-to-peak amplitude of an extracellular action potential is also influenced by the location of an axon relative to the electrode, such that action potential amplitude decreases with increasing distance from the contact point. However, given the small size of the nerve to the rat tibia (size = 37 ± 5 μm, mean ± SD, n=3), and that the contact point on a hook electrode of the sort we have used in this study is relatively large, the effect of fibre-electrode distance is negligible.

Responses of marrow cavity nociceptors to high threshold, noxious mechanical stimulation

Mechanical stimuli were delivered to the marrow cavity by increasing the intra-osseous pressure through infusion of isotonic saline. Thresholds for activation of mechanically sensitive units calculated from our whole-nerve recordings were approximately 3-5 times that of normal intra-osseous pressure in the rat tibia. This is consistent with earlier reports that normal intra-osseous pressure of the canine tibial marrow cavity was in the range of 30-50 mmHg, and that an approximately 3-5 times increase in intra-osseous pressure (to 100-130 mmHg) activated mechanically sensitive units in whole-nerve recordings (Furusawa, 1970; Seike, 1976). However, our findings also show that the threshold for activation of many of the *single* mechanically sensitive Aδ units that we isolated from our whole-nerve recordings were significantly greater than this (up to 230 mmHg). Thus some marrow cavity nociceptors have even higher thresholds for mechanical activation than may have been

appreciated in the past. It is clear that these are high thresholds that are unlikely to be experienced under normal physiological conditions and undoubtedly represent a stimulus that is noxious. Indeed, increases in intra-osseous pressure as little as 3-5 times that of normal intra-osseous pressure are experienced in painful pathological conditions such as intra-osseous engorgement syndromes (Lemperg & Arnoldi, 1978; Arnoldi *et al.*, 1980). In intra-osseous engorgement syndrome, pain can be relieved by fenestration, suggesting that it is the increased pressure in the marrow cavity that produces the pain. These findings strongly suggest that the Aδ units we have recorded from in this study are mechano-nociceptors, and are consistent with the notion that mechanical stimulation of nerve terminals within bone produces pain, not innocuous mechano-sensibility (Rowe *et al.*, 2005).

Our study is the first to report single unit responses to changes in intra-osseous pressure applied to bone. Single unit responses fell into one of two categories. Phasic units had a burst of activity (< 2 Hz) in the dynamic phase of the stimulus and then adapted early in the static phase (Fig 4A, dark grey). In contrast, phasic-tonic units had a larger initial burst of activity (≥ 5 Hz) in the dynamic phase, and continued to fire action potentials with low frequency (< 3 Hz) during the static phase, often lasting for the duration of the pressure stimulus. The difference in the response profiles of phasic and phasic-tonic units to noxious mechanical stimulation suggests that each class is able to signal different aspects of intra-osseous pressure. Phasic-tonic units signal the intensity of pressure during both the dynamic and the static phase of the stimulus (intensity coding), whereas phasic units signal the rate of change in intra-osseous pressure only in the dynamic phase (rate coding). It is therefore likely that phasic-tonic units code for pain associated with pathologies that involve sustained increases in pressure, for example intra-osseous engorgement syndrome (Lemperg & Arnoldi, 1978; Arnoldi *et al.*, 1980). In contrast, phasic units are likely to signal pain associated with pathologies that involve rapid changes in pressure within the marrow cavity, for example during needle aspiration of bone marrow or emergency intra-osseous vascular access (Cooper *et al.*, 2007; Ngo *et al.*, 2009).

Nociceptors exhibit fatigue in response to high intensity repetitive stimulation with heat, mechanical and chemical stimuli (LaMotte & Campbell, 1978; Slugg *et al.*, 2000; Liang *et al.*, 2001). Fatigue likely occurs as a result of changes in stimulus transduction and/or spike initiation and is important for adjusting response sensitivities of nociceptors to an appropriate stimulus range. However, it might also negatively impact on electrophysiological studies because it can affect the reproducibility of data (LaMotte & Campbell, 1978; Torebjork *et al.*,

1984; Reeh *et al.*, 1987; Slugg *et al.*, 2000; Peng *et al.*, 2003). In the present study, we observed fatigue of Aδ mechano-nociceptors in the bone marrow at inter-stimulus time intervals of 30 sec, 2 and 5 min, and that the fatigue completely resolved by 10 minutes. Thus in order to obtain stable and highly reproducible responses in marrow cavity nociceptors, we used at least 10 min between pressure stimuli to allow for recovery from fatigue. Interestingly, whilst phasic-tonic and phasic units both exhibited fatigue, the magnitude and time-course of the fatigue was different for each. This suggests that Aδ nociceptors with phasic-tonic and phasic responses to mechanical stimulation in bone may utilise different mechanisms for stimulus transduction and/or spike initiation.

Sensitization of mechanically sensitive marrow cavity nociceptors

Sensitization of peripheral bone nociceptors has been used to explain, in part, increased sensitivity of patients to mechanical stimuli in a variety of bony pathologies (Portenoy *et al.*, 1999; Honore & Mantyh, 2000; Haegerstam, 2001). However, direct evidence of sensitization of peripheral bone nociceptors to mechanical stimulation is scarce. Some studies have reported increased spontaneous activity and reduced heat (but not mechanical) thresholds in peripherally recorded C-fibre afferents in an animal model of cancer induced bone pain (Cain *et al.*, 2001; Uhelski *et al.*, 2013). However, in both of these studies, the tumour cells were not clearly confined to the bone, and the C fibres recorded were cutaneous afferents, not bone nociceptors. In the present study, we report that a quarter of the Aδ mechano-nociceptors that innervate the bone marrow have reduced thresholds for activation and increased discharge frequency in response to pressure after the application of capsaicin. Thus at least some Aδ nociceptors in bone can be sensitized by capsaicin, and likely also other algesic substances.This is consistent with our recent finding that a substantial proportion of sensory (DRG) neurons labeled with injections of a retrograde tracer into the marrow cavity of the rat tibia express the capsaicin receptor TRPV1 (Nencini *et al.*, 2017).Capsaicin is known to sensitize Aδ and/or C fibre nociceptors to mechanical stimulation (Ren *et al.*, 2005; Zagorodnyuk *et al.*, 2007; Li *et al.*, 2008; Wang *et al.*, 2011). However, the mechanisms by which this occurs remain to be determined. TRPV1 is a nonselective ligand-gated cation channel that integrates many physical and chemical stimuli, including noxious heat (>43°C), capsaicin and other inflammatory mediators (Tominaga *et al.*, 1998). Capsaicin-induced mechanical sensitization occurs in TRPV1 expressing sensory neurons and blocking conduction in these neurons in rats prevents mechanical hyperalgesia

(Brenneis *et al.*, 2013). However, TRPV1 is not thought to transduce mechanical stimuli, and so it is not clear how capsaicin alters mechanical sensitivity in TRPV1 expressing nociceptors. It is possible that capsaicin-induced activation of TRPV1 indirectly enhances mechanical sensitivity through phosphorylation of other, mechanically sensitive transduction molecules (Sowa *et al.*, 2010), or that enhanced membrane permeability to cations following TRPV1 activation could lower the voltage threshold for action potential initiation, making these neurons more sensitive to mechanical stimulation. Alternatively, Aδ mechano-nociceptors could be sensitized by capsaicin-induced local inflammation, or by inflammatory mediators released from peptidergic C fibre nerve terminal endings in response to stimulation by capsaicin (Li *et al.*, 2008).

The capsaicin-sensitized phasic units reported in the present study had high thresholds for activation to mechanical stimulation, long latencies to peak activation and low discharge frequencies before capsaicin was applied. This suggests that under normal conditions (in the absence of a sensitizing agent) they are not well suited to coding noxious mechanical stimuli applied to the bone marrow. In other tissue systems, nociceptors with these properties are classically described as mechanically insensitive afferents (MIAs) or "silent" nociceptors that under normal conditions are not activated by mechanical stimuli, but after inflammation or chemical stimulation, can become sensitive to a number of different stimulus types, including mechanical stimuli (Meyer *et al.*, 1991; Michaelis *et al.*, 1996). There is also evidence that many capsaicin-sensitive nociceptors can be desensitized to mechanical stimuli by (often systemic) injections of relatively high doses of capsaicin (Holzer, 1991). A relevant example includes group III and IV knee joint afferents that are desensitized by bolus injections, into blood vessels surrounding the knee, of capsaicin at concentrations $>10^{-4}$ M (He *et al.*, 1990). However, we found no evidence of this using a lower concentration (10^{-7}M) applied directly to the marrow cavity in the current study.We have recently reported that another inflammatory mediator, NGF, rapidly activates and sensitizes Aδ mechano-nociceptors in bone (Nencini *et al.*, 2017). It will be important in future studies to determine if thermal sensitization of bone nociceptors occurs in response to capsaicin, NGF and/or other inflammatory mediators.

Is Piezo2 responsible for mechano-transduction in Aδ mechano-nociceptors in bone?

The Piezo2 gene is highly expressed in mouse DRG, and knockdown of Piezo2 using RNA interference in mouse cultured DRG neurons results in suppression of mechanically activated currents in rapidly adapting neurons (Coste *et al.*, 2010; Coste *et al.*, 2012). There is now significant evidence that Piezo2 is the transducer for low threshold mechanical stimuli in Merkel cells (Ikeda *et al.*, 2014; Ikeda & Gu, 2014; Ranade *et al.*, 2014; Woo *et al.*, 2014) and proprioceptors (Woo *et al.*, 2015; Florez-Paz *et al.*, 2016). However, only a few studies have suggested it might also be involved in nociception. Mechanically activated Piezo2 currents are enhanced by bradykinin, an algogenic peptide that drives mechanical hyperalgesia associated with inflammation (Dubin *et al.*, 2012). Piezo2 knock-down in DRG inhibits CFA-induced mechanical but not thermal hyperalgesia in mouse skin (Singhmar *et al.*, 2016) and attenuates visceral sensation to both innocuous and noxious stimuli in rats (Yang *et al.*, 2016). Piezo2 is expressed in a sub-population of primary afferent neurons that likely mediates responses to noxious mechanical stimulation in the cornea (Bron *et al.*, 2014; Alamri *et al.*, 2015). Taken together, these findings suggest a role for Piezo2 in the transduction of noxious mechanical stimuli.

In the present study, we have provided evidence that most small and medium sized myelinated sensory neurons that innervate the bone marrow express Piezo2. This size profile and myelination status is consistent with a classification for these neurons as Aδ nociceptors. Interestingly Piezo2 knockout affects the sensitivity (threshold for activation) of some Aδ fiber mechano-receptors identified in the skin-nerve preparation (Ranade *et al.*, 2014), further reinforcing a role for Piezo2 in mechano-transduction in Aδ mechano-receptors. We therefore suggest that Piezo2 is the mechano-transducer in the Aδ mechano-nociceptors we have reported in the present study. However, there are no pharmacological agents currently available to specifically block Piezo2 signalling, and so we are unable to confirm whether Piezo2 is indeed responsible for mechano-transduction in bone nociceptors using our *in vivo* preparation. Furthermore, we cannot rule out the involvement of other molecules in the mechano-transduction process, alongside or independently of Piezo2.

CONCLUSIONS

Our findings indicate that Aδ mechano-nociceptors are likely to play an important role in generating and maintaining pain in response to bony pathologies that involve a

mechanical disturbance or increased intra-osseous pressure, and imply that Piezo2 signalling may be involved in mechano-transduction in these receptors.

ADDITIONAL INFORMATION SECTION

Competing interests

The authors do not have any competing interests

Author contributions

Both JI and SN were involved in conception and design of the work; acquisition, analysis, and interpretation of data for the work; and drafting the work and revising it critically for important intellectual content. Both JI and SN approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; and confirm all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work was supported by funding from the Australian National Health and Medical Research Council, a Clive and Vera Ramaciotti Foundation Establishment Grant, and a Medical Research and Technology Grant from Australian and New Zealand Charitable Trustees.

Acknowledgements

The authors acknowledge Associate Professor James Brock for his comments on the manuscript.

FIGURES LEGENDS

Figure 1

Experimental set-up of the *in vivo* bone-nerve preparation and spike discrimination

A, Schematic diagram of the electrophysiological set-up. Scissors represent locations where nerves were cut. B, Whole-nerve recording and rasters of single unit activity in response to a ramp-and-hold pressure stimulus applied to the marrow cavity. C, Action potentials from single mechanically activated units were discriminated on the basis of their amplitude and duration using Spike Histogram software.

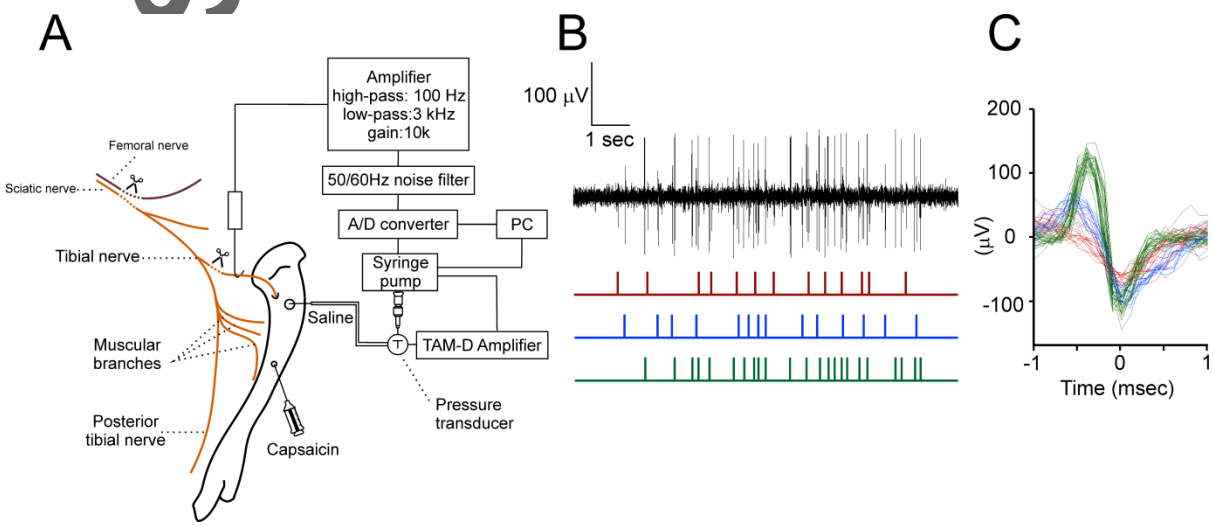
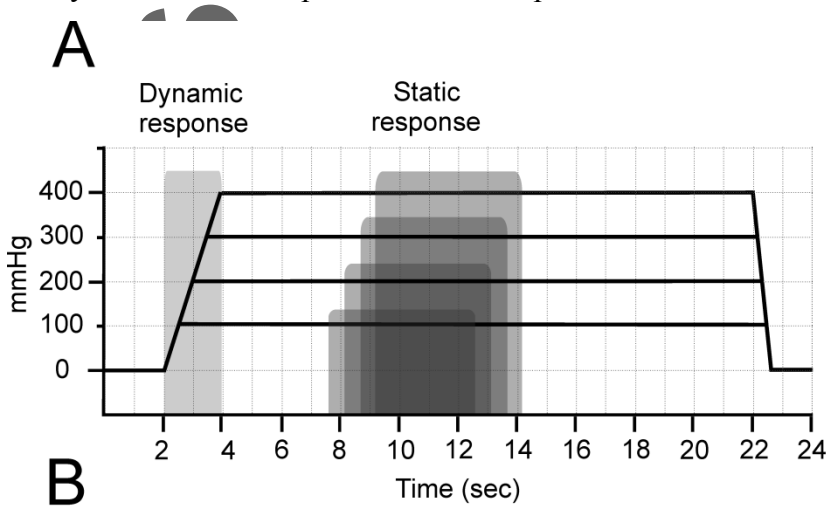


Figure 2

Mechanical stimulation paradigms.

A, Series of four pressure ramps with intensities of 100, 200, 300 and 400 mmHg and a fixed flow rate (8ml/min) were used to explore the stimulus intensity coding properties of single mechanically evoked units. B, Series of three pressure ramps applied with saline infusion rates of 6, 8, 10 ml/min and a fixed intensity of pressure of 300 mmHg were used to test the rate coding properties of single mechanically evoked units. Stimuli were presented at 10-min inter-stimulus intervals. Representations of the periods that were used to assess effects during the dynamic and static phase of each ramp-and-hold stimulus are indicated by the grey areas.



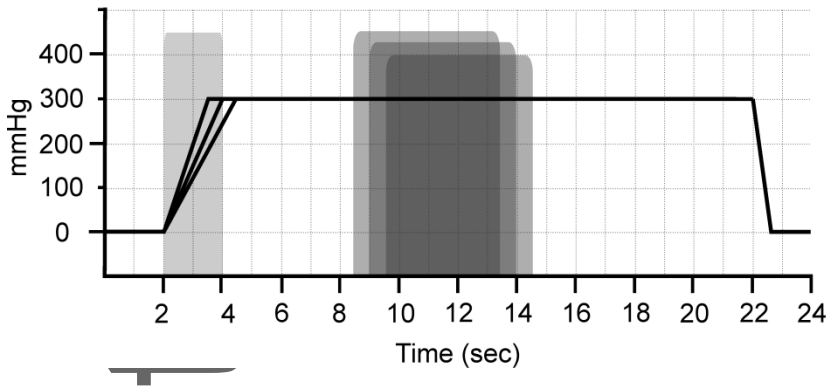


Figure 3

Relationship between conduction velocity and amplitude of action potentials

Action potential amplitude (μV; peak-to-peak) was plotted against conduction velocity (m/sec) for 30 single units activated by high-intensity intra-osseous pressure stimuli (≥ 300 mmHg). Units that also responded to stimulation of surrounding tissues (white circles) had very large action potential amplitudes (>120 μV) and conducted in the Aβ range (≥14.3 m/sec). Units with conduction velocities in the C fibre range (<2 m/sec) had the smallest action potential amplitudes (<40 μV peak-to-peak)(grey circles). All other units, with action potential amplitudes greater than 40 μV peak-to-peak and that did not respond to stimulation of surrounding tissues, had Aδ conduction velocities (black circles).

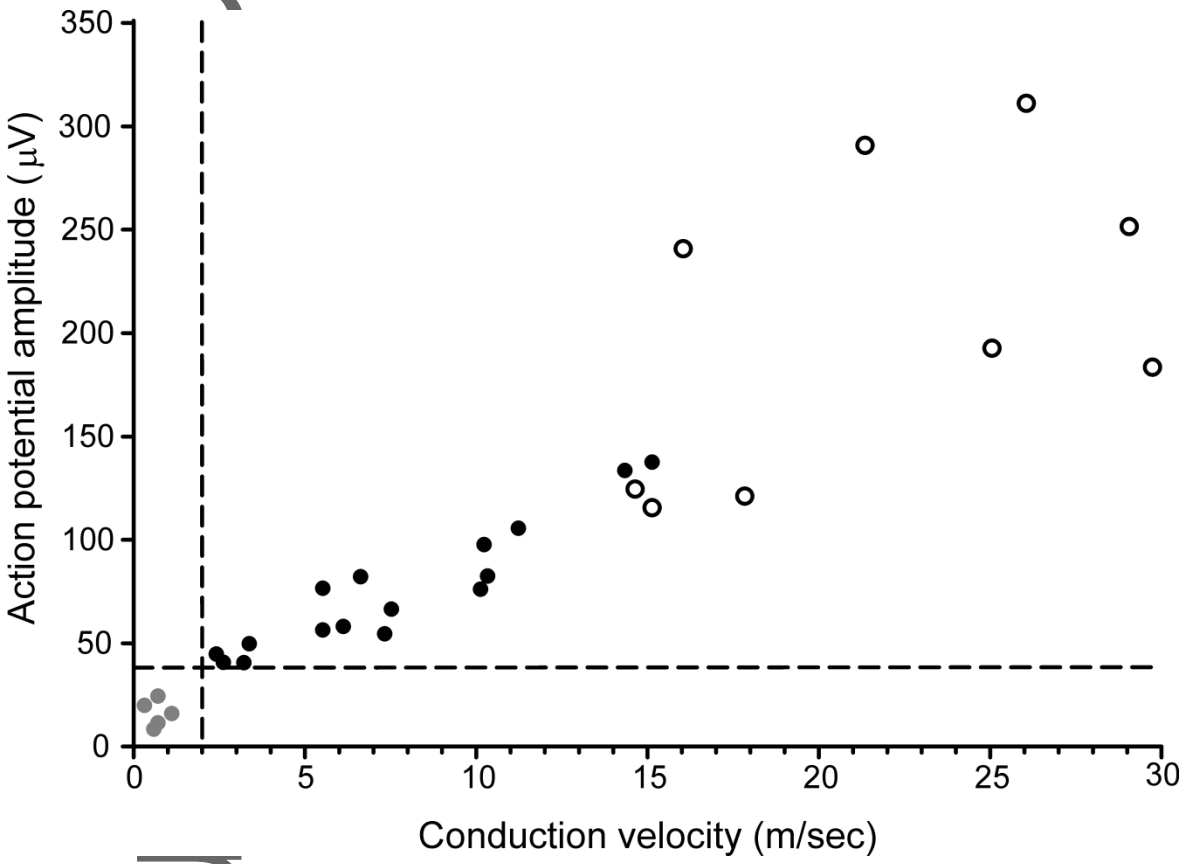


Figure 4

Mechanically sensitive units were distinguished on the basis of their response to increased intra-osseous pressure (300 mmHg, 8ml/min).

A, Average response to three consecutive pressure ramps of one phasic-tonic (light grey) and one phasic (dark grey) unit. The number of impulses evoked per 0.5 sec bin were counted and plotted as spike frequency histograms. B, Instantaneous discharge frequency was plotted as a function of time for the response of one representative phasic-tonic (upper panel) and one phasic (lower panel) unit. The responses were fitted with a one-phase exponential decay. C, Distribution of time constants (τ) for the adaptation of phasic-tonic and phasic units. Phasic units (dark grey) adapted more rapidly to the pressure than phasic-tonic units (light grey)(t-test; $p \leq 0.0001$).

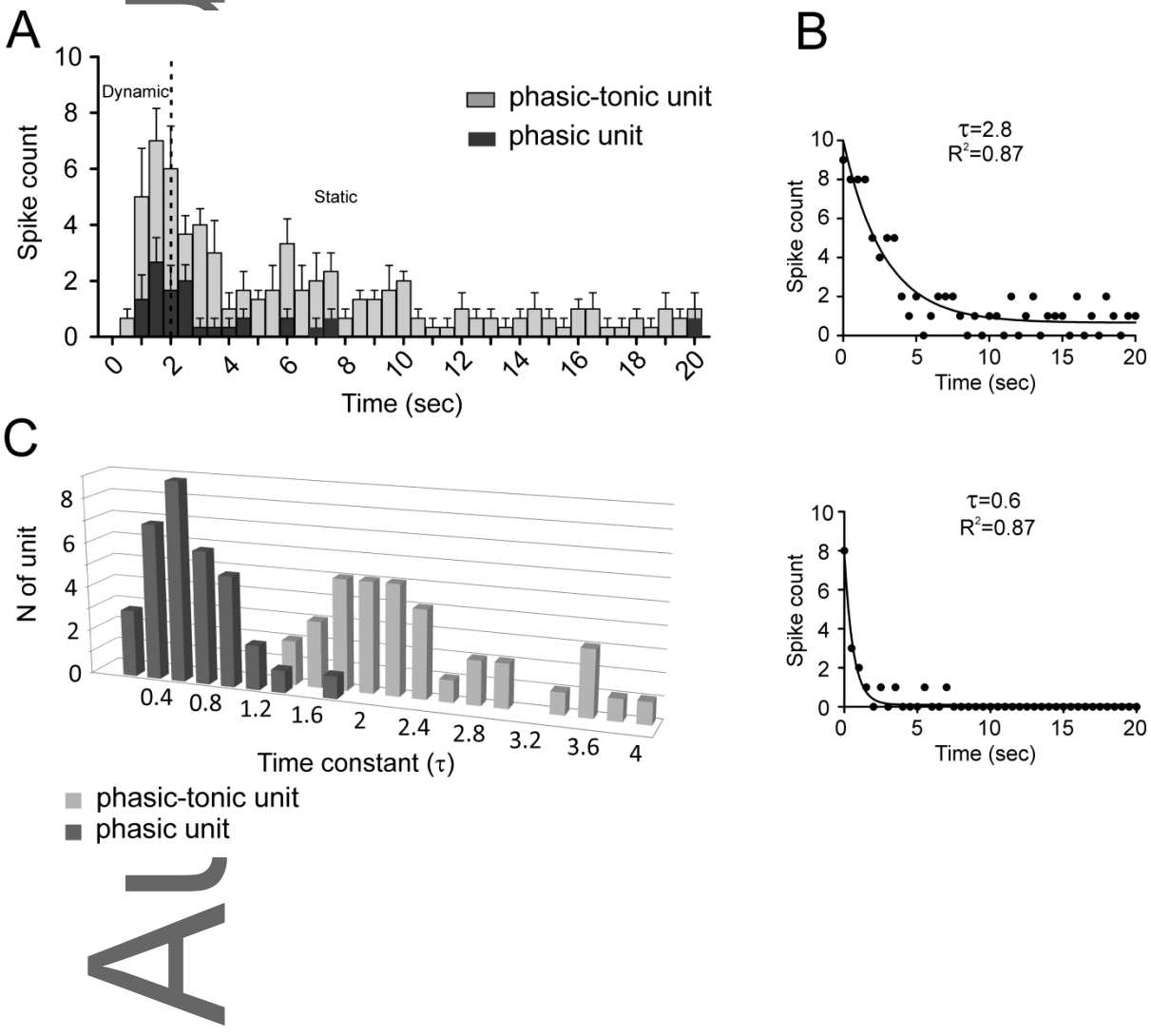


Figure 5

Repetitive stimulation produces fatigue.

Discharge frequency at different ISIs in the dynamic phase of the ramp-and-hold stimulus. The discharge frequency at the 2nd stimulus is expressed as a percentage of that at the first stimulus. A, Phasic-tonic units showed significant reductions in discharge frequency in the dynamic phase at 30 sec and 2 min ISIs. B, Phasic units showed significant reductions in discharge frequency in the dynamic phase at ISIs ≤ 5 min. C, Average responses for both phasic-tonic and phasic units at each of the ISIs tested. At ISIs of 30 sec and 2 min, fatigue was significantly greater in phasic units than in phasic-tonic units. **** p≤0.0001, *** p≤0.001, ** p≤0.01. Data are presented as mean ± SEM.

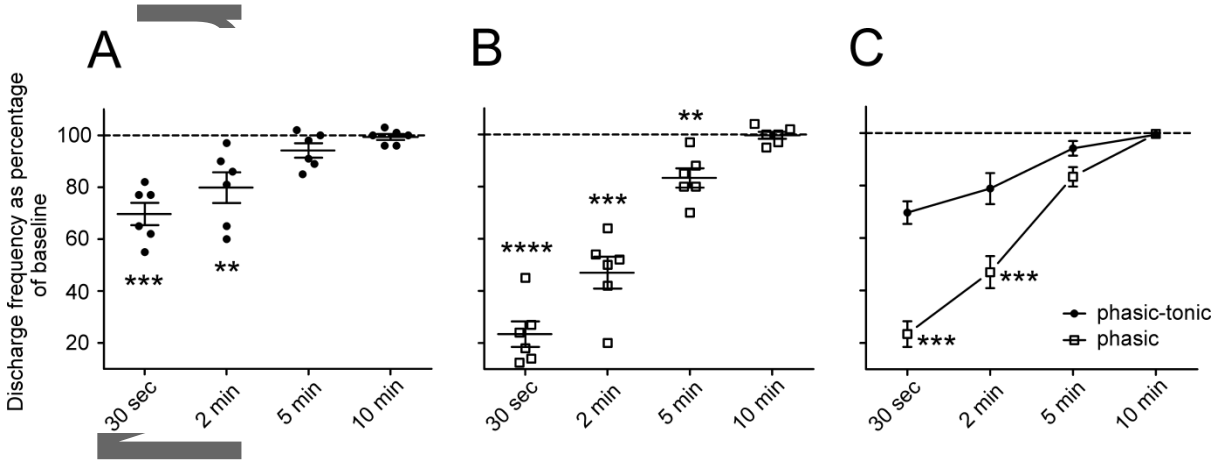


Figure 6

Phasic-tonic units are more responsive and better able to code for different intra-osseous pressures than phasic units.

Mean discharge frequency of phasic-tonic and phasic units tested with the intensity-coding protocol. Phasic-tonic units are represented by black circles. Phasic units are represented by white squares. A, Discharge frequency during the whole ramp-and-hold pressure stimulus (total response). The discharge frequency of phasic-tonic units increased with each step of pressure. At 200, 300 and 400 mmHg, it was significantly greater than at 100 mmHg, and at 400 and 300 mmHg it was significantly greater than at 200 mmHg. The threshold for activation of phasic units was always above 100 mmHg. The discharge frequency of phasic units increased to 300 mmHg and then plateaued at higher intensities. At 300 and 400 mmHg, their discharge frequency was significantly greater than at 200 mmHg. B, Discharge frequency in the dynamic phase of the stimulus. Phasic-tonic units increased their discharge frequency for each step of pressure, but phasic units did not. C, Discharge frequency in the static phase of the stimulus. There was an increase in the discharge frequency of phasic-tonic, but not phasic units, in response to increased pressure. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$. Data are presented as mean ± SEM.

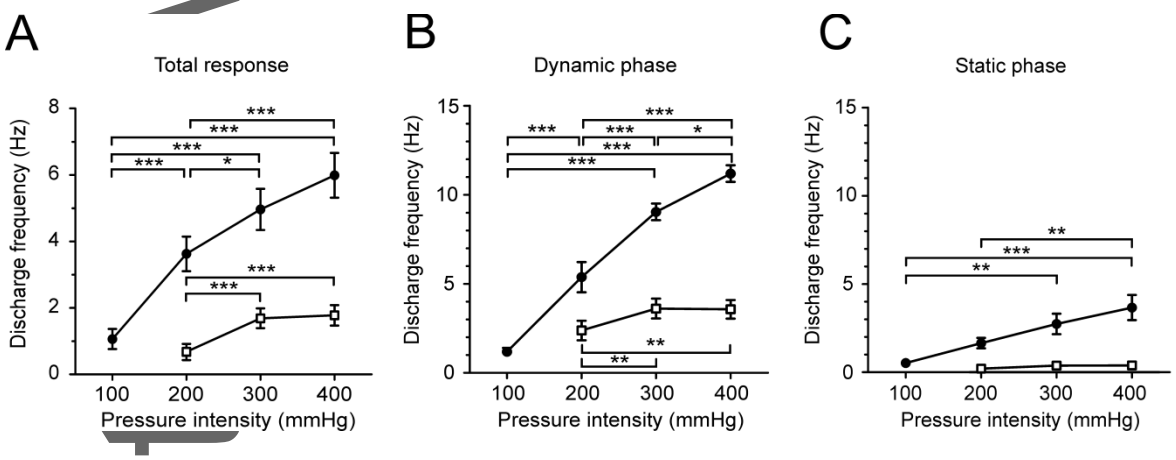


Figure 7

Phasic (but not phasic-tonic) units are able to code for the rate of change in intra-osseous pressure.

Mean discharge frequency of phasic-tonic and phasic units tested with the rate coding protocol (6, 8, 10ml/min pressure ramps). Phasic-tonic units are represented by black circles. Phasic units are represented by white squares. A, Discharge frequency during the whole ramp-and-hold pressure stimulus (total response). There was a rate dependant increase in discharge frequency in phasic, but not phasic-tonic units. B, Mean discharge frequency during the dynamic phase of the stimulus. There was a rate dependant increase in the discharge frequency of phasic, but not phasic-tonic units in the dynamic phase of the stimulus. C, Mean discharge frequency during the static phase of the stimulus. There was no change in the discharge frequency of either phasic or phasic-tonic units, at any rate tested, in the static phase. *** $p \leq 0.001$, ** $p \leq 0.01$. Data are presented as mean ± SEM.

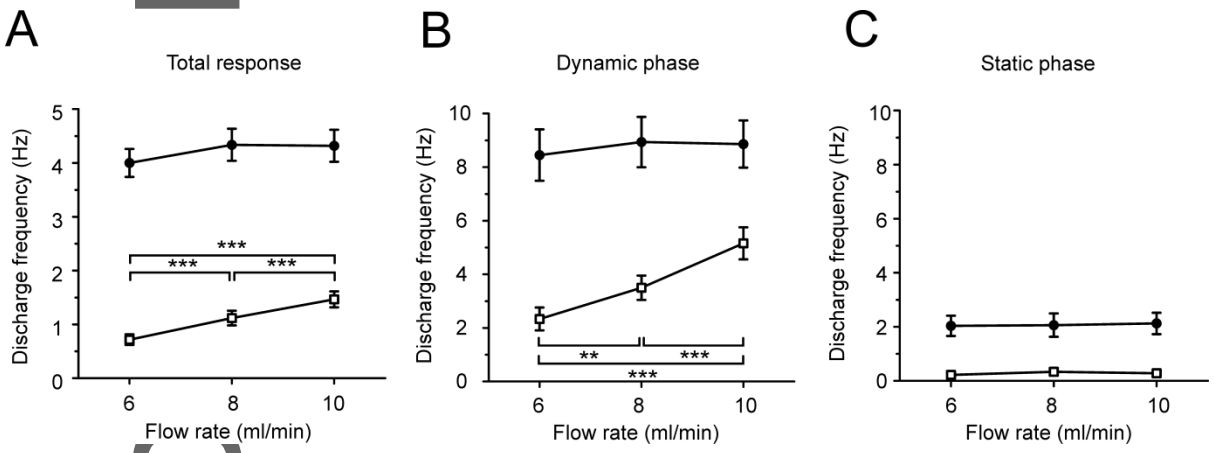


Figure 8

Capsaicin sensitizes mechanically activated units.

Threshold for activation of phasic-tonic units (A) and phasic units (B). Capsaicin reduced the threshold for mechanical activation in 6/13 phasic units, but not in the 10 phasic-tonic units (Student's t-test; $p>0.05$ compared to saline). Discharge frequency of phasic-tonic units (C) and phasic units (D). There was an increase in discharge frequency in response to IOP after injection of capsaicin. The increase was only evident in phasic units that responded to capsaicin with reduced activation thresholds. Threshold for activation and discharge frequency of each unit were expressed as percentage of baseline. *** $p\leq0.001$. Data are presented as mean ± SEM.

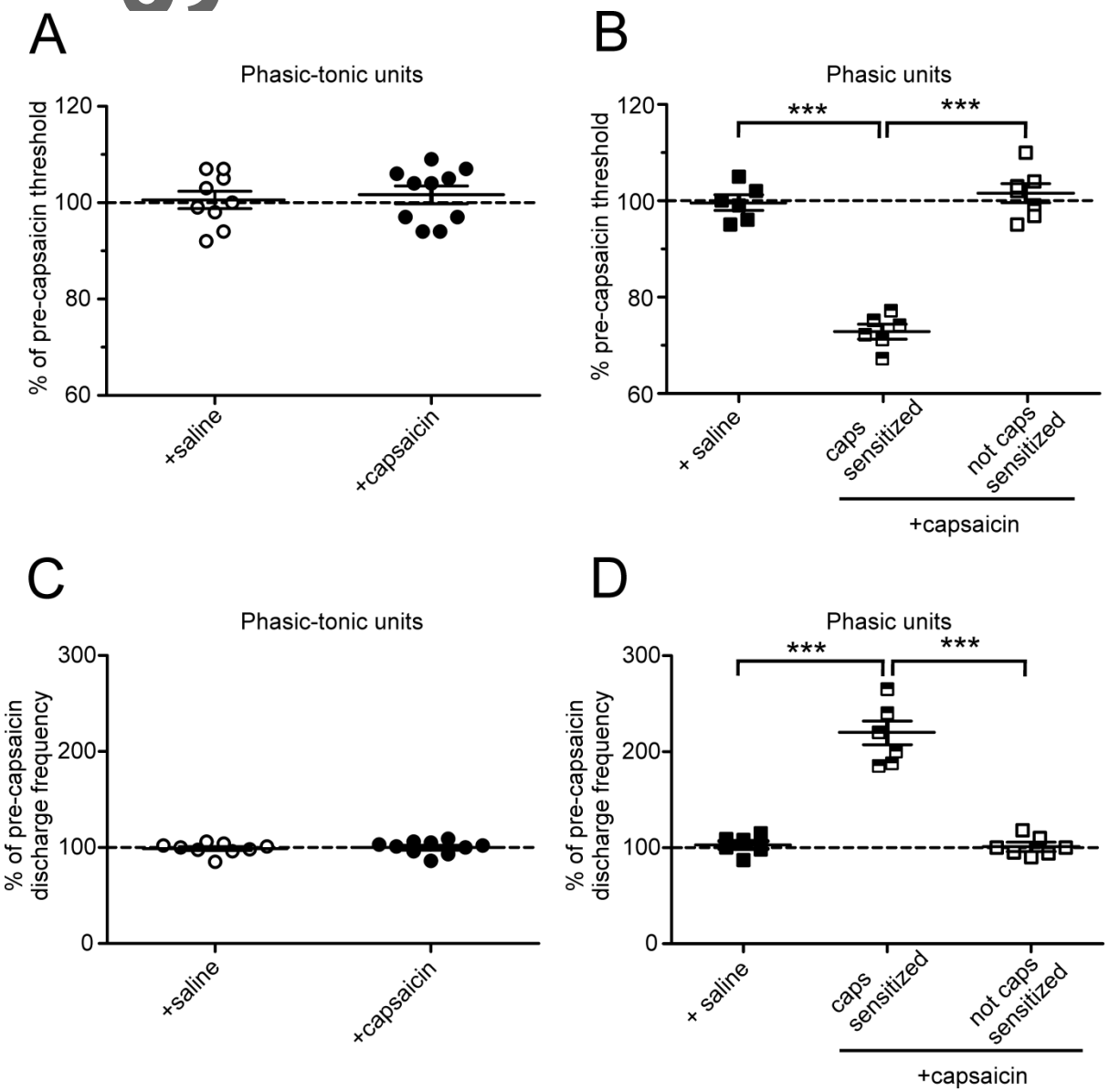


Figure 9

Phasic-tonic units, capsaicin sensitized phasic units and phasic units not sensitized by capsaicin could be distinguished on the basis of their response to mechanical stimulation before application the capsaicin.

A, Threshold for activation (in mmHg). Phasic-tonic units, capsaicin sensitized phasic units and phasic units not sensitized by capsaicin had significantly different thresholds for activation. B, Mean discharge frequency calculated in the dynamic phase of the pressure (Hz). Phasic-tonic units had significantly greater discharge frequencies than capsaicin sensitized phasic units and phasic units not sensitized by capsaicin. C, Latency to peak activation (sec). Phasic-tonic units, capsaicin sensitized phasic units and phasic units not sensitized by capsaicin had significantly different latencies to peak activation. D, Peak-to-peak action potential amplitude (μV). Phasic units not sensitized by capsaicin had significantly smaller action potential amplitudes than phasic-tonic units and phasic units not sensitized by capsaicin. *** p≤0.001, *p≤0.05. Data are presented as mean ± SEM.

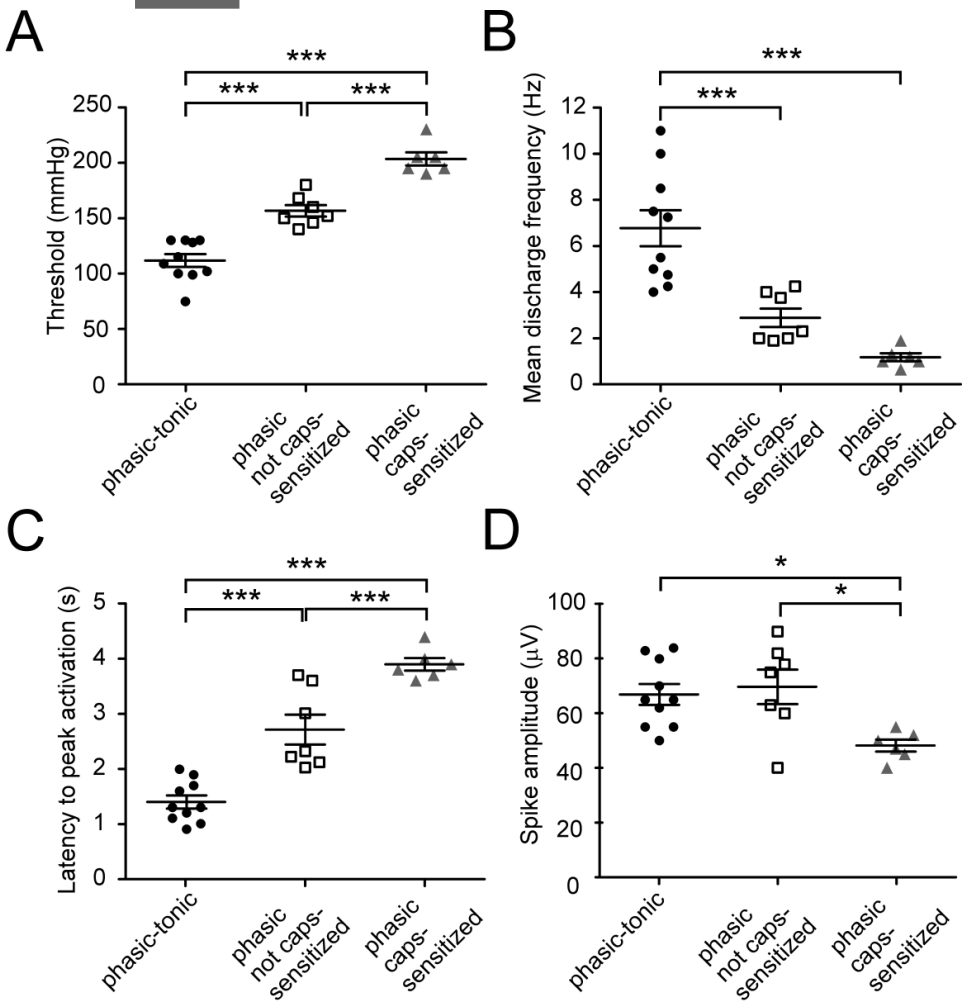


Figure 10

Piezo2 is expressed in small and medium sized myelinated bone afferent neurons.

A-D are images of the same field of a single section of the L3 dorsal root ganglion, showing retrograde labelled bone afferent neurons in blue (A), NF200 immuno-labelling of myelinated afferent neurons in green (B), Piezo2 immuno-labelling in red (C), and a merge of all three channels (D). Arrowheads identify retrograde labelled bone afferent neurons throughout. Asterisks (*) indicate bone afferent neurons that are NF200+, and hashes (#) indicate bone afferent neurons that are Piezo2+. Scale bars = 100 μm. E shows the size/frequency distribution of all retrograde labelled bone afferent neurons analysed in this study (black), those that are also myelinated (grey), and those that are myelinated and express Piezo2 (white). Most myelinated bone afferent neurons express Piezo2 and have a size profile consistent with an Aδ classification.

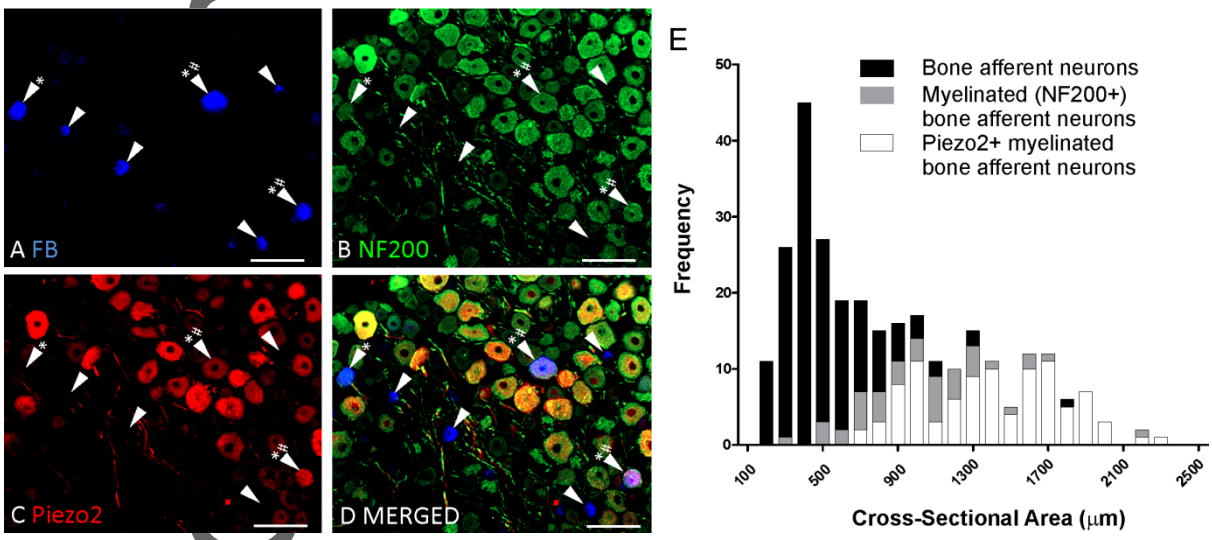


Table 1. Source and concentrations of the primary and secondary antisera used in this study

Primary Antibody Antigen	**Immunogen**	**Manufacturer**	**Dilution used**
Rabbit α Piezo2 (Piezo2)	human PIEZO2 protein (residues 1600-1650)	Novus Biologicals; Colorado, US; # NBP1-78624	1:100
Mouse α Neurofilament 200 (NF200)	carboxyterminal tail segment of pig neurofilament H-subunit	Sigma; Missouri, US; Mouse monoclonal; #N0142	1:1000
Secondary antibody	**Manufacturer**	**Dilution used**	
Donkey α Rabbit Alexa594	Molecular probes, Invitrogen; #A21207	1:200	
Donkey α mouse Alexa488	Molecular probes, Invitrogen; #A11001	1:200	

REFERENCES

Alamri A, Bron R, Brock JA & Ivanusic JJ. (2015). Transient receptor potential cation channel subfamily V member 1 expressing corneal sensory neurons can be subdivided into at least three subpopulations. *Frontiers in neuroanatomy* **9**, 71.

Arnoldi CC, Djurhuus JC, Heerfordt J & Karle A. (1980). Intraosseous phlebography, intraosseous pressure measurements and 99mTC-polyphosphate scintigraphy in patients with various painful conditions in the hip and knee. *Acta Orthop Scand* **51**, 19-28.

Bove SE, Flatters SJ, Inglis JJ & Mantyh PW. (2009). New advances in musculoskeletal pain. *Brain Res Rev* **60**, 187-201.

Brenneis C, Kistner K, Puopolo M, Segal D, Roberson D, Sisignano M, Labocha S, Ferreiros N, Strominger A, Cobos EJ, Ghasemlou N, Geisslinger G, Reeh PW, Bean BP & Woolf CJ. (2013). Phenotyping the function of TRPV1-expressing sensory neurons by targeted axonal silencing. *J Neurosci* **33**, 315-326.

Bron R, Wood RJ, Brock JA & Ivanusic JJ. (2014). Piezo2 expression in corneal afferent neurons. *J Comp Neurol* **522**, 2967-2979.

Cain DM, Wacnik PW, Eikmeier L, Beitz A, Wilcox GL & Simone DA. (2001). Functional interactions between tumor and peripheral nerve in a model of cancer pain in the mouse. *Pain Med* **2**, 15-23.

Cooper BR, Mahoney PF, Hodgetts TJ & Mellor A. (2007). Intra-osseous access (EZ-IO) for resuscitation: UK military combat experience. *J R Army Med Corps* **153**, 314-316.

Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, Petrus MJ, Dubin AE & Patapoutian A. (2010). Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science* **330**, 55-60.

Coste B, Xiao B, Santos JS, Syeda R, Grandl J, Spencer KS, Kim SE, Schmidt M, Mathur J, Dubin AE, Montal M & Patapoutian A. (2012). Piezo proteins are pore-forming subunits of mechanically activated channels. *Nature* **483**, 176-181.

Dubin AE, Schmidt M, Mathur J, Petrus MJ, Xiao B, Coste B & Patapoutian A. (2012). Inflammatory signals enhance piezo2-mediated mechanosensitive currents. *Cell Rep* **2**, 511-517.

Florez-Paz D, Bali KK, Kuner R & Gomis A. (2016). A critical role for Piezo2 channels in the mechanotransduction of mouse proprioceptive neurons. *Sci Rep* **6**, 25923.

Furusawa S. (1970). A neurophysiological study on the sensibility of the bone marrow. *Nippon Seikeigeka Gakkai Zasshi* **44**, 365-370.

Grundy D. (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. *The Journal of physiology* **593**, 2547-2549.

Haegerstam GA. (2001). Pathophysiology of bone pain: a review. *Acta Orthop Scand* **72**, 308-317.

Hakansson CH. (1956). Conduction velocity and amplitude of the action potential as related to circumference in the isolated fibre of frog muscle. *Acta Physiol Scand* **37**, 14-34.

He X, Schepelmann K, Schaible HG & Schmidt RF. (1990). Capsaicin inhibits responses of fine afferents from the knee joint of the cat to mechanical and chemical stimuli. *Brain Res* **530**, 147-150.

Holzer P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* **43**, 143-201.

Honore P & Mantyh PW. (2000). Bone cancer pain: from mechanism to model to therapy. *Pain Med* **1**, 303-309.

Ikeda R, Cha M, Ling J, Jia Z, Coyle D & Gu JG. (2014). Merkel cells transduce and encode tactile stimuli to drive Abeta-afferent impulses. *Cell* **157**, 664-675.
Ikeda R & Gu JG. (2014). Piezo2 channel conductance and localization domains in Merkel cells of rat whisker hair follicles. *Neurosci Lett* **583**, 210-215.
Ivanusic JJ. (2009). Size, neurochemistry, and segmental distribution of sensory neurons innervating the rat tibia. *J Comp Neurol* **517**, 276-283.
Jimenez Andrade JM & Mantyh P. (2010). Cancer Pain: From the Development of Mouse Models to Human Clinical Trials. In *Translational Pain Research: From Mouse to Man*, ed. L. Kruger & Light AR. CRC Press, Boca Raton, FL.
Kidd BL, Photiou A & Inglis JJ. (2004). The role of inflammatory mediators on nociception and pain in arthritis. *Novartis Found Symp* **260**, 122-133.
Kim SE, Coste B, Chadha A, Cook B & Patapoutian A. (2012). The role of Drosophila Piezo in mechanical nociception. *Nature* **483**, 209-212.
LaMotte RH & Campbell JN. (1978). Comparison of responses of warm and nociceptive C-fiber afferents in monkey with human judgments of thermal pain. *J Neurophysiol* **41**, 509-528.
Lemperg RK & Arnoldi CC. (1978). The significance of intraosseous pressure in normal and diseased states with special reference to the intraosseous engorgement-pain syndrome. *Clin Orthop Relat Res*, 143-156.
Li D, Ren Y, Xu X, Zou X, Fang L & Lin Q. (2008). Sensitization of primary afferent nociceptors induced by intradermal capsaicin involves the peripheral release of calcitonin gene-related Peptide driven by dorsal root reflexes. *J Pain* **9**, 1155-1168.
Liang YF, Haake B & Reeh PW. (2001). Sustained sensitization and recruitment of rat cutaneous nociceptors by bradykinin and a novel theory of its excitatory action. *The Journal of physiology* **532**, 229-239.
Mach DB, Rogers SD, Sabino MC, Luger NM, Schwei MJ, Pomonis JD, Keyser CP, Clohisy DR, Adams DJ, O'Leary P & Mantyh PW. (2002). Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. *Neuroscience* **113**, 155-166.
Mantyh PW. (2014). The neurobiology of skeletal pain. *Eur J Neurosci* **39**, 508-519.
Meyer RA, Davis KD, Cohen RH, Treede RD & Campbell JN. (1991). Mechanically insensitive afferents (MIAs) in cutaneous nerves of monkey. *Brain Res* **561**, 252-261.
Michaelis M, Habler HJ & Janig W. (1996). Silent afferents: a separate class of primary afferents? *Clin Exp Pharmacol Physiol* **23**, 99-105.
Milner TE, Stein RB, Gillespie J & Hanley B. (1981). Improved estimates of conduction velocity distributions using single unit action potentials. *J Neurol Neurosurg Psychiatry* **44**, 476-484.
Nencini S & Ivanusic JJ. (2016). The Physiology of Bone Pain. How Much Do We Really Know? *Frontiers in physiology* **7**, 157.
Nencini S, Ringuet M, Kim D, Chen Y, Greenhill C & Ivanusic J. (2017). Mechanisms of NGF signaling in bone nociceptors and in an animal model of inflammatory bone pain. *Mol Pain* **(in press)**.
Ngo AS, Oh JJ, Chen Y, Yong D & Ong ME. (2009). Intraosseous vascular access in adults using the EZ-IO in an emergency department. *Int J Emerg Med* **2**, 155-160.
Niv D, Gofeld M & Devor M. (2003). Causes of pain in degenerative bone and joint disease: a lesson from vertebroplasty. *Pain* **105**, 387-392.
Peng YB, Ringkamp M, Meyer RA & Campbell JN. (2003). Fatigue and paradoxical enhancement of heat response in C-fiber nociceptors from cross-modal excitation. *J Neurosci* **23**, 4766-4774.

Portenoy RK, Payne D & Jacobsen P. (1999). Breakthrough pain: characteristics and impact in patients with cancer pain. *Pain* **81**, 129-134.

Ranade SS, Woo SH, Dubin AE, Moshourab RA, Wetzel C, Petrus M, Mathur J, Begay V, Coste B, Mainquist J, Wilson AJ, Francisco AG, Reddy K, Qiu Z, Wood JN, Lewin GR & Patapoutian A. (2014). Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature* **516**, 121-125.

Reeh PW, Bayer J, Kocher L & Handwerker HO. (1987). Sensitization of nociceptive cutaneous nerve fibers from the rat's tail by noxious mechanical stimulation. *Exp Brain Res* **65**, 505-512.

Ren Y, Zou X, Fang L & Lin Q. (2005). Sympathetic modulation of activity in Adelta- and C-primary nociceptive afferents after intradermal injection of capsaicin in rats. *J Neurophysiol* **93**, 365-377.

Rowe MJ, Tracey DJ, Mahns DA, Sahai V & Ivanusic JJ. (2005). Mechanosensory perception: are there contributions from bone-associated receptors? *Clin Exp Pharmacol Physiol* **32**, 100-108.

Seike W. (1976). Electrophysiological and histological studies on the sensibility of the bone marrow nerve terminal. *Yonago Acta Med* **20**, 192-211.

Singhmar P, Huo X, Eijkelkamp N, Berciano SR, Baameur F, Mei FC, Zhu Y, Cheng X, Hawke D, Mayor F, Jr., Murga C, Heijnen CJ & Kavelaars A. (2016). Critical role for Epac1 in inflammatory pain controlled by GRK2-mediated phosphorylation of Epac1. *Proc Natl Acad Sci U S A* **113**, 3036-3041.

Slugg RM, Meyer RA & Campbell JN. (2000). Response of cutaneous A- and C-fiber nociceptors in the monkey to controlled-force stimuli. *J Neurophysiol* **83**, 2179-2191.

Sowa NA, Street SE, Vihko P & Zylka MJ. (2010). Prostatic acid phosphatase reduces thermal sensitivity and chronic pain sensitization by depleting phosphatidylinositol 4,5-bisphosphate. *J Neurosci* **30**, 10282-10293.

Starr AM, Wessely MA, Albastaki U, Pierre-Jerome C & Kettner NW. (2008). Bone marrow edema: pathophysiology, differential diagnosis, and imaging. *Acta Radiol* **49**, 771-786.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI & Julius D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21**, 531-543.

Torebjork HE, LaMotte RH & Robinson CJ. (1984). Peripheral neural correlates of magnitude of cutaneous pain and hyperalgesia: simultaneous recordings in humans of sensory judgments of pain and evoked responses in nociceptors with C-fibers. *J Neurophysiol* **51**, 325-339.

Uhelski ML, Cain DM, Harding-Rose C & Simone DA. (2013). The non-selective cannabinoid receptor agonist WIN 55,212-2 attenuates responses of C-fiber nociceptors in a murine model of cancer pain. *Neuroscience* **247**, 84-94.

Urch C. (2004). The pathophysiology of cancer-induced bone pain: current understanding. *Palliat Med* **18**, 267-274.

Wang J, Cao DY, Guo Y, Ma SJ, Luo R, Pickar JG & Zhao Y. (2011). Octreotide inhibits capsaicin-induced activation of C and Adelta afferent fibres in rat hairy skin in vivo. *Clin Exp Pharmacol Physiol* **38**, 521-527.

Woo SH, Lukacs V, de Nooij JC, Zaytseva D, Criddle CR, Francisco A, Jessell TM, Wilkinson KA & Patapoutian A. (2015). Piezo2 is the principal mechanotransduction channel for proprioception. *Nat Neurosci* **18**, 1756-1762.

Woo SH, Ranade S, Weyer AD, Dubin AE, Baba Y, Qiu Z, Petrus M, Miyamoto T, Reddy K, Lumpkin EA, Stucky CL & Patapoutian A. (2014). Piezo2 is required for Merkel-cell mechanotransduction. *Nature* **509**, 622-626.

Yang J, Zhang J, Yang H, Li K, Lei X & Xu C. (2016). The potential role of Piezo2 in the mediation of visceral sensation. *Neurosci Lett* **630**, 158-163.

Zagorodnyuk VP, Gibbins IL, Costa M, Brookes SJ & Gregory SJ. (2007). Properties of the major classes of mechanoreceptors in the guinea pig bladder. *The Journal of physiology* **585**, 147-163.