Evidence for a Dense and Intimate Innervation of the Bone Tissue, Including Glutamate-Containing Fibers

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The recent demonstration in bone cells of receptors for glutamate (Glu), a major neuromediator, suggests that Glu may also act as a signaling molecule in bone and regulate bone cell metabolism. Although bone is known to be innervated, the distribution and characteristics of nerve fibers in this tissue have not been well documented. We have studied the anatomical distribution of nerve fibers and the presence of glutamate-immunoreactive ones in sections of long bones from neonatal, 15-, and 25-day-old rats, using immunocytochemistry with antibodies directed against several neuronal markers and Glu. We showed by electron microscopy that bone is rich in nerve-like processes running along vessels adjacent to bone trabeculae, in the vicinity of hematopoietic cells and bone cells. Immunocytochemical studies at the tissue and cellular level confirmed the presence of a dense network of thin nerve processes immunolabeled for neurofilament 200, tyrosine hydroxylase, and microtubule associated protein-2, three markers of nerve fibers. Some of these nerve processes showed local dilatations in contact with medullary cells and bone cells that were immunolabeled for synaptophysin, a nerve terminal marker. Glu was largely expressed in these thin nerve processes in proximity to bone cells. These findings show evidence for a dense and intimate network of nerve processes in bone, some of which were containing Glu, suggesting glutamatergic innervation in bone. (Bone 25:623–629; 1999) © 1999 by Elsevier Science Inc. All rights reserved.

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Introduction

Pioneer studies, using light microscopy with methylene blue staining or silver impregnation have demonstrated that bone is innervated. Nevertheless, the distribution of nerve fibers in bone has not been well documented, and the discrimination of fibers representing the sensory, sympathetic, and parasympathetic nervous system was not possible with conventional histological techniques. Duncan and Shim have later shown the presence of sympathetic nerve fibers in bone tissue by visualizing noradrenaline. More recently, nerve fibers immunoreactive for neuropeptides and tyrosine hydroxylase (TH) have been demonstrated in bone, indicating the presence of both sympathetic and sensory fibers. These studies have shown a high degree of peptidergic innervation in bone tissue in regions of high osteogenic activity. However, immunocytochemistry using antibodies directed against specific neuronal markers has not been performed at the cellular level in bone, and the distribution of nerve fibers in the direct vicinity of bone cells is still not well known.

Glutamate (Glu) is a major neuromediator of both the central and peripheral nervous system. The recent identification of a neuronal glutamate transporter in bone, as well as the demonstration of the expression of different subtypes of Glu receptors by both bone resorbing osteoclasts and bone forming osteoblasts, suggest that Glu might be an important local regulator of bone cell functions. The origin of Glu in bone is unknown, and one hypothesis is that its presence is due to innervation of this tissue. Clinical observations, as well as experimental and in vitro studies, have already established the involvement of the peripheral nervous system in the regulation of bone development and bone remodeling.

The purpose of this study was to examine the anatomical distribution of nerve fibers in bone and to identify Glu-immunoreactive fibers. We used immunocytochemistry on long bone sections from neonatal, 15- and 25-day-old rats, with antibodies directed against several specific neuronal markers and Glu. We have demonstrated the presence of a dense network of nerve processes in the vicinity of bone cells, some of which contained Glu, suggesting the existence of a glutamatergic innervation in bone.

Materials and Methods

Animals and Tissue Processing

Neonatal (few hours), 15- and 25-day-old Wistar rats were used for this study. Animals were killed, tibia and femurs were rapidly excised, roughly cleaned of adherent tissues and immediately immersed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Bones were cut transversely at the diaphysis level, then longitudinally, and fixed for 24 h. After several washings in sodium cacodylate buffer, all specimens were post-fixed with potassium ferrocyanide-reduced osmium tetroxyde for 2 h. Undecalcified samples were immediately dehydrated through a graded ethanol series and embedded in Epon, whereas some samples were partially decalcified in 4.13% disodium ethylenediamine-tetra-acetic acid (EDTA) for 5 days before dehydration and embedding in Epon.

Semithin sections (0.8–1 μm) were cut on a Reichert Ultracut E Microtome (Leica, Leitz, Austria). Some sections were stained with azur-methylene blue to enable selection of samples for
immunocytochemistry at tissue or cellular levels as well as for transmission electron microscopy (TEM).

For TEM, thin sections (80 nm to 100 nm) were cut and stained with uranyl acetate and lead citrate. Sections were examined using a Jeol 1200EX electron microscope (Jeol Ltd., Akishina, Tokyo, Japan) at 80 kV.

**Antibodies.** The monoclonal antibodies directed against neurofilament 200 (NF 200), synaptophysin and the microtubule-associated protein-2 (MAP2) were obtained from Sigma (St Louis, MO). The rabbit anti-tyrosine hydroxylase (TH) and anti-glutamate polyclonal antibodies were purchased from Chemicon (Temecula, CA). The antibody used in this study recognizes free Glu. The polyclonal antibody directed against human α1 (I) carboxy-telopeptide of type I collagen (LF-67) was provided by Dr. L. Fisher (NIH, Bethesda, MD). The polyclonal antibody directed against rat type III collagen was given by Dr. Hartmann (Novotec, Lyon, France). The anti-VIP polyclonal antibody was a gift from Dr. J. L. Saffar (Paris, France). Controls were performed by using a monoclonal antibody directed against the Kappa chains of immunoglobulins (MOPC21) or purified immunoglobulins G from nonimmune rabbit serum.

**Semithin sections.** Sections from decalcified samples were laid on sylanized slides (aminopropyltriethoxyalsilane, 2% in acetone) and dried overnight at 37°C. Epon was removed with 13.3% (w/v) potassium hydroxide in a methanol/propylene oxide (2/1) mixture and rehydrated. Sections were treated for 20 min with 100 mM glycine and 50 mM ammonium chloride in Tris buffer, pH 7.6 in order to saturate free aldehydic groups. Endogenous peroxydase activity was inhibited by incubation for 15 min with 1.3% (w/v) potassium hydroxide in a methanol/proplylene oxide (2/1) mixture and rehydrated. Sections were treated for 20 min with 100 mM glycine and 50 mM ammonium chloride in Tris buffer, pH 7.6 in order to saturate free aldehydic groups. Sections were incubated overnight with primary antibodies diluted in TBS containing 1% NGS (dilutions of antibodies: anti-NF200: 30 μg/mL; anti-MAP2: 1 μg/mL; anti-synaptophysin: 6 μg/mL, anti-Glu: 1/1000; anti-TH: 1/15000; anti-VIP: 1/2000; LF-67: 1/5000; anti-type III collagen (batch 217): 1/1000). Control sections were incubated with nonimmune rabbit serum or with the monoclonal antibody MOPC21 which has no known hapten or antigen binding activity. Antigen-antibody complexes were detected with a peroxydase streptavidin system (Dako, Copenhagen, Denmark) and revealed with 3-3′ dianinobenzidine tetrahydrochloride (DAB) (Sigma) in Tris buffer containing 0.01% H2O2. All washings were done with
Figure 2. Immunohistochemical localization of several nerve markers in long bones of growing rats. (a) Metaphysis of neonatal rat femur. NF 200-positive nerve processes (arrows), showing local enlargements (arrow heads), are running along bone trabeculae (t) and vessels (V). They are close to bone cells (Oc: osteoclast. Ob: osteoblast) and hematopoietic cells (H). Original magnification: ×1000. (b) Deep metaphysis of neonatal rat femur at the level of the afferent vessel. Tyrosine hydroxylase is present in these nerve processes (arrows) showing local dilatations (arrow heads) in contact with hematopoietic cells (H). Oc: osteoclast. Original magnification: ×1500. (c) Deep metaphysis of neonatal rat femur. MAP2-positive nerve processes showing dilatations (arrow heads) in contact with hematopoietic cells (H) and osteoclasts (Oc). Original magnification: ×1000. (d) Diaphysis of 15-day-old rat tibia. NF200 is present in nerve endings (arrow heads) in contact with hematopoietic cells (H). Original magnification: ×1000.

Figure 3. Immunolabelings for NF 200 and type I or III collagen are mutually exclusive in long bone sections. (a) and (b) Consecutive sections of neonatal rat femur (metaphysis). Type I collagen (a) is visualized in bone matrix, osteoblasts (Ob) and preosteoblasts (POb) (arrows). NF200-positive nerve processes (b) are running in bone marrow close to osteoblasts (Ob), hematopoietic cells (H) and vessels (arrow heads). The labelling profile obtained in (b) is completely distinct from that obtained in (a). Original magnification: ×1000. (c) and (d) Consecutive sections of neonatal rat femur (metaphysis). Type III collagen (c) is observed in the remaining cartilage matrix of the bone trabeculae (double arrow) and in collagen fibrils in the bone marrow (arrows). NF200-positive nerve processes (arrow heads) are located near bone cells and vessels. The labeling profile obtained in (d) is completely distinct from that obtained in (c). Original magnification: ×400.

TBS containing 0.02% Triton X100. Sections were counter-stained with Meyer’s hematoxylin (diluted 1:3 in distilled water), dehydrated, and mounted in Xam (Gurr-BDH Laboratory, Poole, UK).

Ultrathin sections. Sections from decalcified and undecalcified samples were collected on nickel grids covered with a formvar film. Grids were floated for 10 min on a drop of 50 mM glycine in 0.05 M Tris buffer pH 7.4, transferred for 30 min on a drop of Tris buffer containing 1% BSA and 1% NGS, then incubated for 90 min at room temperature on a drop of the specific primary antibodies (dilutions of antibodies: anti-NF200: 60 μg/mL; anti-MAP2: 1 μg/mL; anti-TH: 1/5000; anti-Glu: 1/500). After incubation, sections were rinsed on drops of Tris buffer/BSA, placed on biotinylated anti-mouse or anti-rabbit antibodies (dilution: 1/300 in Tris buffer/BSA, pH 7.4), then laid on gold streptavidin (Jansen, Tebu, France) at pH 8.2. After careful washing, grids were transferred to 1% glutaraldehyde, rinsed in distilled water, and conventionally
stained with uranyl acetate and lead citrate before examination by TEM.

Some semithin sections from decalcified samples were immunolabeled for NF200 without Meyer’s hematoxylin counterstaining. These sections were dehydrated in ethanol, impregnated with a mixture of propylene oxide-Epon (v/v), and re-embedded in Epon. Ultrathin sections were cut and observed without counterstaining.

**Results**

**Localization of Nerve Fibers in Long Bones of Growing Rats**

Electron microscopy studies of long bones from neonatal, 15- and 25-day-old rats revealed the presence of nerve fibers either in the vicinity of the periosteum and surrounding connective tissues or in the muscles attached to it. Some of them were running along veins and arteries. The diaphyseal nerve trunk, entering bone along nutrient vessels, comprised mainly nonmyelinated fibers in 15-day-old rats (Figure 1a). In the periosteum of 15- and 25-day-old rats, nerve fibers were generally unmyelinated. Whereas in the endosteum, some myelinated fibers were observed in proximity to osteoblasts, more numerous in 25-day-old rats (Figure 1b). In bone marrow, only unmyelinated nerve fibers running between hematopoietic cells were demonstrated (Figure 1c). Myelinated nerve fibers were never present in the endosteum or bone marrow of neonatal rats.

In addition to these sparse morphologically clearly identified nerve fibers, abundant thin nerve-like processes were observed in long bone sections. These ran along morphologically close identified bone trabeculae in the vicinity of hematopoietic and bone cells and often showed enlarged endings in close contact with these cells. They were always devoid of basement membrane. Neither Schwann cells nor myelin sheath were observed at their periphery. The structural features of these nerve-like processes were slightly different for neonatal and 15-day-old rats. In neonatal rats, nerve-like processes contained only hyaloplasm and rare organelles (Figure 1d). In older rats, more organelles were observed, such as small mitochondria, glycogen particles, and dense granules. Microfilaments, microtubules, or microvesicles, usually abundant in nerve fibers, were often visible in these cell processes (Figure 1e). In contrast, they contained no golgi apparatus and no endoplasmic tubular structures, generally described in stromal cells.

**Immunoreactivity of these Nerve Processes For Several Specific Neuronal Markers**

An antibody directed against NF200, used as a general nerve marker, revealed that the epiphysis of neonatal rats consisted only of cartilage at this stage of development, and was devoid of innervation. In contrast, in all the metaphysis of neonatal rats and in the diaphyseal area containing the nutrient vessels, a dense innervation was observed that appeared as a prominent network of thin cytoplasmic cell processes in close relation with blood vessels (Figure 2a). Nerve endings, corresponding to dilatations of these nerve processes, were found in contact with medullary cells and bone cells (Figure 2a). Hematopoietic cells, endothelial cells, and bone cells were always negative for this marker in any part of the bone. These nerve processes were also labeled for TH (Figure 2b) and MAP2 (Figure 2c), two other markers of nerve fibers. Control sections, in which specific antibodies were replaced by MOPC21 or by purified immunoglobulins G from rabbit, show no specific immunoreactivity (data not shown). In femurs and tibia of 15- and 25-day-old rats, immunolabeling for neuronal markers stained the same network of nerve processes adjacent to blood vessels, bone marrow and bone cells. Its distribution was slightly different for these later stages of development, because nerve processes were observed in the epiphysis in the secondary ossification center. They were localized in proximity to bone cells lining trabeculae facing the growth plate. The deeper part of the epiphysis appeared less innervated. In the metaphysis immediately under the growth plate, nerve processes were sparse. Further away from the growth plate, they formed an expanded network between bone trabeculae, although the intensity of the labeling was not homogeneous from one trabecula to another. In the diaphysis, nerve processes were seen under cortical bone as well as in periosteocytic lacunae; a few nerve

![Figure 4. Electron micrographs of rat long bone sections after immunocytochemical labeling for various nerve markers. (a,b,c,d) Immunolabelings of neonatal rat femur (a,c,d) or 15 day-old rat tibia (b) metaphysis, followed by biotin streptavidin-gold complex staining. For NF200 (a,b), TH (c), and MAP2 (d), gold particles (arrows) are localized in nerve processes running in bone marrow close to preosteoblasts (pOB), or in nerve endings (containing microvesicle: asterisk) in contact with hematopoietic cell (H). The inset in 4c points out gold particles (arrow). Bars: 200 nm. (e) Low magnification electron micrograph of a reembedded semithin section immunostained for NF200. Positive profiles (arrows) contain few organelles and were morphologically similar to those observed on ultrathin sections immunolabeled with gold particles (Fig. 4a,b).]
endings were also evident in the center of the diaphysis, in contact with hematopoietic cells (Figure 2d).

The distribution of nerve markers was different from those of type I and III collagens. Immunolabelings for NF200 and type I or type III collagen, performed on consecutive bone sections, were found to be mutually exclusive. Type I collagen was visualized in bone matrix, in osteoblasts, in some stromal cells probably preosteoblasts (Figure 3a), but never in cell processes immunolabeled for NF200 (Figure 3b). Type III collagen was present in the remaining cartilage matrix of the bone trabeculae and in extracellular collagen fibrils abundant at stromal cell periphery (Figure 3c), but was not expressed in the network of cell processes containing NF200 (Figure 3d).

The electron-microscopic analysis, using colloidal gold immunolabeling for neuronal markers, confirmed that nerve processes were expressing NF200 (Figure 4a,b), TH (Figure 4c), and MAP2 (Figure 4d). The labeling was abundant in nerve processes running in bone marrow and in their widened parts in contact with hematopoietic cells (Figure 4a) and bone cells (Figure 4b,c,d). A low magnification electron micrograph of a reembedded semithin section immunostained for NF200 (Figure 4e) showed that the immunolabeled profiles contained rare organelles and were morphologically similar to those observed on ultrathin sections immunostained for NF200 with colloidal gold (Figure 4a,b).

Synaptophysin, one of the major proteins present in synaptic vesicles, was detected in long bone sections. The tissue distribution of this protein was similar to other nerve markers, but not identical. Labeling for synaptophysin was more restricted and mainly localized to short nerve structures in contact to bone cells, endothelial, and bone marrow cells (Figure 5a,b,c). VIP immunoreactivity was also found in bone and the tissue distribution of this neuropeptide was identical to synaptophysin (Figure 5d).

**Figure 5.** Immunocytochemical localizations of synaptophysin and VIP in long bones of growing rats. (a) Metaphysis of neonatal rat femur. Synaptophysin is present in enlargements of nerve processes in contact with blood vessels (arrows), osteoclasts (asterisk) and hematopoietic cells (arrow heads). (b) Diaphysis of 25-day-old rat tibia. Synaptophysin is present in nerve endings (arrow heads) in contact with osteoblasts underlying cortical bone (c). Some peristeoctic lacunae contain positive endings (arrow) in contact with osteocytes. (c) and (d) Consecutive sections of neonatal rat femur metaphysis, near afferent vessels. Synaptophysin (c) is visualized in nerve processes close to hematopoietic cells (arrows) and in the wall of the artery (a) (arrow heads). VIP (d) is coexpressed in the same nerve profiles. Original magnification: ×1000.

**Figure 6.** Immunocytochemical localization of glutamate in long bones of growing rats. (a) Metaphysis of neonatal rat. Glu (arrows) is present in nerve processes running in bone marrow and showing enlargements in contact with hematopoietic cells (H). Original magnification: ×800. (b,c) Electron micrographs of 15-day-old rat tibia sections immunolabeled for Glu. Glu (arrows) is observed in nerve processes in the vicinity of osteoblasts (Ob) as well as in the afferent nerve trunk. S: Schwann cell; A: unmyelinated axon.
Expression of Glutamate in Long Bones of Growing Rats

In the metaphysis and diaphysis of neonatal rat femurs, nerve processes, running in bone marrow close to bone cells and blood vessels, contained Glu (Figure 6a). Immunolabeling for Glu was particularly abundant in the endosteal part of the metaphysis and was localized in the same expanded network of thin processes previously shown to be immunoreactive for nerve markers. A few other cells, mainly osteoblastic cells, also contained Glu in varying amounts, whereas osteoclasts, endothelial, and hematopoietic cells were always negative.

In 15- and 25-day-old rat tibia, Glu had also the same tissue distribution as neuronal markers, highly expressed in nerve processes in proximity to blood vessels and bone cells.

The electron microscopic immunocytochemistry analysis confirmed that Glu was largely present in nerve processes in the vicinity of or in contact with bone cells (Figure 6b), as well as in some axons of the afferent nerve trunk (Figure 6c).

Discussion

The present study has demonstrated the existence, in long bones of neonatal, 15- and 25-day-old rats, of an extensive network of cytoplasmic cell processes immunostained for several nerve markers. NF200 is a general neuronal marker specific of neurocytoplasmic cell processes immunostained for several nerve fibers, various neuropeptides used by either sensory or postganglionic sympathetic nerve fibers previously described previously,25,31 were also present in rat long bones and could be sensory nerve processes because this neuronal marker is primarily expressed in the dendraoscopic compartment and excluded from the axons of most nerve fibers.19 Our results confirm the presence of both sensory and sympathetic nerve fibers previously documented in bone and suggest that these two types of innervation may form parallel networks with a close distribution in bone tissue. Immunoreactivities for these specific nerve markers were only observed in this network of particular cell processes and in well-defined nerve fibers. Stromal cells expressing type I and III collagens, osteoblastic cells, hematopoietic cells, endothelial cells, and osteoclasts were always negative for each of these neuronal markers, as demonstrated by light and electron microscopy.

This dense network of thin varicose nerve processes running with medullary sinusoids deeply in metaphysis and ending in contact with bone cells or medullary cells constitutes a novel observation not emphasized in previous studies. Morphologically well-defined unmyelinated and myelinated nerve fibers, as described previously,6,25 were also observed in 15- and 25-day-old rat long bone sections, but not in neonatal bones. In contrast, the network of nerve-like processes was already present in neonatal rats and the identification of TH, VIP, and MAP2-immunoreactive processes in this network confirmed that rat bone is already supplied at birth with sympathetic and sensory nerve fibers.32

This localization of nerve processes at proximity to bone and medullary cells is indicative of a possible role of nerve supply in the regulation of bone cell activities, in agreement with clinical observations in patients with neurological disorders and experimental denervation studies.10,11,15 However, although some of these nerve terminals were in contact with bone or bone marrow cells, our electron-microscopic observations did not detect typical synapses in the nerve endings which did not contain many vesicles. Hara-Irie et al.14 have also demonstrated the presence of CGRP-positive nerve fibers in close contact with osteoclasts, at epiphyseal trabeculae facing the growth plate of rat femurs; these structures were not typical synapses in agreement with our own findings. Further studies will be needed to clarify the presence of synaptophysin in bone in the absence of synaptic structures.

The nature of neurotransmitters present in bone has not been clearly identified. In addition to noradrenergic sympathetic nerve fibers, various neuropeptides used by either sensory or postganglionic sympathetic nerve fibers have been demonstrated in bone, suggesting a possible role for these neuromediators in skeletal metabolism.19,20,22,23

This is the first study to reveal glutamate-immunoreactive nerve processes in bone. Glu is the primary excitatory neuromediator in both the central nervous system and the peripheral nervous system.25 We have shown that Glu was widely expressed in nerve processes running in bone marrow in the vicinity of bone or bone marrow cells, suggesting that it might also act as a neurotransmitter in bone. In addition, Glu was detected in some osteoblasts and preosteoblasts, probably in relation to cell metabolism. Our observations have clearly demonstrated some contacts between Glu-immunoreactive nerve terminals and bone cells, but no synaptic vesicles were observed in these structures. Although our study did not show any evidence for a release of Glu from these nerve terminals, we can consider that significant local concentrations of Glu might be present in the vicinity of bone cells.

Glu acts on a variety of different ligand-gated ion cell surface receptors, classified in two categories, mainly, NMDA receptors and non-NMDA receptors. These receptors, as well as glutamate transporters, have been shown to be expressed by bone cells.25,31 Furthermore, we have recently demonstrated that NMDA subtype Glu receptors expressed by osteoclasts are functional and that Glu elicits current in these cells.7,9

The cellular origin of Glu and the mechanisms leading to its release in bone are unknown. One proposed hypothesis is that Glu is released from bone cells and has a role in paracrine intercellular communications in bone.25 Another possibility is that Glu, which is the most largely distributed neuromediator in the nervous system, is released from nerve fibers present in the vicinity of bone cells. Our results, which demonstrate in long bones of growing rats an extensive network of nerve processes containing Glu in proximity to bone cells, strengthen this last hypothesis, and provide initial data for the existence of a glutamatergic innervation of bone.

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