IS BONE A TARGET-TISSUE FOR THE NERVOUS SYSTEM? NEW ADVANCES ON THE UNDERSTANDING OF THEIR INTERACTIONS

José M. García-Castellano, M.D., Ph.D.*
Pilar Díaz-Herrera, M.D., Ph.D.*
José A. Morcuende, M.D., Ph.D.**

ABSTRACT

Bone cells respond in specific ways to various hormones and growth factors, but the biology of skeletal innervation and its physiologic significance in bone metabolism is poorly understood. With the introduction of immunohistochemical staining techniques and new molecular biology tools, the knowledge in this field has significantly improved. In this review, we update current understanding of the effects of neuropeptides on bone metabolism, specifically vasoactive intestinal peptide (VIP) and calcitonin-gene related peptide (CGRP). In addition, new information concerning the role of growth factors, such as neurotrophins, is also discussed. There is strong evidence to suggest that bone can be a target of the nervous system. Further investigations in this field will allow us to answer questions related to pre-natal development, bone growth, fracture healing, osteoporosis, osteoarthritis or neoplasias of mesoderm origin.

INTRODUCTION

The importance of the nervous system on body homeostasis including the immune, endocrine and hematopoietic systems has been previously described.[3] It has also been suggested that organogenesis and tissue repair are under neuronal control. Although bone has some innervation, little knowledge concerning the neural influence on bone metabolism has been accumulated. However, it seems reasonable that neural control could also apply to bone tissue, and several clinical and experimental observations support this concept, including Charcot's neuropathy and the exhuberant callus formation after diaphyseal fractures on head injured patients.[6, 55]

*Departamento de Morfología, Facultad de Medicina, Universidad de Las Palmas de Gran Canaria, Spain
**Department of Orthopaedic Surgery,University of Iowa, Iowa City, IA, USA

Address correspondence and reprint requests:
José M. García-Castellano, c/ Anton Dvorak 13-9
35016 Las Palmas de Gran Canaria, España.
Telephone: + (34) –928 - 33.42.47
Fax: + (34) –928 - 33.70.29
E-mail: jmgc_61@yahoo.com

The lack of knowledge concerning the physiologic significance of bone innervation is mainly due to two reasons. One, the relatively few number of nerve fibers in bone compared with other tissues has been interpreted as the nervous system playing a minor role in the skeleton. Second, it has been very difficult to identify nerves in mineralized tissue. Methodologically, histologic studies to demonstrate nerves in bone have used routine staining techniques, such as gold chloride-osmic acid and silver staining, but these techniques provide limited morphological information.[14,98] In the last few years, the introduction of immunohistochemical and molecular biology have greatly expanded our knowledge in this field.

In this review, we will update the current understanding of the effects of neuropeptides on bone metabolism, specifically vasoactive intestinal peptide (VIP) and calcitonin-gene related peptide (CGRP). In addition, new information on the role of growth factors such as neurotrophins is discussed.

Anatomy and physiology of bone innervation

Anatomy. The distribution of nerves in bone, specifically those with neuropeptide-containing fibers, has been extensively studied. These nerves are most frequently found in metabolically-active bone. In contrast to local factors, neuropeptides in the sensory and autonomic nervous systems are synthesized in dorsal root or local sympathetic/parasympathetic ganglia and then transported along the axon by means of dense vesicles to their storage site in bone.[54]

The majority of nerves in bone are found along blood vessels. Both sensory and autonomic fibers have been demonstrated in the vessels of the periosteum, Volkmann's canals, bone marrow, osteochondral junction of the growth plate and the attachment of the synovial membrane.[8,12,42,46] The anatomy of the autonomic sympathetic vasomotor nerve supply of bone has also been extensively studied in rabbits. Adrenergic nerves profusely supply intraosseous vessels. These nerves probably contain neurotransmitter substances that function as a neuro-vaso-muscular synapse.[18] On the surface of the bone, peptidergic periosteal nerves are more numerous at the epiphysis than in the mid-shaft region

of bones. Additionally, neuropeptide-like molecules can be produced and/or released by many non-neuronal cells in bone, which act synergistically with nerve fibers, e.g., vasoactive intestinal peptide-like peptides (VIP) by mast cells and neuropeptide Y by megakaryocytes/platelets.[54]

Neuropeptides are not only present in bone under normal conditions; changes in neuropeptide-containing nerves in various pathological experimental situations suggest that they are actively involved in local disease processes such as bone growth, repair, and remodeling.[16,31,38,54,63,73,86] Moreover, clinical situations have shown that patients with neurologic disorders exhibit localized bone changes and altered fracture healing with excessive callus formation.[29,32,39]

Physiology . Although there are few nerve fibers in bone, their presence may represent sophisticated and specialized regulatory elements able to deliver time- and site-specific stimuli according to demand [54]. The distribution of different nerves during bone formation, combined with the observed effects of transmitters on bone metabolism *in vitro*, suggest that there is neuroendocrine regulation of bone physiology. This fact is crucial, not only for local bone physiology, but also for skeletal ontogeny and pathology.[6]

Essentially, bone nerves have been implicated in two different roles: as regulators of bony mechanical forces and as a source of trophic factors essential for structure and bone function. According to Wolff's law, different grades of physical activity are converted into changes in bone mass. In this case, bone nerves may represent the "organ" able to perceive mechanical strain and stresses, process this information and then transform this physical signal into cellular and biochemical responses.[54] The perception of stretch, pressure, and position of the bone nerves may contribute to the overall mechanism of coordinated movement of the limbs and bone modeling.[68]

On the other hand, bone is a living and continuously remodeling tissue. Neuro-related molecules appear to have trophic effects on normal bone metabolism. Recently, it has become more evident that at least some neural influences on bone may be mediated by neuropeptides released from the sensory nerve fibers and from the post-ganglionic autonomic nerve fibers. Release of neuropeptides from bone nerves seems to be related to the stimulation of those nerves. Non-stimulated nerves do not seem to release their peptides to any great extent, but under diverse situations of stress, the nerve terminals liberate neuropeptides resulting in significant local concentrations of these molecules.[54] In addition, during the ontogeny of sensory and autonomic nerves in the hindlimb of the rat, neuropeptide expression coincided with the mineralization process.[91,92] It has also been observed that the nerves are predominantly located in areas of high osteogenic activity, such as the periosteum and osteochondral junction of the growth plate.

It is widely known that bone cell physiology and repair is controlled by various systemic and local factors, and some of these molecules are deposited in the bone matrix and bound to different extracellular matrix components. Following bone resorption or fracture, growth factors are released into their surrounding environment, where they reach significant concentrations. It has been suggested that neuropeptides can affect the bone remodeling cycle in a similar fashion.[54] Several neuropeptides, such as substance P, neuropeptide Y, neurokinin A, VIP and CGRP, have been involved in bone physiology, but the best characterized at present are calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP). We will discuss in a subsequent section the effects of these molecules on bone.

Bone patho-physiology and the nervous system

Many morpho-functional studies indicate a role of the nervous system on bone physiology. We will describe the clinical processes in which an influence of the nervous system has been observed, including fracture healing,[64,73] bone growth,[16,31] sciatic denervation,[42] heterotopic bone formation,[104] arthropathies[33,94] and limb regeneration.[20]

1. Fracture healing. In animal models, fibular fractures failed to unite after removal of proprioceptive receptors by periosteal stripping.[1] In human samples with delayed union or nonunion of diaphyseal fractures, the most remarkable finding was the insufficiency or total lack of peripheral innervation. Although mechanical stability is considered the main factor underlying this situation, lack of neural control leading to a delayed fracture is an attractive hypothesis. Supporting these concepts are the observations that patients with neurologic disorders exhibit altered fracture healing and excessive callus formation.[21,29,39] In addition, Dyck et al[19] showed that patients with neuropathic arthropathy due to subclinical sensory neuropathy also suffer from recurrent long bone fractures.

This difference in healing may imply that in fractures with an abnormal nerve supply the sensory innervation does not recognize anomalous movement of the fracture and, with unstable fixation, nerves may mediate signals that lead to altered bone healing. Under conditions of altered nerve supply, Retief and Dreyer observed that connective tissue proliferation from the damaged bone is non-osteogenic and prevents healing of experimental cortical defects in the rat mandibulae.[81]

Other studies have shown that fracture calluses are bigger, less dense, and mechanically weaker compared with controls after sciatic section.[72] In addition, Becker showed that the time of denervation in relation to the time of fracture is a critical factor in the influence of denervation on the rate of healing in the rat fibula[4]. In paraplegic rats, the fracture callus showed delayed accumulation of calcium and incomplete maturation of woven new bone.[2]

2. Bone growth. The effect of division of peripheral nerves on longitudinal bone growth has been studied by a number of authors. Experimental studies have demonstrated controversial results ranging from a decrease in growth[51] to minimal change,[77] to stimulation of growth.[83] After sciatic denervation in rats, using measurements of bones exclusively innervated by the sciatic nerve (such as the metatarsals), Garcés and Santandreu showed that the denervated side grew about 3% to 5% less than on the control side.[31] Clinical cases of sciatic nerve injury in childhood indicate that limb denervation produces decreased growth of the foot.[26]

3. Denervation. A number of experimental studies suggest direct neuronal influence on local bone metabolism. In sympathetically denervated rats, quantitative autoradioagraphic analysis using ^{3}H-proline showed reduced osteoblastic activity, and morphometric analysis indicated an increased number of osteoclasts and increased resorption activity after sympathectomy.[40] Sensory denervation, on the other hand, was associated with a decrease in the number of osteoclasts [55].

How denervation influences bone metabolism is unclear. Some authors suggest that the bone response after denervation is not only due to a local effect, but also to a systemic response. Accordingly, some authors have shown that after neurectomy there is an alteration in bone mass of the contralateral femur compared to the sham-operated limb.[64] This supports studies that have found increased callus formation, a greater and more rapid healing response, and heterotopic ossification in patients with head injuries.[93] *In vitro* studies also seem to support these findings. Kurereret al demonstrated the possible existence of a circulating humoral factor. Sera drawn from patients with head injury and from paraplegic patients with heterotopic ossification have increased osteoblast activity *in vitro*.[57]

4. Heterotopic ossification. When heterotopic bone formation is induced by the implantation of demineralized bone matrix in the abdominal muscles of the rat, nerves immunoreactive to substance P, CGRP, neuropeptide Y, VIP and neurokinin A appear very early after implantation. Several days later, interleukin-1-positive nerves are also observed. Sensory and autonomic nerves could be seen among differentiating chondroblasts in the fibrous tissue developing around and also within the implants. These observations suggest participation of the nervous system in the early development of bone.[8,56] However, which cells are responding and through what mechanisms is still unknown.

5. Arthropathies. Many observations implicate the nervous system in the pathophysiology of arthritis. For example, there is a symmetric, neurotome-like involvement of joints in rheumatoid arthritis. In patients with hemiplegia who develop rheumatoid arthritis, the paralytic side shows little or no sign of joint inflammation.[33,94] It is known that antidromic stimulation of primary afferents evokes vasodilation and increased vascular permeability, resulting in extravasation of plasma proteins into the surrounding tissues. Sympathetic denervation has been shown to cause an increase in bone blood flow.[97] Because of this, CGRP is recognized as the most potent vasodilator among sensory neuropeptides.

6. Limb regeneration. During the process of limb regeneration in animals it has been observed that nerves supply trophic factors that act on mesenchymal tissues.[89] Experimentally, amputation of a newt arm coincident with excision of the fourth spinal nerve resulted in a significantly smaller limb with significant skeletal deficiencies in all four digits when compared to the unoperated control regenerate arm. In addition, like experimental fractures, the timing of denervation is crucial to limb regeneration. When this neurotrophic factor is depleted by denervation at the time of amputation, regeneration does not occur. If the denervation occurs several weeks before amputation, the limb will regenerate.[103] If the nerve is sectioned after the critical post-amputation period, regeneration occurs although the resulting limb is reduced in size.[90] These results suggest that the absence of available neurotrophic factors at the amputation stump may affect the limb regeneration process.

Neuropeptides Involved in Bone Metabolism

As cited previously, many neuropeptides have been found in bone, but the best studied are calcitonin-gene related peptide (CGRP) and vasoactive intestinal peptide (VIP). In this section, we will review their effects on bone.

1. Calcitonin-gene related peptide (CGRP). CGRP is a 37 amino acid peptide produced by tissue-specific alternative processing of the primary RNA transcripts of the calcitonin gene. Two isoforms of CGRP, CGRP-I and CGRP-II, differ only in three amino acids.[54] The distribution of CGRP-positive nerve fibers has been widely described. During rat femur development, the number of CGRP-positive nerve fibers increased in the

metaphysis until the tenth postnatal day, when the animals started to use their limbs. After this period of time, innervation increases in the epiphysis but decreases in the metaphysis. In addition, CGRP-positive nerve fibers were more abundant in the epiphysis than in the metaphysis, and these nerve fibers ran along the epiphyseal trabeculae facing the growth plate.[38] Interestingly, Schwab et al., using immunohistochemistry staining, described the presence of CGRP-immunoreactive nerve fibers in the outer layer of articular cartilage and in contact with chondrocytes in the knee joints of newborn and adult rats.[87]

CGRP-innervation of bone has also been studied under different experimental situations. In sciatic-denervated rats, it has been shown that the number of CGRP-positive nerve fibers is markedly decreased along epiphyseal trabeculae facing the growth plate. Furthermore, the osteoclasts were localized in close contact with the bone surface and showed intense tartrate-resistant acid phosphatase activity. Additionally, the cartilage extracellular matrix almost disappeared from the epiphyseal trabeculae facing the growth plate. These results suggest that the high bone turnover at this site resulted in rapid absorption of mixed trabeculae with subsequent replacement by bone spicules without calcified cartilage.[38]

After a fracture, CGRP-immunoreactive fibers have been widely depicted. Periosteal CGRP-immunoreactive fibers showed dense ramifications and terminal sprouting after seven days. In addition to the periosteum, these nerve fibers were found in the middle of the callus interspersed with inflammatory cells. At days 14 and 21, many tortuous nerves were found in the periosteum, but not in mid-callus. Interestingly, mast cells have been observed in close proximity to CGRP-immunoreactive nerve fibers, especially at 21 days after trauma. Mast cells are powerful inflammatory mediator cells that may interact synergistically with the nerve fibers.[11]

CGRP-immunoreactive fibers have been shown to serve three functions. Acting on the vasculature, CGRP is the most potent vasodilator among sensory neuropeptides.[97] On bone cells, CGRP has been reported to stimulate osteogenesis,[5] either by activating stem cell mitosis or osteoprogenitor cell differentiation, or both.[88] Bernard and Shih reported that CGRP increased the number and size of bone colonies *in vitro*. This effect appeared to be dose-dependent. A similar finding was obtained by intravenous injection of CGRP 2 hours before bone marrow cells were harvested.[5]

The effect of CGRP on osteogenesis is mediated via $CGRP_1$ and $CGRP_2$ receptors which are distinct from calcitonin C receptors. The signal transduction mechanisms of calcitonin receptors have been extensively studied. It has been shown that CGRP receptors activate the cAMP or the protein kinase C (PKC) pathways.[54] These two transduction pathways required guanosine triphosphate (GTP)-binding proteins (G proteins) and led to opposite biological responses. Moreover, selective activation of one or the other pathway was cell cycle-dependent. Therefore, CGRP may induce different cell responses depending on their stage in the cell cycle. This type of modulation could be important in rapidly growing cell populations such as during embryogenesis, growth and tumor formation.[10]

Other authors have found a different mechanism of action not linked to the cAMP-signaling pathway. Data from Kawase et. al indicate that CGRP transiently increases in the intracellular pool of Ca^{2+}, in part by release of Ca^{2+} from intracellular stores.[50] This action is apparently not mediated via the cAMP-signaling pathway, because they are not replicated by forskolin. During bone resorption, CGRP inhibits osteoclastic function.[17, 47,75,85]

2. Vasoactive intestinal peptide (VIP). VIP is a ubiquitous 28 amino acid cleavage product of pre-pro-VIP, originally isolated from porcine intestine, that has been shown to be a potent activator of adenylate cyclase in many organ systems.[54] VIP-immunoreactive nerves are distributed in bones in a similar pattern to CGRP-related fibers. Several reports have shown that VIP-immunoreactive fibers are sympathetic in origin.[43] VIP-neurons could produce high VIP concentrations locally in bone, but unlike some other vascular structures, bone and periosteal vessels are not responsive to the vasodilatory effects of VIP alone.[44] These studies have suggested that vasodilation is probably not the primary action of VIP in bone.[43]

The prevalent function of VIP-immunoreactive nerves in bone seems to be the stimulation of resorption.[55] In experimental models inducing heterotopic bone formation, VIP-immunoreactive nerves have been found among differentiating chondroblasts in the fibrous tissue developing around, and also within, the implants.[8,56] In human osteosarcoma cell lines, osteoblasts respond to VIP by expression of specific cell surface receptors that also have been observed in bone organ cultures.[45,55]

The effects of VIP on bone are mediated by receptors coupled to two types of proteins belonging to the G family:[7,27,62] G_s protein and G_{plc} protein. VIP receptors linked to G_s protein stimulate bone resorption via a cAMP-dependent mechanism.[44] It has been shown that osteoblastic osteosarcoma cells respond to nanomolar concentrations of VIP with increases in cAMP, and whole mouse calvariae are resorbed under the influence of VIP.[44]

Although the effects on bone by neuropeptides trans-

ported by sensory and autonomic nerves is not totally elucidated, Vignery et al demonstrated that the neuropeptide CGRP increases both the accumulation of mRNA encoding IGF-I and the production of IGF-I peptide by osteoblasts.[101] Also, the neuropeptide VIP may take part in bone resorption by stimulating prostaglandin E_2.[80] Interleukin-1 (IL-1) immunoreactive nerve fibers have been seen at a late stage of heterotopic bone formation induced by allogenic bone matrix. The IL-1 immunoreactive nerve fibers stimulate release of prostaglandin E_2, which is known to promote bone resorption. IL-1 immunoreactive nerve fibers were also observed several days after immunoreactive-nerves to CGRP appeared. Both types of nerves have been related in the early development of bone.[7,56] These observations are important since they may lead to a better understanding of the interactions between nerves and bone function.

Neurotrophins and bone metabolism

The protein nerve growth factor (NGF) is a complex molecule that contains three different subunits. In neural tissues, it has been shown that NGF is an important factor required for the development and maintenance of peripheral sensory and post-ganglionic sympathetic nerves.[59] Moreover, during vertebrate development, survival and differentiation of many neurons depend on their target cells.

The role of NGF on target tissues has been demonstrated by studying the skin. The arrival of sensory fibers in developing mouse skin coincides precisely with the initiation of NGF synthesis in this area. This temporal association suggests that the arrival of sensory fibers might initiate NGF synthesis in their target tissues.[84] NGF also appears to evoke response in certain non-classical neural tissues, e.g. embryonic cartilage rudiments.[22] NGF, demonstrated by immunohistochemistry during craniofacial development in the mouse, appears in premuscular and precartilaginous mesenchyme as well as in the teeth. Cartilaginous expression of NGF supports the view that this molecule could play a role in the regulation of preskeletal differentiation.[61] Moreover, Frenkel et. al showed that NGF is not only present in chick embryo cartilage, but also in bone.[30] In accordance with the previous findings, more than twenty years ago, Varon and Bunge advanced that "it is possible that several of these responsive tissues are not 'physiological' targets of NGF but, rather, recognize it as an 'analog' of their own trophic agents."[99]

However, neurotrophins are also produced in the peripheral nervous system by non-neural target cells.[25] In the case of bone morphogenesis, bone-associated neurons have been considered to regulate bone differentiation through synaptic interaction between neuronal cells and bone forming cells.[41] Furthermore, Nakanishi et al[69] suggest that osteoblast-derived neurotrophins may support not only survival and differentiation of neural cells, but also the proliferation of osteoblasts themselves in bone tissue *in vivo*, finally leading to bone formation.

During bone proliferative phase and differentiation there is an increase in the level of NGF mRNA.[70] By addition of neurotrophin-3, Nakanishi et al[69] observed that bone cells became more elongated, condensed and overlapped, suggesting a stimulation of cell proliferation. Interestingly, it has been observed that the presence of neurons increased thymidine incorporation into non-neuronal cells by up to 400 %.[66] These results suggest that NGF could induce osteoblastic cell stimulation and may reflect the progressive interaction between bone cells and bone-associated neurons in the bone differentiation phase.[70]

The exact mechanism by which neurotrophins work on bone remains unknown, but several factors have been suggested. Proteoglycans are the binding site for many growth factors, and the binding of neurotrophin-6 to cell surface and/or extracellular matrix proteoglycans seems to protect neurotrophin-6 from proteolytic degradation.[35] In addition, it has been suggested that the anchoring of neurotrophin-6 to the proteoglycans might spatially restrict the action of this molecule and serve as an extracellular storage form, as has been shown for fibroblast growth factor (FGF) bound to heparan sulfate proteoglycans.[82,102] It remains to be established whether neurotrophin-6 requires the presence of heparin or a specific heparan sulfate proteoglycan for optimal biological activity, as is the case with the fibroblast growth factor.[74,107]

Yada et al[106] suggest that NGF acts on cultured osteoblastic cells by endogenous PGE_2. Other authors, however, have observed that cell lines from the osteoblastic lineage respond to the presence of $1,25(OH)_2$-D_3 by an increase in NGF mRNA levels.[49] On the other hand, Nakanishi et al have shown that addition of varied concentrations of TGF-ß to culture cells at the end of the monolayer stage enhanced the expression of neurotrophin genes[70]. TGF-ß is considered to control the expression of NGF mRNA in osteoblastic cells during osteogenesis, relevant to the stimulating effect of TGF-ß reported on osteogenesis of periosteal bone in vivo.[71]

The signal transduction of NGF has been studied using ^{125}I-NGF. When ^{125}I-NGF is added to bone cells in culture, osteoblastic cells display the properties of a low affinity NGF receptor.[49] Furthermore, Nakanishi et. al showed that neurotrophins, especially neurotrophin-3,

stimulated the proliferation of native non-neural osteoblastic cells via trkC.[69]. Signal transducible neurotrophin receptors have been identified as the trk protooncogens.[53,65,58] These receptors are selectively recognized by neurotrophins.[13,52,58] The expression level of trkC was higher during the growth phase than during the early differentiation phase. The co-expression of trkC and its ligand in the proliferating cell suggests that neurotrophin-3 plays an important role in the proliferation of osteoblastic cells in an autocrine manner. After activation of these receptors, NGF induces the expression of immediate-early genes, such as NGFI-A, c-myc, c-fos and c-jun in PC12 cells.[15,67,105] These immediate-early genes encode transcription factors that regulate the induction of late genes. Among immediate-early genes, overexpression of c-fos, c-jun and c-myc leads to cellular transformation, suggesting that these genes promote cellular proliferation. However, no evidence of NGF receptors or functional responses has been found in chick bone cells *in vitro*.[28]

NGF has also been noted in several physiologic and pathologic conditions. Sensory nerves also appear to be important in normal bone metabolism and in bone fracture repair.[47] Immunostaining for NGF was seen in skeletal muscle fibers, periosteal osteoprogenitor cells and superficial osteocytes in cortical bone. In addition, increased sensory and sympathetic innervation of fracture calluses has been reported in animal experiments.[37,47] NGF staining was almost complete in the whole tissues related with the fracture site, except in some chondrocytes and deep osteocytes in trabecular or cortical bone and osteoclasts. An important finding is that, in calluses, periosteal matrix stained heavily for NGF when juxtaposed to cartilage and less obviously when associated with new bone.[36]

It has been suggested that some cellular components of the fracture callus produce NGF which in turn increases the innervation.[37,72] NGF detected in chondrocytes and osteoblasts of embryonic chick skeletal tissue suggests that these cells may govern innervation in the embryo by synthesizing and secreting NGF.[30] Since fracture callus is initially composed of similar embryonal-like skeletal cells, it was therefore expected that cells of the callus could similarly contain NGF.[48] Grills et. al reported that most chondrocytes of the callus stained for NGF, indicating that NGF appears at a particular stage during chondrocytic differentiation in callus development.[36] Fibrous and cartilaginous matrices did not exhibit immunostaining for NGF, a finding similar to that in the embryonic chick study.[30] In addition, the presence of NGF in periosteal matrices contiguous to cartilage and new bone may also indicate that reinnervation of the periosteum (a highly innervated tissue) is important in fracture repair, as opposed to non-innervated cartilage or bone matrices.[43] This observation may also indicate that NGF, like some other growth factors (e.g., IGF-I), can be stored in certain matrices, and thus be made available for physiologic responses by release from such tissue.[79]

Eppley et. al observed that after producing nerve gaps in rat mandibular bone, nerve axon regeneration was induced by the topical administration of NGF.[23] This incidental finding suggest that bone formation was stimulated around the NGF-induced regenerating axons. Another important finding is the enhanced bone formation induced by NGF when it is specifically administered topically in areas of onlay bone grafts. It has been shown that NGF exhibited a beneficial effect on maintenance of onlay bone graft volume.[24] In addition, topical application of NGF to fractured rat ribs increases the stiffness and breaking strains of the bone callus, thereby suggesting stimulation of bone cells.[37]

Finally, another role of neurotrophins in the musculoskeletal system seems to be related to neoplastic processes. In fact, the implantation of mesenchymal tumors into chick embryos was the starting point for the discovery and investigation of NGF.[60] In Ewing's sarcomas, cells bear high-affinity receptors for NGF, and utilize signal pathways similar to NGF receptors on PC12 cells.[96] In addition, Fas/APO-1, a member of the NGF/TNF receptor superfamily, is expressed on the cell-surface of normal and malignant cells. It is known that Fas/APO-1 is associated with cell death induced by apoptosis, however, human osteosarcoma cell lines are resistant to the apoptosis-inducing effects of anti-Fas.[76] However, Thompson et al. found that the expression of human NGF receptors in non-neurogenic mesenchymal tumors was generally negative: 0 of 5 chondrosarcomas, 0 of 6 malignant fibrous histiocytomas, and 1 of 8 leiomyosarcomas.[95]

In summary, there is indirect evidence that strongly suggests that bone, and even cartilage, could be targets of the nervous system. Further research in this field will allow a better understanding of the basic mechanisms of neural control on skeletal cells, and could provide new pathways for the study of skeletal development and growth, fracture healing, osteoporosis, arthropathies or even neoplasias.

REFERENCES

1. **Aro, H.:** Development of nonunions in the rat fibula after removal of periosteal neural mechanorecptors. *Clin Orthop Rel Res* 1985; 199:292-9
2. **Aro, H., Eerola, E., Aho, A.J.:** Fracture healing in paraplegic rats. *Acta Orthop Scand* 1985; 56:228-32.
3. **Basedovsky, H.O., DelRey, A.:** Immuno-neuroendocrine interactions: facts and hypotheses. *Endocr Rev* 1996; 17:64-102.
4. **Becker, R.O.:** The significance of bioelectric potentials. *Bioelectrochem Bionerg*, 1974; 1:187.
5. **Bernard, G.W., Shih, C.:** The osteogenic stimulating effect of neuroactive calcitonin gene-related peptide. *Peptides* 1990; 11:625-32.
6. **Bjurholm, A.:** Neuroendocrine peptides in bone. *Int Orthop* 1991; 15:325-29.
7. **Bjurholm, A., Kreicbergs, A., Dahlbergm L., Schultzberg, M.:** The occurrence of neuropeptides at different stages of DBM-induced heterotopic bone formation. *Bone Miner* 1990; 10:95-107.
8. **Bjurholm, A., Kreicbergs, A., Schultzberg, M.:** Substance P- and CGRP-immunoreactive nerves in bone. *Peptides* 1988; 9:165-171.
9. **Brenneman, D.E.,** and **Eiden, L.E.:** Vasoactive intestinal peptide and electrical activity influence neuronal survival. *Proc Natl Acad Sci U.S.A.* 1986; 83:1159-62.
10. **Chakraborty, M., Chatterjee, D., Kellokumpu, S., Rasmussen, H., Baron, R.:** Cell cycle-dependent coupling of the calcitonin receptor to different G proteins. *Science* 1991; 251:1078-82.
11. **Coderre, T.J., Basbaum, A.I., Levine, J.D.:** Neural control of vascular permeability: interactions between primary afferents, mast cells, and sympathetic efferents. *J Neurophysiol* 1989; 62:48-58.
12. **Cooper, R.R.:** Nerves in cortical bone. *Science* 1968, 160:327-328.
13. **Cordon-Cardo, C., Tapley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F., Barbacid, M.:** The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3 *Cell* 1991; 66:173-83.
14. **DeCastro, F.:** Technique pour la coloration du systeme nerveux quand il est pourvu de ses etius. *Trav Lab Rech Biol* 1925 ; 23: 429-446.
15. **DeFranco, C., Damon, D.H., Endoh, M., Wagner, J.A.:** Nerve growth factor induces transcription of NGFIA through complex regulatory elements that are also sensitive to serum and phorbol 12-myristate 13-acetate. *Mol Endocrinol* 1993; 7:365-79.
16. **Dietz, F.R**.: Effect of denervation on limb growth. *J Orthop Res* 1989; 7:292-303.
17. **D'Souza, S.M., MacIntyre, I., Girgis, S.I., Mundy, G.R.:** Human synthetic calcitonin gene-related peptide inhibits bone resorption in vitro. *Endocrinology*,1986; 119:58-61.
18. **Duncan, C.P.:** The autonomic nerve supply of bone. An experimental study of the intraosseous adrenergic nervi vasorum in the rabbit. *J Bone Joint Surg* 1977; 59-B:323-30.
19. **Dyck, P.J., Stevens, J.C., O'Brien, P.C., Oviatt, K.F., Lais, A.C., Coventry, M.B., Beabout, J.W.:** Neurogenic arthropathy and recurring fractures with subclinical inherited neuropathy. *Neurology* 1983; 33:357.
20. **Egar, M., McCredie, J., Singer, M.:** Newt forelimb cartilage regeneration after partial denervation. *Anat Rec* 1982; 204:131-136.
21. **Eichenholtz SN.** Management of long-bone fractures in paraplegic patients *J Bone Joint Surg* 1963; 45-A:299-310.
22. **Eisenbarth, G.S., Drezner, M.K., Lebovitz, H.E.:** Inhibition of chondromucoprotein synthesis: an extraneuronal effect of nerve growth factor. *J Pharmacol Exp Ther* 1975; 192:630-4
23. **Eppley, B.L., Snyders, R.V., Winkelmann, T., Delfino, J.J., Sadove, A.M.:** Effects of nerve growth factor on craniofacial onlay bone graft survival: preliminary findings. *J Craniofac Surg* 1992; 2:174-80.
24. **Eppley, B.L., Snyders, R.V., Winkelmann, T., Roufa, D.G.:** Efficacy of nerve growth factor in regeneration of the mandibular nerve: a preliminary report. *J Oral Maxillofac Surg* 1991; 49:61-8.
25. **Ernfors, P., Wetmore, C., Olson, L., Persson, H.:** Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 1990; 5:511-26.
26. **Esteban, B., Díaz, J.:** Lesiones del nervio ciático postinyección glutea. 1. Estudio clínico. *Rev Orthop Traum* 1981; 25:67-80.
27. **Fatatis, A., Holtzclaw, L.A., Avidor, R., Brenneman, D.E., Russell, J.T.:** Vasoactive intestinal peptide increases intracellular calcium in astroglia: synergism with a-adrenergic receptors. *Proc Natl Acad Sci USA* 1994; 91:2036-40.
28. **Finkelman, R.D., Lau, K.H., Abraham, S.M., Baylink, D.J.:** Evidence for a lack of functional receptors for nerve growth factor (NGF) in chick bone cells in vitro. *Mol Cell Biochem* 1992; 115:129-36.
29. **Freehafer, A.A., Mast, W.A.:** Lower extremity fractures in patients with spinal cord injury. *J Bone Joint Surg* 1965; 47:683-694.

30. **Frenkel, S.R., Guerra, L.A., Mitchell, O.G., Singh, I.J.:** Nerve growth factor in skeletal tissues of the embryonic chick. *Cell Tissue Res* 1990; 260:507-11.
31. **Garcés, G.L., Santandreu, M.E.:** Longitudinal bone growth after sciatic denervation in rats. *J Bone Joint Surg* 1988; 70-B:315-8.
32. **Gillespie, J.A.:** The nature of bone changes associated with nerve injuries and disuse. *J Bone Joint Surg* 1963; 36:464-473.
33. **Glick, E.N.:** Asymmetrical rheumatoid arthritis after poliomyelitis. *BMJ* 1967; 3:26-29.
34. **Goldring, S.R., Goldring, M.B.:** Cytokines and skeletal physiology. *Clin Orthop* 1996; 324:13-23.
35. **Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., Thoenen, H.:** Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* 1994; 372:266-9.
36. **Grills, B.L., Schuijers, J.A.:** Immunohistochemical localization of nerve growth factor in fractured and unfractured rat bone. *Acta Orthop Scand* 1998; 69:415-9.
37. **Grills, B.L., Schuijers, J.A., Ward, A.R.:** Topical application of nerve growth factor improves fracture healing in rats. *J Orthop Res* 1997; 15:235-242.
38. **Hara-Irie, F., Amizuka, N., Ozawa, H.:** Immunohistochemical and ultrastructural localization of CGRP-positive nerve fibers at the epiphyseal trabecules facing the growth plate of rat femurs *Bone* 1996; 18:29-39.
39. **Hardy, A.G., Dickson, J.W.:** Pathological ossification in traumatic paraplegia. *J Bone Joint Surg* 1963; 45:76-87.
40. **Herskovits, M.S., Singh, I.J.:** Effect of guanethidine-induced sympathectomy on osteoblastic activity in the rat femur evaluated by ^{3}H-proline autoradiography. *Acta Anat* 1984; 120:151-5.
41. **Hill, E.L., Elde, R.:** Calcitonin gene-related peptide-immunoreactive nerve fibers in mandibular periosteum of rat: evidence for primary afferent origin. *Neurosci Lett* 1988; 85:172-8.
42. **Hill, E.L., Elde, R.:** Distribution of CGRP-, VIP-, Dbd, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. *Cell Tissue Res* 1991; 264:469-480.
43. **Hohmann, E.L., Elde, R.P., Rysavy, J.A., Einzig, S., Gebhard, R.L.:** Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing nerve fibers. *Science* 1986; 232:868-71.
44. **Hohmann, E.L. Levin, L., Tashjian, A.H., Jr.:** Vasoactive intestinal peptide stimulates bone resorption via a cyclic adenosine 3',5'-monophosphate-dependent mechanism. *Endocrinology* 1983; 112:1233-39.
45. **Hohmann, E.L., Tashjian, A.H., Jr.:** Functional receptors for vasoactive intestinal peptide on human osteosarcoma cells. *Endocrinology* 1987; 114:1321-2.
46. **Hukkanen, M., Konttinen, Y.T., Rees, R.G., Gibson, S.J., Santavirta, S., Polak, J.M.:** 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.
47. **Hukkanen, M., Konttinen, Y.T., Santavirta, S., Paavolainen, P., Gu, X-H., Terenghi, G., Polak, J.M.:** Rapid proliferation of calcitonin gene-related peptide-immunoreative nerves during healing of rat tibial fracture suggests neural involvement in bone growth and remodelling. *Neuroscience* 1993; 54:969-79.
48. **Hulth, A.:** Current concepts of fracture healing. *Clin Orthop* 1989; 249:265-84.
49. **Jehan, F., Naveilhan, P., Neveu, I., Harvie, D., Dicou, E., Brachet, P., Wion, D.:** Regulation of NGF, BDNF and LNGFR gene expression in ROS 17/2.8 cells. *Mol Cell Endocrinol* 1996; 116:149-56.
50. **Kawase, T., Howard, G.A., Roos, B.A., Burns, D.M.:** Diverse actions of calcitonin gene-related peptide on intracellular free Ca^{2+} concentrations in UMR 106 osteoblastic cells. *Bone* 1995; 16:379S-84S.
51. **Kikuchi, M., Lu, C-H., Sebata, M., Yamamoto, Y.:** The mandibular development of the rat after the denervation of the masseteric nerve. *Bull Tokyo Dent Coll* 1978; 19:78-86.
52. **Klein, R., Nanduri, V., Jing, S.A., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K.R., Reichardt, L.F., Barbacid, M.:** The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* 1991; 66:395-403.
53. **Klein, R., Parada, L.F., Coulier, F., Barbacid, M.:** trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J* 1989 ; 8:3701-9.
54. **Konttinen, Y.T., Imai, S., Suda, A.:** Neuropeptides and the puzzle of bone remodeling. State of the art. *Acta Orthop Scand* 1996; 67:632-39.
55. **Kreicbergs A.** Neuropeptides in bone. *Curr Opinion Orthop* 1997; 8:71-79.
56. **Kreicbergs, A., Ahmed, M., Ehrnberg, A., Schultzberg, M., Svensson, S.B., Bjurholm, A.:** Interleukin-1 immunoreactive nerves in heterotopic bone induced by DBM. *Bone* 1995; 17:341-345.

57. **Kurerer, M.H.J., Khoker, M.A., Dandona, P.:** Human osteoblast stimulation by sera from paraplegic patients with heterotopic ossification. *Paraplegia* 199230:165-168.
58. **Lamballe, F., Klein, R., Barbacid, M.:** trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* 1991; 66:967-79.
59. **Levi-Montalcini, R.:** The nerve growth factor 35 years later. *Science* 1987; 237:1154-62.
60. **Levi-Montalcini, R., Hamburger, V.:** Selective growth stimulating effects of mouse sarcoma on sensory and sympathetic nervous system of the chick embryo. *J Exp Zool* 1951; 116:321-62.
61. **Louryan, S., Biermans, J., Flemal, F.:** Nerve growth factor in the developing craniofacial region of the mouse embryo. *Eur J Morpho*l 1995; 33:415-9.
62. **Lutz, W.M., Sheward, W.J., West, K.M., Morrow, J.A., Fink, G., Harmar, A.J.:** The VIP_2 receptor: molecular characterization of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett* 1993; 334:3-8.
63. **Maden, M.:** The limb bud-part two. *Nature* 1994; 371:560-1
64. **Madsen, J.E., Aune, A.K., Falch, J.A., Hukkanen, M., Konttinen, Y.T., Santavirta, S., Nordsletten, L.:** Neural involvement in post-traumatic osteopenia: an experimental study in the rat. *Bone* 1996; 18:411-16.
65. **Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., Barbacid, M.:** Molecular and biochemical characterization of the human trk proto-oncogene. *Mol Cell Biol* 1989; 9:24-33.
66. **McCarthy, K., Parlow, L.:** Neuronal stimulation of ^{3}H-thymidine incorporation by primary cultures of highly purified non-neuronal cells. *Brain Res* 1976; 114:415-26.
67. **Milbrandt, J.:** Nerve growth factor rapidly induces c-fos mRNA in PC12 rat pheochromocytoma cells. *Proc Natl Acad Sci U S A* 1986; 83:4789-93
68. **Miller, M.R., and Kasahara, M.:** Observations on the innervation of human long bones *Anat Rec* 1963; 145:13.
69. **Nakanishi, T., Ohyama, K., Aoki, C., Kudo, A., Hattori, T., Takahashi, K., Taniguchi, S., Takigawa, M.:** Expression of trkC in a mouse osteoblastic cell line and its response to neurotrophin-3. *Bioch Biophy Res Commun* 1994; 203:1268-74.
70. **Nakanishi, T., Takahashi, K., Aoki, C., Nishikawa, K., Hattori, T., Taniguchi, S.:** Expression of NGF family neurotrophins in a mouse osteoblastic cell line. *Bioch Biophy Res Commun* 1994; 198:891-7.
71. **Noda, M., Camilliere, J.J.:** In vivo stimulation of bone formation by transforming growth factor-beta. *Endocrinology* 1989; 124: 2991-4
72. **Nordsletten, L., Madsen, J.E., Halse, J., Konttinen, Y.T., Hukkanen, M., Santavirta, S.:** The neuronal regulation of fracture healing. Effects of sciatic nerve resection in rat tibia. *Acta Orthop Scand* 1994; 65:299-304.
73. **Nordstrom, D., Satntavirta, S., Seitsalo, ss, Hukkanen, M., Polak, J., Nordsletten, L., Konttinen, Y.T.:** Symptomatic lumbar spondylosis: neuroimmu-nological studies. *Spine* 1994; 19:2752-8.
74. **Nurcombe, V., Fordm M.D., Wildschut, J.A., Bartlett, P.F.:** Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science* 1993; 260:103-6.
75. **Owan, I., Ibaraki, K.:** The role of calcitonin gene-related peptide (CGRP) in macrophages: the presence of functional receptors and effects on proliferation and differentiation into osteoclast-like cells. *Bone Miner* 1994; 24:151-64.
76. **Owen-Schaub, L.B., Angelo, L.S., Radinsky, R., Ware, C.F., Gesner, T.G., Bartos, D.P.:** Soluble Fas/APO-1 in tumor cells: a potential regulator of apoptosis? *Cancer Lett* 1995; 94:1-8.
77. **Pennock, J.M., Kalu, D.N., Clark, M.B., Foster, G.V., Doyle, F.H.:** Hypoplasia of bone induced by immobilization. *Br J Radiol* 1972; 45:641-6
78. **Perosio, P.M., Brooks, J.J.:** Expression of nerve growth factor receptor in paraffin-embedded soft tissue tumors. *Am J Pathol* 1988; 132:152-60.
79. **Pfeilschifter, J., Laukhuf, F., Muller-Beckman, B., Blum, W.S., Pfister, T., Ziegler, R.:** Parathyroid hormone increase the concentration of insulin-like growth factor I and transforming growth factor beta I in rat bone. *J Clin Invest* 1995; 96:767-74.
80. **Rahman, S., Dobson, P.R., Bunning, R.A., Russell, R.G., Brown, B.L.:** The regulation of connective tissue metabolism by vasoactive intestinal polypeptide. *Regul Pept* 1992; 37:111-21.
81. **Retief, D.H., Dreyer, C.J.:** Effects of neural damage on the repair of bony defects in the rat. *Arch Oral Biol* 1967; 12:1035.
82. **Rifkin, D.B., Moscatelli, D.:** Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol* 1989; 109:1-6.
83. **Ring, P.A.:** The influence of the nervous system upon the growth of bones. *J Bone Joint Surg* 1961; 43-B:121-40.

84. **Rohrer, H.:** The synthesis of nerve growth factor (NGF) in developing skin is independent of innervation. *Dev Biol* 1988; 128:240-4.
85. **Roos, B.A., Fischer, J.A., Pignat, W., Alander, C.B., Raisz, L.G.:** Evaluation of the in vivo and in vitro calcium-regulating actions of noncalcitonin peptides produced by calcitonin gene expression. *Endocrinology* 1986; 18:46-51.
86. **Santavirta, S., Konttinen, Y.T., Nordström, D., Mäkelä, A., Sorsa, T., Hukkanen, M., Rokkanen, P.:** Immunologic studies of nonunited fractures. *Acta Orthop Scand* 1992; 63:579-85.
87. **Schwab, W., Bilgicyildirim, Funk, R.H.W.:** Microphotography of the autonomic nerves in the rat knee: a fluorescent microscopic study. *Anat Rec* 1997; 247:109-118.
88. **Shih, C., Bernard, G.W.:** Calcitonin gene-related peptide enhances bone colony development in vitro. *Clin Orthop* 1997; 334:335-44.
89. **Singer, M.:** The influence of the nerve in regeneration of the amphibian extremity. *Q Rev Biol* 1952; 27:169-200.
90. **Singer, M., Craven, L.:** The growth and morphogenesis of the regenerating forelimb of adult *Triturus* following denervation at various stages of development. *J Exp Zool* 1948; 108:279-308.
91. **Sisask, G., Bjurholm, A., Ahmed, M., Kreicbergs, A.:** Ontogeny of sensory nerves in the developing skeleton. *Anat Rec* 1995; 243:234-240.
92. **Sisask, G., Bjurholm, A., Ahmed, M., Kreicbergs, A.:** The development of autonomic innervation in bone and joints of the rat. *J Auton Nerv Syst* 1996; 59:27-33.
93. **Spencer, R.F.:** The effect of head injury on fracture healing. A quantitative assessment. *J Bone Joint Surg* 1987; 69-B:525-8.
94. **Thompson, M., Bywaters, E.G.L.:** Unilateral rheumatoid arthritis following hemiplegia. *Ann Rheum Dis* 1966; 21:370-377.
95. **Thompson, S.J., Schatteman, G.C., Gown, A.M., Bothwell, M.A.:** Monoclonal antibody against nerve growth factor receptor. Immunohistochemical analysis of normal and neoplastic human tissue. *Am J Clin Pathol* 1989; 92:415-23.
96. **Thomson, T.M., Pellicer, A., Greene, L.A.:** Functional receptors for nerve growth factor on Ewing's sarcoma and Wilm's tumor cells. *J Cell Physiol* 1989;141:60-4.
97. **Trotman, N.M., Kelly, W.D.:** The effect of sympathectomy on blood flow to bone. *JAMA* 1963; 183:121-2.
98. **Variot, G., Remy, C.:** Sur les nerfs de la modelle des os. *J lánat Physiol* 1980;21:273-284.
99. **Varon, S.S.** and **Bunge, R.P.:** Trophic mechanisms in the Peripheral Nervous System. *Ann Rev Neurosci* 1978; 1:327-61.
100. V**eenstra, T.D., Fahnestock, M., Kumar, R.:** An AP-1 site in the nerve growth factor promoter is essential for 1, 25-dihydroxyvitamin D_3-mediated nerve growth factor expression in osteoblasts. *Biochemistry* 1998; 37:5988-94.
101. **Vignery, A., McCarthy, T.L.:** The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts. *Bone* 1996; 18:331-5.
102. **Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P., Fuks, Z.:** Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? *Trends Biochem Sci* 1991; 16:268-71
103. **Wallace, H., Watson, A., Egar, M.:** Regeneration of subnormally innervated axolotl arms. *J Embryool Exp Morphol* 1981; 62:1-11.
104. **Wildburger, R., Zarkovic, N., Egger, G., Petek, W., Zarkovic, K., Hofer, H.P.:** Basic fibroblast growth factor (bFGF) immunoreactivity as a possible link between head injury and impaired bone fracture healing. *Bone Miner* 1994; 27:183-92.
105. **Wu, D.K., Maciag, T., de Vellis, J.:** Regulation of neuroblast proliferation by hormones and growth factors in chemically defined medium. *J Cell Physiol* 1988; 136:367-72.
106. **Yada, M., Yamaguchi, K., Tsuji, T.:** NGF stimulates differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* 1994; 205:1187-93.
107. **Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., Ornitz, D.M.:** Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 1991; 64:841-8.