ABSTRACT

BACKGROUND: Pathologies that affect the bone marrow have a significant inflammatory component; however, it is not clear how inflammatory mediators affect nociceptive nerve terminals within the marrow cavity.

METHODS: In this study, an *in vivo* bone-nerve preparation was used to directly record the physiological response properties of bone marrow nociceptors innervating the tibial marrow cavity of rats, before and after application of the inflammatory agent carrageenan. In addition, endogenous artemin was sequestered by application of an artemin neutralizing antibody to determine if this could prevent the inflammation-induced physiological changes observed.

RESULTS: A single injection of carrageenan administered into the tibial marrow cavity produced rapid changes in weight bearing (pain-like behavior) in conscious animals. Carrageenan, but not saline, activated bone marrow nociceptors in whole-nerve recordings and sensitized a subtype of A δ -bone marrow nociceptors to mechanical stimulation. The activation and sensitization had a rapid time course that matched that of pain-like behaviors. Sequestration of endogenous artemin significantly reduced carrageenan-induced increases in ongoing activity and completely abolished sensitization of bone marrow nociceptors to mechanical stimulation.

CONCLUSIONS: These observations indicate that inflammation affects the activity and sensitivity of bone marrow nociceptors; that artemin plays a role in these changes; and that artemin *might be a promising target for pharmacological manipulations in the treatment of inflammatory bone pain.*

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TITLE:

**SEQUESTRATION OF ARTEMIN REDUCES INFLAMMATION-INDUCED
ACTIVATION AND SENSITIZATION OF BONE MARROW NOCICEPTORS IN A
RODENT MODEL OF CARRAGEENAN-INDUCED INFLAMMATORY BONE
PAIN**

RUNNING TITLE: Artemin sequestration and acute inflammatory bone pain

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SIGNIFICANCE STATEMENT:

Most pathologies that affect the bone marrow have an inflammatory component. We have used a model of carrageenan-induced inflammation to show that sequestration of artemin reduces inflammation-induced activation and sensitization of bone marrow nociceptors. Our findings suggest that artemin signaling is a target for the treatment of inflammatory bone pain.

ABSTRACT

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METHODS: In this study, an in vivo bone-nerve preparation was used to directly record the physiological response properties of bone marrow nociceptors innervating the tibial marrow cavity of rats, before and after application of the inflammatory agent carrageenan. In addition, endogenous artemin was sequestered by application of an artemin neutralizing antibody to determine if this could prevent the inflammation-induced physiological changes observed.

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CONCLUSIONS: These observations indicate that inflammation affects the activity and sensitivity of bone marrow nociceptors; that artemin plays a role in these changes; and that

artemin might be a promising target for pharmacological manipulations in the treatment of inflammatory bone pain.

INTRODUCTION

Pain is associated with a number of different bony pathologies, including bone marrow edema syndromes, cancers, fractures, osteoarthritis, and osteomyelitis. Many of these pathologies have a significant inflammatory component, and inflammation is a likely trigger for the pain (Bennett 1988; Goldring 2000; Haegerstam 2001; Starr et al., 2008). Agents known to act by reducing inflammatory processes (e.g. non-steroidal anti-inflammatory drugs, NSAIDs) are generally used to treat mild to severe bone pain (Honore and Mantyh 2000). However, they are often inadequate and linked to undesirable side effects with prolonged use. In particular, they can impact on bone remodeling and/or healing (Bove et al., 2009; Chrastil et al., 2013; Pountos et al., 2012). There is a clear need for new therapies to treat inflammatory bone pain.

A number of animal models of bone pathology are currently being used to explore the pathophysiological mechanisms that contribute to bone pain and to test the therapeutic potential of novel molecular targets to treat it. Inoculation of tumor cells or injection of experimental inflammatory agents such as complete Freund's adjuvant (CFA) into the bone marrow produce pain-like behaviors, accompanied by signs of inflammation (e.g. invasion of neutrophils and granulocytes; prominent fibrinous exudates; fibrosis; osteoclast proliferation and hypertrophy) (Honore et al., 2000; Jimenez-Andrade et al., 2010; Nencini et al., 2017; Yang et al., 2011). By analogy with what happens in other tissue systems, we predict that chemical mediators released from immune cells in the marrow cavity during inflammation could activate and/or enhance the excitability of bone marrow nociceptors, ultimately contributing to pain (Nencini and Ivanusic 2016). However, there is at present no direct evidence that inflammation physiologically activates and/or sensitizes peripheral bone marrow nociceptors, or that acute experimentally induced inflammation of the bone marrow produces pain-like behavior in rodents.

The glial cell line-derived neurotrophic factor (GDNF) family of ligands (GDNF, artemin and neurturin) have been implicated as endogenous regulators of inflammation and pain (Malin et al., 2006; Schmutzler et al., 2009; Schmutzler et al., 2011). We have recently provided evidence that artemin, in particular, acts as a strong mediator of bone pain (Nencini et al., 2018). A substantial proportion of peripheral sensory neurons that innervate the bone marrow express the artemin receptor GFR α 3 (~40%), injection of artemin into the tibial marrow cavity of rats produces rapid pain-like behaviors and changes in the activity and sensitivity of bone marrow nociceptors, and sequestration of endogenous artemin prevents the development of CFA-induced inflammatory bone pain (Nencini et al., 2018).

Here, we present a new model of acute experimental inflammatory bone pain induced by injection of carrageenan directly into the marrow cavity of the rat tibia. We use this model to show that the electrophysiological response properties of bone marrow nociceptors are rapidly altered by acute inflammation, and that sequestering endogenous artemin can prevent inflammation-induced changes in the activity and sensitivity of bone marrow nociceptors.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing between 200 and 250 g were used in this study. The study was confined to male rats to avoid potential increased variance related to the effects of cyclic changes in estrogen level on neuronal activity. Animals were housed in pairs 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.

Pain-like behavior in response to application of carrageenan

Animals were anesthetized with isoflurane (4% induction; 2.5% maintenance; O₂: 2 ml/min). A skin incision was made on the medial aspect of the tibia and a hole placed through the underlying cortical bone using a sterile 26-gauge needle. A Hamilton syringe was used to apply carrageenan (λ -carrageenan, Sigma-Aldrich, Australia; 2%, 10 μ l) or saline (10 μ l, volume control) directly into the marrow cavity through the hole. This concentration of carrageenan is in the range that produces rapid pain-like responses and nociceptor sensitization when applied to other tissues (Carpenter et al., 2003; Chakrabarty et al., 2011; Koltzenburg et al., 1999; Loram et al., 2007). The hole was sealed with bone wax and the

skin was closed with stainless steel autoclips. Recovery from isoflurane anesthesia occurred within a few minutes from the end of the surgery, allowing behavioral testing to be performed within 15 min of surgery. Changes in static weight bearing, assessed with an incapitance meter (IITC Life Science Inc., California, US), were used to assay pain-like behaviors in response to injection of carrageenan or saline at 15 and 30 mins, 1, 2 and 5 hours, and 1, 2 and 3 days after injection (Figure 1A). Testing was always performed at the same time each day and by the same experimenter. Animals were first habituated with exposure to the equipment for 15 mins per day over three days prior to surgery. At each testing time-point, five weight bearing readings of 3 sec duration were collected. The weight bearing on the injected hind-limb of each animal was then calculated as a percentage of the total weight bearing, using the following equation:

$$\frac{\text{Average weight on injected hindlimb}}{\{\text{Average weight on injected hindlimb} + \text{Average weight on un-injected hindlimb}\}} \times 100$$

Animals were killed by CO₂ asphyxiation at the end of the behavioral testing.

Electrophysiological recordings using an in vivo bone-nerve preparation

Recording configuration and spike discrimination

To determine how bone marrow nociceptors responded to experimentally induced inflammation and artemin sequestration, we used an in vivo bone-nerve electrophysiological preparation recently developed in our lab (Nencini and Ivanusic 2017; Nencini et al., 2017; Nencini et al., 2018). 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 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 marrow nociceptors by raising intra-osseous pressure in the marrow cavity. This was achieved by injection of heparinized 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 (ATM-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 mmHg of pressure delivered for 15-sec duration during the hold phase. Carrageenan and/or anti-artemin antibodies were delivered to the marrow cavity through a second hole using a Hamilton syringe attached with polyethylene tubing. These agents were infused slowly and there were no changes in the pressure recorded in the marrow cavity in response to injections of carrageenan, anti-artemin or their control solutions (saline or isotype control antibody).

Mechanically evoked action potentials originating from single units were discriminated from whole-nerve recordings according to their amplitude and duration using Spike Histogram software (LabChart 8, ADInstruments). 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 units as C, A δ or A β on the basis of previously published experiments 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 (Nencini and Ivanusic 2017; Nencini et al., 2018). During active conduction, large fast conducting axons produce greater extracellular current flux at the site of recording than small slow conducting axons, and so for fast conducting axons the extracellularly recorded action potentials have larger amplitudes than those of slowly conducting axons (Hakansson 1956; Milner et al., 1981). In our most recent study (Nencini et al., 2018), we have shown that units with small amplitude action potentials (<40 μ V) conducted slower than 2.5 m/sec. Units with medium action potential amplitudes (range: 40-145 μ V) conducted between 2.5 to 12.5 m/sec. Units with large action potential amplitudes (>120 μ V) conducted faster than 12.5 m/sec, and on probing of surrounding tissue, displayed low-threshold receptive fields outside the marrow cavity. It is possible that the latter 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. On the basis of these findings, for our recording configuration, the 40 μ V peak-to-peak amplitude provides a consistent cut-off to distinguish units with conduction velocities above and below 2.5 m/sec. The 2.5 m/sec cut-off

conduction velocity is somewhat arbitrary and is in part informed by our ability to classify impulses with peak-to-peak amplitudes above and below 40 μ V as originating from single units with distinguishable conduction velocities, but it is consistent with a cut-off that would distinguish between rodent A δ and C fiber nociceptors in other tissue systems (eg. Djouhri and Lawson 2004; Paintal 1967; Villiere and McLachlan 1996). Thus, in the present study, we define action potentials with peak-to-peak amplitudes of between 40-145 μ V as being derived from A δ fibers, and those with peak-to-peak amplitudes of less than 40 μ V as being derived from C fibers.

Recording protocols and analysis

For analysis of ongoing activity, a continuous whole-nerve recording was made from 5 minutes before to 60 minutes after carrageenan or saline injection. All action potentials with positive and/or negative peaks clearly above noise were sampled from the whole-nerve recordings. The number of spikes before and after the injection was reported in a frequency histogram generated using 20 sec bin widths. The onset of carrageenan-induced activity was defined as the time at which the mean frequency of discharge in two consecutive 20 sec bins exceeded that of the maximum mean frequency of discharge in all pre-injection bins. The frequency of discharge (Hz) was also averaged over 5 min intervals starting at the time of injection (0 min), and at 20 and 40 mins after the injection. This analysis was performed on data derived from all spikes in the whole-nerve recordings, regardless of whether they belonged to A δ or C fiber units, and on data derived from spikes with amplitudes consistent with A δ or C conduction velocities. For analysis of sensitivity to mechanical stimulation, single mechanically-activated A δ -bone marrow nociceptors were isolated from whole-nerve recordings made during application of a ramp-and-hold intra-osseous pressure stimulus of 15-sec duration and 300 mmHg. Discharge frequency and thresholds for mechanical activation during the pressure stimulus were determined before and at 15, 30, 45 and 60 mins after application of carrageenan or saline. Discharge frequency was reported over the entire 15 sec ramp-and-hold pressure stimulus (total response), for the ramp phase of the stimulus (defined as the first 2 sec of the ramp-and-hold pressure stimulus, dynamic response), and for the hold phase of the stimulus (defined as the last 5 sec of the ramp-and-hold pressure stimulus, static response). Both discharge frequency and threshold for activation were expressed as a percentage of the pre-injection baseline values. Units with increases in total discharge frequency greater than 20% were defined as sensitized. We were not able to isolate individual C fiber units using our recording configuration and so cannot comment on whether single C

fiber units were sensitized to mechanical stimulation. Previously we have reported that there are at least two different classes of mechanically-activated A δ -bone marrow nociceptors that can be distinguished on the basis of their adaptation profile: phasic units and phasic-tonic units (Nencini and Ivanusic 2017). In the present study, for each of the isolated bone marrow nociceptors, the instantaneous discharge frequency during the static phase of the stimulus was plotted as a function of time, the decay phase was fitted to a mono-exponential function, and the decay time constant tau (i.e. the reciprocal of the decay constant calculated from the regression) was calculated as a measure of adaptation (GraphPad Prism, GraphPad Software, La Jolla, CA, USA). Units were classified as phasic if they adapted quickly ($\tau < 1.3$) or as phasic-tonic if they adapted slowly ($\tau \geq 1.3$) (Nencini and Ivanusic 2017). This classification was done before application of carrageenan or saline to define the adaptation profile of each unit before experimental manipulation.

To determine if sequestering artemin could prevent carrageenan-induced changes in activity or sensitivity of bone marrow nociceptors, a polyclonal artemin neutralizing antibody (AF1085; R&D Systems; 20 μ g/ml, 10 μ l injection) or an isotype control antibody (AB108C, R&D Systems; 20 μ g/ml, 10 μ l injection) was delivered at the same time as carrageenan directly into the marrow cavity. This concentration of the artemin neutralizing antibody has previously been used to produce functional block of artemin signaling in a growth assay of peripheral sensory neurons in vitro (Damon et al., 2007), and in our previous study to prevent the induction of pain behavior in a rat model of CFA-induced inflammatory bone pain (Nencini et al., 2018). Analysis of ongoing activity and sensitivity to mechanical stimulation was performed as above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Comparison of treatment effects for carrageenan or anti-artemin on the activation and sensitization of bone marrow nociceptors, and for carrageenan on pain behavior, were assessed using a Two-way ANOVA with repeated measures over time. Pairwise comparisons were made with Bonferroni's post hoc testing only if analysis of variance showed significance. Comparison of the tau constants of phasic vs phasic-tonic units before carrageenan application was made using a Student's unpaired t-test, and for phasic units before vs after carrageenan application using a paired t-test. $P < 0.05$ was used to define statistical significance.

RESULTS

Carrageenan injected into the marrow cavity produces a rapid and transient change in weight bearing behavior in rats

To test whether carrageenan-induced inflammation can produce pain behavior in conscious animals, we applied carrageenan to the marrow cavity of anesthetized rats and following recovery of consciousness, we determined the percentage of total weight bearing on the injected hindlimb (Figure 1). Two-way ANOVA with repeated measures revealed significant treatment effects in animals injected with carrageenan ($n=9$) relative to those injected with saline ($n=6$) ($F(1;13)=20.04$, $p=0.0006$; Figure 1B). At 15, 30 mins and 1 hour after carrageenan injection, the percentage of weight bearing on the injected hindlimb was significantly lower than the values for saline-injected animals (Bonferroni's post hoc tests, $p<0.05$), suggesting that pain-like behaviors are due to carrageenan injection and not to surgery. This effect disappeared by 2 hours (Figure 1B).

Carrageenan injected into the marrow cavity activates bone marrow nociceptors in whole-nerve recordings

Application of carrageenan to the marrow cavity increased ongoing activity in whole-nerve recordings (Figure 2). There was a rapid increase in whole-nerve discharge frequency that started between 30 sec and 80 sec of carrageenan application (58 ± 9.12 sec, mean $\pm$ SEM, $n=5$; Figure 2B). The increase in whole-nerve activity was robust and lasted for the entire 60 min recording, with no clear peak in activity over this time. Saline injection resulted in no change in activity ($n=5$) (Figure 2B). Figure 2C shows group data for the 0-5, 20-25 and 40-45 min bins. Two-way ANOVA with repeated measures revealed significant differences in whole-nerve discharge frequency in animals injected with carrageenan, relative to those injected with saline ($F(1;8)=88.56$, $p<0.0001$). Carrageenan injection produced a significant increase in whole-nerve discharge frequency, relative to saline injection, at each of the three post-injection time-points (Bonferroni's post hoc tests, $p<0.05$; Figure 2C). Analysis of frequency histograms generated using action potentials derived from A δ - or C-fiber units (on the basis of their action potential amplitude; see methods) revealed that carrageenan injection resulted in increased ongoing activity of both fiber types (Figure 2D).

Carrageenan injected into the marrow cavity sensitizes mechanically-activated A δ -bone marrow nociceptors

Application of carrageenan to the marrow cavity sensitized single A δ -bone marrow nociceptors to mechanical stimulation delivered by ramp-and-hold pressure stimuli (Figure 3). A total of 18 single, mechanically activated A δ -bone marrow nociceptors were isolated from whole-nerve recordings and were classified as either phasic (8/18) or phasic-tonic (10/18) units (eg. Figure 3A). The phasic units had tau constants that were significantly different from phasic-tonic units (0.47 ± 0.076 vs 2.6 ± 0.48 , mean $\pm$ SEM, unpaired t-test, $p=0.0015$). Two-way ANOVA with repeated measures revealed significant differences in the total discharge frequency during the ramp-and-hold stimulus between phasic units exposed to carrageenan, phasic-tonic units exposed to carrageenan and all units exposed to saline ($F(2;25)=22.48$, $p<0.0001$; Figure 3B, left panel). In particular, carrageenan increased the discharge frequency of phasic, but not phasic-tonic units for up to 45 mins after injection (Bonferroni's post hoc tests, $p<0.05$; Figure 3B). In addition, carrageenan significantly prolonged the adaptation kinetics for these phasic units as revealed by their increased tau constants calculated before and 15 mins after carrageenan injection (before: 0.47 ± 0.076 , after: 1.34 ± 0.183 , paired t-test, $p=0.0008$). Two-way ANOVA with repeated measures also revealed significant differences in the threshold for activation between phasic units exposed to carrageenan, phasic-tonic units exposed to carrageenan and all units exposed to saline ($F(2;25)=46.08$, $p<0.0001$; Figure 3C). In this case, carrageenan decreased the activation threshold of phasic, but not phasic-tonic units, at every time-point tested (Bonferroni's post hoc tests, $p<0.05$, up to 60 min; Figure 3C). Figure 3D shows the average discharge frequency of the single units during the dynamic (left panel) and static (right panel) phases of the pressure stimulus, after carrageenan or saline treatment, plotted as a percentage of their pre-treatment response. Significant differences between phasic units exposed to carrageenan, phasic-tonic units exposed to carrageenan and all units exposed to saline were observed for both the dynamic ($F(2;25)=10.46$, $p=0.0005$; Figure 3C, left panel) and static ($F(2;25)=31.15$, $p<0.0001$; Figure 3C, right panel) discharge frequency. For both dynamic and static discharge frequency, carrageenan increased the discharge frequency of phasic, but not phasic-tonic units, for up to 45 mins after injection (Bonferroni's post hoc tests, $p<0.05$). Thus carrageenan sensitized phasic (but not phasic-tonic) bone marrow nociceptors by reducing their threshold for activation and increasing their discharge frequency during both the dynamic and static phases of the pressure stimulus.

Sequestration of artemin reduces carrageenan-induced changes in activity and prevents carrageenan-induced changes in mechanical sensitivity of bone marrow nociceptors

Sequestration of artemin reduces carrageenan-induced changes in whole-nerve ongoing activity (Figure 4A and B). There was a clear reduction in the number of spikes recorded in animals co-injected with the anti-artemin antibody (n=8) relative to those co-injected with an isotype control antibody (n=6) that lasted for at least 60 mins (Figure 4A). Two-way ANOVA with repeated measures revealed significant differences in whole-nerve discharge frequency after application of carrageenan in animals that had artemin sequestered relative to those that had not ($F(1;12)=126.7$, $p<0.0001$). There was a significantly reduced discharge frequency at 0-5, 20-25 and 40-45 mins after co-injection of carrageenan with anti-artemin antibody relative to co-injection of carrageenan with isotype control antibody (Bonferroni's post hoc tests, $p<0.05$; Figure 4B). Whilst artemin sequestration clearly reduced the carrageenan-induced changes in the activity of bone marrow nociceptors, the increase in ongoing activity was not entirely blocked by the anti-artemin treatment, particularly in the first 5-10 mins after application (Figure 4A and B).

Sequestration of artemin entirely prevented carrageenan-induced sensitization of single, mechanically-activated A δ bone marrow nociceptors with phasic adaptation profiles (Figure 4C and D, left panels). Two way ANOVA with repeated measures revealed a significant treatment effect of the artemin neutralizing antibody on phasic units, relative to its isotype control antibody, for both discharge frequency ($F(1,10)=21.04$, $p=0.001$; Figure 4C, left panel) and threshold for activation ($F(1,10)=23.85$, $p=0.0006$; Figure 4D, left panel). There was a significant reduction in discharge frequency (Figure 4C) and increase in threshold for activation (Figure 4D) in phasic units recorded from animals co-injected with anti-artemin relative to those co-injected with its isotype control antibody. Phasic-tonic units were not sensitized by carrageenan and so co-injection with anti-artemin had no effect (Figures 4C and D, right panels).

DISCUSSION

Carrageenan-induced inflammation rapidly activates and sensitizes bone marrow nociceptors

Lambda (λ)-carrageenan is a sulfated polysaccharide that is used to induce acute inflammatory reactions accompanied by hyperalgesia (Henriques et al., 1987; Jain et al., 2001; Petersson et al., 2001; Posadas et al., 2004; Radhakrishnan et al., 2003; Sugishita et al., 1981). Localized peripheral injections of carrageenan result in infiltration of neutrophils and plasma extravasation of proteins at the site of injection (Capasso et al., 1975; Posadas et al., 2004; Vinegar et al., 1976), and a rapid increase in vascular permeability that is associated with tissue edema (Posadas et al., 2004). In non-compliant tissues such as the bone, inflammation-induced edema could contribute to increased pressure (Di Rosa et al., 1971), and it is possible that this would mechanically activate bone marrow nociceptors (Nencini and Ivanusic 2017). However, we did not observe changes in intra-osseous pressure at any time following carrageenan injection, so this is unlikely to be the case in our model. Furthermore, there is no evidence that carrageenan can directly act on nociceptive terminals. Rather, it is believed that carrageenan-induced changes in nociceptor function are a consequence of the release from activated immune cells of a number of inflammatory mediators (including histamine, prostaglandins, NGF and bradykinin) which are known to interact with peripheral sensory neurons (Al-Haboubi and Zeitlin 1983; Capasso et al., 1975; Costello and Hargreaves 1989; Di Rosa et al., 1971; Fernandez-Duenas et al., 2008; Koltzenburg et al., 1999; Tsuchida et al., 2015; Valenti et al., 2010).

A number of studies have provided clear evidence that carrageenan-induced inflammation increases the spontaneous activity of cutaneous and muscular nociceptors (Berberich et al., 1988; Diehl et al., 1993; Koltzenburg et al., 1999; Xu et al., 2000). However, the recordings of spontaneous activity in these studies were made between 2 and 5 hours after application of carrageenan, at which point changes in spontaneous activity had already been established. They do not resolve the latency to onset of carrageenan-induced activity in the peripheral nociceptors they record from. We could find only a single study that made recordings in short blocks of time immediately before and after application of carrageenan to the receptive fields of peripheral nociceptors (Ozaktay et al., 1994). This study provided evidence of a very short latency to onset of carrageenan-induced activity in nerves innervating the lumbar facet joints of the rabbit. In our own study, we were able to record continuously before, during and immediately after carrageenan injection into the marrow cavity, and up to one hour afterwards. We also found a very short latency to onset of changes in the activity of bone marrow nociceptors, occurring within only a few minutes of carrageenan application, that lasted for the entire one-hour recording period. Thus carrageenan-induced inflammation

rapidly activates bone marrow nociceptors and produces changes in activity that last for at least one hour. This could result from direct activation of bone marrow nociceptors by a number of different inflammatory mediators, including bradykinin or histamine released by activated immune cells, as these have been shown to activate nociceptors in other tissue systems (Belcher 1979; Koda and Mizumura 2002; Mense and Meyer 1988; Sann 1998). It could also result from sensitization of the bone marrow nociceptors by prostaglandins released from activated immune cells, which are known to increase the sensitivity of peripheral nociceptors to other inflammatory mediators (Belcher 1979; Birrell et al., 1993; Koda and Mizumura 2002; St-Jacques and Ma 2014).

Carrageenan-induced inflammation has also been reported to sensitize cutaneous, muscular and joint nociceptors to both heat and mechanical stimuli (Berberich et al., 1988; Kirchhoff et al., 1990; Kocher et al., 1987; Koltzenburg et al., 1999; Lu et al., 2017; Moon et al., 2016) and the sensitization is mostly confined to A δ - and C-fiber polymodal nociceptors that can be activated by known algescic substances, including capsaicin (Kocher et al., 1987; Mitchell et al., 2014; Takeuchi et al., 2004). Using our bone-nerve preparation, we have shown that carrageenan sensitizes phasic, but not phasic-tonic, A δ -bone marrow nociceptors to mechanical stimulation, and that the time course of sensitization is rapid. Under normal conditions, phasic A δ -bone marrow nociceptors have very high thresholds for mechanical activation (typically >200 mmHg of intra-osseous pressure) and generate only a short burst of activity during the ramp phase of the pressure stimulus (Nencini and Ivanusic 2017). For these phasic units, carrageenan decreased the threshold for mechanical activation, and increased the frequency of discharge during both the dynamic and static phases of the pressure stimulus. They also adapted more slowly to sustain pressure stimulation and so had longer tau constants. In a previous study, we showed that many phasic A δ -bone marrow nociceptors could be sensitized by capsaicin, suggesting that they are polymodal nociceptors (Nencini and Ivanusic 2017). Thus carrageenan-induced inflammation is likely to affect polymodal nociceptors innervating the bone marrow, as it does in other tissue systems.

We have also shown that carrageenan injection into the marrow cavity induces pain-like behavior with a time course consistent with the rapid changes in the ongoing activity and mechanical sensitivity of bone marrow nociceptors. This suggests that acute carrageenan-induced inflammatory pain can be explained at least in part by the rapid activation and sensitization of bone marrow nociceptors. Rapid changes in pain behaviors following

carrageenan-induced inflammation were reported by other studies and especially in relation to mechanically-induced hyperalgesia (Bonet et al., 2013; Watanabe et al., 2015). However, in bone, the duration of carrageenan-induced pain was shorter than in other tissue systems (Bonet et al., 2013; Hedo et al., 1999; Poisbeau et al., 2005), for which peak behavioral changes are often noted the day after application of carrageenan. This is likely due to the rapid clearance of carrageenan from the marrow cavity through the vasculature and subsequent resolution of the inflammation.

Inflammation-induced increases in the activity of peripheral nociceptors provide the spinal cord with an increased afferent input that has been suggested to be the basis of resting pain (Hedo et al., 1999; Okun et al., 2012; Schaible and Schmidt 1985; 1988). Inflammation-induced sensitization also makes peripheral sensory neurons hyper-excitable, a feature that is thought to contribute to hyperalgesia (Millan 1999; Woolf and Ma 2007). Both resting pain and mechanical hyperalgesia are common features of many bone pathologies, including those that have a significant inflammatory component (Honore and Mantyh 2000; Houghton et al., 1997; Mercadante et al., 2004; Mitchell et al., 2014; Portenoy and Hagen 1990). Thus our findings that the changes in activity and sensitivity of bone marrow nociceptors occur in conjunction with altered pain behavior during inflammation of bone marrow suggests that they may also contribute to pain experienced by patients with bone pathology that involves inflammation.

Artemin contributes to carrageenan-induced activation and sensitization of bone marrow nociceptors

Increased expression of artemin in peripheral tissues occurs in animal models of cutaneous inflammation and migraine (Albers et al., 2014; Ikeda-Miyagawa et al., 2015; Malin et al., 2006; Shang et al., 2016; Thornton et al., 2013), and in patients with chronic pancreatitis and burning mouth syndrome (Ceyhan et al., 2007; Shinoda et al., 2015). Taken together, these studies suggest that artemin signaling pathways could serve as a novel intervention point for therapies directed at alleviating inflammatory pain. In experimental animals, anti-artemin therapy has already been shown to block the development of, and reverse, already established bladder pain (DeBerry et al., 2015), as well as transiently reduce CFA-induced mechanical hyperalgesia of the footpad (Thornton et al., 2013). In addition, a number of studies have shown altered activity and/or sensitivity of peripheral nociceptors in response to

overexpression or direct injection of artemin in both skin and bone (Albers et al., 2014; Elitt et al., 2006; Ikeda-Miyagawa et al., 2015; Lippoldt et al., 2013; Nencini et al., 2018; Thornton et al., 2013), providing clues as to the mechanistic basis for action of therapies directed against artemin signaling in inflammatory pain.

We have now extended these findings to show a role for artemin signaling and anti-artemin therapy in inflammatory bone pain. We previously reported that artemin activates and sensitizes a substantial proportion of bone marrow nociceptors, and produces pain-like behavior within minutes of exogenous application to the rat tibial marrow cavity (Nencini et al., 2018). In addition, sequestering artemin prevents the development of CFA-induced inflammatory bone pain, showing that endogenous artemin is involved, at least in a model of inflammatory bone pain that develops over days (Nencini et al., 2018). Here we have now also shown that artemin sequestration reduces inflammation-induced activation, and prevents inflammation-induced mechanical sensitization, of bone marrow nociceptors, that occurs within 15 minutes of carrageenan application. Thus endogenous artemin is involved in the pathogenesis of acute inflammatory bone pain. The rapid effect is likely to result from direct actions on nerve terminal endings of bone marrow nociceptors. Interestingly, anti-artemin treatment was not able to entirely prevent the early activation of bone marrow nociceptors in whole-nerve recordings. This could be explained by the slow kinetics of anti-artemin antibody binding to its target. Alternatively, it could be attributed to the action on bone marrow nociceptors of other pro-inflammatory mediators released from immune cells upon carrageenan induced-inflammation (Ahmad et al., 2014; Hajhashemi et al., 2015; Koltzenburg et al., 1999; McMahon et al., 1995; Valenti et al., 2010). Taken together, our findings suggest that artemin is involved in the pathogenesis of inflammatory bone pain and could be a target for pharmacological manipulation to treat it. It will be interesting and important in the future to test whether artemin sequestration abrogates altered weight bearing behavior in carrageenan inflamed animals and other models of acute inflammatory bone pain, and if sequestering any of the other GDNF Family Ligands has similar effects.

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AUTHOR CONTRIBUTIONS:

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JL, SN and JT made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; drafted the article or revised it critically for important intellectual content; and approved the final of the version of the manuscript.

FIGURES LEGEND

Fig 1 Intra-osseous injection of carrageenan produces pain-like behaviors in animals. A.

Pain-like behaviors induced by intra-osseous injection of carrageenan (2%, 10 μ l) or saline (10 μ l) were measured using a weight bearing assay (with an incapacitance meter; upper panels). Each animal was habituated to the experimental apparatus 3 days prior to surgery and assessed before and after treatments (saline or carrageenan; lower panel). At each testing time-point, five weight bearing readings of 3 secs duration were collected. B. There was a significant reduction in weight bearing in the injected hindlimb, relative to un-injected hindlimb, at 15, 30 mins and 1 hour (but not at later time-points) after carrageenan injection (n=9) compared to saline-injected animals (n=6) (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, *p<0.05).

Fig 2 Carrageenan rapidly activates bone marrow nociceptors in whole-nerve recordings. A. Raw data for a whole-nerve recording in response to application of carrageenan to the marrow cavity. Each trace represents a 1 min segment of the recording before (baseline), and at 15, 30, 45 and 60 mins after application of carrageenan. B. Average spike frequency histograms of the responses of bone marrow nociceptors before and up to 60 mins after carrageenan (n=5, black bars) or saline (n=5, grey bars) injection. Each bin represents the mean ($\pm$ SEM) of impulses evoked per 20 secs. Carrageenan produced a rapid increase in whole-nerve ongoing activity that lasts for the entire 60 min recorded. Saline did not alter whole-nerve activity at any time point. C. Group data showing frequency of whole-nerve discharge over 5 min periods immediately after injection (0-5 mins), and at 20-25 mins and 40-45 mins after carrageenan (n=5) or saline (n=5) injection. Carrageenan increased whole-nerve discharge frequency, relative to saline, at each time point (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, *p<0.05). D. Average spike frequency histograms for spikes derived from A δ (> 40mV peak-to-peak amplitude; left panel) and C (<40 mV peak-to-peak amplitude; right panel) fiber units, in the whole-nerve

recordings, before and up to 60 mins after carrageenan injection (arrows). Each bin represents the mean ($\pm$ SEM) of impulses evoked per 20 secs. Carrageenan produced a rapid increase in whole-nerve activity for spikes derived from both A δ and C fiber bone marrow nociceptors.

Fig 3 Carrageenan sensitizes phasic, but not phasic-tonic A δ -bone marrow nociceptors.

A. Example of rasters of activity for two single, isolated units in response to a 300mmHg ramp and hold pressure stimulus before (top), 15 (middle), and 30 mins (bottom) after application of carrageenan to the marrow cavity. The phasic unit (blue), but not the phasic-tonic unit (green), had increased discharge frequency and decreased threshold for mechanical activation after carrageenan injection. B-C. The time course of changes in total discharge frequency (B) and threshold for activation (C) of single mechanically-activated units expressed as a percentage of pre-injection. D-E. The time course of changes in discharge frequency of single mechanically-activated units expressed as a percentage of pre-injection values during the ramp phase (dynamic discharge frequency, D) and the hold phase (static discharge frequency, E) of the pressure stimulus. In all experiments where carrageenan was applied, single units were separated into those that responded with a phasic discharge pattern (n= 8) and those that responded with a phasic-tonic discharge pattern (n=10). In all phasic units, but in none of the phasic-tonic units, carrageenan increased the total, dynamic and static discharge frequency at 15, 30 and 45 min relative to saline (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, *p<0.05). Carrageenan also reduced the threshold for activation of phasic units at 15, 30, 45 and 60 mins, relative to saline (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, *p<0.05). Saline did not alter the discharge frequency of any of the units tested. Data in B-E are represented as mean $\pm$ SEM.

Fig 4 Sequestration of artemin reduces carrageenan-induced increases whole-nerve activity, and prevents carrageenan-induced mechanical sensitization of bone marrow nociceptors.

Artemin was sequestered by co-injection of the artemin neutralizing antibody, AF1085 (20 μ g/10 μ l), with carrageenan into the marrow cavity. A. Average spike frequency histograms of the responses of bone marrow nociceptors before and up to 60 mins after co-injection of anti-artemin and carrageenan (n=8; green bars), or isotype control antibody and carrageenan (n=6; black bars). Each bin represents the mean ($\pm$ SEM) of impulses evoked per 20 secs. Sequestration of artemin reduced carrageenan-induced activation of bone marrow nociceptors. B. Group data showing frequency of whole-nerve discharge measured over 5

min intervals immediately after (0-5 mins), at 20-25 mins and 40-45 mins after co-injection of anti-artemin and carrageenan (n=5), or isotype control antibody and carrageenan (n=5). Sequestration of artemin resulted in a reduction in carrageenan-induced whole-nerve discharge frequency, relative to isotype control antibody, at each time point tested (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, * $p < 0.05$). C-D. Total discharge frequency (C) and threshold for activation (D), of single mechanically-activated phasic (left panels) and phasic-tonic (right panels) units, expressed as a percentage of pre-injection values. Sequestration of artemin resulted in a reduction in carrageenan-induced sensitization of phasic units to mechanical stimulation, relative to isotype control antibody, at the 15, 30 and 45 min time-points (Two-way ANOVA with repeated measures; Bonferroni's multiple comparisons, * $p < 0.05$, left panels). Phasic-tonic units were not sensitized by carrageenan and so co-injection with anti-artemin had no effect (C and D, right panels).

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