Published in final edited form as:

Neurosci Lett. 2007 November 12; 427(3): 148–152. doi:10.1016/j.neulet.2007.08.055.

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

Carl D. Martin¹, Juan Miguel Jimenez-Andrade¹, Joseph R. Ghilardi², and Patrick W. Mantyh^{1,2}

¹Neurosystems Center and Department of Diagnostic and Biological Sciences, University of Minnesota, Minneapolis, MN 55455. USA

²Research Service, VA Medical Center, Minneapolis, MN 55417, USA

Abstract

Although bone fracture frequently results in significant pain and can lead to increased morbidity and mortality, it is still not clearly understood how sensory neurons are organized to detect fracture pain. In the present report we focused on the periosteum, as this thin tissue is highly innervated and tightly adherent to the outer surface of bone. To define the organization and distribution of the sensory and sympathetic fibers in the mouse femoral periosteum, we used whole mount preparations, transverse sections, immunofluorescence, and laser scanning confocal microscopy. While both the outer fibrous layer and the inner more cellular cambium layer of the periosteum receive an extensive innervation by calcitonin gene related peptide (CGRP) and 200-kDa neurofilament (NF200) positive sensory fibers as well as tyrosine hydroxylase (TH) positive sympathetic fibers, there is a differential organization of sensory vs. sympathetic fibers within the periosteum. In both layers, the great majority of TH+ fibers are closely associated with CD31+ blood vessels and wind around the larger vessels in a corkscrew pattern. In contrast, the majority of CGRP+ and NF200+ sensory fibers in both layers lack a clear association with CD31+ blood vessels and appear to be organized in a dense net-like meshwork to detect mechanical distortion of periosteum and bone. This organization would explain why stabilization/fixation causes a marked attenuation of movement-evoked fracture pain. Understanding the organization, plasticity and molecular characteristics of sensory and sympathetic nerve fibers innervating the skeleton may permit the development of novel mechanism-based therapies for treating non-malignant skeletal pain.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Corresponding Author: Patrick W. Mantyh, Neurosystems Center, 18-208 Moos Tower, University of Minnesota, 515 Delaware Street SE, Minneapolis, MN 55455, Phone: (612) 626-0180, Fax: (612) 626-2565, manty001@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

non-malignant skeletal pain; blast injury; poly-trauma; mechanotransducers

Skeletal pain is the most common reason individuals seek medical attention. In frequent conditions such as osteoarthritis, osteoporosis, rheumatoid arthritis, and skeletal fracture, pain not only causes the patient significant suffering but chronic skeletal pain can dramatically impact mobility, effective rehabilitation and ultimately the lifespan of the individual [2, 10, 15].

A key reason skeletal pain is so difficult to fully control is that in large part there is a lack of understanding of the organization and function of sensory fibers in the normal bone, how these sensory fibers are activated, and how their phenotype is altered in skeletal disease. Recent studies have shown that mineralized bone, marrow and periosteum (the thin fibrous and cellular sheath that covers the outer surface of the mineralized bone) are all innervated by primary afferent sensory neurons and post-ganglionic sympathetic neurons [7, 11]. In previous studies we focused on innervation of the marrow and mineralized bone as we were interested in defining the mechanisms that drive bone cancer pain [11]. In the current study we focus on the sensory and sympathetic innervation of the periosteum, as this thin fibrous sheath may be pivotally involved in the generation and maintenance of fracture pain [3, 19]. Using several histochemical and imaging techniques, we show that the sensory fibers innervating the periosteum are organized in a unique net-like meshwork and suggest that these sensory fibers may be distinctively organized to detect mechanical distortion of the periosteum and underlying mineralized bone.

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and were in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. Naïve male C57/BL/6J mice (weighing 18–25 g, n=20, Jackson Laboratories, Bar Harbor, ME) were euthanized with CO₂ and tissue was processed for immunohistochemistry as previously described [11]. For bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) experiments, anesthetized male C57/BL/6J mice (n=6) were implanted subcutaneously with one osmotic pump (model # 1003D, Alzet, Cupertino, CA) containing 100 µl of 200 mg/ml BrdU solution. Mice were sacrificed 24 hours post-pump implantation and processed for immunohistochemistry.

Periosteum from the femoral shaft was removed as a whole mount and processed as free floating preparation as previously described [11]. Small- to medium-diameter peptidergic primary afferent sensory nerve fibers were immunostained for the neurotransmitter calcitonin gene-related peptide (CGRP) (polyclonal rabbit anti-rat CGRP, 1:15,000, Sigma-Aldrich, St. Louis, MO). Myelinated primary afferent sensory nerve fibers were immunostained for 200 kD neurofilament H (NF200) (polyclonal chicken anti-mouse NF200, 1:2,000, Chemicon, Temecula, CA). Noradrenergic postganglionic sympathetic nerve fibers were immunostained for the enzyme tyrosine hydroxylase (TH) (polyclonal rabbit anti-rat TH, 1:2,000, Chemicon, Temecula, CA). Blood vessels were immunostained for the platelet endothelial cell adhesion molecule CD31 (monoclonal rat anti-mouse CD31, 1:500, BD Pharmingen, San Diego, CA). For double labeled immunohistochemistry, tissue

was incubated with CGRP/CD31, NF200/CD31 or TH/CD31 overnight at room temperature (RT).

Sections were then washed in phosphate buffer saline (PBS) and incubated for three hours at RT with secondary antibodies conjugated to fluorescent markers (Cy2 1:200, Cy3 1:600; Jackson ImmunoResearch, West Grove, PA). Sections were washed in PBS and counterstained with DAPI (4', 6-diamidino-2-phenyl-indole, dihydrochloride, 1:40,000, Molecular Probes, Carlsbad, CA) for 5 minutes. Finally tissue was washed in PBS and dehydrated through an alcohol gradient (70, 90 and 100%), cleared in xylene, mounted (attached muscle layer in contact with the slide) on gelatin-coated slides and coverslipped with DPX. To confirm the specificity of the primary antibodies, controls included preabsorption with the corresponding synthetic peptide and omission of the primary antibody.

For histology and immunohistochemistry in periosteal cross sections, mouse femora were decalcified in 10% EDTA at 4°C for no more than two weeks. After complete bone demineralization, bones were embedded in paraffin. Femoral sections, 5µm-thick (for hematoxylin and eosin staining, H&E) and 40µm-thick (for BrdU immunostaining) were cut on the longitudinal axis. These sections were pretreated with HCl at 37°C for 30 minutes and washed with borate buffer. Then, sections were incubated with a polyclonal sheep anti-BrdU antibody (1:500, US Biological, Swampscott, MA). Further immunohistochemical processing was performed as described above.

Images of periosteal whole-mount preparations were captured using a BX62 microscope equipped with the Fluoview FV1000 confocal imaging software 5.0 (Olympus America Inc, Melville, NY). Density of DAPI+ cells was used as a reference to acquire confocal images of the nerve fibers and blood vessels in each periosteal layer. The first confocal z-series (0–30µm) was considered the cambium layer as a high density of DAPI+ cells was observed. The second confocal z-series (30–60µm) was considered to be the fibrous layer as determined by the lower density of DAPI+ cells and presence of fibroblast-like nuclear morphology. Then, digital confocal grayscale images (200x magnification) for each scan were compiled from 120 optical sections spaced 0.25µm apart in the z-plane. Light and differential interference contrast (DIC) photomicrographs were acquired using an Olympus BX51 microscope fitted with an Olympus DP70 digital CCD camera.

Quantification of CGRP+, NF200+ and TH+ fibers in periosteal whole mounts from mice was performed as previously described [8]. Briefly, digital confocal images for each periosteal layer (400x magnification; one random section per mouse) were acquired as described above. Images were viewed on a high-resolution monitor and the number of intersections between nerve fibers and the vertical grids (7.35µm spacing, Adobe Photoshop software v. 7.0) was quantified. Results were expressed as number of intersections per mm² (n=10).

The association of the CGRP+ and TH+ nerve fibers with the CD31+ blood vessels was quantified using a method adapted from Ruocco et al.[16]. Briefly, any nerve fiber (longer than 100 µm) within 15µm of a CD31+ blood vessel for a length of at least 50µm was

considered to be in close association with blood vessels. Four random $250\text{ }\mu\text{m} \times 250\text{ }\mu\text{m}$ areas of periosteum per mouse ($n=10$) were used to make this quantitative analysis.

Structure of the periosteum

Three methods were used to determine the layers of the periosteum in this study. H&E histological staining and DIC light microscopy of unstained tissue illustrate the different morphologies of the two layers (Fig. 1B–C). DAPI nucleic counterstaining also clearly shows the existence of two periosteal layers (Fig. 1D). The first $30\text{ }\mu\text{m}$ (z-series 0– $30\text{ }\mu\text{m}$) was found to be highly cellular and was considered to be the cambium layer. The cambium was composed of a layer of bone lining cells, likely osteoblasts and osteoclasts, and a proliferative layer (Fig. 1B,D). The proliferative layer was determined by the presence of BrdU+ nuclei. The BrdU+ nuclei formed a layer several cells thick spread evenly across the periosteum (Fig. 1D) and was found to be contained in the external half of the cambium (z-series 15– $30\text{ }\mu\text{m}$). The second $30\text{ }\mu\text{m}$ (z-series 30– $60\text{ }\mu\text{m}$) was found to be composed of loose fibrous tissue and relatively few spindle-shaped fibroblast-like cells and was considered to be the fibrous layer (Fig. 1B). The nuclei of the fibrous layer exhibited the morphology of fibroblastic cells and no BrdU+ immunoreactivity was observed (1D).

Fibrous Layer

CD31+ blood vessels ($2\text{--}50\text{ }\mu\text{m}$ in diameter) densely vascularized the fibrous layer (2208 ± 747 intersections/ mm^2) and were present in a dense and branched network (Fig. 2 D–F). TH+ sympathetic fibers which innervated the fibrous layer were closely associated with CD31+ blood vessels, $80 \pm 3\%$, forming tightly woven corkscrew-like baskets around the blood vessels (Fig. 2 D, Table 1B). Although TH+ fibers are dense within the fibrous layer, 3138 ± 157 intersections/ mm^2 , the distribution of the fibers was mainly to the areas surrounding CD31+ blood vessels (Fig. 2 A, D).

CGRP+ and NF200+ sensory nerve fibers exhibited a very different morphology as compared to TH+ fibers. The sensory populations studied within the fibrous layer were laid out in a meshwork of bundled fibers covering the entire periosteum (Fig. 2 B–C). CGRP+ and NF200+ nerve fibers had densities of 2664 ± 127 intersections/ mm^2 and 2679 ± 233 intersections/ mm^2 , respectively. In addition having a more widespread innervation than TH+ fibers, the CGRP+ and NF200+ fibers showed little association with CD31+ blood vessels, $26 \pm 6\%$ and $20 \pm 8\%$ respectively (Fig. 2 E–F, Table 1B). Within the fibrous layer, CGRP+ and NF200+ nerve fibers had similar distribution and density, but showed little co-localization (10%).

Cambium Layer

The CD31+ vascularization of the cambium layer was also dense, 1486 ± 529 intersections/ mm^2 (Fig. 3 D–F). TH+ sympathetic fibers sparsely innervated the cambium, 907 ± 31 intersections/ mm^2 (Fig. 3 A), and while the complex corkscrew morphology observed in the fibrous layer was absent, the fibers present still closely associated with CD31+ blood vessels, $77 \pm 3\%$ (Fig. 3 D, Table 1B).

As seen in the fibrous layer, the sensory nerve population studied had a very different morphology than the TH+ sympathetic fibers. CGRP+ and NF200+ nerve fibers innervated the cambium layer in a dense net-like meshwork (Fig. 3 B–C), 3089 ± 92 intersections/mm² and 2506 ± 60 intersections/mm² respectively. The meshwork covered the entire periosteum and was comprised mainly of single fibers. As seen in the fibrous layer, the sensory fibers studied showed a lack of association with CD31+ blood vessels, $22 \pm 4\%$ and $20 \pm 2\%$ respectively (Fig. 3 E–F, Table 1B). Within the cambium layer, CGRP+ and NF200+ nerve fibers had similar distribution and density, but showed little co-localization (10%).

In the present report we demonstrate that, in contrast to sensory fibers that innervate the marrow or mineralized bone [4, 7, 11, 14] as well as sympathetic fibers that innervate the marrow, mineralized bone and periosteum, sensory fibers that innervate the periosteum are organized in a distinct net-like meshwork and these periosteal sensory fibers frequently lack association with CD31+ blood vessels. Given this unique organization, a key question is whether this organization has functional significance and if so whether it sheds light on the human perception of pain following skeletal fracture.

Following fracture of the human femur, patients frequently report that they experience an immediate acute, stabbing and excruciating (“worst imaginable”) pain that is referred to the fractured bone [17]. Within minutes-hours following the initial fracture, if movement of the fractured bone is reduced, the pain decreases to a severe, dull aching pain [17]. However, further movement of the fractured bone at this point results as a pain that can be as or more intense than the pain experienced upon initial fracture [17, 19]. This clinical picture would suggest that there must be a set of mechanosensitive nociceptors that are uniquely positioned to detect mechanical distortion of the mineralized bone.

These observations, as well as those made in the present report, would suggest that the sensory nerve fibers that innervate the periosteum are ideally placed to participate in such a role. Mechanical distortion of periosteal nerve fibers driving fracture pain is suggested by experimental studies performed in human volunteers [9]. In these studies direct mechanical stimulation of the periosteum in awake humans elicited an immediate sharp arresting pain which possessed a significantly lower threshold compared to stimulation of the ligaments, fibrous capsule of the joints, tendons, fascia and muscle. These results, along with the clinical observation that stabilization of the bone produces a marked reduction of fracture pain [1, 6], suggests that periosteal nociceptors may play a unique role in driving mechanically induced fracture pain.

The present results demonstrate that there is a dense net-like innervation of CGRP+ and NF200+ sensory fibers in the periosteum of the femur. Based on the organization of these sensory fibers, along with the clinical picture of painful human fractures [17], the effectiveness of bone stabilization in reducing fracture pain [1, 6] and experimental stimulation of human periosteum [9], it is suggested that sensory nerve fibers that innervate the periosteum are uniquely organized to detect mechanical distortion of the periosteum and underlying mineralized bone. It should be noted that sensory fibers in the marrow and mineralized bone may also contribute to the pain following fracture of the skeleton [5, 11, 18]. Similarly, as sensory neuron mechanotransduction pathways have been shown to be

sensitized by nerve growth factor and other compounds released upon tissue injury [12, 13], chemical mediators released upon injury to bone probably also participate in driving fracture pain. Understanding how sensory and sympathetic fibers are organized to both sense bone pain and regulate bone remodeling may allow the development of mechanism based therapies for treating skeletal pain.

References

1. Bone LB, Johnson KD, Weigelt J, Scheinberg R. Early versus delayed stabilization of femoral fractures: a prospective randomized study. 1989. Clin Orthop Relat Res. 2004;11–6. [PubMed: 15187827]
2. Empana JP, Dargent-Molina P, Breart G. Effect of hip fracture on mortality in elderly women: the EPIDOS prospective study. J Am Geriatr Soc. 2004; 52:685–90. [PubMed: 15086646]
3. Greenfield, GQ. Orthopaedic Pain. In: Boswell, MV.; Cole, BE., editors. Weiner's pain management: a practical guide for clinicians. CRC Press; Boca Raton: 2006. p. 465–476.
4. Gronblad M, Liesi P, Korkala O, Karaharju E, Polak J. Innervation of human bone periosteum by peptidergic nerves. Anat Rec. 1984; 209:297–9. [PubMed: 6205609]
5. Haegerstam GA. Pathophysiology of bone pain: a review. Acta Orthop Scand. 2001; 72:308–17. [PubMed: 11480611]
6. Hedequist D, Starr AJ, Wilson P, Walker J. Early versus delayed stabilization of pediatric femur fractures: analysis of 387 patients. J Orthop Trauma. 1999; 13:490–3. [PubMed: 10513971]
7. Hill EL, Elde R. Distribution of CGRP-, VIP-, DbH-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. Cell Tissue Res. 1991; 264:469–480. [PubMed: 1714353]
8. Hukkanen M, Kontinen YT, Rees RG, Gibson SJ, Santavirta S, Polak JM. Innervation of bone from healthy and arthritic rats by substance P and calcitonin gene related peptide containing sensory fibers. J Rheumatol. 1992; 19:1252–1259. [PubMed: 1383542]
9. Inman VT, Saunders JB. Referred pain from skeletal structures. J Nerve Ment Dis. 1944; 99:660–7.
10. Kidd BL. Osteoarthritis and joint pain. Pain. 2006; 123:6–9. [PubMed: 16714085]
11. 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; 113:155–66. [PubMed: 12123694]
12. Malik-Hall M, Dina OA, Levine JD. Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. Eur J Neurosci. 2005; 21:3387–94. [PubMed: 16026476]
13. McMahon, SB.; Bennett, DLH.; Bevan, S. Inflammatory mediators and modulators of pain. In: Wall, PD.; McMahon, SB.; Koltzenburg, M., editors. Wall and Melzack's Textbook of Pain. Elsevier/Churchill Livingstone; Philadelphia: 2006. p. 49–72.
14. Miller MR, Kasahara M. Observations on the innervation of human long bones. Anat Rec. 1963; 145:13–23.
15. Praemer A, Furner S, Rice D. Musculoskeletal Conditions in the United States. American Academy of Orthopaedic Surgeons. 1999:182.
16. Ruocco I, Cuello AC, Parent A, Ribeiro-da-Silva A. Skin blood vessels are simultaneously innervated by sensory, sympathetic, and parasympathetic fibers. J Comp Neurol. 2002; 448:323–36. [PubMed: 12115696]
17. Santy J, Mackintosh C. A phenomenological study of pain following fractured shaft of femur. J Clin Nurs. 2001; 10:521–7. [PubMed: 11822500]
18. Sherman MS, McFarland G. Mechanism of pain in osteoid osteomas. Southern Medical Journal. 1965; 58:163–166. [PubMed: 14246937]
19. Yates, D.; Smith, M. Orthopaedic pain after trauma. In: Wall, PD.; Melzack, R., editors. Textbook of pain. Churchill Livingstone; Edinburgh; New York: 1994. p. 409–421.

Figure 1.

Periosteum (the thin fibrous sheath that surrounds the outer surface of the mineralized bone) is composed of two primary layers. Five μm -thick longitudinal cross section of femoral bone stained with hematoxylin and eosin (H&E) displays the anatomical localization of the periosteum (A). High power magnification of H&E-stained section of mid-diaphysis shows the periosteal layers which are localized between the underlying cortical bone and muscle (B). Note that the inner cambium layer of the periosteum has a high density of rounded cells including osteoblast-like cells and multinucleated osteoclasts. In contrast, the outer fibrous layer has a low density of spindle-shaped fibroblast-like cells. The differential cell composition and appearance of periosteal layers are also observed in the differential interference contrast micrograph (C) and high magnification confocal photomicrograph from the same section (40 μm -thick) counter stained with a nuclear cell marker (DAPI) colored violet (D). Note that the cambium layer of the periosteum has several layers of BrdU+ nuclei colored yellow (proliferative zone) localized approximately 15–30 μm from bone lining cells.

Figure 2.

Association of sensory and sympathetic nerve fibers with blood vessels in the outer fibrous layer of the mouse femoral periosteum. Representative confocal photomicrographs of periosteal whole-mount preparations show tyrosine hydroxylase positive (TH+) sympathetic nerve fibers (A) which form tightly woven corkscrew-like baskets around CD31 positive blood vessels that vascularize the fibrous layer (D). In contrast, 200-kDa neurofilament positive (NF200+) (B) calcitonin gene related peptide positive (CGRP+) sensory nerve fibers (C) show a relative lack of association with CD31 positive blood vessels (E, F respectively). Note the sensory nerve fiber bundles (arrowheads) and single fibers (arrows) form a dense net-like meshwork over the mineralized bone. Confocal images (30 μm-thick section) were projected from 120 optical sections acquired at 0.25 μm intervals.

Figure 3.

Sensory and sympathetic nerve fibers have a differential association with CD31 positive blood vessels in the inner cambium layer of the mouse periosteum. Representative confocal photomicrographs of periosteal whole-mounts show the cambium layer has a low density of tyrosine hydroxylase positive (TH+) sympathetic single nerve fibers (A) which are closely associated with CD31 positive blood vessels (D). In contrast, there is a high density of 200-kDa neurofilament positive (NF200+) (B) and calcitonin gene related peptide positive (CGRP+) sensory nerve fibers (C) which are occasionally associated with CD31 positive blood vessels (E,F respectively). Note that in the cambium, there again appears to be a meshwork of single (arrows) and bundled fibers (arrowheads) similar to that observed in the fibrous layer. Confocal images (30 μm-thick section) were projected from 120 optical sections acquired at 0.25 μm intervals.

Table 1A

Distribution of periosteal nerve fibers and vasculature*

	Cambium Layer	Fibrous Layer
CGRP+ sensory fibers	3089±92	2664±127
NF200+ sensory fibers	2506±60	2679±233
TH+ sympathetic fibers	907±31	3138±157
CD31+ blood vessels	1486±529	2208±747

* Values represented as mean number of intersections per mm² ± SEM

Table 1BPercent of nerve fiber association with CD31+ blood vessels within the periosteal layers^{*}

	Cambium Layer	Fibrous Layer
CGRP+ sensory fibers	22±4	26±6
NF200+ sensory fibers	20±2	20±8
TH+ sympathetic fibers	77±3	80±3

^{*} Values represented as mean percentage of association ± SEM