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A phenotypically restricted set of primary afferent nerve fibers innervate the bone versus skin: therapeutic opportunity for treating skeletal pain

Juan Miguel Jimenez-Andrade^a, William G. Mantyh^a, Aaron P. Bloom^a, Kevin Haili Xu^a, Alice S. Ferng^b, Gregory Dussor^a, Todd W. Vanderah^a, and Patrick W. Mantyh^{a,c,d}

^aDepartment of Pharmacology, University of Arizona, Tucson, AZ, 85724, USA

^bPhysiological Sciences, College of Medicine, University of Arizona, Tucson, AZ, 85724, USA

^cArizona Cancer Center, University of Arizona, Tucson, AZ, 85724, USA

^dResearch Service, VA Medical Center, Minneapolis, MN 55417, USA

Abstract

Although musculoskeletal pain is one of the most common causes of chronic pain and physical disability in both developed as well as developing countries, relatively little is known about the nerve fibers and mechanisms that drive skeletal pain. Small diameter sensory nerve fibers, most of which are C-fiber nociceptors, can be separated into two broad populations: the peptide-rich and peptide-poor nerve fibers. Peptide-rich nerve fibers express substance P (SP) and calcitonin gene related peptide (CGRP). In contrast, the peptide-poor nerve fibers bind to isolectin B4 (IB₄) and express the purinergic receptor P₂X₃ and *Mas-related G protein-coupled receptor member d* (*Mrgprd*). In the present report, we used mice in which the *Mrgprd*⁺ nerve fibers express genetically encoded axonal tracers to determine the peptide-rich and peptide-poor sensory nerve fibers that innervate the glabrous skin of the hindpaw as compared to the bone marrow, mineralized bone and periosteum of the femur. Whereas the skin is richly innervated by CGRP⁺, SP⁺, P₂X₃⁺ and *Mrgprd*⁺ sensory nerve fibers, the bone marrow, mineralized bone and periosteum receive a significant innervation by SP⁺ and CGRP⁺, but not *Mrgprd*⁺ and P₂X₃⁺ nerve fibers. This lack of redundancy in the populations of C-fibers that innervate the bone may present a unique therapeutic opportunity for targeting skeletal pain, as the peptide-rich and peptide-poor sensory nerve fibers generally express a different repertoire of receptors and channels to detect noxious stimuli. Thus, therapies that target the specific types of C-nerve fibers that innervate the bone may be uniquely effective in attenuating skeletal pain as compared to skin pain.

Keywords

periosteum; bone marrow; mineralized bone; analgesia; trauma

Corresponding author: Patrick W. Mantyh, Ph D, Department of Pharmacology, College of Medicine, University of Arizona, 1656 E. Mabel, Rm. #119, PO Box 245215, Tucson, AZ 85724, Phone: (520) 626-0742, Fax: (520) 626-4182, pmantyh@email.arizona.edu.

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Introduction

Musculoskeletal diseases such as osteoarthritis, low back pain, and trauma- or osteoporosis-related fracture are leading causes of long-term disability and chronic pain [1–4]. Given the burden that musculoskeletal pain places on society, it is surprising how little is known about the specific primary afferent sensory nerve fibers that drive musculoskeletal pain.

Primary afferent sensory neurons are the gateway by which sensory information is transmitted from the peripheral tissues to the spinal cord and brain [5,6]. The cell bodies of primary afferent sensory nerve fibers are located in the dorsal root ganglia (DRG) and trigeminal ganglia. Anatomically, there are two broad groups of sensory nerve fibers: myelinated A-fibers and smaller diameter, unmyelinated C-fibers [5,6]. The majority of primary afferent neurons that transmit noxious stimuli are C-fibers which can be divided into two different classes based on the content of neuropeptides/receptors/channels they express, the site within the spinal cord where their central projections terminate and the neural growth factors they respond to in adulthood [5,6]. In the adult, peptide-rich C-fibers express neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), are regulated by nerve growth factor (NGF) and express tyrosine receptor kinase A (trkA), the cognate receptor for NGF [6–8]. These fibers are largely nociceptive and terminate more superficially within the dorsal horn. In contrast, the peptide-poor C-fibers contain the enzyme fluoride-resistant acid phosphatase and selectively bind the lectin *Griffonia simplicifolia* isolectin B4 (IB₄) [6,8,9]. Additionally these nerve fibers express the purinergic receptor known as P₂X₃ [10,11], and respond to glial cell line-derived neurotrophic factor (GDNF) as they express the GDNF receptor complex, which includes glial cell line-derived factor receptor (GFR- α) subunits and receptor tyrosine kinase c-Ret (c-RET) [8,9]. These peptide-poor nerve fibers terminate almost exclusively within the deeper parts of lamina II of the spinal dorsal horn [6,12].

Recently developed animal models are beginning to provide insights into the mechanisms that drive skeletal pain [13–15]. However, what remains unclear is whether the full repertoire of C-fibers that innervate the skin also innervate the bone, since skin is often used as a generic surrogate to investigate musculoskeletal pain. Previous reports employing retrograde tracer studies have suggested that peptide-rich CGRP⁺, and to a lesser extent, the peptide-poor IB₄⁺ nerve fibers innervate both cutaneous and deep tissues [16–19]. However, interpreting these results is problematic as endothelial cells [20] and keratinocytes [21,22] also bind IB₄, making it difficult to define whether IB₄⁺ nerve fibers are present and if so what specific structures and cell types these nerve fibers innervate. Recently, a family of sensory neuron-specific G protein-coupled receptors called Mas related G protein-coupled receptors (*Mrgprs*: *Mrgpra*, *Mrgprb*, *Mrgprc*, *Mrgprd*, *MrgX*) were cloned and found to be expressed in a subset of rodent and human DRG neurons [12,23–25]. Genetically modified animals were developed to express encoded axonal tracers from the *Mrgprd* locus and previous results showed that *Mrgprd*⁺ nerve fibers constitute 75% of the peptide-poor IB₄⁺ population of C-fibers in the epidermis of the mouse glabrous skin [12]. In the present study we use these mice to determine the specific populations of peptide-rich (SP⁺ and CGRP⁺) and peptide-poor (P₂X₃⁺ and *Mrgprd*⁺) nerve fibers which innervate the bone marrow, mineralized bone and periosteum in the mouse femur.

Material and Methods

Animals

Experiments were performed on 12 femurs obtained from 6 female *Mrgprd* Δ^{EGFPf} mice (a generous donation from Dr. David Anderson, California Institute of Technology, Pasadena, CA, USA) weighing 20–25g. In the knock-in *Mrgprd* Δ^{EGFPf} mice the *Mrgprd* locus has been deleted and replaced with a gene encoding for enhanced green fluorescent protein (EGFPf)

[12]. These transgenic mice were used since the *Mrgprd* receptor is exclusively expressed in peptide-poor nerve fibers innervating the skin [12]. In these mice, nearly all *Mrgprd*⁺ DRG neurons were labeled with the non-peptidergic nociceptive marker (IB₄) and expressed low to undetectable levels of the peptidergic nociceptive marker CGRP [12]. Homozygous female mice (−/−) were used in this study since the pattern of neurochemical expression in the DRG and glabrous skin is similar between homozygous and heterozygous mice [12]. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Immunohistochemistry on whole-mount preparations of the femoral periosteum

The *Mrgprd*Δ^{EGFPf} mice were sacrificed with CO₂ and perfused intracardially with 20 ml of 0.1 M phosphate buffered saline (PBS) followed by 20 ml of 4% formaldehyde/12.5% picric acid solution in 0.1M PBS. For whole-mount periosteal preparations, the left femurs of *Mrgprd*Δ^{EGFPf} mice were harvested, post-fixed for 4 hours in the perfusion fixative and then placed in PBS solution. In the present study, evaluation of the expression of different neurochemical markers in the periosteum was performed in whole-mount preparations as it is relatively easy to visualize the mesh-like networks formed by sensory nerve fibers compared to a cross sectional analysis of the periosteum attached to the bone. Periosteum from the left diaphyseal shaft was removed for a whole mount and processed for immunohistochemistry according to the following procedure adapted from previous studies [26]. Excess muscle was removed from the femur using surgical scissors without disturbing the bone and attached periosteum. Periosteum was harvested from the distal growth plate region to immediately below the third trochanter. Periosteum was removed from the bone by tracing the lower and upper limits of the desired area with a micro scalpel blade and a vertical cut was then performed along the posterior surface of the bone. Under a dissecting microscope, the periosteum was removed by gently scraping against the bone using the edge of forceps [27]. In our hands, the technique described above resulted in maximal preservation and minimal damage of both the cambium and fibrous layers of the periosteum. During periosteum removal, femurs were continually irrigated with PBS to prevent tissue dehydration. The size of the periosteal whole mount preparation and its attached thin muscle layer used for immunohistochemistry was approximately: width=6 mm; length=6 mm; thickness=0.5 mm.

The whole mount preparations of periosteum were then washed in 0.1M PBS three times for 10 minutes each, incubated for 60 minutes at room temperature (RT, 22°C) in a blocking solution of 3% normal donkey serum in PBS with 0.3% Triton-X 100 and then incubated overnight at RT with primary antibodies. Peptide-rich primary afferent sensory neurons were labeled with polyclonal rabbit anti-rat CGRP [28,29] (1:15,000; Sigma Chemical Co., St. Louis, MO, USA; Catalog Number: C8198) and polyclonal rabbit anti-rat SP [30,31] (1:1000; ImmunoStar, Hudson, WI, Catalog Number: 20064). Peptide-poor primary afferent neurons were labeled with polyclonal guinea pig anti-P₂X₃ [11,32] (1:10000; Neuromics, Edina, MN, USA; Catalog number GP10108). To visualize GFP⁺ neurons in *Mrgprd*Δ^{EGFPf} mice, a GFP antibody (rabbit anti-GFP; 1:1000; Invitrogen; Catalog number A11122 [12]) was used to amplify the constitutive GFP signal. Identification of peptide-poor nerve fibers in skin and periosteum using an antibody against IB₄ was not performed due to confounding IB₄ staining of keratinocytes in skin [21,22] and IB₄ staining of blood vessels in periosteum [26].

Preparations were then washed in PBS and incubated for three hours at RT with secondary antibodies conjugated to fluorescent markers (Cy3 1:600 and Cy2 1:200; Jackson ImmunoResearch, West Grove, PA, USA). Preparations were counterstained with DAPI (4', 6-diamidino-2-phenyl-indole, dihydrochloride, 1:30000, Molecular Probes, OR, USA) for 5 minutes. Finally, tissue was washed in PBS and dehydrated through an alcohol gradient (70, 80, 90 and 100%), cleared in xylene, mounted (with attached muscle layer in contact with the

slide) on gelatin-coated slides and coverslipped with di-n-butylphthalate-polystyrene-xylene (Sigma Chemical Co., St. Louis, MO, USA).

Immunohistochemistry on sectioned tissue: skin, DRGs, bone

The lumbar 4 (L4) DRG and glabrous skin of the hindpaw were removed following perfusion of mice as previously described [33]. Tissue was post-fixed for 4 hours in the perfusion fixative, and cryoprotected for 24 hours in 30% sucrose in 0.1M PBS, all at 4°C, and then processed for immunohistochemistry. Serial frozen DRG sections (15µm thick) and glabrous skin of the hindpaw sections (40µm thick) were cut on a cryostat and thaw-mounted on gelatin-coated slides for processing.

In order to evaluate the expression of peptide-poor nerve fibers in the other compartments of the mouse femur, a cross sectional analysis of the right femurs from *Mrgprd Δ^{EGFPf}* mice with the periosteum attached to the bone were processed as follows. Once right femurs were post-fixed for 4 hours, they were washed in PBS and decalcified in 10% EDTA at 4°C for no more than two weeks. After complete bone demineralization, determined radiographically, bones were cryoprotected in 30% sucrose at 4°C for at least 48 hours and serially sectioned on a cryostat along the longitudinal axis at a thickness of 40µm so that the anterior posterior plane was visualized.

Sectioned glabrous skin, L4 DRG, and bone were incubated overnight at RT in the same primary antisera used for whole mount preparations. Additionally, bone sections from *Mrgprd Δ^{EGFPf}* mice were incubated with a cocktail of rabbit anti-GFP (1:1000; Invitrogen; Carlsbad, CA, USA; Catalog number A11122) and chicken anti-neurofilament 200Kd (NF200) [34] (an antibody that labels myelinated primary afferent sensory nerve fibers 1:1000; Chemicon, Temecula, CA, USA; Catalog number AB5539). The NF200 antibody was used as positive control in the double immunofluorescence protocol to evaluate whether the decalcification protocol resulted in loss of antigenicity. Further immunohistochemical steps were performed as described above.

Laser scanning confocal microscopy

Laser scanning confocal microscopy of the whole mount preparations was performed with a BX-61 microscope equipped with the Fluoview 1000 imaging software 5.0 (Olympus America Inc, Melville, NY). Confocal z-series at 0.5µm (sectioned DRG) and 1.0µm (whole mount periosteal preparations, sectioned skin and sectioned bone) intervals were acquired for each observation area. For tissue processed with the double label immunofluorescence protocol, sequential acquisition mode was used to reduce bleed-through. Image threshold and channel pseudocolors were adjusted with Adobe Photoshop CS and thereafter assembled in Adobe Illustrator CS.

Semi-quantitative analysis of the sensory innervation in the bone

Briefly, sections were examined under an Olympus BX-51 epi-fluorescence microscope using a 40X objective, under which it was possible to distinguish individual axons. The metaphyseal region in the distal end of the femur (a 1.5 mm-long metaphyseal region starting 1 mm from the distal femoral growth plate) was selected for evaluation. It is worthy to note that for the *Mrgprd⁺* and *P2X3⁺* nerve fibers, the whole cross section area was examined and there were no detectable *Mrgprd⁺* and *P2X3⁺* nerve fibers in any aspect of the femur. Two 40X objective fields of the metaphyseal region per mouse section from 6 mice were used for evaluation. Density of nerve fibers in the same field of view was graded by two independent observers well-versed in immunofluorescence microscopy. We used a scale of – to ++++ for the semi-quantitative assessment of the sensory innervation. The – indicates non-detectable nerve fibers; + = 1–2 nerve fibers; ++ = 3–5 nerve fibers; +++ = 6–10 nerve fibers and ++++ > 10 nerve

fibers. When two observers obtained the same grade, it was taken as the final value. However, when observers obtained different readings (~20% of the readings), the sections were re-examined by the two observers and a consensus grade was taken as the final reading.

Results

Peptidergic neurochemical markers are expressed by primary afferent neurons innervating skin and femoral periosteum

To define the neurochemical phenotype of the sensory C-fibers that innervate skin and periosteum from naïve mice, we have used immunohistochemistry and confocal microscopy. We have utilized antibodies against CGRP and SP, all of which are neurochemical markers widely used to label peptide-rich sensory neurons [28,30,31,35,36]. As a positive control, we have evaluated the expression of these antibodies in lumbar DRG, which housed the cell bodies of the peripheral sensory neurons.

In the mouse L4 DRG, CGRP immunostaining was observed in the cell bodies and their axons. CGRP⁺ cell bodies were small to medium size DRG cell bodies (Fig 1A). Qualitative immunohistochemical analysis for SP in DRG revealed that this neuropeptide is expressed in fewer neurons as compared to CGRP and its expression is restricted to small to medium size primary afferent neurons (Fig 2A).

The periosteum is a thin, cellular and fibrous tissue that tightly adheres to the outer surface of all but the articulated surface of bone [37] and is thought to play a pivotal role in driving fracture pain [38–40]. Confocal photomicrographs of periosteal whole mount preparations show that CGRP⁺ (Fig 1B) and SP⁺ (Fig 2B) nerve fibers appear as single, thin fibers or bundles of nerve fibers. CGRP⁺ and SP⁺ nerve fibers in the periosteum form a mesh-like network that may be involved in detecting algogenic (pain-causing) substances as well as mechanical distortion of the underlying mineralized bone following fracture.

To examine the presence of peripheral projections of peptide-rich sensory neurons at a site further away than periosteum, we evaluated the expression of CGRP⁺ and SP⁺ nerve fibers in the hindpaw glabrous skin as this tissue is highly innervated by peptide-rich nerve fibers. In the glabrous skin, numerous thick bundles of CGRP⁺ nerve fibers, present in the dermal layers, divide and result in thinner nerve fascicles as they approach the dermoepidermal junction (Fig 1C). Single thin CGRP⁺ nerve fibers penetrate into the epidermis and some of these fibers terminate in superficial layers. In the glabrous skin, SP⁺ nerve fibers have a similar pattern of expression in the dermis and epidermis as compared to CGRP⁺ nerve fibers (Fig 2C). Semi-quantitative analysis indicated that there were fewer SP⁺ nerve fibers as compared to CGRP⁺ nerve fibers (Fig 2C, Table 1) in the glabrous skin.

Non-peptidergic neurochemical markers are expressed by primary afferent neurons innervating skin but not by primary afferent neurons innervating femoral periosteum

To test whether peptide-poor nerve fibers are present in periosteum, we have evaluated the expression of the P₂X₃ receptor (marker of the peptide-poor subpopulation of nociceptors [32,36]) in periosteum and skin using immunohistochemistry. Furthermore, we have evaluated the expression of the neuronal tracer GFP in periosteum and skin from *Mrgprd*^{EGFPf} mice.

In the L4 DRG of *Mrgprd*^{EGFPf} mice, *Mrgprd*⁺ neurons (small to medium size DRG neurons) and *Mrgprd*⁺ axons were found (Fig 1D). Small to medium size DRG cell bodies expressed the receptor P₂X₃⁺. Consistent with previous reports [12], double labeling of DRG from *Mrgprd*^{EGFPf} mice for GFP and P₂X₃ revealed that the majority of *Mrgprd*⁺ neurons were also labeled for P₂X₃ (Fig 3A–C) indicating GFP expression in *Mrgprd*^{EGFPf} mice is expressed primarily in peptide-poor DRG sensory neurons.

Immunohistochemical analysis in whole mount preparations revealed that peptide-rich CGRP⁺ and SP⁺ nerve fibers highly innervate the periosteum (Fig 1B, 2B). In contrast, virtually no P₂X₃⁺ and *Mrgprd*⁺ nerve fibers were found in these preparations (Fig 1E, Fig 2E, Table 1). It is worth noting that the immunohistochemical protocols used to label these P₂X₃⁺ and *Mrgprd*⁺ nerve fibers in whole mount preparations were also used to detect these peptide-poor nerve fibers in skin and DRG.

In the glabrous skin of the mouse hindpaw, numerous bundles of *Mrgprd*⁺ nerve fibers were found in the dermis (Fig 1F). These bundles of nerve fibers became single nerve fibers as they penetrated into the epidermis. As reported previously, *Mrgprd*⁺ nerve fibers and CGRP⁺ nerve fibers have different termination zones. While most *Mrgprd*⁺ axons appear in a more superficial layer of the epidermis (in the border of the stratum granulosum and corneum) [12], CGRP⁺ axons finish in the stratum spinosum [12,29] of the epidermis. In glabrous skin, P₂X₃⁺ nerve fibers have a similar pattern of distribution as *Mrgprd*⁺ nerve fibers (Fig 2F); however, P₂X₃⁺ nerve fibers were less abundant (Table 1).

CGRP⁺, SP⁺, NF200⁺ but neither *Mrgprd*⁺ nor P₂X₃⁺ nerve fibers are present in the mineralized bone and bone marrow of the mouse femur

Studies evaluating the existence and/or distribution of peptide-poor nerve fibers in bone have been hampered by the fact that IB₄ staining is observed in endothelial cells of blood vessels [20,26,41]. In this study, using genetically encoded axonal tracers to label *Mrgprd*⁺ neurons (the majority of IB₄⁺ peptide-poor neurons express *Mrgprd*), we evaluated the existence of peptide-rich and peptide-poor nerve fibers in different compartments of the bone using cross-sectional analysis. As a positive control, we performed double immunostaining for NF200 (marker for myelinated nerve fibers) and GFP using antibodies raised in different species.

Within the mouse femur of *Mrgprd*^{ΔEGFPf} mice, NF200⁺ nerve fibers were detected in the periosteum, mineralized bone and bone marrow (Fig 4A, Table 1). In order to illustrate sensory innervation in the three compartments of the bone, the distal nutrient foramen of the femur is presented (Fig 4). Bundles, or thin fascicles, of NF200⁺ nerve fibers in the periosteum run longitudinally along the longest axis of the bone (Fig 4A). Thinner fascicles of sensory nerve fibers penetrate into the mineralized bone and bone marrow through the nutrient foramen (Fig 4A). SP⁺ and CGRP⁺ nerve fibers have a similar pattern of distribution in the periosteum, bone marrow, and mineralized bone of the femur (Table 1). In contrast to NF200⁺, SP⁺ and CGRP⁺ nerve fibers, *Mrgprd*⁺ nerve fibers were not observed in any aspect of the bone marrow, mineralized bone or periosteum (Fig 4B, Table 1). Additionally, P₂X₃⁺ nerve fibers were not found in mineralized bone, bone marrow or periosteum of the femur (Table 1).

Discussion

In the present study we used transgenic mice in which the *Mrgprd*⁺ primary afferent nerve fibers express a genetically encoded axonal tracer, green fluorescent protein. In addition, we performed immunohistochemical localization of CGRP⁺, SP⁺, P₂X₃⁺ and NF200⁺ nerve fibers on the same tissue to define the populations of sensory C-fibers that innervate the glabrous skin versus periosteum, mineralized bone and marrow of the femur. In the glabrous surface of the mouse skin there is extensive innervation by *Mrgprd*⁺, P₂X₃⁺, CGRP⁺, SP⁺, and NF200⁺ nerve fibers. In contrast, while CGRP⁺, SP⁺, and NF200⁺ sensory nerve fibers richly innervate the marrow, mineralized bone and periosteum, few if any *Mrgprd*⁺ or P₂X₃⁺ nerve fibers could be detected in these same tissues. Previous studies using retrograde tracing combined with histochemical localization of IB₄ or CGRP have similarly suggested that while there is a significant innervation by CGRP⁺ nerve fibers, there are relatively few IB₄⁺ sensory nerves that innervate the rat hip joint, wrist and vertebrae [16,18,19,42,43]. Similarly, immunohistochemical examination suggests that while there is a significant innervation by

CGRP⁺ nerve fibers in the human intervertebral disc, there are very few IB₄⁺ sensory nerve fibers in this same tissue [17].

The present results provide further evidence that in examined skeletal tissues (hip, femur, wrist and vertebral body) [16–18,42,43] the repertoire of C-fibers that innervate the skeleton is quite different from the C-fibers that innervate the glabrous skin. Thus, whereas the glabrous skin is richly innervated by peptide-poor IB₄⁺/*Mrgprd*⁺ nerve fibers [6,12], this population of sensory nerve fibers appears to be largely absent in bone (Fig 5). While this difference in innervation is interesting from both a developmental and neuroanatomical perspective, it may have significant functional and clinical relevance in developing a mechanism-based understanding of which populations of sensory nerve fibers generate and maintain skeletal pain. For example, it has recently been shown that in the mouse glabrous skin, genetic ablation of *Mrgprd*⁺ nerve fibers results in a reduction of the response to noxious mechanical stimuli without affecting the response to noxious heat or cold stimuli [44]. These results suggest that *Mrgprd*⁺ nerve fibers play a significant role in transmitting painful mechanosensation from the skin. However, as demonstrated and discussed here, bone appears to lack a significant innervation by *Mrgprd*⁺ fibers. We recently reported that capsaicin-induced depletion of CGRP⁺ sensory nerve fibers results in a 50% reduction in fracture-induced pain behaviors [45]. Furthermore, many common painful skeletal conditions such as fracture, osteoarthritis and bone cancer are partially driven and exacerbated by mechanical stimuli [46–48]. In light of these observations, we may suggest that peptidergic CGRP⁺ and the myelinated NF200⁺, but not the *Mrgprd*⁺, sensory nerve fibers appear to play a significant role in transmitting painful mechanosensation from the skeleton.

The fact that bone lacks a significant innervation by *Mrgprd*⁺ nerve fibers, and that the peptidergic C-fibers and Aβ/Aδ fibers are the major fiber types signaling pain from the bone, may present a unique therapeutic opportunity for developing novel pharmacological therapies for treating skeletal pain. Thus, in light of the absence of *Mrgprd*⁺ nerves in bone and the corresponding lack of “nociceptor redundancy” in bone, one might predict that therapies that specifically target noxious mechanical stimuli conveyed by CGRP⁺ peptidergic C-fibers and/or NF200⁺ Aβ/Aδ fibers will be more efficacious at reducing skeletal versus skin pain. Indeed, recent data suggests that this is true. For example, most CGRP⁺ sensory nerve fibers, but not *Mrgprd*⁺ sensory nerve fibers, express the TrkA receptor and are excited and/or sensitized by NGF [7,49–52]. Recent studies show that therapies that sequester NGF or prevent the activation of the TrkA receptor result in a 50% reduction in mouse bone fracture pain [13,15] as well as human osteoarthritis-related pain [53]. In contrast, the same therapy does not have a significant analgesic effect in blocking mechanical hypersensitivity due to injury of the skin [54]. A major reason why anti-NGF therapy may be more effective in relieving mechanosensitive pain in the skeleton versus the skin is that the skeleton lacks the nociceptor redundancy provided by the *Mrgprd*⁺/P₂X₃⁺ population of nerve fibers that is present in the skin. These data suggest that models of skin pain are not adequate surrogates for assessing therapies to treat skeletal pain. Furthermore, the understanding of the specific populations of sensory nerve fibers that innervate the skeleton, and how these nerve fibers change following skeletal injury, disease and aging will advance the development of novel therapies that may be uniquely effective in attenuating skeletal pain.

The present study has several potential limitations. First, while the present study has focused on the mouse femur, additional studies will be needed to determine whether the same restricted repertoire of sensory neurons innervates other long and flat bones in both rodents and humans. Second, a possible reason why we did not observe the *Mrgprd*⁺ nerve fibers in the femur of the transgenic animals was that axonal transport of the receptors or the genetically encoded tracer from the DRG to bone tissue, may be adversely impacted in the genetically modified mice. However, the skin of the hindpaw, which did show evidence of a rich innervation by

Mrgprd⁺, P₂X₃⁺ nerve fibers, is an even further distance from the DRG cell bodies than the femur, thereby suggesting that lengthy axonal transport alone is not a significant issue in the present study. Third, decalcification of the bone using EDTA may decrease the antigenicity of P₂X₃ receptors and the genetically encoded tracer GFP. However, in the present study we also used whole mount preparations of the periosteum which did not require decalcification and were processed by immunohistochemistry in an identical manner to skin. In these non-decalcified preparations, nerve fibers expressing P₂X₃ receptors and/or the genetically encoded tracer GFP also were not observed. Last, this study was performed under physiological conditions in normal, healthy bones of young adult mice. Whether the populations and phenotype of sensory nerve fibers that innervate the bone changes with age, injury or disease, remains an important but largely unanswered question.

Conclusion

The present data suggests that bone receives a significant innervation by the peptide-rich population of C-fibers but lacks a significant innervation by the peptide-poor population of C-fibers that bind the lectin IB₄ and express P₂X₃ and *Mrgprd*. This lack of C-fiber “redundancy” in bone versus skin suggests that fewer classes of C-fibers will need to be targeted to attenuate bone versus skin pain. In addition, this lack of “nociceptor redundancy” in the sensory innervation of the bone presents a unique therapeutic opportunity as unlike the skin there are no *Mrgprd*⁺ nerve fibers. As a result, therapies targeting the peptide-rich C-fiber and/or the NF200⁺ population of Aδ/Aβ sensory fibers, which together comprise the great majority of sensory nerve fibers that innervate the bone, may be uniquely effective in alleviating skeletal versus skin pain.

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References

- Brooks PM. The burden of musculoskeletal disease-a global perspective. *Clin Rheumatol* 2006;25:778–781. [PubMed: 16609823]
- Morrison RS, Magaziner J, McLaughlin MA, Orosz G, Silberzweig SB, Koval KJ, Siu AL. The impact of post-operative pain on outcomes following hip fracture. *Pain* 2003;103:303–311. [PubMed: 12791436]
- Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. *Bull World Health Organ* 2003;81:646–656. [PubMed: 14710506]
- Kidd BL. Osteoarthritis and joint pain. *Pain* 2006;123:6–9. [PubMed: 16714085]
- Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature* 2001;413:203–210. [PubMed: 11557989]
- Hunt SP, Mantyh PW. The molecular dynamics of pain control. *Nat Rev Neurosci* 2001;2:83–91. [PubMed: 11252998]
- Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci* 1995;7:1484–1494. [PubMed: 7551174]
- Priestley JV, Michael GJ, Averill S, Liu M, Willmott N. Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor. *Can J Physiol Pharmacol* 2002;80:495–505. [PubMed: 12056559]
- Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 1997;19:849–861. [PubMed: 9354331]

10. Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R. Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology* 1997;36:1229–1242. [PubMed: 9364478]
11. Bradbury EJ, Burnstock G, McMahon SB. The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* 1998;12:256–268. [PubMed: 9828090]
12. Zylka MJ, Rice FL, Anderson DJ. Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* 2005;45:17–25. [PubMed: 15629699]
13. Jimenez-Andrade JM, Martin CD, Koewler NJ, Freeman KT, Sullivan LJ, Halvorson KG, Barthold CM, Peters CM, Buus RJ, Ghilardi JR, Lewis JL, Kuskowski MA, Mantyh PW. Nerve growth factor sequestering therapy attenuates non-malignant skeletal pain following fracture. *Pain* 2007;133:183–196. [PubMed: 17693023]
14. Freeman KT, Koewler NJ, Jimenez-Andrade JM, Buus RJ, Herrera MB, Martin CD, Ghilardi JR, Kuskowski MA, Mantyh PW. A fracture pain model in the rat: adaptation of a closed femur fracture model to study skeletal pain. *Anesthesiology* 2008;108:473–483. [PubMed: 18292685]
15. Koewler NJ, Freeman KT, Buus RJ, Herrera MB, Jimenez-Andrade JM, Ghilardi JR, Peters CM, Sullivan LJ, Kuskowski MA, Lewis JL, Mantyh PW. Effects of a Monoclonal Antibody Raised Against Nerve Growth Factor on Skeletal Pain and Bone Healing Following Fracture of the C57BL/6J Mouse Femur. *J Bone Miner Res* 2007;22:1732–1742. [PubMed: 17638576]
16. Ozawa T, Aoki Y, Ohtori S, Takahashi K, Chiba T, Ino H, Moriya H. The dorsal portion of the lumbar intervertebral disc is innervated primarily by small peptide-containing dorsal root ganglion neurons in rats. *Neurosci Lett* 2003;344:65–67. [PubMed: 12781923]
17. Ozawa T, Ohtori S, Inoue G, Aoki Y, Moriya H, Takahashi K. The degenerated lumbar intervertebral disc is innervated primarily by peptide-containing sensory nerve fibers in humans. *Spine* 2006;31:2418–2422. [PubMed: 17023849]
18. Ohtori S, Inoue G, Koshi T, Ito T, Yamashita M, Yamauchi K, Suzuki M, Doya H, Moriya H, Takahashi Y, Takahashi K. Characteristics of sensory dorsal root ganglia neurons innervating the lumbar vertebral body in rats. *J Pain* 2007;8:483–488. [PubMed: 17382597]
19. Aoki Y, Ohtori S, Takahashi K, Ino H, Douya H, Ozawa T, Saito T, Moriya H. Expression and co-expression of VR1, CGRP, and IB4-binding glycoprotein in dorsal root ganglion neurons in rats: differences between the disc afferents and the cutaneous afferents. *Spine* 2005;30:1496–1500. [PubMed: 15990662]
20. Laitinen L. Griffonia simplicifolia lectins bind specifically to endothelial cells and some epithelial cells in mouse tissues. *Histochem J* 1987;19:225–234. [PubMed: 3597137]
21. Brabec RK, Peters BP, Bernstein IA, Gray RH, Goldstein IJ. Differential lectin binding to cellular membranes in the epidermis of the newborn rat. *Proc Natl Acad Sci U S A* 1980;77:477–479. [PubMed: 6928640]
22. Rice FL. Structure, vascularization, and innervation of the mystacial pad of the rat as revealed by the lectin Griffonia simplicifolia. *J Comp Neurol* 1993;337:386–399. [PubMed: 8282849]
23. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* 2001;106:619–632. [PubMed: 11551509]
24. Lembo PM, Grazzini E, Groblewski T, O'Donnell D, Roy MO, Zhang J, Hoffert C, Cao J, Schmidt R, Pelletier M, Labarre M, Gosselin M, Fortin Y, Banville D, Shen SH, Strom P, Payza K, Dray A, Walker P, Ahmad S. Proenkephalin A gene products activate a new family of sensory neuron-specific GPCRs. *Nat Neurosci* 2002;5:201–209. [PubMed: 11850634]
25. Grazzini E, Puma C, Roy MO, Yu XH, O'Donnell D, Schmidt R, Dautrey S, Ducharme J, Perkins M, Panetta R, Laird JM, Ahmad S, Lembo PM. Sensory neuron-specific receptor activation elicits central and peripheral nociceptive effects in rats. *Proc Natl Acad Sci U S A* 2004;101:7175–7180. [PubMed: 15118101]
26. Mach DB, Rogers SD, Sabino MC, Luger NM, Schwei MJ, Pomonis JD, Keyser CP, Clohisy DR, Adams DJ, O'Leary P, Mantyh PW. Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. *Neuroscience* 2002;113:155–166. [PubMed: 12123694]
27. Brownlow HC, Reed A, Joyner C, Simpson AH. Anatomical effects of periosteal elevation. *J Orthop Res* 2000;18:500–502. [PubMed: 10937640]

28. McCarthy PW, Lawson SN. Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. *Neuroscience* 1990;34:623–632. [PubMed: 2352644]
29. Kruger L, Silverman JD, Mantyh PW, Sternini C, Brecha NC. Peripheral patterns of calcitonin-gene-related peptide general somatic sensory innervation: cutaneous and deep terminations. *J Comp Neurol* 1989;280:291–302. [PubMed: 2784448]
30. Hokfelt T, Kellerth JO, Nilsson G, Pernow B. Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. *Brain Res* 1975;100:235–252. [PubMed: 1104079]
31. McCarthy PW, Lawson SN. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* 1989;28:745–753. [PubMed: 2469036]
32. Vulchanova L, Riedl MS, Shuster SJ, Stone LS, Hargreaves KM, Buell G, Surprenant A, North RA, Elde R. P2X3 is expressed by DRG neurons that terminate in inner lamina II. *Eur J Neurosci* 1998;10:3470–3478. [PubMed: 9824460]
33. Peters CM, Ghilardi JR, Keyser CP, Kubota K, Lindsay TH, Luger NM, Mach DB, Schwei MJ, Sevcik MA, Mantyh PW. Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. *Exp Neurol* 2005;193:85–100. [PubMed: 15817267]
34. Lawson SN, Waddell PJ. Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. *J Physiol* 1991;435:41–63. [PubMed: 1770443]
35. Chen Y, Willcockson HH, Valtschanoff JG. Increased expression of CGRP in sensory afferents of arthritic mice - effect of genetic deletion of the vanilloid receptor TRPV1. *Neuropeptides* 2008;42:551–556. [PubMed: 18789524]
36. Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM. Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. *J Neurosci* 2002;22:4057–4065. [PubMed: 12019325]
37. Allen MR, Hock JM, Burr DB. Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 2004;35:1003–1012. [PubMed: 15542024]
38. Martin CD, Jimenez-Andrade JM, Ghilardi JR, Mantyh PW. Organization of a unique net-like meshwork of CGRP+ sensory fibers in the mouse periosteum: implications for the generation and maintenance of bone fracture pain. *Neurosci Lett* 2007;427:148–152. [PubMed: 17950532]
39. Yates, D.; Smith, M. Orthopaedic pain after trauma. In: Wall, PD.; Melzack, R., editors. *Textbook of pain*. 3rd ed.. Edinburgh ; New York: Churchill Livingstone; 1994. p. 409-421.
40. Greenfield, GQ. Orthopaedic Pain. In: Boswell, MV.; Cole, BE., editors. *Weiner's pain management : a practical guide for clinicians*. 7th ed.. Boca Raton: CRC Press; 2006. p. 465-476.
41. Benton RL, Maddie MA, Minnillo DR, Hagg T, Whittemore SR. Griffonia simplicifolia isolectin B4 identifies a specific subpopulation of angiogenic blood vessels following contusive spinal cord injury in the adult mouse. *J Comp Neurol* 2008;507:1031–1052. [PubMed: 18092342]
42. Kuniyoshi K, Ohtori S, Ochiai N, Murata R, Matsudo T, Yamada T, Ochiai SS, Moriya H, Takahashi K. Characteristics of sensory DRG neurons innervating the wrist joint in rats. *Eur J Pain* 2007;11:323–328. [PubMed: 16807014]
43. Nakajima T, Ohtori S, Yamamoto S, Takahashi K, Harada Y. Differences in innervation and innervated neurons between hip and inguinal skin. *Clin Orthop Relat Res* 2008;466:2527–2532. [PubMed: 18704614]
44. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc Natl Acad Sci U S A* 2009;106:9075–9080. [PubMed: 19451647]
45. Jimenez-Andrade JM, Bloom AP, Mantyh WG, Koewler NJ, Freeman KT, Delong D, Ghilardi JR, Kuskowski MA, Mantyh PW. Capsaicin-sensitive sensory nerve fibers contribute to the generation and maintenance of skeletal fracture pain. *Neuroscience* 2009;162:1244–1254. [PubMed: 19486928]
46. Santy J, Mackintosh C. A phenomenological study of pain following fractured shaft of femur. *J Clin Nurs* 2001;10:521–527. [PubMed: 11822500]
47. Zeppetella G. Impact and management of breakthrough pain in cancer. *Curr Opin Support Palliat Care* 2009;3:1–6. [PubMed: 19365156]

48. Lafeber FP, Intema F, Van Roermund PM, Marijnissen AC. Unloading joints to treat osteoarthritis, including joint distraction. *Curr Opin Rheumatol* 2006;18:519–525. [PubMed: 16896294]
49. Molliver DC, Radeke MJ, Feinstein SC, Snider WD. Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *J Comp Neurol* 1995;361:404–416. [PubMed: 8550888]
50. McMahon SB, Armanini MP, Ling LH, Phillips HS. Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* 1994;12:1161–1171. [PubMed: 7514427]
51. Bennett DL, Dmietrieva N, Priestley JV, Clary D, McMahon SB. TrkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neurosci Lett* 1996;206:33–36. [PubMed: 8848275]
52. Shu X, Mendell LM. Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. *J Neurophysiol* 2001;86:2931–2938. [PubMed: 11731549]
53. Schnitzer, TJ.; Lane, NE.; Smith, MD.; Brown, MT. 12th World Congress of Pain. Scotland, UK: Glasgow; 2008. Efficacy and Safety of Tanezumab (PF04383119), an Anti-Nerve Growth Factor (NGF) Antibody, for Moderate to Severe Pain Due to Osteoarthritis (OA) of the Knee: A Randomized Trial; p. PT 214
54. Zahn PK, Subieta A, Park SS, Brennan TJ. Effect of blockade of nerve growth factor and tumor necrosis factor on pain behaviors after plantar incision. *J Pain* 2004;5:157–163. [PubMed: 15106128]
55. McMahon, SB.; Bennett, DLH.; Bevan, S. Inflammatory mediators and modulators of pain. In: McMahon, SB.; Koltzenburg, M., editors. *Wall and Melzack's textbook of pain*. 5th ed.. Philadelphia: Elsevier/Churchill Livingstone; 2006. p. 49-72.
56. Lewin GR, Moshourab R. Mechanosensation and pain. *J Neurobiol* 2004;61:30–44. [PubMed: 15362151]

Figure 1.

Whereas CGRP⁺ and *Mrgprd*⁺ cell bodies and nerve fibers are abundant in the DRG and glabrous skin, only CGRP⁺ nerve fibers, but not *Mrgprd*⁺ nerve fibers, are detectable in the periosteum. Confocal images showing calcitonin gene-related peptide (CGRP) immunofluorescence (A, B, C) and amplified green fluorescence protein (GFP) driven off a *Mrgprd* promoter (D, E, F) in neurons and nerve fibers in sectioned L4 dorsal root ganglia (DRG, A, D), in whole mount preparations of the femoral periosteum (B, E) and in sections of the glabrous skin of the hindpaw (C, F) in 2 month-old female *Mrgprd*^{ΔEGFPf} mice. Note that CGRP and *Mrgprd* immunoreactivity is abundant in both the L4 DRG and glabrous skin, while only CGRP⁺ nerve fibers are present in the periosteum of the femur. This data suggests that while periosteum is richly innervated by the CGRP⁺ peptide-rich population of sensory nerve fibers, *Mrgprd*⁺ nerve fibers representing the peptide-poor population of C-fibers, are virtually absent in the periosteum. The confocal images in A–F were acquired at 0.5 μm (A, D) and 1 μm (B, C, E, F) z-plane intervals and the total z-plane was 14 μm (A, D), 120 μm (B, E) and 40 μm (C, F). Scale bar= 50 μm.

Figure 2.

In the mouse femoral periosteum, substance P positive (SP⁺) nerve fibers, but not nerve fibers expressing the purinergic receptor (P₂X₃⁺) are readily detectable. Confocal images showing the expression of SP, which is a marker of peptide-rich sensory nerve fibers (A–C), and P₂X₃, which is a marker of peptide-poor nerve fibers (D–F). The images shown here were obtained from histological sections of the mouse L4 dorsal root ganglia (L4 DRG, A and D), whole mount preparations of the femoral periosteum (B and E) and sectioned glabrous skin of the hindpaw (C and F). Note that both SP and P₂X₃ are expressed in neuronal DRG cell bodies and their peripheral nerve terminals innervating the glabrous skin (C&F). In contrast, in the whole mount preparations of the periosteum, only SP⁺ nerve fibers are detectable whereas P₂X₃⁺ nerve fibers are virtually absent. The confocal images in A–F were acquired at 0.5 μ m (A, D) and 1 μ m (B,C,E,F) z-plane intervals and the total z-plane was 14 μ m (A, D), 120 μ m (B, E) and 40 μ m (C, F). Scale bar= 50 μ m.

Figure 3.

Most sensory neurons that express GFP under the *Mrgprd* promoter (*Mrgprd*⁺) also express the purinergic P₂X₃ receptor. Whereas GFP expression is driven by the *Mrgprd* promoter, the purinergic receptor was colocalized in these sections with an antibody that recognizes the P₂X₃ receptor. Confocal images were taken of L4 DRG sections of *Mrgprd*^{ΔEGFPf} mice stained for GFP (green in A), P₂X₃ (red in B) and cells that co-express GFP and the P₂X₃ receptor (yellow in C, arrowheads). Note that the great majority of *Mrgprd*⁺ neurons also express P₂X₃, suggesting the *Mrgprd*⁺ neurons from these transgenic mice constitute a significant percentage of the peptide-poor class of nociceptors. The confocal images in A–C were acquired at 0.5 μm z-plane intervals and the total z-plane was 14 μm.

Figure 4.

Confocal images of the mouse bone showing that whereas the periosteum, mineralized bone and bone marrow are all innervated by myelinated sensory nerve fibers (labeling with NF200), there is a relative absence of peptide-poor C-fibers that constitutively express green fluorescent protein under the *Mrgprd* promoter (B). This is a confocal image of a femoral section (40 μm-thick) from a *Mrgprd*^{ΔEGFPf} mouse that has been stained with antibodies against neurofilament 200Kd (NF200, A) and green fluorescent protein (GFP, B) to amplify the constitutive GFP signal. Background images were taken using the DAPI channel in order to provide orientation in the different compartments of the bone. In (A) the nerve fibers in the periosteum are traversing the mineralized bone in the distal nutrient foramen. While NF200⁺ fibers are present in periosteum, mineralized bone and bone marrow, there is virtually no *Mrgprd*⁺ nerve fibers observable in any of these different bone compartments in the same section. The confocal images in A–B were acquired at 1.0 μm z-plane intervals and the total z-plane was 40 μm.

Figure 5.

Pie charts summarizing the populations of primary afferent sensory nerve fibers that appear to innervate the glabrous skin versus the periosteum, marrow and mineralized bone. Note that skin is innervated by both thickly (Aα/β) and thinly (Aδ) myelinated nerve fibers that express the 200Kd neurofilament (NF200) as well as unmyelinated C-fibers that are peptide-rich and peptide-poor (nerve fibers that bind isolectin B4 (IB₄) and express *Mas-related G protein-coupled receptor member d* (*Mrgprd*)). In contrast, the periosteum, mineralized bone and marrow appear to be innervated by myelinated A-fibers and peptide-rich sensory nerve fibers that express calcitonin gene related peptide (CGRP) while lacking a significant innervation by the peptide poor IB₄⁺/P₂X₃⁺/*Mrgprd* C-fibers. It is worth noting that there are some nerve fibers that co-express CGRP/NF200 and some that co-express CGRP/IB₄ as depicted by the CGRP overlaid on NF200 or IB₄ portions of the pie charts. Pie chart showing sensory innervation in skin is adapted from previous studies [55,56].

Table 1

A summary of the type and density of sensory nerve fibers in the mouse femur compartments and glabrous skin of the mouse.

	CGRP	NF200	SP	Mrgprd	P ₂ X ₃
Periosteum (femur)	++	+++	+	-	-
Bone marrow (femur)	+	++	+	-	-
Mineralized bone (femur)	+	+	+	-	-
Glabrous skin of the hindpaw	++++	++++	+++	++++	+++

- = not detectable nerve fibers; + = 1-2 nerve fibers; ++ = 3-5 nerve fibers; +++ = 6-10 nerve fibers; ++++ > 10 nerve fibers. Calcitonin gene related peptide, CGRP; neurofilament 200Kd, NF200; substance P, SP; *Mas-related G protein-coupled receptor member 4*, *Mrgprd*.